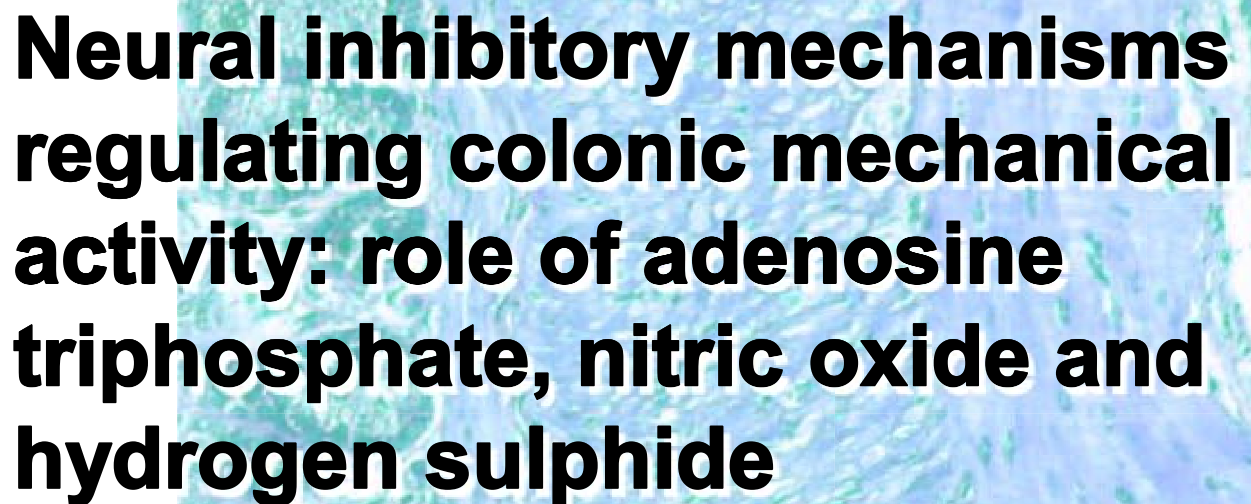

Neural inhibitory mechanisms regulating colonic mechanical activity: role of adenosine triphosphate, nitric oxide and hydrogen sulphide

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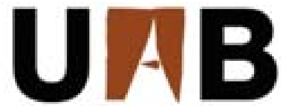

Neural inhibitory mechanisms regulating colonic mechanical activity: role of adenosine triphosphate, nitric oxide and hydrogen sulphide

By

Víctor Gil Folch

A dissertation submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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I certify that the dissertation entitled: "Neural inhibitory mechanisms regulating colonic mechanical activity: role of adenosine triphosphate, nitric oxide and hydrogen sulphide" submitted by **VÍCTOR GIL FOLCH** 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 defence.

*Faig constar que la tesi titulada "Neural inhibitory mechanisms regulating colonic mechanical activity: role of adenosine triphosphate, nitric oxide and hydrogen sulphide" presentada per **VÍCTOR GIL FOLCH** per optar al grau de Doctor ha estat realitzada sota la meva supervisió i autoritzo la presentació per realitzar la seva defensa oral.*

In witness whereof, I hereby sign this document,

I, perquè així consti, signo aquest document,

Dr. Marcel Jiménez Farrerons, BVSc, PhD

Doctoral Advisor / *Director de tesis*

The harder you work, the luckier you get.

Gary Player

South African Golfer

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Participation in scientific meetings

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Acronyms

2-MeSADP	2-methylthioadenosine-5'-O-diphosphate
2-MeSATP	2-methylthioadenosine-5'-O-triphosphate
3MST	3-mercaptopyruvate sulfurtransferase
5-HT	5-hydroxytryptamine
$5\text{-}HT_3\text{-}R$	5- hydroxytryptamine type 3 receptor
AA	Arachidonic acid
AC	Adenylyl cyclase
ADP	Adenosine-5'-diphosphate
ADPβS	Adenosine 5′-O-2-thiodiphosphate
ADPr	Adenosine-5'-diphosphate ribose
AH	After-hyperpolarization
AMP	Adenosine-5'-monophosphate
ANOVA	Analysis of variance
AOAA	Aminooxyacetic acid
ATP	Adenosine-5'-triphosphate
AUC	Area under the curve
β-NAD	β-nicotinamide adenine dinucleotide
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CNS	Central nervous system
cGMP	Cyclic guanosine monophosphate
CMMC	Colonic migrating motor complexes
Cl_{Ca}	Calcium-activated chloride channels
CO	Carbon monoxide

COS cells	Cells being CV-1 in origin and carrying the SV40 genetic material
CSE	Cystathionine γ-lyase
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
DMEM	Dulbecco's modified Eagle's medium
EDRF	Endothelium-derived relaxing factor
EFS	Electric field stimulation
EJP	Excitatory junction potential
EMNs	Excitatory motor neurons
eNOS	Endothelial nitric oxide synthase
ENS	Enteric nervous system
EPSP	Excitatory postsynaptic potential
Ethe1	Ethylmalonic encephalopathy 1
GC	Guanylate cyclase
GI	Gastrointestinal
GMC	Giant migrating contractions
H_2S	Hydrogen sulphide
HA	Hydroxylamine
HACs	High-amplitude contractions
HEK cells	Human embryonic kidney cells
HO	Heme oxygenase
IC_{50}	Half maximal inhibitory concentration
ICC	Interstitial Cells of Cajal
ICC-MP	Interstitial Cells of Cajal associated with the myenteric plexus
ICC-SMP	Interstitial Cells of Cajal associated with the submuscular plexus
IJP	Inhibitory junction potential

IJPf	Fast component of the inhibitory junction potential
IJPs	Slow component of the inhibitory junction potential
IMNs	Inhibitory motor neurons
iNOS	Inducible nitric oxide synthase
IPAN	Intrinsic primary afferent neurons
IR	Immunoreactivity
K_{2P}	Two-pore domain potassium channels
K_{ATP}	ATP-sensitive potassium channels
LACs	Low-amplitude contractions
LDCs	Long Distance Contractions
L-NNA	N^{ω}-nitro-L-arginine
NaHS	Sodium hydrogen sulphide
NMDA	N-methyl-D-aspartate
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MP	Myenteric plexus
MPOs	Myenteric potential oscillations
NANC	Non-adrenergic non-cholinergic
NO	Nitric oxide
nNOS	Neural nitric oxide synthase
NOS	Nitric oxide synthase
ODQ	1H-[1,2,4]Oxadiazolo[4,3-□]quinoxalin-1-one
PAG	D,L- propargylglycine
PDGFRα^{+} cells	Platelet derived growth factor receptor α-positive cells
PLP	Pyridoxal phosphate

RMP	Resting membrane potential
RNA	Ribonucleic acid
RPCs	Rhythmic phasic contractions
RPMC	Rhythmic propulsive motor complexes
RT-PCR	Real-time polymerase chain reaction
PACAP	Pituitary adenylyl cyclase activating peptide
PKG	Protein kinase G
PLC	Phospholipase C
PS	Propagating sequences
sEPSP	Slow excitatory postsynaptic potential
sIJP	Spontaneous inhibitory junction potential
SD	Standard deviation
SDK	Stretch-dependent potassium channel
SEM	Standard error of the mean
SK_{Ca}	Small-conductance calcium-activated potassium channel
SNP	Sodium nitroprusside
SMCs	Smooth muscle cells
SMP	Submucosal plexus
SQR	Sulphide quinone reductase
TRAAK	TWIK-related arachidonic acid stimulated channel
TREK1	TWIK-related potassium channel 1
TREK2	TWIK-related potassium channel 2
TRPA1	Transient receptor potential cation channel type A1
TRPV1	Transient receptor potential cation channel type V1
TTX	Tetrodotoxin

UDP	Uridine 5'-diphosphate
UDP-glucose	Uridine 5'-diphosphate glucose
UDP-galactose	Uridine 5'-diphosphate galactose
UTP	Uridine 5'-triphosphate
UTPγS	Uridine-5'-(γ-thio)-triphosphate
VDCC	Voltage-dependent calcium channel
VIP	Vasoactive intestinal peptide
ω-CTX	ω-conotoxin

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Abstract

Neural inhibitory mechanisms regulating colonic mechanical activity: role of adenosine triphosphate, nitric oxide and hydrogen sulphide

Inhibitory neuromuscular transmission is involved in the control of colonic motility. Inhibitory neurotransmitters cause membrane hyperpolarization of smooth muscle, that is, an inhibitory junction potential (IJP) which is the electrophysiological basis of muscle relaxation. Thus, adenosine triphosphate (ATP), nitric oxide (NO) and hydrogen sulphide (H_2S) are signalling molecules which can exert this control. The aim of this thesis was to evaluate the role of ATP, NO and H_2S in inhibitory neuromuscular transmission in the rat and human colon. Sodium hydrosulphide (NaHS) was used as the source of H_2S.

Specific $P2Y_1$ receptor antagonists completely inhibited the purinergic component of the IJP in the human and rat colon (potency range: MRS2500> MRS2279> MRS2179). β-NAD produced smooth muscle hyperpolarization and relaxation in the human colon. However, the relaxation was not blocked by $P2Y_1$ antagonists. In the rat colon, it was observed that the release of inhibitory neurotransmitters produced both spontaneous IJP and a sustained muscle hyperpolarization. MRS2500 revealed that $P2Y_1$ receptors were responsible for spontaneous IJP. Nω-nitro-L-arginine (L-NNA) allowed us to establish that the tonic state of muscle hyperpolarization was nitrergic in origin. Apamin, spadin and ODQ were used to assess the involvement of small-conductance calcium-activated potassium channels (SK_{Ca}), TWIK-related potassium channels 1 (TREK1) and the guanylate cyclase (GC) pathway on purinergic and nitrergic neuromuscular transmission. Apamin blocked both components with a different relative sensitivity. ODQ only blocked nitrergic neuromuscular transmission. Furthermore, even though the presence of TREK1 channels was revealed in rat colon, none of the components was blocked by spadin, an effective TREK1 channel inhibitor. The expression of H_2S-producing enzymes was determined by immunohistochemistry in the epithelium, muscle and nerve cells in the rat colon. In addition, it was demonstrated that the rat tissue was able to enzymatically produce H_2S. Although in some cases the effects of H_2S-producing enzymes inhibitors were not specific, they caused muscle depolarization and an increase in colonic mechanical activity. In segments of rat colon, NaHS caused a dual effect on motility patterns when administered via serosa. NaHS inhibited propulsive contractions whereas it caused an increase in the amplitude of ripples. NaHS inhibited cholinergic excitatory junction potentials and hyperpolarized smooth muscle cells. However, NaHS did not

modify the colonic pacemaker located at the level of the submucosal plexus. Luminal addition of NaHS did not modify motility patterns.

Purinergic neurotransmission in the rat and human colon is mediated by $P2Y_1$ receptors. ATP and NO are released by myenteric plexus inhibitory neurons and have complementary functions in the colon. I.e. ATP produces a phasic action on membrane potential and mechanical activity and NO has a tonic effect. Both purinergic and nitrergic responses are in part apamin sensitive suggesting a possible involvement of SK_{Ca} in both pathways. NO effects on membrane potential are produced by GC activation but TREK1 channels are not involved. In the rat colon, H_2S is produced and released endogenously and it might be a signalling molecule modulating action on colonic motility. H_2S mechanism of action involves inhibition of neurally mediated cholinergic responses and muscle hyperpolarization.

Keywords: Colon, smooth muscle cells, inhibitory junction potential, adenosine triphosphate, nitric oxide, hydrogen sulphide, human, rat.

Neural inhibitory mechanisms regulating colonic mechanical activity: role of adenosine triphosphate, nitric oxide and hydrogen sulphide

La neurotransmissió inhibitòria participa en el control de l'activitat motora del còlon. Els neurotransmissors inhibitoris hiperpolaritzen la membrana del múscul llis, generant un potencial d'unió inhibitori (*"Inhibitory Junction Potential*" IJP*)* que és la base electrofisiològica de la relaxació muscular. Així, l'adenosina trifosfat (ATP), l'òxid nítric (NO) i el sulfur d'hidrogen (H_2S) són molècules de senyalització que poden exercir aquest control. L'objectiu d'aquesta tesi doctoral ha estat valorar la participació de l'ATP, el NO i el H_2S en la transmissió neuromuscular inhibitòria en el còlon humà i de rata. El hidrogensulfur de sodi (NaHS) va ser utilitzat com font de H_2S.

Els antagonistes específics dels receptors $P2Y_1$ van inhibir completament el component purinèrgic de l'IJP en el còlon humà i de rata (rang de potència: MRS2500>MRS2279>MRS2179). El β-NAD va produir hiperpolarització i relaxació de la musculatura del còlon humà. Tanmateix, la relaxació no va ser bloquejada pels antagonistes $P2Y_1$. En el còlon de rata es va observar que l'alliberament de neurotransmissors inhibitoris produïa la generació d'IJP espontanis i un potencial de membrana muscular hiperpolaritzat. Mitjançant MRS2500 es va posar de manifest que els receptors $P2Y_1$ participaven en la gènesi dels IJP espontanis. El Nω-nitro-L-arginina (L-NNA) va permetre establir que l'estat de hiperpolarització tònic del potencial de membrana era d'origen nitrèrgic. L'ús d'apamina, spadina i ODQ va permetre valorar la participació dels canals de potassi de baixa conductància activats per calci (SK_{Ca}), els canals de potassi relacionats amb TWIK tipus 1 (TREK1) i la via de la guanilat ciclasa (GC) sobre la neurotransmissió purinèrgica i nitrèrgica. L'apamina va bloquejar tots dos components amb diferents nivells d'afinitat. L'ODQ només va bloquejar la neurotransmissió nitrèrgica. D'altra banda, tot i posar de manifest la presència de canals TREK1 en el còlon de rata, la spadina, un blocador de TREK1, no va bloquejar cap dels components. L'expressió dels enzims de síntesi del H_2S es va determinar immunohistoquímicament en l'epiteli, el múscul i les neurones del còlon de rata. A més, es va demostrar que el teixit de rata era capaç de produir enzimàticament H_2S. Tot i que en alguns casos es van observar efectes no específics dels inhibidors dels enzims de síntesi del H_2S, aquestos van produir despolarització de les cèl·lules musculars i un increment de l'activitat mecànica del còlon. En segments de còlon de rata, el NaHS

administrat per serosa va causar un efecte dual sobre els patrons de motilitat. El NaHS va inhibir les contraccions d'alta amplitud, mentre que va incrementar les contraccions de baixa amplitud. El NaHS va inhibir els potencials d'unió excitador i va hiperpolaritzar el múscul. Tanmateix, l'H_2S no va alterar el marcapassos colònic situat a nivell del plexe submucós. El NaHS via luminal no va modificar la motilitat del còlon.

La neurotransmissió purinèrgica al còlon humà i de rata es produeix a través del receptor $P2Y_1$. L'ATP i el NO són alliberats per les neurones inhibitòries del plexe mientèric i tenen funcions complementàries en el còlon. D'una banda, l'ATP exerceix una acció fàsica sobre el potencial de membrana i l'activitat mecànica, mentre que el NO té una acció tònica. Ambdues respostes nitrèrgica i purinèrgica son en part sensibles a apamina, suggerint que els canals SK_{Ca} poden esta involucrats en les dues vies. El NO exerceix el seu efecte sobre el potencial de membrana a través de l'activació de la via de GC però no activa els canals TREK1. El H_2S és produït i alliberat endògenament en el còlon de rata i pot actuar com una molècula de senyalització amb acció moduladora sobre la motilitat colònica. El seu mecanisme d'acció implica una inhibició de la neurotransmissió colinèrgica i hiperpolarització muscular.

Paraules clau: còlon, cèl·lules musculars llises, potencial d'unió inhibitori, adenosina trifosfat, òxid nítric, sulfur d'hidrogen, humà, rata.

Introduction

Structure and function of the colon

The gastrointestinal (GI) tract is a hollow tubular organ which provides the necessary supply of water, electrolytes and nutrients to the body. The colon is the part comprised between the ileocecal valve and the rectum and in humans it can be divided in ascending, transverse, descending and sigmoid colon. Histologically, it is composed of following five layers from inside outwards: (i) the mucosa, (ii) the submucosa, (iii) the circular muscle layer, (iv) the longitudinal muscle layer, and (v) the serosa (Fig 1) (Young *et al.*, 2006).

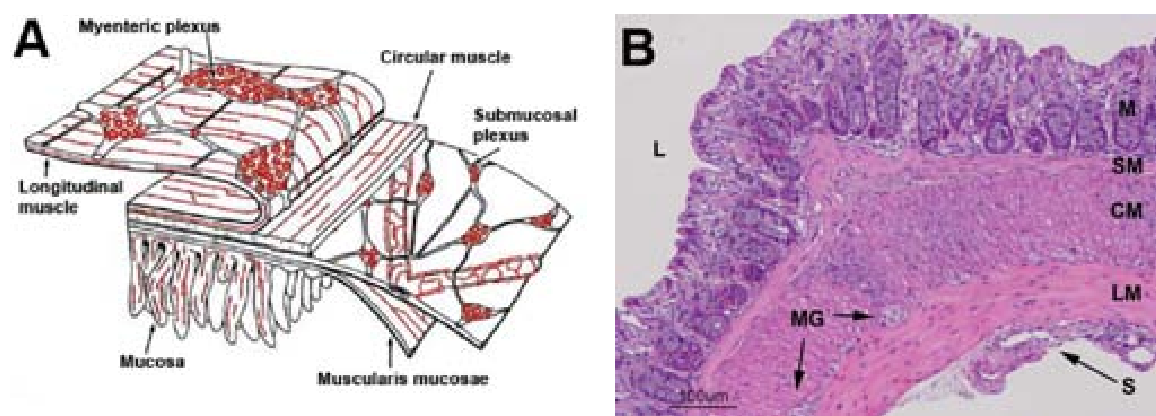

Figure 1. Whole-mount drawing **(A)** and mouse colon cross-section (haematoxylin and eosin staining) **(B)** showing the different layers of the colon. Whole-mount drawing has been adapted from Furness, 2006. The cross-section image has been provided by Dr. Joan Burgueño (Unitat de Fisologia, Facultat de Veterinària, Universitat Autònoma de Barcelona). M, mucosa; SM, submucosa; CM, circular muscle layer; LM, longitudinal muscle layer, S, serosa; L, lumen; MG, myenteric ganglia; SMG, submucosal ganglia.

Unlike the small intestine, the colon does not play a major role in the absorption of nutrients. Conversely, the main functions of the colon are: (i) absorption of water and electrolytes, (ii) formation and storage of solid faeces until defecation, and (iii) propulsion of faeces to the rectum allowing defecation (Guyton & Hall, 2006; Sarna, 2010). Smooth muscle cells (SMCs) generate the contractions required to move and mix colonic content. Multiple and overlapping mechanisms, including intrinsic properties of SMCs, pacemaker activity generated by the networks of interstitial cells of Cajal (ICC), neural regulation and the action of systemic and local hormones, work co-ordinately to orchestrate the mechanical activity in this region of the GI tract. Thus, the intertwined action of these cooperating control systems determines the expression of different motor patterns in the colon (Huizinga & Lammers, 2009).

Colonic motility patterns

Two major patterns can be identified in the colon: (i) small, short duration contractions which produce back and forth movements to mix, stir and turn over the faecal material and (ii) strong, long-lasting contractions which cause propulsion of digesta along the colon. Table 1 summarizes the nomenclature used in the literature to identify both patterns in the colon.

Table 1. Summary of nomenclature commonly used to identify motility patterns present in the colon

Pattern	*Nomenclature*	*Species*	*References*
Mixing	Rhythmic phasic contractions (RPCs)	Human[1]	Sarna, 2006
		Dog[2]	Sarna, 2010
		Rat[2]	Li *et al.*, 2002
	Rhythmic propagating ripples	Rat[3]	Huizinga *et al.*, 2011
	Low-amplitude contractions (LACs)	Mouse[4]	Mule *et al.*, 1999
		Pig[2]	Hipper & Ehrlein, 2001
	High frequency contractions	Guinea pig[4]	Smith *et al.*, 2003
Propulsive	Giant migrating contractions (GMC)	Human[1]	Sarna, 2006
		Dog[2]	Sarna, 2010
		Rat[2]	Li *et al.*, 2002
	Rhythmic propulsive motor complexes (RPMC)	Rat[3]	Huizinga *et al.*, 2011
	Long Distance Contractions (LDCs)	Rat[3]	Chen *et al.*, 2013
	High-amplitude contractions (HACs)	Mouse[4]	Mule *et al.*, 1999
	Colonic migrating motor complexes (CMMC)	Mouse[4]	Fida *et al.*, 1997
	Peristaltic waves	Guinea pig[4]	Smith *et al.*, 2003
		Pig[2]	Hipper & Ehrlein, 2001
	Colonic propagating sequences (PS)	Human[5]	(Dinning *et al.*, 2010)

[1]Colonic manometry.
[2]Strain gauge transducers sewn onto the colonic wall in conscious animals.
[3]In vitro organ bath experiments in isolated colonic segments perfused with physiological solution. Analysis performed with ST-maps obtained from video-recordings of mechanical activity.
[4]In vitro organ bath experiments in isolated colonic segments. Motility measured by using isometric force transducers.
[5]High resolution manometry

Interestingly, in-vitro experiments using colonic strips have revealed similar motor patterns which can be correlated in terms of amplitude and frequency of contractions with mixing and propulsive motor patterns observed in the whole colon. On the one hand, high frequency and low amplitude contractions show similar features to mixing contractions and, on the other hand, low frequency and high amplitude contractions might be related to

propulsive contractions (Fig 2) (Alberti *et al.*, 2005; Gonzalez & Sarna, 2001; Pluja *et al.*, 2001).

Differences in the spatiotemporal distribution of both patterns have been described among species. In rodents propulsive contractions occur at a frequency of about 0.2 – 2 cpm (Alberti *et al.*, 2005; Fida *et al.*, 1997; Huizinga *et al.*, 2011; Li *et al.*, 2002; Mule *et al.*, 1999), whereas in higher species like humans this pattern is observed at lower frequencies (~1 – 10 times a day) (Dinning *et al.*, 2010; Lemann *et al.*, 1995; Narducci *et al.*, 1987; Rao *et al.*, 2001). Interestingly, experiments in rat colon have demonstrated that propulsive contractions show different frequencies in proximal (~1.5 – 2 cpm), mid (~0.6 – 0.7 cpm) and distal (~0.4 – 0.5 cpm) colon (Alberti *et al.*, 2005; Huizinga *et al.*, 2011) (Fig 2). Therefore, propulsive patterns might be involved in the formation of solid faeces, in addition to cause movement of faecal material along the colon. Moreover, two different types of mixing patterns have been described in dog and human colon: (i) short-duration rhythmic phasic contractions (RPCs) and (ii) long-duration RPCs, occurring at frequencies of about 3 – 12 cpm and 0.5 – 2 cpm respectively (Sarna, 2010). Interestingly, only one type of mixing pattern is observed in rodents at a frequency of about 6 – 12 cpm (Alberti *et al.*, 2005; Chen *et al.*, 2013; Gonzalez & Sarna, 2001; Huizinga *et al.*, 2011).

Smooth muscle cells

Smooth muscle cells are the final effectors responsible for motor functions in the GI tract. In SMCs, contractile activity is mainly regulated by phosphorylation/dephosphorylation of the regulatory light chain of myosin. The level of myosin phosphorylation depends on the ratio of activities of myosin light chain phosphatase (MLCP) and the calcium-calmodulin-dependent myosin light chain kinase (MLCK) (Hartshorne *et al.*, 1998; Kamm & Stull, 1985; Taylor & Stull, 1988). Thus, cytosolic calcium concentration ($[Ca^{2+}]_i$) is the main mechanism regulating contraction and relaxation in these cells (Stull *et al.*, 1988). Voltage-dependent calcium channels (VDCC) play a key role in regulating $[Ca^{2+}]_i$ and contraction in SMCs (Somlyo & Somlyo, 1994). These channels are tightly regulated by membrane potential, so that potentials above -40 mV (depolarization) open VDCC (mainly L-type calcium channels), causing calcium influx to increase $[Ca^{2+}]_i$ and

produce contraction whereas more negative potentials (hyperpolarization) close VDCC, reducing calcium influx and $[Ca^{2+}]_i$ to induce relaxation. This regulation of mechanical activity by changes in membrane potential is known as electromechanical coupling (Bolton *et al.*, 1999; Somlyo & Somlyo, 1994).

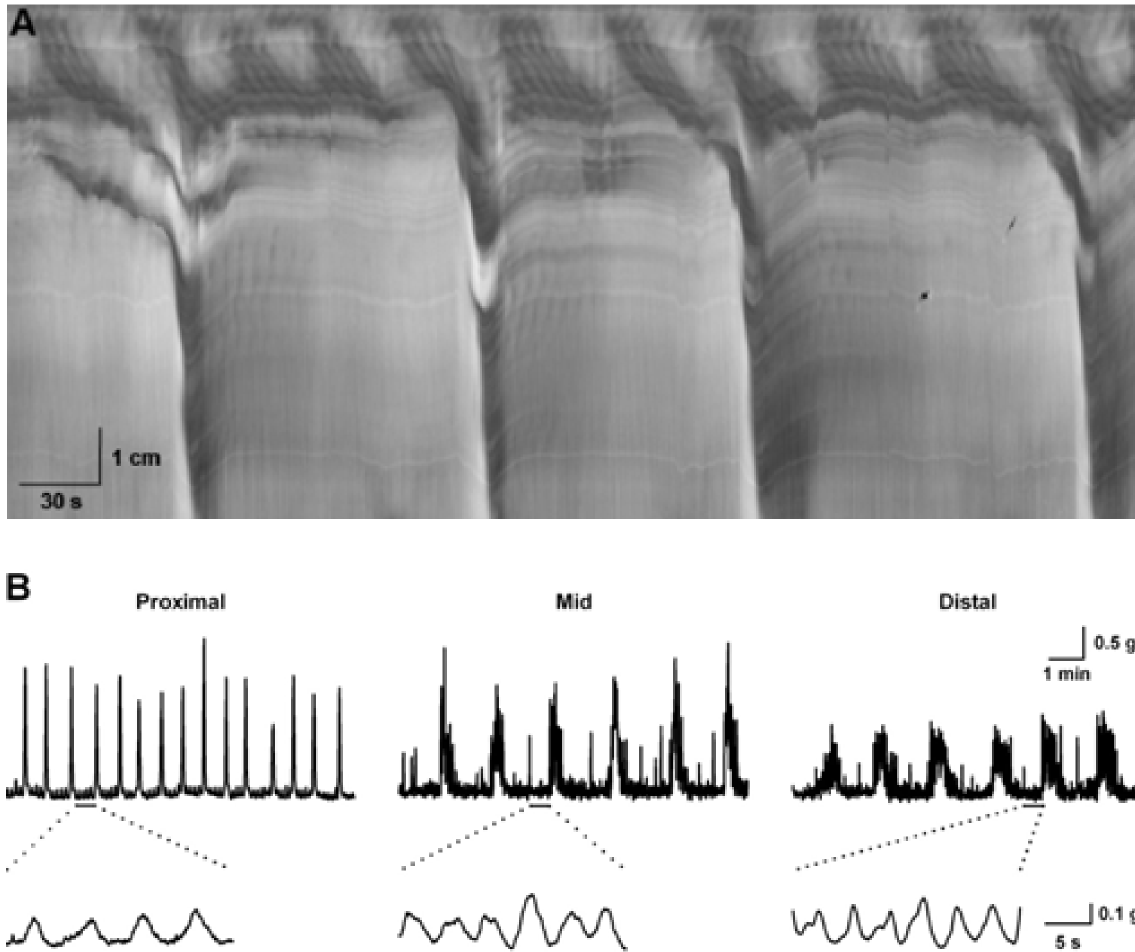

Figure 2. **(A)** Spatio-temporal map showing motility patterns in colonic segments. Note that contraction, that is reduction of the diameter of the lumen, is shown as black colour (for details in methodology see chapter 6). Propulsive contractions propagate aborally and, interestingly, just some of these contractions generated at the proximal edge reach the mid part of the colon. In addition, small contractions (ripples) are observed superimposed on propulsive contractions. Ripples propagate both orally and anally and are more prominent in the proximal colon. **(B)** Muscle bath recording (strips) showing the spontaneous mechanical activity in the proximal (left), mid (centre) and distal (right) colon. Note that, as in segments of colon two distinct types of contraction are observed: low-frequency and high-amplitude contractions and high-frequency contractions and low-amplitude (detailed at the bottom).

A wide variety of ion channels and ion exchangers have been described in the plasma membrane of SMCs regulating their resting membrane potential (RMP) by active and

passive mechanisms (Fleming, 1980; Thorneloe & Nelson, 2005). SMCs show a great variability in their RMP both along the GI tract and across the thickness of the smooth muscle layers (i.e. from -85 to -40 mV) (Farrugia *et al.*, 2003; Sanders, 2008; Sha *et al.*, 2010; Smith *et al.*, 1987a). This variability is explained by the differential expression of ion channels by SMCs and the differences in their relative open probabilities and ionic permeability at rest (Sanders, 2008). Signalling molecules, including neurotransmitters, hormones and inflammatory mediators, modify open probabilities of ion channels present in SMCs, regulating their RMP (Somlyo & Somlyo, 1994). Interestingly, distension or stretch of SMCs can modify the open probability of stretch-sensitive ion channels causing depolarization (i.e. some non-selective cation channels) or even hyperpolarization, i.e. stretch-dependent potassium (SDK) channels or TWIK related potassium 1 (TREK1) channels (Koh & Sanders, 2001; Kraichely & Farrugia, 2007; Sanders & Koh, 2006; Waniishi *et al.*, 1997). Furthermore, the L-type calcium channel itself shows responsiveness to stretch. Therefore, calcium entry and muscle contractibility may be directly modulated by distension of the gut wall (Farrugia *et al.*, 1999; Lyford *et al.*, 2002).

Enteric nervous system

In the small intestine and colon, the enteric nervous system (ENS) consists of a large number of interconnected neurons which are organized in two ganglionated plexuses: (i) the submucosal or Meissner's plexus located between the mucosa and the circular muscle layer and (ii) the myenteric or Auerbach's plexus which is found between the circular and longitudinal muscle layers (Fig 1). Mainly, the submucosal plexus (SMP) regulates secretomotor and vasomotor activities whereas the myenteric plexus (MP) is involved in the modulation of smooth muscle contractile activity. However, it has also been described both direct innervation of the muscle layers from the submucosal plexus and presence of myenteric neurons projecting fibers to the mucosa (Furness, 2006).

Enteric neurons can be functionally classified as (i) intrinsic primary afferent neurons (IPAN) which respond to sensory stimuli decoded by receptors, (ii) interneurons which polarize neuronal inputs from afferent fibbers in both oral and aboral direction and (iii) motor neurons which activate gastrointestinal effectors (SMCs, glands, vasculature, etc.)

(Furness, 2000). The arrangement of these different types of neurons allows the ENS to mediate reflex responses and motor patterns independently of inputs from the central nervous system (CNS) (Costa & Furness, 1976; Spencer, 2001) (Fig 3).

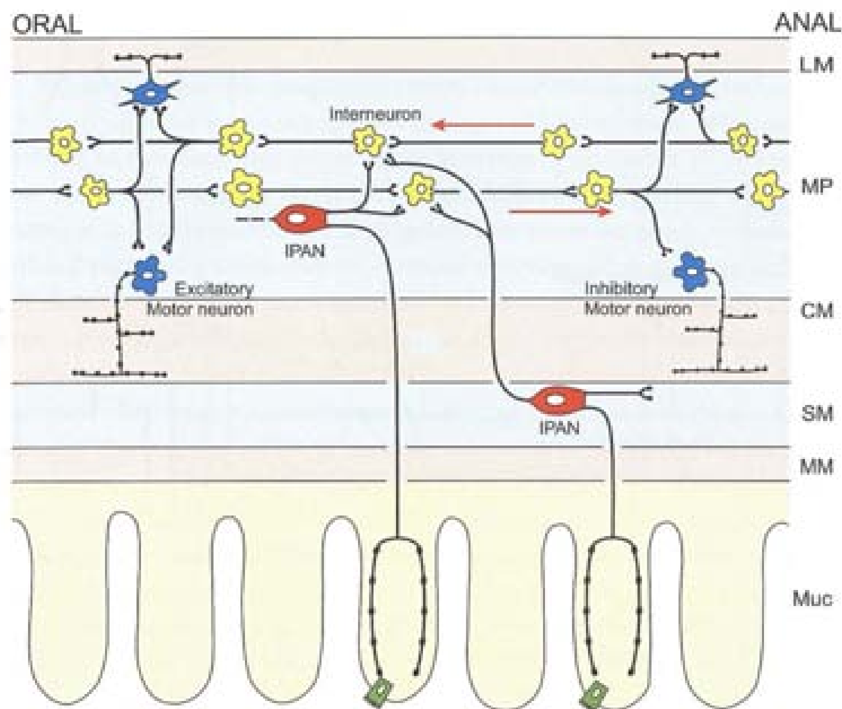

Figure 3. Simplified representation of the circuitry involved in the peristaltic reflex in the gut. Peristaltic reflexes are initiated through IPANs and transduced via interneurons projecting both orally and anally. Activation of excitatory motor neurons causes contraction oral to the side of stimulation. Inhibitory motor neurons mediate relaxation in the area immediately anal to the side in which the stimulus is initiated (receptive relaxation). Note that (1) stretch can also activate IPANs, and (2) in some species, motor neurons innervating muscle layers can present their cell bodies in the submucosal plexus. LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SM, submucosal plexus; MM, muscularis mucosae; Muc, mucosa. Reproduced from Furness, 2006.

Neuromuscular transmission

Excitatory and inhibitory motor neurons have been classified according to their neurochemical code (Furness, 2000). The main neurotransmitters of the excitatory motor neurons (EMNs) are acetylcholine and tachykinins (Rae *et al.*, 1998b; Sang & Young, 1998) whereas nitric oxide (NO), adenosine-5'-triphosphate (ATP) or a related purine, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide

(PACAP) are the most relevant transmitters in inhibitory motor neurons (IMNs) (Gallego *et al.*, 2008b; Gallego *et al.*, 2012; Grider *et al.*, 1994; Keef *et al.*, 1993; Mashimo *et al.*, 1996; Pluja *et al.*, 2000). Carbon monoxide (CO) and hydrogen sulphide (H_2S) have been recently proposed as putative neurotransmitters in the GI tract (Farrugia *et al.*, 1998; Schicho *et al.*, 2006; Teague *et al.*, 2002). Both CO and H_2S, along with NO, belong to the family of gasotransmitters (Linden *et al.*, 2010; Wang, 2002). Release of excitatory neurotransmitters from EMNs causes smooth muscle membrane depolarization which is referred to as excitatory junction potential (EJP). Conversely, an inhibitory junction potential (IJP), leading smooth muscle hyperpolarization, is observed when inhibitory neurotransmitters are released from IMNs. Such changes in the smooth muscle membrane potential regulate the open probability of L-type calcium channels and in turn muscle contractility (see above) (Sanders, 2000).

Electrophysiological studies using sharp microelectrodes have allowed the proper characterization of neurotransmitters and receptors involved in the neuromuscular transmission. Activation of motor neurons by EFS in the colon causes a transient IJP consisting of two components: (i) a fast component mediated by ATP or a related purine followed by (ii) a slow component mediated by NO (Fig 4) (Gallego *et al.*, 2006; Gallego *et al.*, 2008b; Hirst *et al.*, 2004; Keef *et al.*, 1993; Pluja *et al.*, 1999; Shuttleworth *et al.*, 1997). Accordingly, a co-transmission process between these two neurotransmitters has been hypothesized, in which ATP might mediate phasic and transient relaxation whereas NO might mediate tonic and sustained relaxation (Burnstock, 2008; Gallego *et al.*, 2008b).

Within the ENS, motor neurons ultimately regulate membrane potential and contractile activity of SMCs. It has been described that SMCs receive direct neuronal input from motor neurons (Mitsui & Komuro, 2002). Nevertheless, it has been recently hypothesized that another cell, an ICC or a PDGFRα^+ cell (Platelet derived growth factor receptor α-positive cell or Fibroblast-like cell), is interposed between neurons and SMCs mediating neuromuscular transmission in what is known as the “intercalation” hypothesis (Burns *et al.*, 1996; Iino *et al.*, 2009; Kurahashi *et al.*, 2011; Sanders *et al.*, 2010; Ward *et al.*, 2000). However, this is still a controversial issue and data to the contrary have been published as well (Huizinga *et al.*, 2008; Zhang *et al.*, 2010a).

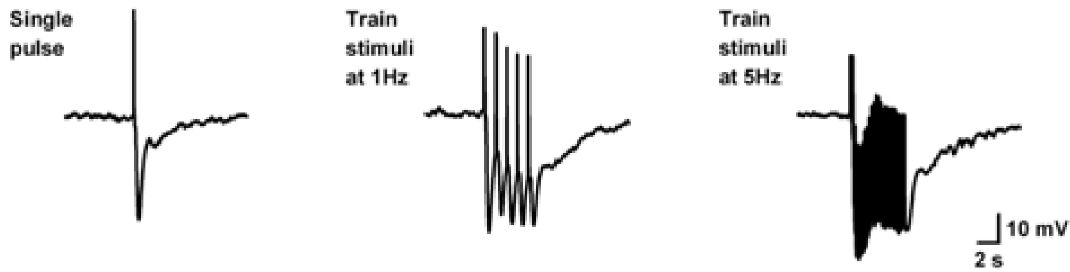

Figure 4. Intracellular microelectrode recordings showing the inhibitory junction potentials (IJP) obtained after electrical field stimulation (EFS) in non-adrenergic non-cholinergic (NANC) conditions. Left: single pulse stimulation causes an IJP characterized by a fast component (sharp negative inflexion) followed by a slow component (note the slow recuperation of membrane potential after the initial sharp hyperpolarization). Middle: train stimulation at 1 Hz during 5 seconds (5 pulses) causes five consecutive hyperpolarizations which are followed by a slow recuperation of membrane potential. Right: train stimulation at 5 Hz during 5 seconds (25 pulses) produces a maintained hyperpolarization state characterized by an initial fast component followed by a sustained one. After the end of the stimulation the membrane potential was slowly recovered. In all cases, EFS performed at supramaximal voltage (32 V) and with 0.3 ms pulse duration.

Purinergic signalling

Neurotransmitter(s) involved in purinergic relaxation

ATP has been recognized as a key compound mediating a wide variety of intracellular processes. Early in the 70s, ATP was proposed as a non adrenergic non cholinergic (NANC) neurotransmitter in the GI tract (Burnstock *et al.*, 1970; Burnstock, 1972). Currently, it is accepted that purinergic neuro-neuronal transmission and neuromuscular transmission play an important role in regulating GI functions (Burnstock, 2008; Duarte-Araujo *et al.*, 2009; Gallego *et al.*, 2006; LePard *et al.*, 1997; Spencer *et al.*, 2000; Wynn *et al.*, 2003). However, the identity of the purine involved in purinergic signalling is still under debate (Goyal, 2011; Hwang *et al.*, 2011). The term “ATP or a related purine” is frequently used in the literature as ATP is quickly hydrolyzed to ADP, AMP and adenosine by several ecto-nucleotidases (Duarte-Araujo *et al.*, 2009; Magalhaes-Cardoso *et al.*, 2003; Zimmermann, 2000). In addition, it has been recently proposed that β-nicotinamide adenine dinucleotide (β-NAD) and/or ADP-ribose might be the endogenous purine released by enteric neurons in the colon (Durnin *et al.*, 2012; Durnin *et al.*, 2013; Hwang *et al.*, 2011; Mutafova-Yambolieva *et al.*, 2007).

Purinergic receptors

Purinergic receptors are classified in two groups: P1 receptors, sensitive to adenosine, and P2 receptors, activated by ATP, ADP, UTP and UDP (Abbracchio *et al.*, 2006; Burnstock, 1976; Burnstock, 2007). In addition, two sub-groups of P2 receptors have been reported: P2X family of ligand-gated ion channel receptors and P2Y family of G protein-coupled receptors (Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998). Currently, seven P2X ($P2X_1$ – $P2X_7$) and eight P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) receptor subtypes have been recognized (Abbracchio *et al.*, 2006; Ralevic & Burnstock, 1998). On the one hand, ionotropic P2X receptors are non-selective cation channels that, after their activation, induce influx of Na^+ and Ca^{2+} inside the cell leading to depolarization and activation of intracellular calcium-dependent signalling cascades. On the other hand, P2Y receptors are metabotropic G-coupled protein receptors which activate several intracellular signalling cascades including activation of phospholipase C (PLC) and adenylyl cyclase (AC) (Zimmermann, 2006).

Several P2Y receptors have been identified in the colon of different species (Table 2) and, therefore, they might potentially participate in the mediation of inhibitory neural signals to smooth muscle cells.

Purinoreceptors in inhibitory neuromuscular transmission: Pharmacological tools

Specific subtype receptor antagonists have been essential to characterize purinergic receptors involved in neuromuscular transmission in the GI tract. MRS2179, a selective $P2Y_1$ receptor antagonist (Alexander *et al.*, 2011; Boyer *et al.*, 1998; Camaioni *et al.*, 1998), has been an excellent pharmacological tool to demonstrate that the fast IJP is mediated by this receptors 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, the inhibition caused by MRS2179 is variable between species and areas of the GI tract. In rodents, high concentrations of MRS2179 (10 µM) are needed to inhibit the fast IJP both in the internal anal sphincter (McDonnell *et al.*, 2008) and caecum (Zizzo *et al.*, 2007). Therefore, it has been hypothesized that other P2Y receptors might also mediate the fast component of the IJP in these species (McDonnell *et al.*, 2008; Zizzo *et al.*, 2007). Interestingly, new competitive antagonists of the $P2Y_1$ receptor,

MRS2279 and MRS2500, have been discovered (Boyer *et al.*, 2002; Kim *et al.*, 2003). MRS2279 and MRS2500 show a higher affinity for $P2Y_1$ receptors than MRS2179, being MRS2500 the one with the lowest estimated equilibrium dissociation constant (pK_B) (Table 3). In addition, as MRS2179, these compounds do not show non-selective effects on other P2Y receptors (Boyer *et al.*, 1998; Boyer *et al.*, 2002; Camaioni *et al.*, 1998; Cattaneo *et al.*, 2004; Kim *et al.*, 2003). Therefore, such pharmacological tools might be useful to clarify the role of $P2Y_1$ receptors in the purinergic neuromuscular transmission and clearly determine the putative participation of other P2Y receptors.

Table 2. P2Y receptor subtypes identified in the colon

Receptor	***Species***	***Method***	***Cellular type***	***References***
$P2Y_1$	Human	IHQ	SMCs and enteric neurons	(Gallego *et al.*, 2006)
	Mouse	RT-PCR	SMCs	(Monaghan *et al.*, 2006)
			$PDGFR\alpha^+$ cells	(Kurahashi *et al.*, 2011)
		IHQ	SMCs and enteric neurons	(Zhang *et al.*, 2010b) (Giaroni *et al.*, 2002)
	Rat	IHQ	SMCs	(Van Crombruggen & Lefebvre, 2004)
	Guinea Pig	IHQ	SMCs	(King & Townsend-Nicholson, 2008)
$P2Y_2$	Mouse	IHQ	SMCs	(Giaroni *et al.*, 2006)
	Rat	IHQ	Enteric neurons (MP)	(Van Crombruggen & Lefebvre, 2004)
	Guinea Pig	IHQ	Enteric neurons	(Xiang & Burnstock, 2005)
$P2Y_4$	Mouse	RT-PCR	SMCs	(Monaghan *et al.*, 2006)
		IHQ	SMCs	(Giaroni *et al.*, 2006)
	Rat	IHQ	EGCs	(Van Crombruggen & Lefebvre, 2004)
	Guinea Pig	IHQ	EGCs and ICC-SMP	(Van Nassauw *et al.*, 2006)
$P2Y_6$	Rat	IHQ	EGCs and SMCs	(Van Crombruggen & Lefebvre, 2004)
$P2Y_{11}$	Rat	IHQ	EGCs	(Van Crombruggen & Lefebvre, 2004)
	Guinea Pig	IHQ	SMCs	(King & Townsend-Nicholson, 2008)
$P2Y_{12}$	Rat	IHQ	Enteric neurons	(Van Crombruggen & Lefebvre, 2004)
$P2Y_{14}$	Rat	IHQ	Enteric neurons (SMP)	(Van Crombruggen & Lefebvre, 2004)

Note that the expression of P2Y receptors in cellular types present in the mucosa and submucosa has not been included in this table. IHQ, Immunohistochemistry; RT-PCR. Reverse transcription polymerase chain reaction; SMCs, Smooth Muscle Cells; $PDGFR\alpha^+$ cells, Platelet Derived Growth Factor receptor α-positive cells; MP, Myenteric plexus; EGCs, Enteric Glial Cells; ICC-SMP, Interstitial Cells of Cajal associated with the submuscular plexus in the colon; SMP, Submucosal plexus.

Another way to demonstrate the possible involvement of one receptor in a specific function is by the use of specific agonists which mimic the endogenous response.

Furthermore, it is also worth to note that all the above mentioned endogenous purinergic signalling molecules have been identified as agonists of some of the purinoreceptors described in the colon, if not of all of them (i.e. ATP). A summary of the pharmacologic profile of these purinoreceptors is shown in table 4.

Table 3. Summary of antagonist affinities at $P2Y_1$ receptors

Antagonists	*Turkey $P2Y_1$ receptor*	*Human $P2Y_1$ receptor*	*References*
MRS2179	6.99 (102.33 nM)	-	(Boyer *et al.*, 1998)
MRS2279	7.75 (17.78 nM)	8.10 (7.94 nM)	(Boyer *et al.*, 2002)
MRS2500	-	8,76 (1.74 nM)	(Kim *et al.*, 2003)

Summary table showing the estimated equilibrium dissociation constants of MRS2179, MRS2279 and MRS2500 on $P2Y_1$ receptors expressed in turkey erythrocyte membranes and human 1321N1 astrocytoma cells. Values are calculated by evaluating different concentrations of the antagonists on the phospholipase C activity induced by the $P2Y_1$ agonist 2MeSADP (Schild plot method). Values are expressed as pK_B and K_B (in brackets).

Table 4. Pharmacologic profile of P2Y receptors [1]

Receptor	*Endogenous agonists*	*Synthetic agonists*	*Antagonists*
$P2Y_1$	ADP > ATP > β-NAD (Mutafova-Yambolieva *et al.*, 2007; Palmer *et al.*, 1998); ADPr (Durnin *et al.*, 2012)	MRS2365 (Chhatriwala *et al.*, 2004); 2-MeSADP; 2-MeSATP; ADPβS (Ralevic & Burnstock, 1998)	MRS2500 (Kim *et al.*, 2003); MRS2279 (Boyer *et al.*, 2002); MRS2179 (Boyer *et al.*, 1998)
$P2Y_2$	UTP = ATP (Nicholas *et al.*, 1996)	MRS2768 (Ko *et al.*, 2008); 2-thioUTP (El-Tayeb *et al.*, 2006); UTPγS (Lazarowski *et al.*, 1996)	-
$P2Y_4$	UTP > ATP (Kennedy *et al.*, 2000; Nicholas *et al.*, 1996)[2]	MRS4062 (Maruoka *et al.*, 2011); UTPγS (Lazarowski *et al.*, 1996)	ATP (Kennedy *et al.*, 2000)[2]
$P2Y_6$	UDP > UTP > ADP >> ATP (Communi *et al.*, 1996; Lazarowski *et al.*, 2001)	3-phenacyl-UDP (El-Tayeb *et al.*, 2006); 5-iodoUDP (Besada *et al.*, 2006)	MRS2578 (Mamedova *et al.*, 2004)
$P2Y_{11}$	ATP (Communi *et al.*, 1997); β-NAD (Moreschi *et al.*, 2006)	ARC67085; ATPγS; BzATP; 2-MeSATP (Communi *et al.*, 1999);	NF157 (Ullmann *et al.*, 2005)
$P2Y_{12}$	ADP >> ATP (Hollopeter *et al.*, 2001)	2-MeSADP (Hollopeter *et al.*, 2001)	Cangrelor (AR-C69931MX) (Ingall *et al.*, 1999)
$P2Y_{13}$	ADP >> ATP (Communi *et al.*, 2001; Marteau *et al.*, 2003)	2-MeSADP; ADPβS (Communi *et al.*, 2001; Marteau *et al.*, 2003)	MRS2211 (Kim *et al.*, 2005); Cangrelor (AR-C69931MX) (Marteau *et al.*, 2003)
$P2Y_{14}$	UDP-glucose > UDP-galactose (Chambers *et al.*, 2000; Freeman *et al.*, 2001)	MRS2690 (Ko *et al.*, 2007)	-

[1] Adapted from Alexander et al., 2011 [2] ATP behaves as an agonist of rat $P2Y_4$ receptors, whereas it shows antagonistic effect on human $P2Y_4$ receptors.

SK_{Ca} channels in purinergic neuromuscular transmission

Apamin, a specific small conductance calcium-activated potassium channels (SK_{Ca}) inhibitor, has been widely used to differentiate between the fast and the slow component of the IJP in several species and areas of the gut. Thus, the term "apamin sensitive" vs. "apamin insensitive" IJP is extensively used in the literature to distinguish between purinergic and nitrergic components, respectively (Bennett, 1997; Pluja *et al.*, 1999; Serio *et al.*, 2003; Zagorodnyuk & Maggi, 1994; Zagorodnyuk *et al.*, 1996). Nevertheless, it has also been described that apamin can also mediate nitrergic neuromuscular transmission and, therefore, this drug would not allow such a differentiation (Keef *et al.*, 1993; Xue *et al.*, 1999). In addition, it has also been reported that the purinergic IJP is in part apamin insensitive, both in the human (~ 70%) and mouse (~ 55%) colon (Gallego *et al.*, 2006; Zhang *et al.*, 2010b). Thus, the relative contribution of SK_{Ca} channels in mediating both in purinergic and nitrergic neuromuscular transmission in the colon is still unknown.

Gasotransmitters

The term gasotransmitter is used for gas molecules involved in cell signalling processes (Linden *et al.*, 2010). Unlike other signalling molecules, gasotransmitters are not stored in vesicles and they are synthesized on demand by enzymes subjected to tight regulatory mechanisms (Mustafa *et al.*, 2009b). Gasotransmitters are characterized by (i) being gas compounds, (ii) being endogenously and enzymatically produced in a regulated manner, (iii) causing well-defined physiologic and/or pathophysiological effects, (iv) acting at specific cellular and molecular targets and (v) employing a specific mechanism of degradation (Li & Moore, 2007; Linden *et al.*, 2010; Wang, 2002).

Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S) are part of this family of signalling molecules (Li & Moore, 2007; Linden *et al.*, 2010; Pouokam *et al.*, 2011; Wang, 2002). They are small compounds freely permeable through cell membranes which activate membrane receptor-independent signalling mechanisms (Li & Moore, 2007; Wang, 2002). All of them have been described to be synthesized in the GI tract with physiological and pathological functions (Gibbons & Farrugia, 2004; Shah *et al.*, 2004; Toda & Herman, 2005; Wallace, 2010).

Nitric oxide

At the end of the 80s, NO was described as the endothelium-derived relaxing factor (EDRF) mediating relaxation in blood vessels (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) and in the early 90s it was proposed as a new NANC inhibitory neurotransmitter in the gut (Bult *et al.*, 1990). NO has a wide variety of functions in the GI tract, including modulation of gastrointestinal motility, mucosal function, inflammatory responses and control of vascular perfusion of the gut (Shah *et al.*, 2004).

NO is generated from the amino acid L-arginine, with citrulline formed as a by-product, by the action of a family of enzymes called nitric oxide synthases (NOS) (Kerwin, Jr. *et al.*, 1995). Three different NOS isoforms have been described: neural (nNOS) and endothelial (eNOS) isoforms are calcium-calmodulin activated and constitutively expressed in neurons and endothelial cells respectively whereas the inducible (iNOS) isoform is expressed during inflammatory responses (Stuehr, 1999).

Gastrointestinal motility is largely controlled by the NO released from the inhibitory motor neurons expressing nNOS. Neural NO mediates the slow component of the IJP and causes smooth muscle relaxation in several areas of the GI tract and different species (Gallego *et al.*, 2008b; Keef *et al.*, 1993; Lecea *et al.*, 2011; Mashimo *et al.*, 1996; Opazo *et al.*, 2011; Pluja *et al.*, 1999; Wang *et al.*, 2007; Xue *et al.*, 1999).

At the cellular level, NO activates soluble guanylate cyclase (GC) by binding to the heme group present in the enzyme, causing an increase of cyclic guanosine monophosphate (cGMP), which in turn activates cGMP-dependent kinases, ion channels or phosphodiesterases (Arnold *et al.*, 1977; Bellamy & Garthwaite, 2002; Cosyns *et al.*, 2013; De Man *et al.*, 2007; Dhaese *et al.*, 2008; Friebe & Koesling, 2009; Groneberg *et al.*, 2011). However, cGMP-independent mechanisms have also been described for NO, although, their physiological role is still a question of debate (Bolotina *et al.*, 1994; Friebe & Koesling, 2009; Jaffrey *et al.*, 2001). Several ion channels have been described to mediate the effect of NO on smooth muscle membrane potential. NO activates different types of potassium channels, causing potassium efflux and the subsequent membrane hyperpolarization (Keef *et al.*, 1993; Koh *et al.*, 1995; Xue *et al.*, 1999). Recently, it has been suggested that TREK1 channels, a member of the mammalian two-pore domain K^+

channels (K_{2P}) (Honore, 2007), might mediate nitrergic neurotransmission in the gastrointestinal tract (Koh *et al.*, 2001; Sanders & Koh, 2006). However, the activation of these channels by NO/cGMP signal cascade remains still unclear (Lloyd *et al.*, 2009; Zhang *et al.*, 2010c). In addition, calcium-activated chloride channels (Cl_{Ca}) might also participate in the nitrergic IJP. However, in this case NO closes constitutively active Cl_{Ca} channels reducing the chloride efflux outside the cell to produce membrane hyperpolarization (Zhang & Paterson, 2002; Zhang & Paterson, 2003).

Carbon monoxide

CO is synthesized from the metabolism of heme by the activity of heme oxygenases (HO), being biliverdin iron and hydrogen peroxide other products of this catabolic reaction. Similarly to NOS, inducible (HO-1) and constitutively expressed (HO-2) isoforms of HO have been described (Maines, 1997). A putative third isoform has been proposed (HO-3), however, its existence as an independent isoform is still unclear (Hayashi *et al.*, 2004).

HO-2 expression has been demonstrated in neurons, ICC and SMCs of the GI tract (Farrugia *et al.*, 1998; Miller *et al.*, 1998; Miller *et al.*, 2001; Ny *et al.*, 1997; Piotrowska *et al.*, 2003). As observed with NO, CO effects are also caused by an increase in cGMP levels as result of GC activation (Farrugia *et al.*, 1998; Gibbons & Farrugia, 2004). However, as in the case of NO, cGMP-independent mechanisms of action have been described as well (Althaus *et al.*, 2009; Wang *et al.*, 1997).

In the GI tract, CO causes hyperpolarization of SMCs by increasing outward potassium currents (Farrugia *et al.*, 1993; Farrugia *et al.*, 1998). Accordingly, CO has been defined as an endogenous hyperpolarizing factor in the GI tract (Szurszewski & Farrugia, 2004). It has been observed that CO plays a major role in maintaining a transwall membrane potential gradient of smooth muscle layers in the stomach, small intestine and colon. Such a voltage gradient regulates smooth muscle contractility across the thickness of the gut wall allowing only the contraction of the more depolarised SMCs in response to excitatory inputs (Farrugia *et al.*, 2003; Sha *et al.*, 2007; Sha *et al.*, 2010).

Hydrogen sulphide

The biology of H_2S is an emerging topic of research and several biological functions have been recently attributed to this gaseous molecule in a vast number of areas including the central nervous, cardiovascular, urogenital, respiratory and digestive systems (Abe & Kimura, 1996; d'Emmanuele di Villa Bianca *et al.*, 2009; Patacchini *et al.*, 2004; Trevisani *et al.*, 2005; Wallace, 2010; Yang *et al.*, 2008). In mammalian cells, two pyridoxal phosphate (PLP) dependent enzymes are mainly responsible for H_2S synthesis from the amino acid L-cysteine: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Braunstein *et al.*, 1971; Cavallini *et al.*, 1962; Stipanuk & Beck, 1982; Yang *et al.*, 2008). In addition, a third route of H_2S synthesis involves 3-mercaptopyruvate sulfurtransferase (3MST) in combination with cysteine aminotransferase (CAT) (Shibuya *et al.*, 2009a; Shibuya *et al.*, 2009b; Stipanuk & Beck, 1982) (Fig 5).

Although the mechanisms regulating H_2S release remain unclear, it has been proposed that H_2S might be synthesized on demand or alternatively released from sulphur stores in response to physiologic signals. Selective activation of CSE by calcium-calmodulin has been demonstrated (Yang *et al.*, 2008), but release of H_2S in response to reducing conditions has been reported as well (Ishigami *et al.*, 2009; Kimura, 2010). In the latter case, H_2S might be stored in the cytoplasm as bound sulfane sulphur, a divalent sulphur bound with other sulphur atoms present in intracellular proteins (Ishigami *et al.*, 2009; Kimura, 2010).

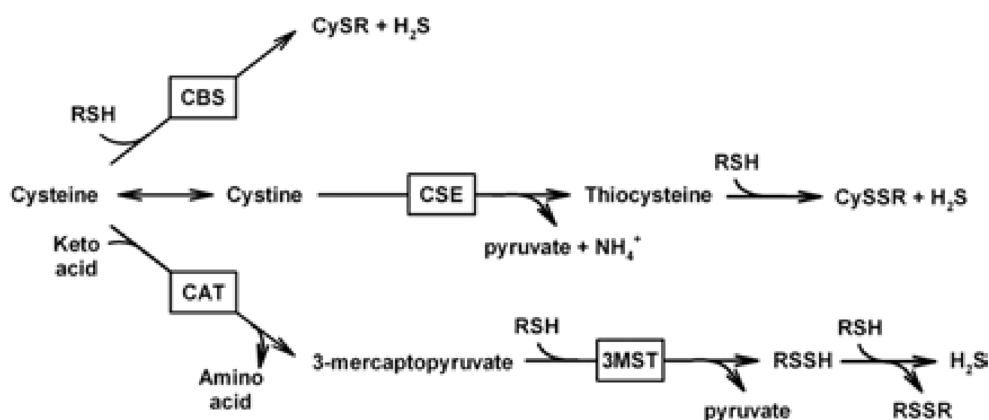

Figure 5. Enzymatic pathways involved in H_2S synthesis in mammalian cells. CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CAT, cysteine aminotransferase; 3MST, 3-mercaptopyruvate sulfurtransferase; RSH, thiol; RSSH, persulphide; RSSR, disulphide. Adapted from Stipanuk & Beck, 1982.

CBS and CSE have been localized along the entire GI tract (Martin *et al.*, 2010). Both enzymes have been detected immunonhistochemically in the muscle and ephitelial layers of rat colon (Hennig & Diener, 2009). CSE-immunoreactivity (IR) has been localised in neurons of the mouse and guinea-pig myenteric plexus and in neurons of the guinea-pig and human submucosal plexus as well as in certain subclasses of ICC in the guinea-pig colon (Linden *et al.*, 2008; Schicho *et al.*, 2006). CBS-IR has been detected in guinea-pig myenteric and human submucosal plexus (Schicho *et al.*, 2006). These data suggest that several cell structures are able to synthesize H_2S in the GI tract.

Several compounds are used to inhibit H_2S-producing enzymes. The most commonly used ones in the study of H_2S biology are: D,L-propargylglycine (PAG), an inhibitor of CSE and both amino-oxyacetic acid (AOAA) and hydroxylamine (HA), inhibitors of CBS (Linden *et al.*, 2010; Szabo, 2007; Wang, 2002). AOAA and HA are non-selective PLP-dependent enzymes inhibitors whereas PAG is an irreversible inhibitor of CSE with a certain degree of specificity (Jimenez, 2010; John & Charteris, 1978; Linden *et al.*, 2010; Sun *et al.*, 2009).

In the large intestine, luminal bacteria also represent a potential source of H_2S (Blachier *et al.*, 2010). However, despite the fact that high concentrations of H_2S are present in the colon (mM range), the vast majority of these H_2S is bound to the luminal contents (Jorgensen & Mortensen, 2001; Levitt *et al.*, 2002). Thus, low levels (~ 60 µM in the human colon) of free H_2S are available in the colonic lumen (Jorgensen & Mortensen, 2001; Mimoun *et al.*, 2012). Furthermore, luminal H_2S is quickly oxidized to thiosulphate by colonic epithelial cells (Furne *et al.*, 2001; Goubern *et al.*, 2007; Mimoun *et al.*, 2012; Ramasamy *et al.*, 2006). Therefore, under physiological conditions, the amount of H_2S that reaches the submucosa and the muscle layers is negligible. However, significant levels of H_2S might reach the neuromuscular apparatus when the epithelial barrier is disrupted and/or the detoxification mechanisms are impaired under pathological conditions.

In addition, enzymes involved in the degradation of H_2S are also important in the termination of the H_2S signalling. Thus, it has been demonstrated that H_2S can be metabolised to thiosulphate by the serial action of three mitochondrial enzymes: sulphide quinone reductase (SQR), sulphur dioxygenase [ethylmalonic encephalopathy 1 (Ethe1)]

and sulphur transferase (Hildebrandt & Grieshaber, 2008; Tiranti *et al.*, 2009). This functional unit of enzymes has been described in the mitochondria of colonic epithelial cells and it catabolises the degradation of the luminal H_2S as described above (Mimoun *et al.*, 2012). Interestingly, SQR has been identified in the muscle layer and the myenteric plexus in the mouse colon. In addition, pharmacological blockade of SQR induces an increase of the tissue levels of H_2S (Linden *et al.*, 2012). However, it is important to note that Ethe1 and sulphur transferase have not been detected in colonic muscle cells and, therefore, it might be possible that other downstream enzymes may exist for the degradation of H_2S in this tissue (Linden *et al.*, 2012).

Sodium hydrosulphide (NaHS) is a hydrogen sulphide sodium salt widely used to study the biological effects of H_2S (Hosoki *et al.*, 1997; Linden *et al.*, 2010; Szabo, 2007; Wang, 2002). In the GI tract NaHS exerts pro-secretory effects both through neural-mediated mechanisms and by direct stimulation of the intestinal epithelium (Hennig & Diener, 2009; Krueger *et al.*, 2010; Pouokam & Diener, 2011; Schicho *et al.*, 2006). Both antinociceptive and pronociceptive effects have been observed in response to NaHS administrated intraperitoneally and intracolonically respectively (Distrutti *et al.*, 2006; Matsunami *et al.*, 2009; Schemann & Grundy, 2009). Anti-inflammatory properties have been described for H_2S and administration of NaHS accelerates healing of gastric ulcers and significantly contributes to the resolution of colitis (Wallace *et al.*, 2007; Wallace *et al.*, 2009; Wallace *et al.*, 2012). Regarding its role modulating gastrointestinal motility both contractile and relaxant responses have been observed. For example in the guinea-pig and mouse stomach NaHS causes a dual effect, producing contraction at low concentrations and relaxation at high concentrations (Han *et al.*, 2011; Zhao *et al.*, 2009). NaHS concentration-dependently relaxed prostaglandin $F_{2\alpha}$-contracted circular muscle strips of mouse fundus and distal colon (Dhaese & Lefebvre, 2009; Dhaese *et al.*, 2010). NaHS also exerted relaxant effects on guinea-pig, rabbit and rat ileum and jejunum preparations (Hosoki *et al.*, 1997; Kasparek *et al.*, 2012; Nagao *et al.*, 2011; Nagao *et al.*, 2012; Teague *et al.*, 2002). Furthermore, NaHS inhibits peristaltic activity in the mouse small intestine and colon (Gallego *et al.*, 2008a). Spontaneous circular smooth muscle contractions observed in rat and human colonic strips are also concentration-dependently inhibited by NaHS (Gallego *et al.*, 2008a).

NaHS exerts its biological effects through a wide variety of mechanism of action which include activation of cAMP-dependent pathways (Kimura, 2000) and myosin light chain phosphatase (MLCP) (Dhaese & Lefebvre, 2009; Nagao *et al.*, 2012), opening of ATP-sensitive potassium channels (K_{ATP}) channels (Gallego *et al.*, 2008a; Nagao *et al.*, 2012; Zhao *et al.*, 2009); SK_{Ca} channels (Gallego *et al.*, 2008a), Na(V)1.5 voltage-dependent sodium channels (Strege *et al.*, 2011); Cav3.2-T-type channels (Matsunami *et al.*, 2009), transient receptor potential (TRP) cation channels types V1 (TRPV1) and A1 (TRPA1) (Krueger *et al.*, 2010; Macpherson *et al.*, 2007; Schicho *et al.*, 2006) and inhibition of phosphodiesterase activity (Bucci *et al.*, 2010). It has been hypothezised that sulfhydration, a covalent modification of existing thiol residues present in proteines, might explain the "promiscuity" of H_2S (Mustafa *et al.*, 2009a; Mustafa *et al.*, 2009b). However, some authors have claimed that such nonselective mechanism of action would not allow to classify H_2S as a signalling molecule (Linden *et al.*, 2010).

Interstitial cells of Cajal

Interstitial cells of Cajal (ICC) are non-muscular cells of mesenchymal origin widely distributed in the GI tract which were firstly described by Dr. Santiago Ramon y Cajal more than one hundred years ago (Kluppel *et al.*, 1998; Ramón y Cajal, 1904). According to their location, ICC can be classified in the colon as (i) ICC-SMP, ICC locate along the submucosal surface of the circular muscle layer; (ii) ICC-MP, ICC associated with the myenteric plexus; and (iii) ICC-IM, ICC intermixed with smooth muscle fibers (Huizinga *et al.*, 2011; Sanders *et al.*, 2006).

Morphological and physiological studies have suggested several functions for ICC in the GI tract (Daniel & Posey-Daniel, 1984; Sanders *et al.*, 2006; Thuneberg, 1982). ICC expresses c-kit and ANO-1 both proteins allow the localization of ICC with immunohistochemical techniques (Gomez-Pinilla *et al.*, 2009; Huizinga *et al.*, 1995; Ward *et al.*, 1994). ICC are spontaneously active and generate rhythmic depolarizations suggesting that they may serve as pacemaker cells (Langton *et al.*, 1989). Accordingly, ICC-SM and ICC-SMP are located in the two pacemaker areas described in the colon (see below) (Pluja *et al.*, 2001; Smith *et al.*, 1987a). ICC may also participate in the active propagation of electrical events (Sanders, 1989; Smith *et al.*, 1987b). ICC are in close

apposition with nerve varicosities and they may mediate neuromuscular transmission (see above) (Bayguinov *et al.*, 2010a; Huizinga *et al.*, 2011; Sanders *et al.*, 2010). Accordingly, mutant animals with ICC depletion (mainly ICC-IM) show defective 77neuromuscular transmission (Alberti *et al.*, 2007; Burns *et al.*, 1996; Ward *et al.*, 2000).

Colonic pacemaker activity

Colonic motility patterns are orchestrated by means of rhythmic oscillations in the resting membrane potential of SMCs which organize the open probability of VDCC to generate a phasic contractile activity. Mainly, two different types of cyclic electrical activity are observed in the colon of many species which are generated from two main pacemaker areas: (i) slow waves which are observed at a frequency similar to mixing contractions and generated at the submucosal surface of the circular muscle layer (Dickson *et al.*, 2010; Pluja *et al.*, 2001; Rae *et al.*, 1998a; Smith *et al.*, 1987b; Yoneda *et al.*, 2002) and (ii) cyclic depolarizations, also called myoelectric complexes, with a similar frequency to propagating contractions which are originated in the region between the circular and longitudinal muscle layers (Bywater *et al.*, 1989; Lyster *et al.*, 1995; Martin *et al.*, 2004; Pluja *et al.*, 2001; Powell *et al.*, 2003; Yoneda *et al.*, 2002) (Fig 6). Interestingly, a third pattern of cyclic electrical behaviour has been described in the SMCs of the dog and human colon. This pattern is characterized by rapid oscillations of smooth muscle membrane potential at a frequency of about 20 cycles per minute generated in the myenteric region and it has been termed myenteric potential oscillations (MPOs) (Rae *et al.*, 1998a; Sanders, 1989; Smith *et al.*, 1987a).

Dissection experiments have shown that removal of pacemaker areas causes lack of rhythmic electrical oscillations and regular mechanical activity (Pluja *et al.*, 2001; Smith *et al.*, 1987a; Smith *et al.*, 1987b). Therefore, such electric oscillations are not primarily developed by SMCs themselves, although intrinsic properties of these cells might modulate them (see above). Neural and ICC networks are extensively distributed in the pacemaker areas of the colon and both type of cells have been hypothesized as pacemaker cells in this organ (Alberti *et al.*, 2007; Bayguinov *et al.*, 2010a; Bayguinov *et al.*, 2010b; Huizinga *et al.*, 2011; Sanders, 1989; Spencer, 2001).

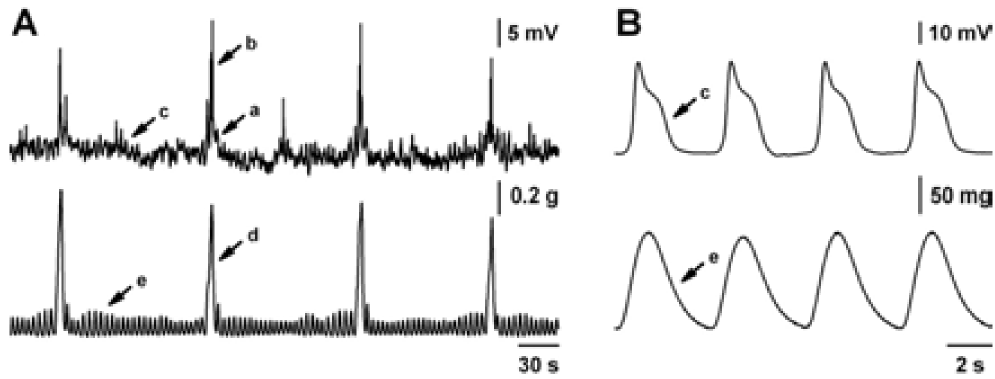

Figure 6. **(A)** Simultaneous microelectrode and mechanical recordings showing how cyclic depolarizations **(a)** with upper-imposed spike activity **(b)** and slow waves **(c)** (top) are correlated with low-frequency and high-amplitude contractions **(d)** and high-frequency and low-amplitude contractions **(e)**. (B) Detail of slow wave activity **(c)** (top) and high-frequency and low-amplitude contractions **(e)** (bottom). Note that the recordings showed in **(A)** and **(B)** are obtained from different experiments (differences in the slow wave are probably related to the impalement site).

Pacemaker cells responsible for slow waves have been identified as ICC-SM in the mouse colon by neurobiotin injection and immunohistochemical examination (Yoneda *et al.*, 2002). Furthermore, calcium transients associated with slow waves are originated in ICC-SM and then propagated to the bulk of circular SMCs (Lee *et al.*, 2009). In addition, regular slow waves are not observed in Ws/Ws mutant rats which present a complete lack of ICC-SMP (Alberti *et al.*, 2007).

Cyclic depolarizations and propulsive contractions have been claimed to be neurogenic in origin because they are abolished in the presence of tetrodotoxin (TTX), an inhibitor of voltage-gated sodium channels which blocks action potentials in neurons, or hexametonium, a blocker of nicotinic receptors involved in the neuro-neuronal transmission (Bywater *et al.*, 1989; Li *et al.*, 2002; Lyster *et al.*, 1995; Smith *et al.*, 2003). However, this effect has not been observed in all the experimental conditions, i.e. in muscle strips of rat colon, TTX increases mechanical activity (Alberti *et al.*, 2005; Gonzalez & Sarna, 2001). In addition, cyclic depolarizations have been recorded in colonic strips, in which the majority of the enteric neural circuitry is absent (Alberti *et al.*, 2007; Pluja *et al.*, 2001; Yoneda *et al.*, 2002). Interestingly, it has been recently described in segments of rat colon that rhythmic propulsive contractions are restored after neural blocked with muscarinic agonists suggesting that the ENS provides an excitatory input but is not the pacemaker generator (Chen *et al.*, 2013; Huizinga *et al.*, 2011). Moreover,

calcium imaging experiments have shown that ICC-MP are activated by excitatory nerves (Bayguinov *et al.*, 2010a). Therefore, it is possible that ICC-MP and myenteric neurons work cooperatively to generate propulsive contractions in the way that ICC-MP are the pacemaker cells but depending their activity on the stimulus provided by the ENS (Chen *et al.*, 2013; Huizinga *et al.*, 2011).

Objectives

The aim of the present work was to investigate neural mechanisms which regulate smooth muscle relaxation in the human and rat colon. In particular we evaluated the role of adenosine-5'-triphosphate (ATP), nitric oxide (NO) and hydrogen sulphide (H_2S) as inhibitory neurotransmitters which modulate colonic motility.

Therefore, the main objectives of this study were as follows:

1. Pharmacological characterisation of the transmitters, receptors, pathways and channels underlying purinergic and nitrergic neuromuscular transmission in the colon. Including the study of the spontaneous inhibitory neural tone.
2. Study of H_2S as the putative third inhibitory gasotransmitter modulating motility in the colon
3. Investigate putative interactions between these three signalling molecules.

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Publications

Chapter 1

$P2Y_1$ receptors mediate inhibitory neuromuscular transmission in the rat colon

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RESEARCH PAPER

P2Y$_1$ receptors mediate inhibitory neuromuscular transmission in the rat colon

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Background and purpose: Inhibitory junction potentials (IJP) are responsible for smooth muscle relaxation in the gastrointestinal tract. The aim of this study was to pharmacologically characterize the neurotransmitters [nitric oxide (NO) and adenosine triphosphate (ATP)] and receptors involved at the inhibitory neuromuscular junctions in the rat colon using newly available P2Y$_1$ antagonists.
Experimental approach: Organ bath and microelectrode recordings were used to evaluate the effect of drugs on spontaneous mechanical activity and resting membrane potential. IJP and mechanical relaxation were studied using electrical field stimulation (EFS).
Key results: N$^{\omega}$-nitro-L-arginine (L-NNA) inhibited the slow component of the IJP and partially inhibited the mechanical relaxation induced by EFS. MRS2179, MRS2500 and MRS2279, all selective P2Y$_1$ receptor antagonists, inhibited the fast component of the IJP without having a major effect on the relaxation induced by EFS. The combination of both L-NNA and P2Y$_1$ antagonists inhibited the fast and the slow components of the IJP and completely blocked the mechanical relaxation induced by EFS. Sodium nitroprusside caused smooth muscle hyperpolarization and cessation of spontaneous motility that was prevented by oxadiazolo[4,3-α]quinoxalin-1-one. Adenosine 5′-O-2-thiodiphosphate, a preferential P2Y agonist, hyperpolarized smooth muscle cells and decreased spontaneous motility. This effect was inhibited by P2Y$_1$ antagonists.
Conclusions and implications: The co-transmission process in the rat colon involves ATP and NO. P2Y$_1$ receptors mediate the fast IJP and NO the slow IJP. The rank order of potency of the P2Y$_1$ receptor antagonists is MRS2500 greater than MRS2279 greater than MRS2179. P2Y$_1$ receptors might be potential pharmacological targets for the regulation of gastrointestinal motility.
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Keywords: smooth muscle; gastrointestinal; inhibitory neuromuscular transmission; P2Y$_1$ receptors; nitric oxide; MRS2179; MRS2279; MRS2500

Abbreviations: ADPβS, adenosine 5′-O-2-thiodiphosphate; AUC, area under the curve; EFS, electrical field stimulation; GI, gastrointestinal; IJP, inhibitory junction potential; IJPf, fast component of the IJP; IJPs, slow component of the IJP; L-NNA, N$^{\omega}$-nitro-L-arginine; ODQ, oxadiazolo[4,3-α]quinoxalin-1-one; SNP, sodium nitroprusside

Introduction

Stimulation of inhibitory motor neurones by electrical field stimulation (EFS) causes the release of inhibitory neurotransmitter(s). Smooth muscle responds with a transient inhibitory junction potential (IJP), which is the electrophysiological base of smooth muscle relaxation. IJP usually display two components: a fast (IJPf) followed by a slow (IJPs) component. These two components suggest the release of at least two inhibitory neurotransmitters (Crist *et al.*, 1992; Lyster *et al.*, 1992; He and Goyal, 1993; Keef *et al.*, 1993; Pluja *et al.*, 1999; Gallego *et al.*, 2006). Experiments with new pharmacological tools are essential to characterize the co-transmission process where nitric oxide (NO) and ATP might be involved (Burnstock, 2008; Gallego *et al.*, 2008a). It has been clearly demonstrated that the IJPs is abolished by treatments that block NO synthesis (Keef *et al.*, 1993; Pluja *et al.*, 1999; Wang *et al.*, 2007); this indicates that the IJPs is mediated by NO release from inhibitory motor neurones. In contrast, NO synthase inhibition does not alter the IJPf, showing that this component is not mediated by NO.

ATP, or a related purine, is an inhibitory neurotransmitter in the gut (Burnstock *et al.*, 1970; Ralevic and Burnstock, 1998). There are two families of purine receptors: P2X and P2Y. P2X receptors are ligand-gated ion channels, and P2Y

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receptors are G-protein coupled receptors. Eight distinct P2Y receptor subtypes, $P2Y_{1,2,4,6,11,12,13,14}$ and seven P2X receptor subtypes, $P2X_{1–7}$, have been reported (Burnstock, 2007).

MRS2179 is a selective antagonist of $P2Y_1$ receptors that has been recently used to characterize purinergic inhibitory neurotransmission in the gastrointestinal (GI) tract (Boyer *et al.*, 1998; Camaioni *et al.*, 1998; Alexander *et al.*, 2008). However, findings are not conclusive, and the effect of MRS2179 might vary between species and areas of the GI tract. In the human colon (Gallego *et al.*, 2006; 2008a), guinea pig ileum (Wang *et al.*, 2007) and pig small intestine (Gallego *et al.*, 2008b), the IJPf is sensitive to MRS2179. In rodents, the non-nitrergic relaxation is partially sensitive to MRS2179; the response of circular muscle strips of mouse jejunum are affected by MRS2179 at a concentration of 1 μM (De Man *et al.*, 2003). Moreover, adenosine 5′-O-2-thiodiphosphate (ADPßS), a preferential P2Y agonist, induces MRS2179-sensitive relaxations in the rat distal colon. It has thus been postulated that post-junctional $P2Y_1$ receptors located in smooth muscle mediate the non-nitrergic relaxation in murine tissues (Giaroni *et al.*, 2002; Van Crombruggen *et al.*, 2007). Higher concentrations of MRS2179 (10 μM) are needed to inhibit the IJPf in the mouse internal anal sphincter (McDonnell *et al.*, 2008) and caecum (Zizzo *et al.*, 2007). These results suggest that other P2Y receptors might be involved in inhibitory neurotransmission (McDonnell *et al.*, 2008), or, alternatively, pre-junctional $P2Y_1$ receptors might mediate this effect (Zizzo *et al.*, 2007).

Specific P2Y receptor antagonists are essential pharmacological tools for the proper characterization of purinergic inhibitory neurotransmission (Bornstein, 2008). In this study, we investigated inhibitory neurotransmission in the rat mid-colon using MRS2179 (Camaioni *et al.*, 1998), and, for the first time, two other recently available $P2Y_1$ antagonists, MRS2279 (Boyer *et al.*, 2002) and MRS2500 (Kim *et al.*, 2001); (Cattaneo *et al.*, 2004). Briefly, we found that the IJPf and the non-nitrergic relaxation evoked by EFS were inhibited in a concentration-dependent manner by MRS2179, MRS2500 and MRS2279, indicating the involvement of $P2Y_1$ receptors in the purinergic inhibitory neurotransmission in the rat mid-colon. Further study of these $P2Y_1$ receptors is crucial to establish them as pharmacological targets in the regulation of gastrointestinal functions, such as secretion and motility (Wood, 2006).

Methods

Animals

Male Sprague-Dawley rats (300–350 g), 8–10 weeks old, were kept at a constant temperature (19–21°C) and humidity (60%), with a lighting cycle of 12 h light/12 h dark and had access to water and food *ad libitum*. The rats were killed by stunning, a sharp blow to the head, before being decapitated and bled. This procedure was approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Tissue preparation

The mid-colon was quickly removed and 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 upward. 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) according to anatomical criteria previously described (Alberti *et al.*, 2005). Mucosal and submucosal layers were carefully removed, and the circular muscle was cut into strips 1 cm long and 0.3 cm wide.

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 the recording commenced. Circular smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl (30–60 MΩ of resistance). 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). EFS was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. The EFS had the following parameters: total duration of train, 100 ms; frequency, 20 Hz; pulse duration, 0.3 ms, and increasing amplitude voltage, 5, 10, 12, 15, 17, 20, 25, 30 and 50 V. The amplitude and the duration of the EFS-induced IJP were measured under control conditions and after infusion of each drug. Resting membrane potential was also measured before and after drug addition. Nifedipine (1 μM) was used to abolish the mechanical activity and obtain stable impalements.

Mechanical studies

Spontaneous mechanical activity was studied in a 10 mL organ bath. Circularly orientated preparations were tied to a support at one end and to an isometric force transducer (Harvard VF-1, Harvard Apparatus Inc., Holliston, MA, USA) at the other using a 2/0 silk thread. 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 A/D converter. A tension of 1 g was applied, and the tissue was allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. In order to study the inhibitory neurotransmitters released, EFS was applied for 4 min (pulse duration, 0.3 ms; frequency, 5 Hz; amplitude, 30 V) through two platinum electrodes placed on the support holding the tissue. To estimate mechanical activity responses to drugs or EFS, the area under the curve (AUC) of contractions from the baseline was measured before and after drug addition and before and during EFS. AUC is expressed as $g{\cdot}min^{-1}$.

Solutions and drugs

The composition of the Krebs solution was (in mM): glucose, 10.10; NaCl, 115.48; $NaHCO_3$, 21.90; KCl, 4.61; NaH_2PO_4,

1.14; $CaCl_2$, 2.50 and $MgSO_4$, 1.16 (pH 7.3–7.4). The Krebs solution (37 ± 1°C) was bubbled with carbogen (95% O_2 and 5% CO_2). In all the experiments, phentolamine, atropine and propanolol 1 µM was added to the Krebs solution to block α- and β-adrenoceptors and muscarinic receptors.

The following drugs were used: nifedipine, N^{ω}-nitro-L-arginine (L-NNA), ADPβS, phentolamine, sodium nitroprusside (SNP) 1H-[1,2,4], oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (Sigma Chemicals, St. Louis, MO, USA), atropine sulphate (Merck, Darmstadt, Germany), propanolol, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS2179), (1R,2S,4S,5S)-4-[2-chloro-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS2279), and (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). 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. Drug and receptor nomenclature conform to the guidelines of the *British Journal of Pharmacology* (Alexander *et al*., 2008)

Data analysis and statistics

Differences in the resting membrane potential before and after infusion of different drugs were compared by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. The duration of the IJP was measured from the beginning of the hyperpolarization to the value of a stable resting membrane potential. The differences between the amplitude and duration of the IJPs before and after drug infusion were compared by two-way ANOVA (drug and voltage).

To normalize mechanical data, the effect of drugs and EFS were calculated as percentage of inhibition, being 100% when a total inhibition of spontaneous motility was recorded after drug administration or during EFS, and 0% when the inhibitory response was completely antagonized. Rebound contraction recorded at the end of the timulus was normalized with the average amplitude of spontaneous contractions before EFS. One-way ANOVA was used (i) to evaluate the effect of drugs on inhibition of spontaneous motility induced by SNP or ADPßS; and (ii) to evaluate the effect of different antagonists on inhibition of spontaneous motility and rebound contraction evoked by EFS. Paired *t*-test was used to compare data when two single groups were studied.

Data are expressed as mean ± SEM. $P < 0.05$ was considered statistically significant; *n* values indicate the number of samples. Statistical analysis was performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

Results

Role of nitric oxide synthase inhibitors in the IJP and relaxation induced by EFS

EFS completely inhibited spontaneous motility, and a rebound contraction (off-contraction) was recorded after the end of the stimulus. The amplitude of the off-contraction was 1.51 ± 0.11 (n = 18) higher than the mean spontaneous contraction. L-NNA (1 mM, n = 11) partially decreased the inhibitory effect induced by EFS by about 23 ± 6% (Figure 1A). However, L-NNA (1 mM, n = 11) did not significantly modify the rebound contraction [control: 1.66 ± 0.14 vs. L-NNA: 1.60 ± 0.07; *t*-test not significant (n.s.)]. As previously reported, EFS caused an IJP with two components: an IJPf (measured as the IJP amplitude) followed by an IJPs (measured as the IJP duration at baseline) (Pluja *et al*., 1999; control tracings of Figures 2 and 4). Both the amplitude and duration of the IJP were voltage dependent. Neither L-NNA (1 mM, n = 4) nor the guanylate cyclase inhibitor ODQ (10 µM, n = 8) modified the amplitude of the IJP, but they did reduce its duration (ANOVA $P < 0.0001$), that is 50 V: control: 3.4 ± 0.4 s versus L-NNA: 2.3 ± 0.2 s and control: 3.7 ± 0.3 s versus ODQ: 2.7 ± 0.3 s. The remaining IJP was the IJPf that is sensitive to $P2Y_1$ antagonists (see below).

Role of $P2Y_1$ antagonists in the IJP and relaxation induced by EFS

Muscle bath experiments demonstrated that MRS2179 (10 µM, n = 7 and 20 µM, n = 5), MRS2279 (1 µM, n = 5) and MRS2500 (1 µM, n = 5) did not modify the inhibitory effect induced by EFS (Figure 1B–D). The amplitude of the rebound contraction was not modified by the different $P2Y_1$ antagonists tested (paired *t*-test): that is control: 1.27 ± 0.17 versus MRS2179 (10 µM) 1.40 ± 0.25, (n = 7, n.s), control: 1.47 ± 0.30 versus MRS2179 (20 µM) 1.48 ± 0.32, (n = 5, n.s), control: 1.28 ± 0.20 versus MRS2279 (1 µM) 0.83 ± 0.23, (n = 5, n.s), and control: 1.35 ± 0.09 versus MRS2500 (1 µM) 0.88 ± 0.16, (n = 5, n.s.). The amplitude of rebound contraction was normalized in relation to spontaneous motility (see *Methods*). The electrophysiological experiments demonstrated that MRS2179 (20 µM; n = 6), MRS2279 (1 µM; n = 4) and MRS2500 (1 µM; n = 8) did not modify the duration but did markedly reduce the amplitude of the IJP (Figure 2). It is important to note that the inhibitory effect was more prominent in experiments performed with MRS2279 (1 µM) and MRS2500 (1 µM) than in those performed with MRS2179 (20 µM). In experiments with MRS2179, the IJPf was recovered after 20 min washout, but in experiments with MRS2279 and MRS2500, the IJPf was only partially recovered after washout.

Role of NOS inhibitors and $P2Y_1$ antagonists in the IJP and relaxation induced by EFS

In order to characterize the neurotransmission process, preparations were first incubated with L-NNA (1 mM), and then the effects of the $P2Y_1$ antagonists were studied. Muscle bath experiments show a concentration-dependent inhibition of EFS-induced relaxation (Figure 3). IC_{50} values were: MRS2179, 3.5 µM; MRS2279, 43.9 nM; MRS2500, 16.5 nM. Note, in the presence of high concentrations of the $P2Y_1$ antagonists, an initial contraction is seen at the onset of the stimulus, contrasting with the relaxation seen in control (Figure 3). In the presence of L-NNA, the rebound contraction was reduced in a concentration-dependent manner by the $P2Y_1$ receptor antagonists. MRS2279 and MRS2500 caused a total inhibition of rebound contraction at 1 µM (Bonferroni's multiple comparison test, control vs. 1 µM, $P < 0.001$ both). In the presence

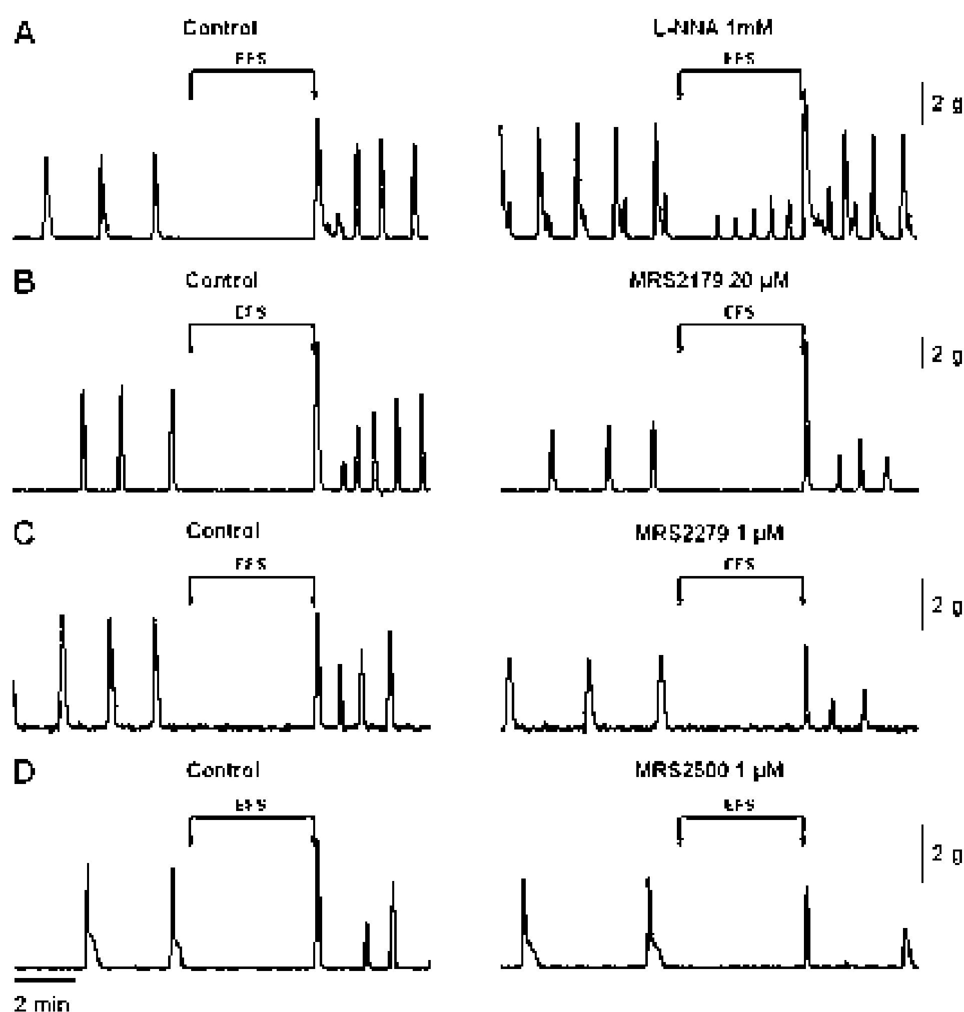

Figure 1 Muscle bath recordings showing the effect of N$^{\omega}$-nitro-L-arginine (L-NNA) 1 mM (A), MRS2179 20 μM (B), MRS2279 1 μM (C) and MRS2500 1 μM (D) on the inhibition of contractile activity induced by electrical field stimulation (EFS).

of both L-NNA and the $P2Y_1$ antagonists, a reduction of both the amplitude and the duration of the IJPs was observed (Figure 4). Concentration-response curves were performed in the presence of L-NNA (Figure 5). IC_{50} values were: MRS2179, 13.1 μM; MRS2279, 17.8 nM; MRS2500, 14.0 nM. Both 1 μM MRS2279 and 1 μM MRS2500 completely blocked the IJP. In contrast, a residual IJP was still recorded with 20 μM MRS2179.

Effect of NO donors on membrane potential and spontaneous motility

Incubation of the tissue with SNP (1 μM), an NO donor, caused a smooth muscle hyperpolarization (–11 ± 0.7 mV; $n = 18$) and a long-lasting (more than 10 min) cessation of spontaneous motility (AUC 11.40 ± 1.94 vs. 0.07 ± 0.05 g·min^{-1}; $n = 4$ *t*-test $P < 0.01$) (Figure 6 A,B respectively). The hyperpolarization induced by SNP 1 μM was not modified by pre-incubation with either L-NNA, MRS2500 or MRS2279 (Figure 6A). ODQ (10 μM) partially antagonized the hyperpolarization induced by SNP (Figure 6A). The inhibition of spontaneous motility induced by SNP (1 μM) was also not modified by either L-NNA, MRS2179, MRS2279 or MRS2500 (Figure 6B). In the presence of these antagonists, SNP (1 μM) caused a total inhibition (100%) of spontaneous motility. SNP caused only a partial inhibition of spontaneous motility (8.4 ± 2.2%; $P < 0.001$) in the presence of ODQ (10 μM) (Figure 6B).

Effect of ADPβS on membrane potential and spontaneous motility

Incubation of the tissue with ADPβS (10 μM) caused a transient (5 min) inhibition of spontaneous motility (AUC: 9.01 ± 1.47 vs. 4.04 ± 0.76 g·min^{-1}; $n = 14$, *t*-test $P < 0.001$) (Figure 7). This inhibitory effect was reversed by MRS2179, MRS2279 and MRS2500, but not by ODQ or L-NNA (Figure 7B). ADPßS (10 μM) induced a hyperpolarization of 9.15 ± 0.71 mV ($n = 25$), which was antagonized by MRS2179, MRS2279 and MRS2500 but not modified by pre-incubation with either L-NNA or ODQ (Figure 7A)

Discussion

In the present study, we have demonstrated that in the rat colon, the fast component of the IJP is sensitive to $P2Y_1$

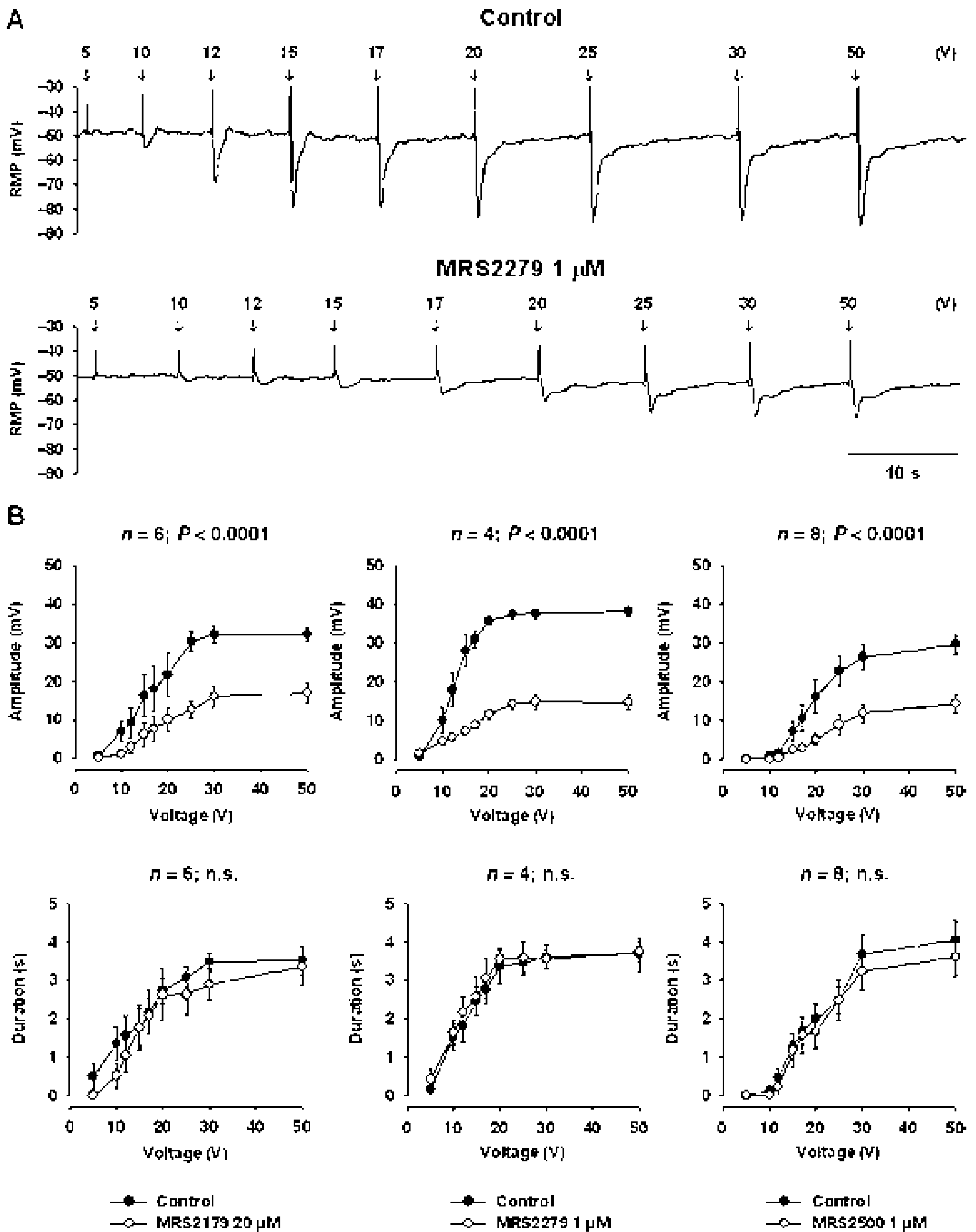

Figure 2 (A) Intracellular microelectrode recordings showing the electrical field stimulation (EFS)-induced inhibitory junction potential (IJP) at different voltages (5, 10, 12, 15, 17, 20, 25, 30 and 50 V) in control conditions and after incubation with MRS2279 (1 μM). (B) Graphs representing the inhibitory effect of MRS2179 (20 μM), MRS2279 (1 μM) and MRS2500 (1 μM) on the amplitude (top) and duration (bottom) of the EFS-induced IJP. All values are expressed as mean ± SEM. Significant differences were assessed using two-way ANOVA.

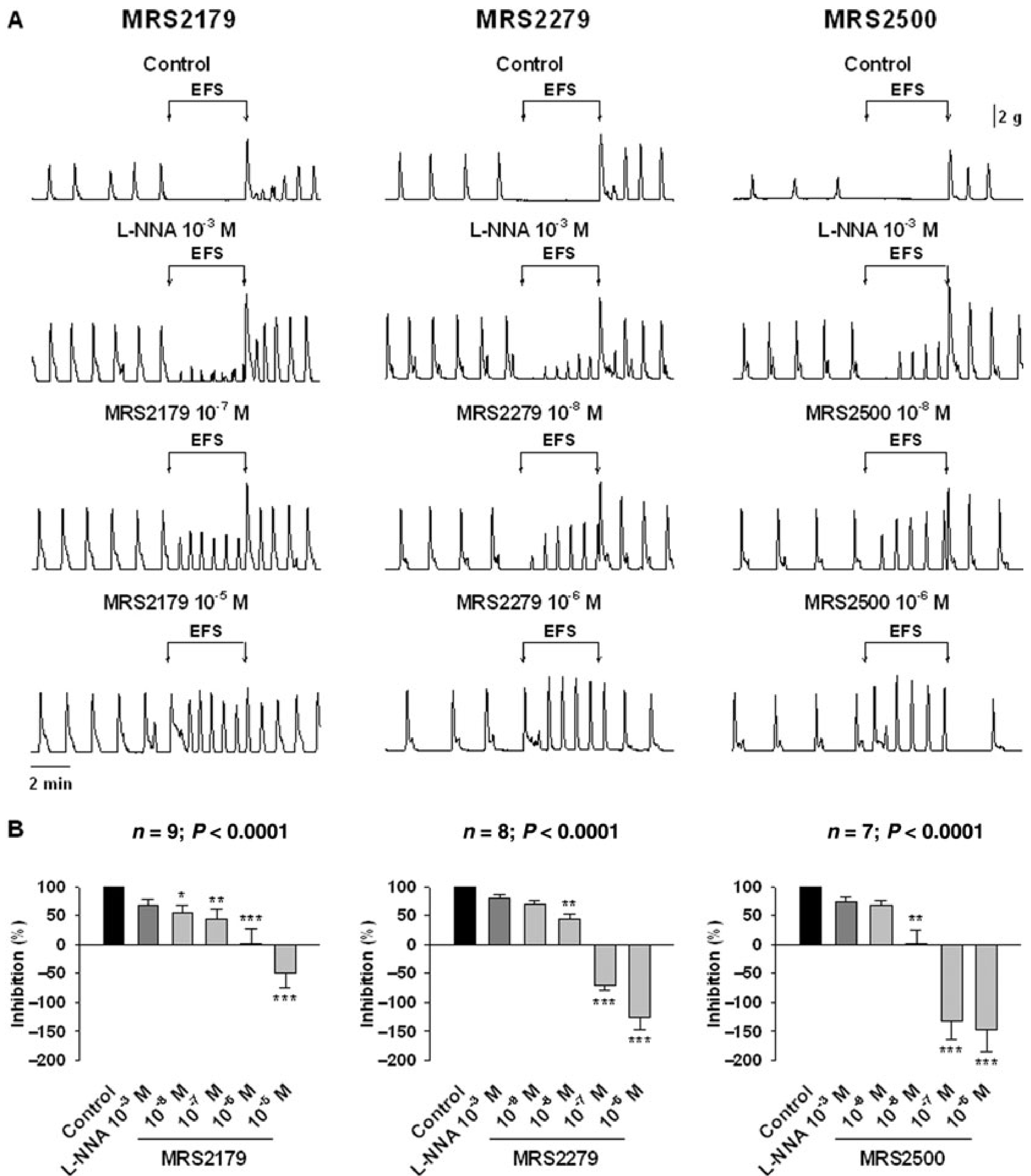

Figure 3 (A) Mechanical recordings showing the effect of MRS2179 (left), MRS2279 (middle) and MRS2500 (right) on the electrical field stimulation (EFS)-induced inhibition of spontaneous motility in the presence of N^{ω}-nitro-L-arginine (1 mM) incubation. (B) Concentration-response histograms showing the percentage of inhibition. Data were calculated using the following formula: 1 – [area under the curve (AUC) during EFS/AUC previous EFS] × 100. Note that 100% is no drug effect (total inhibition of spontaneous motility) and 0% is a complete blockade of the inhibitory response, meaning that spontaneous motility during EFS is equal to spontaneous motility before EFS. Negative data indicate contractile activity during EFS, that is –100% represents a doubling of the spontaneous motility recorded prior to EFS. All values are mean ± SEM. MRS2179 $n = 9$, MRS2279 $n = 8$, MRS2500 $n = 7$. Significant differences were assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significant difference from control.

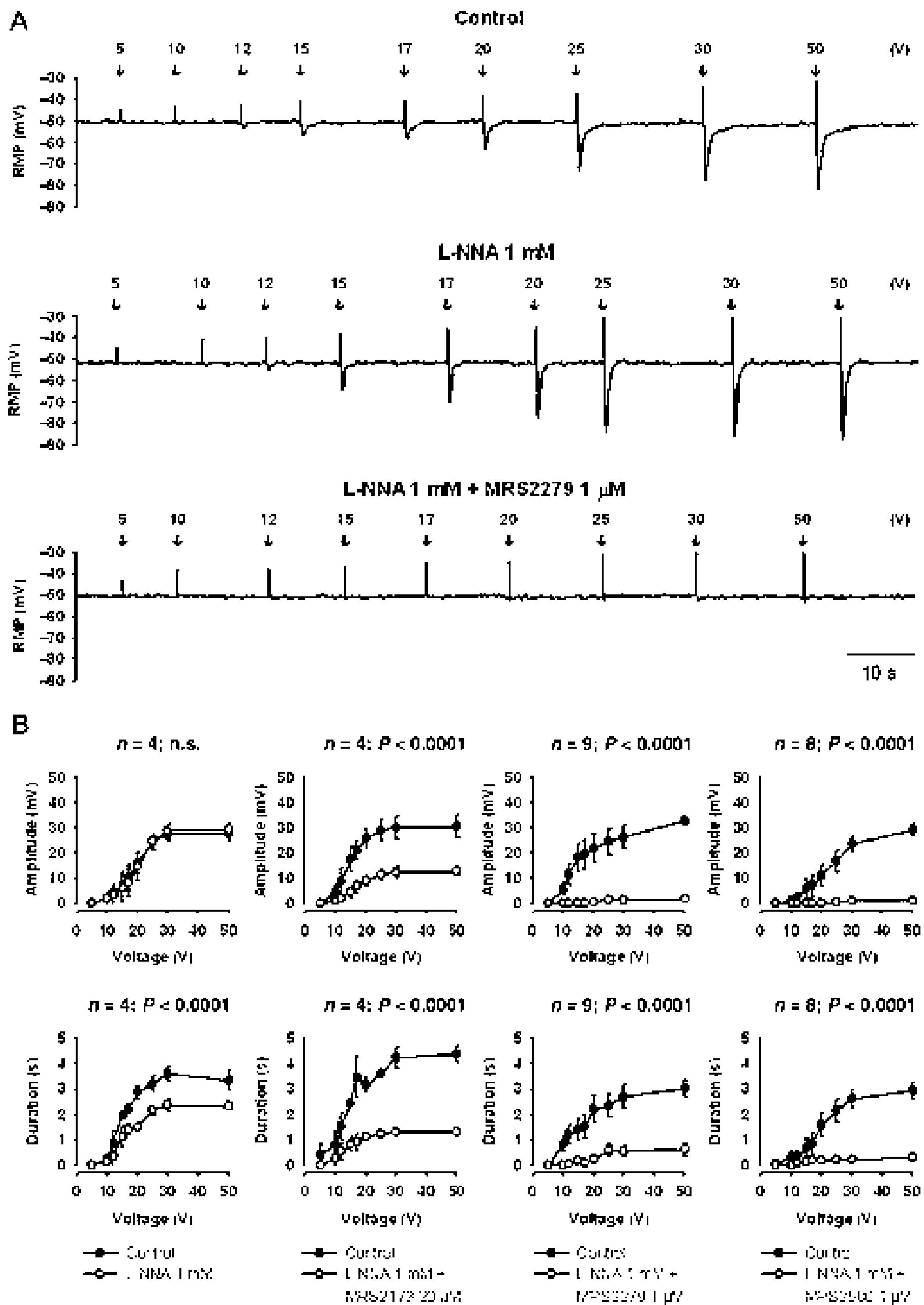

Figure 4 (A) Intracellular microelectrode recordings showing the electrical field stimulation (EFS)-induced inhibitory junction potential (IJP) at different voltages (5, 10, 12, 15, 17, 20, 25, 30 and 50 V) in control conditions and after incubation with N$^{\omega}$-nitro-L-arginine (L-NNA) (1 mM) and L-NNA (1 mM) + MRS2279 (1 μM). (B) Graphs representing the inhibitory effect of L-NNA (1 mM), and L-NNA + P2Y$_1$ antagonists: MRS2179 (20 μM), MRS2279 (1 μM) and MRS2500 (1 μM) on both the amplitude (top) and duration (bottom) of the EFS-induced IJP. All values represent the mean ± SEM. Significant differences were assessed using two-way ANOVA.

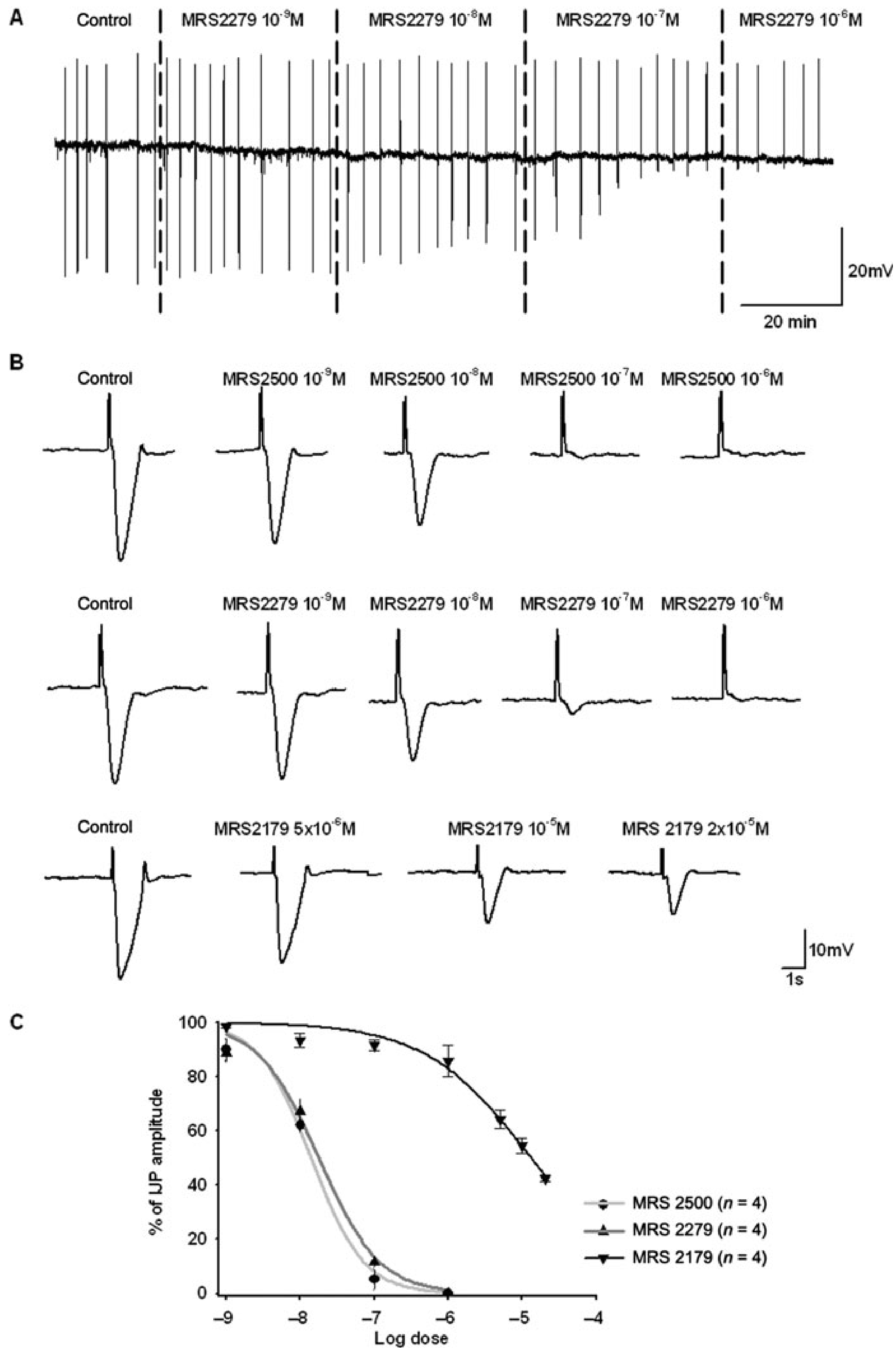

Figure 5 (A,B) Intracellular microelectrode recordings showing the electrical field stimulation-induced supramaximal inhibitory junction potential (IJP) in the presence of N^{ω}-nitro-L-arginine (control) and after cumulative perfusion with the $P2Y_1$ antagonists. (C) Concentration-response curves for the effects of $P2Y_1$ antagonists on the amplitude of the IJP. Note the difference between MRS2179 and MRS2279 or MRS2500.

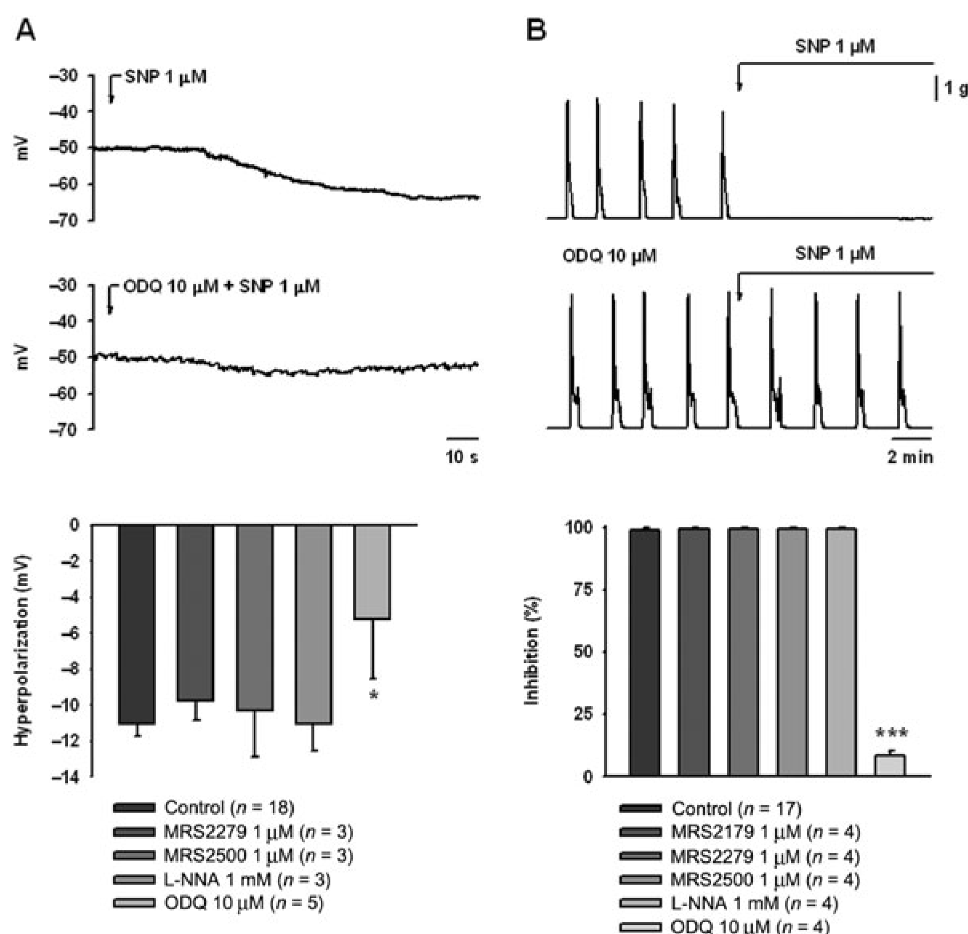

Figure 6 (A) Intracellular microelectrode recordings and histograms showing the hyperpolarization induced by sodium nitroprusside (SNP) (1 μM). oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (10 μM), but not the $P2Y_1$ antagonists, antagonized the hyperpolarization induced by SNP. (B) Inhibition of spontaneous motility by SNP (1 μM). Data were calculated using the following formula: 1 – [area under the curve (AUC) after SNP addition/AUC previous SNP addition] × 100. ODQ (10 μM), but not the $P2Y_1$ antagonists or N^{ω}-nitro-L-arginine (L-NNA), antagonized the inhibitory effect of SNP. All values are mean ± SEM. Significant differences were assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test. *$P < 0.05$; ***$P < 0.001$, significant difference from control.

antagonists, and the slow component to NOS inhibitors. A combination of NOS inhibitors and $P2Y_1$ antagonists completely blocked the inhibition of spontaneous motility induced by EFS and the off-contraction observed at the end of the stimulus. These results suggest that ATP (or a related purine) and NO are two neurotransmitters that act through $P2Y_1$ receptors and the cGMP pathway respectively. The selective $P2Y_1$ receptor antagonists tested in the present study showed different potency, MRS2500 being greater than MRS2279, which was greater than MRS2179.

The present experiments are a continuation of a previously published study where we demonstrated that the IJPf in the rat colon is mainly sensitive to apamin and suramine, and the IJPs is sensitive to NOS inhibitors (Pluja *et al.*, 1999). Suramine is a non-selective purinergic antagonist, and in the present study, we have used newly available pharmacological tools to characterize the co-transmission process.

MRS2179 was the first $P2Y_1$ selective receptor antagonist to be characterized (Camaioni *et al.*, 1998). This antagonist has been used in several biological systems involving $P2Y_1$ receptors, such as an inhibitor of platelet aggregation (Baurand *et al.*, 2001; Baurand and Gachet, 2003) or nucleotide-mediated relaxation in guinea pig aorta (Kaiser and Buxton, 2002). Recently, two other $P2Y_1$ antagonists, MRS2279 and MRS2500, have become available, and they show comparatively higher affinity and potency to the $P2Y_1$ receptor (Kim *et al.*, 2001; Boyer *et al.*, 2002; Cattaneo *et al.*, 2004). All these compounds are essential pharmacological tools for investigating functions involving $P2Y_1$ receptors (King, 2002). This is the first study in which the selective $P2Y_1$ antagonists MRS2279 and MRS2500 have been used to investigate the IJP and non-nitrergic relaxation in the GI tract. Our results show that all the antagonists were able to inhibit the IJPf, the non-nitrergic relaxation and the off-contraction induced by EFS, though with varying potency. MRS2279 and MRS2500 blocked the IJP in the micromolar range, but higher concentrations (up to 20 μM) of MRS2179 were needed to partially inhibit the IJP. Consistent with this result, $P2Y_1$ antagonists had a different range of potency in the non-nitrergic relaxation induced by EFS, MRS2500 being greater than MRS2279, which was greater than MRS2179. The rank order of antagonist potency is similar to that described in the literature (Cattaneo *et al.*, 2004; Von Kugelgen, 2006). The effect of MRS2179 is completely washable, but the effect of MRS2279

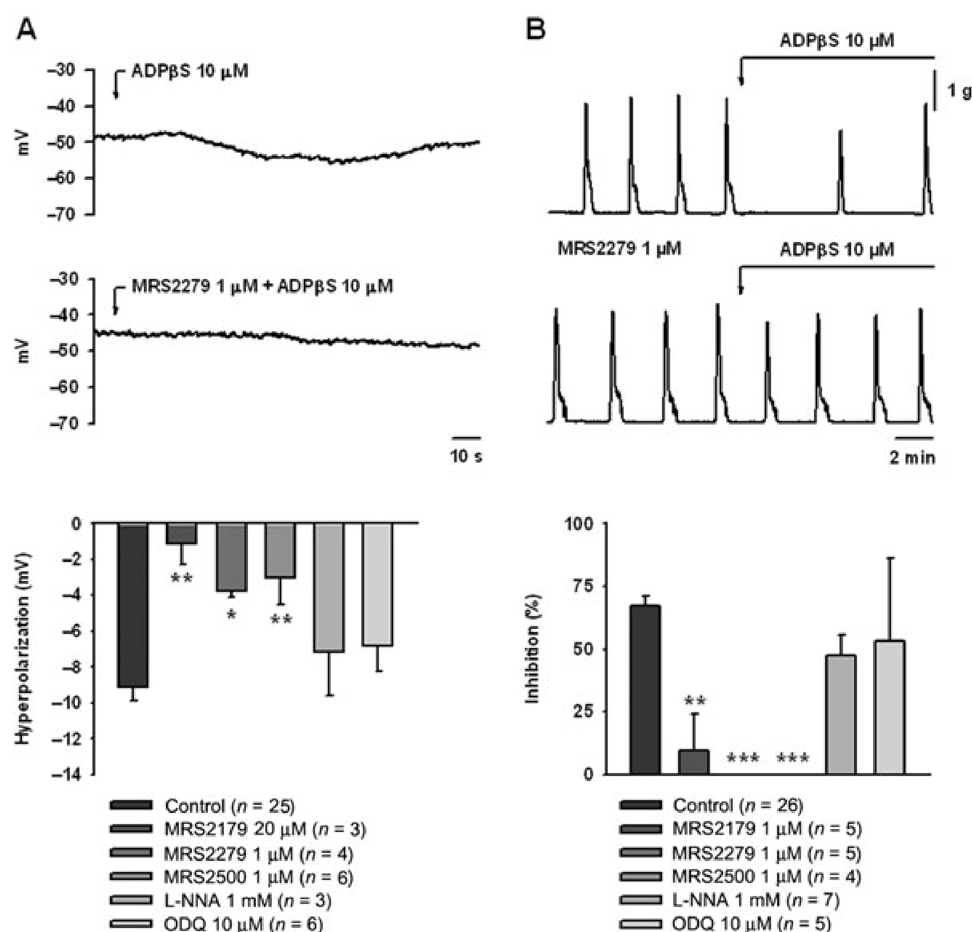

Figure 7 (A) Intracellular microelectrode tracings showing the hyperpolarization induced by ADPβs (10 μM). The hyperpolarization induced by adenosine 5′-O-2-thiodiphosphate (ADPßS) was antagonised by $P2Y_1$ antagonists but not by ODQ (10 mM) or N^{ω}-nitro-L-arginine (L-NNA) 1 mM. (B) Inhibition of spontaneous motility induced by ADPßS (10 μM). Data were calculated using the following formula: 1 – [area under the curve (AUC) after ADPßS addition/AUC previous ADPßS addition] × 100. $P2Y_1$ antagonists, but not L-NNA (1 mM) or ODQ (10 μM), antagonized the inhibition of spontaneous activity. All values are mean ± SEM. Significant differences were assessed using one-way ANOVA, followed by Bonferroni's multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significant difference from control.

and MRS2500 is only partially washable, and this might be due to varying sensitivity to ectonucleases (Ravi *et al.*, 2002). Accordingly, MRS2500 has been used as a stable antithrombotic agent *in vivo* (Hechler *et al.*, 2006).

Differences between species might explain present and previously published data. According to the present results and those from other studies (Zizzo *et al.*, 2007; McDonnell *et al.*, 2008), the inhibitory effect of MRS2179 is less potent in rodents than in humans (Gallego *et al.*, 2008a), pigs (Gallego *et al.*, 2008b) or guinea pigs (Wang *et al.*, 2007). In human colon and pig small intestine, the IC_{50} of MRS2179 is close to 1 μM, but in rats, a higher concentration is needed to inhibit EFS-induced relaxation and off-contraction. MRS2279 and MRS2500 have not been used in human colon, but preliminary data from our laboratory show that this antagonist inhibits the human IJPf and relaxation in the nM range. All these data are consistent with a major role of post-junctional $P2Y_1$ receptors in mediating IJPf and non-nitrergic relaxation. The presence of other P2Y receptors in the internal anal sphincter of mice (McDonnell *et al.*, 2008), or, alternatively, a pre-junctional effect of P2Y receptors in the mouse caecum, has been recently postulated (Zizzo *et al.*, 2007). According to the data presented in the present work, further research is needed with more potent $P2Y_1$ antagonists to determine whether the maximal effect has been reached in these tissues. In the rat colon, only $P2Y_1$ and $P2Y_6$ receptors have been immunolocalized in smooth muscle cells (Van Crombruggen *et al.*, 2007). However, MRS antagonists fail to block the rat $P2Y_6$ receptors expressed in human astrocytoma cells (Boyer *et al.*, 1998). $P2Y_1$ receptors are located in enteric neurones in several species (Gao *et al.*, 2006; Gallego *et al.*, 2008b) and mediate slow excitatory postsynaptic potentials in neurones from the guinea-pig submucous plexus (Hu *et al.*, 2003; Monro *et al.*, 2004) and myenteric plexus (Gwynne and Bornstein, 2009). A pre-junctional effect of $P2Y_1$ receptors inhibiting ATP release is possible, but $P2Y_1$ receptors have not been detected in enteric neurones in the rat colon (Van Crombruggen *et al.*, 2007). In a fine, recent study, $P2Y_1$ and $P2Y_{11}$ receptors were shown to mediate the fast and slow relaxation, respectively, in the guinea-pig taenia coli (King and Townsend-Nicholson, 2008). In the guinea pig taenia coli, both $P2Y_1$ and $P2Y_{11}$ receptors are present in smooth muscle cells and α-β meATP activated $P2Y_{11}$ receptors, causing smooth muscle relaxation. However, MRS2179 did not

antagonize calcium increase induced by purinergic activation of human $P2Y_{11}$ receptors transfected in a human astrocytoma cell line (King and Townsend-Nicholson, 2008). In the rat colon, neither have $P2Y_{11}$ receptors been detected in smooth muscle cells, nor in interstitial cells of Cajal, which might participate in neurotransmission (Van Crombruggen *et al.*, 2007). All these data are consistent with a major role of $P2Y_1$ receptors in the generation of IJPf and non-nitrergic relaxation. However, the possibility that other extrajunctional P2Y receptors are involved in smooth muscle relaxation needs further research.

It is important to note that blockade of $P2Y_1$ receptors alone does not modify the duration of the IJP nor the inhibition induced by EFS. According to our results, the 'non-purinergic' relaxation is mainly nitrergic. This is consistent with recently published data on the human colon where $P2Y_1$ antagonist inhibited IJPf but not the sustained IJP elicited by electrical pulses (Gallego *et al.*, 2008a). In the presence of purinergic blockade, the nitrergic IJP causes smooth muscle hyperpolarization, the membrane potential does not reach the threshold to open calcium channels, and, consequently, spontaneous contractions do not occur. At the end of the stimulus, smooth muscle repolarization probably activates L-type calcium channels, and an off-contraction occurs. In agreement with these results, SNP, an NO donor, causes long-lasting hyperpolarization and smooth muscle relaxation (Pluja *et al.*, 1999), which are sensitive to ODQ, but not to $P2Y_1$ receptor antagonists (present work).

In contrast, NO synthase inhibition reduced the IJPs but not IJPf; a partial effect on the inhibition of spontaneous motility was observed in the presence of L-NNA. In this case, the 'non-nitergic' IJP is mainly purinergic through $P2Y_1$ receptors. It is possible that the reduction in the IJP duration would result in smooth muscle cells transiently reaching the threshold to open calcium channels, and this could explain the partial contractions present during EFS. A similar result has been reported in the human colon (Gallego *et al.*, 2008a).

In conclusion, the present work demonstrates that in the rat colon, a co-transmission process that involves ATP through $P2Y_1$ receptors, and NO is present. As described in other species, $P2Y_1$ receptors are responsible for the IJPf, and NO is responsible for the IJPs. The rank order of potency for the $P2Y_1$ receptor antagonists in the rat colon is MRS2500 greater than MRS2279, which is greater than MRS2179. It would be interesting to investigate the potencies of these antagonists in human colonic tissue.

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Chapter 2

Pharmacological characterization of purinergic inhibitory neuromuscular transmission in the human colon

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Pharmacological characterization of purinergic inhibitory neuromuscular transmission in the human colon

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Abstract

Background *In the present study, we further characterize the purinergic receptors mediating the inhibitory junction potential (IJP) and smooth muscle relaxation in the human colon using a new, potent and selective agonist (MRS2365), and antagonists (MR2279 and MRS2500) of the* $P2Y_1$ *receptor. The* $P2Y_{12}$ *antagonist AR-C66096 was tested as well. Using this pharmacological approach, we tested whether β-nicotinamide adenine dinucleotide (β-NAD) fulfilled the criteria to be considered an inhibitory neurotransmitter in the human colon.* ***Methods*** *We carried out muscle bath and microelectrode experiments on circular strips from the human colon and calcium imaging recordings on HEK293 cells, which constitutively express the human* $P2Y_1$ *receptor.* ***Key Results*** *Both the fast component of IJP and non-nitrergic relaxation was concentration-dependently inhibited by MRS2279 and MRS2500. This antagonism was confirmed in HEK293 cells. However, AR-C66096 did not modify the inhibitory responses. Adenosine 5′-O-2-thiodiphosphate and MRS2365 caused a smooth muscle hyperpolarization and transient inhibition of spontaneous motility that was antagonized by MRS2279 and MRS2500. β-Nicotinamide adenine dinucleotide inhibited the spontaneous motility* (IC_{50} *= 3.3 mmol* L^{-1}*). Nevertheless, this effect was not antagonized by high concentrations of* $P2Y_1$ *antagonists.* ***Conclusions & Inferences*** *Inhibitory purinergic neuromuscular transmission in the human colon was pharmacologically assessed by the use of new* $P2Y_1$ *receptor antagonists MRS2179, MRS2279, and MRS2500. The rank order of potency of the* $P2Y_1$ *antagonists is MRS2500 > MRS2279 > MRS2179. We found that β-NAD partially fulfills the criteria to be considered an inhibitory neurotransmitter in the human colon, but the relative contribution of each purine (ATP/ADP vs β-NAD) requires further studies.*

Keywords *gastrointestinal, inhibitory junction potential,* $P2Y_1$ *receptors,* $P2Y_{12}$ *receptors, smooth muscle.*

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INTRODUCTION

Purinergic and nitrergic neurotransmission are the main inhibitory mechanisms causing smooth muscle relaxation in the gastrointestinal (GI) tract. However, the lack of specific agonists and antagonists has made it difficult to establish the identity of the receptors involved in purinergic relaxation. In 1998, Camaioni *et al.* described the compound MRS2179 (the N^6-methyl modification of 2′-deoxyadenosine 3′,5′-bisphosphate) as a potent $P2Y_1$ receptor antagonist.[1] This purinergic antagonist is currently considered competitive and specific for the $P2Y_1$ receptor.[2] Using MRS2179 it has been shown, in several species and regions of the GI tract that $P2Y_1$ receptors mediate purinergic neurotransmission.[3–7] We have recently demonstrated that $P2Y_1$ receptors mediate the fast component of the inhibitory junction potential (IJPf) and therefore phasic smooth muscle relaxation both in pig small intestine and in rat and human colon.[3,7,8] However, it has also been postulated that other P2Y receptors might also be involved in neuromuscular transmission.[9,10] Recent studies suggest that both $P2Y_1$ and $P2Y_{11}$ receptors mediate fast and slow relaxations in the guinea-pig tenia coli.[11] $P2Y_4$ receptors

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expressed in interstitial cells of Cajal might also participate in inhibitory neurotransmission.[12–14]

Several pharmacological modifications of MRS2179 have lead to the development of a new agonists and new antagonists for the $P2Y_1$ receptors.[15,16] MRS2279 and MRS2500 are selective $P2Y_1$ antagonists that inhibit purinergic neurotransmission in the rat colon and internal anal sphincter.[8,17,18] MRS2365 is a preferential $P2Y_1$ agonist, whereas ADPβS is a stable purinergic agonist acting on $P2Y_1$, $P2Y_{11}$ and $P2Y_{12}$ receptors. $P2Y_{12}$ receptors are pharmacological targets to treat thrombosis in humans[19] and so, it is important to know whether these receptors participate in neuromuscular transmission in the human GI tract. AR-C66096 is a preferential antagonist of $P2Y_{12}$ receptors.

The neurotransmitter responsible for purinergic neurotransmission is still under debate. Classically, the terminology 'ATP or a related purine' is employed in the majority of articles because ATP is quickly degraded by ectonucleases to ADP, AMP, and adenosine.[20] It has been recently proposed that β-nicotinamide adenine dinucleotide (β-NAD) is potentially an inhibitory purinergic neurotransmitter in the murine GI tract, possibly acting on $P2Y_1$ receptors.[14] β-Nicotinamide adenine dinucleotide might also activate $P2Y_{11}$ receptors in human granulocytes.[21] Thus, two putative families of compounds: β-NAD and ATP and their respective products of degradation are possible candidates to be identified as inhibitory neurotransmitters in the GI tract.[14] While, we were preparing the manuscript it has been proposed that β-NAD as inhibitory neurotransmitter in the human colon.[22]

According to these results, it is important to translate data from animals to humans and the main aim of the present work was to study the purinergic inhibitory neurotransmission in the human colon using these new available pharmacological tools. We also wanted to test whether β-NAD fulfills the criteria to be considered an inhibitory neurotransmitter in the human colon. Pharmacological characterization of receptors and neurotransmitters involved in human colonic smooth muscle relaxation mediated by purines might help to develop pharmacological strategies to treat colonic motility disorders with impaired purinergic neurotransmission as has been described in animal models.[23]

MATERIALS AND METHODS

Tissue preparation

Specimens of distal and sigmoid colon (n = 39) were obtained from patients (aged 46–91 years) during colon resections for neoplasm. Colon segments from macroscopically normal regions were collected and transported to the laboratory in cold saline buffer. The tissue was placed in Krebs solution on a dissection dish, and the mucosal layer was gently removed. Circular muscle strips, 10 × 4 mm, were cut. The experimental procedure was approved by the Ethics Committee of the Hospital of Mataró (Barcelona, Spain).

Mechanical experiments

Circular muscle strips were studied in a 10 mL organ bath filled with Krebs solution containing phentolamine, atropine, and propanol (all 1 μmol L^{-1}) at 37 ± 1 °C. An isometric force transducer (Harvard VF-1; Harvard Apparatus Inc., Holliston, MA, USA) connected to a computer through an amplifier was used to record the mechanical activity. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an ISC-16 A/D card installed in the computer. A tension of 4 g was applied and the tissue was allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. Electrical field stimulation (EFS) was applied for 2 min (pulse duration 0.4 ms, frequency 2 Hz, and amplitude 50 V). The area under the curve (AUC) of contractions from the baseline was used to measure the spontaneous motility.

Electrophysiological experiments

Muscle strips were dissected parallel to the circular muscle and placed in a Sylgard-coated chamber continuously perfused with Krebs solution containing phentolamine, atropine, propranolol (all 1 μmol L^{-1}), and L-NNA (1 mmol L^{-1}) at 37 ± 1 °C. Strips were meticulously pinned in a cross-sectioned slab allowing microelectrode recordings from circular muscle. This procedure was previously reported in the canine ileum[24] and human colon.[3] Preparations were allowed to equilibrate for approximately 1 h before experiments started. Circular muscle cells were impaled with glass microelectrodes (40–60 MΩ) filled with 3 mol L^{-1} KCl. Membrane potential was measured 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) using PowerLab 4/30 system and Chart 5 software for Windows (all from AD Instruments, Castle Hill, NSW, Australia). Electrical field stimulation 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. Resting membrane potential was measured before and after drug addition. The amplitude of IJPs was measured under control conditions and after infusion of each drug. To obtain stable smooth muscle cell impalements, nifedipine (1 μmol L^{-1}) was used to abolish its mechanical activity.

Calcium imaging technique

HEK293 cells, which constitutively express the human $P2Y_1$ receptor[25] were studied by using the calcium imaging technique. HEK 293 cells were grown in Dulbecco's Modification of Eagles Medium (DMEM) (37 °C, pH 7.4) and seeded in a culture dish with a coverglass 48 h before the experimental procedure. Cells were loaded with Fluo-4 AM (5 μmol L^{-1}, room temperature, 45 min) in extracellular medium solution. The coverglass containing the Fluo-4 AM loaded HEK 293 cells was placed over

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a metal ring, immobilized by a rubber o-ring and transferred to a chamber. Fluo-4 AM was used to monitor changes in cytosol calcium level. After washing out the remaining dye, cells were incubated in the recording medium. Cells were imaged with IX-FLA Camera connected to an Olympus IX70 microscope and scanned using CELL^{R} software (Olympus Biosystems, Heidelberg, Germany). In preliminary experiments, optimal UV light intensity was set to obtain sufficient image quality, whereas minimizing phototoxicity and bleaching. Cells were perfused in a constant flow of extracellular medium solution (1 mL min^{-1}) at RT and a manual valve ALA VM-8 channel bath perfusion system (npi Electronic Instruments, Tamm, Germany) allowed switching between normal and drug-containing solutions. Changes in Fluo-4 fluorescence were recorded at 2.5Hz with a spatial resolution of 512×480 pixels. At the end of the experiments, images were analyzed over time using regions of interest (ROIs). Fluorescence intensity of ROIs ($\Delta F/F$) analysis was performed and plotted over time.

Solutions and drugs

The composition of the Krebs solution was (in mmol L^{-1}): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3–7.4). It was maintained at 37 ± 1 °C and bubbled with a mixture of 5% CO_2 and 95% O_2. In all the experiments, phentolamine, atropine and propranolol (1 μmol L^{-1}) were added to the Krebs solution to block α- and β-adrenoceptors and muscarinic receptors. The composition of the extracellular medium solution was (in mmol L^{-1}): 140 NaCl, 4.8 KCl, 1 $MgCl_2$ $6H_2O$, 1.8 $CaCl_2$ $2H_2O$, 10 glucose.

The following drugs were added to the Krebs solution: Nifedipine, N_ϵ-nitro-L-arginine (L-NNA), adenosine 5′-O-2-thiodiphosphate (ADPβS), β-nicotinamide adenine dinucleotide sodium salt (β-NAD) and phentolamine (Sigma Chemicals, St Louis, MO, USA); tetrodotoxin (TTX) (Latoxan, Valence, France); atropine sulphate (Merck, Darmstadt, Germany); propranolol, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS2179), (1R,2S,4S,5S)-4-[2-Chloro-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS2279), (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), [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3 dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365), 2-(Propylthio)adenosine-5′-*O*-(β,γ-difluoromethylene)triphosphate tetrasodium salt (AR-C66096) (Tocris, Bristol, UK); and Fluo-4 AM (Teflabs Inc. Texas, USA). Stock solutions were made by dissolving drugs in distilled water except for nifedipine, which was dissolved in ethanol (96%), L-NNA was dissolved in Krebs solution by sonication and Fluo-4 AM was dissolved in DMSO (0.002% final concentration).

Data analysis and statistics

One-way analysis of variance (ANOVA) was used to evaluate the effect of MRS2279 or MRS2500 on: (i) the inhibition of spontaneous motility induced by ADPβS and MRS2365 and (ii) EFS-induced relaxation. Two-way ANOVA was used to analyze the effect of MRS2279 or MRS2500 on: (i) the inhibition of spontaneous mechanical activity caused by β-NAD, and (ii) the amplitude of the IJPs (drug and voltage). Paired *t*-test was used to evaluate the effect of MRS2279 or MRS2500 on the hyperpolarization induced by ADPβS.

To normalize data, the inhibitory effect of ADPβS, MRS2365, β-NAD, and EFS on spontaneous rhythmic colonic contractions was calculated as percentage of inhibition using the following formula: 1 − [area under the curve (AUC) during drug addition or EFS/AUC previous drug addition or EFS] × 100. When motility was completely abolished, inhibition was considered 100%, whereas inhibition was 0% when no changes in AUC were observed. To calculate IC_{50} and Hill slopes, data were fitted to a sigmoid concentration-response curve of variable slope: $Y = 100/(1 + 10^{((LogEC50-X)*Hill\ slope)})$, where X is the logarithm of concentration and Y is the response.

All image analysis was performed with the CELL^{R} sofware (Olympus Biosystems). Regions of interest were drawn over each cell, fluorescence intensity was normalized to the basal fluorescence at the onset of the recording for each ROI, and peaks were analyzed. Paired student's *t*-test or ANOVA test was used before and after drug addition.

A $P < 0.05$ was considered statistically significant. '*n*' values indicate the number of samples from different patients or the number of cells analyzed (using at least three to four different plates). Statistical analysis was performed with GRAPHPAD PRISM version 4.00, (GraphPad Software, San Diego, CA, USA).

RESULTS

Effect of $P2Y_1$ antagonists on the inhibition of spontaneous motility induced by EFS

Two minutes EFS caused complete cessation of spontaneous motility. Incubation with MRS2279 (1 μmol L^{-1}) or MRS2500 (1 μmol L^{-1}) did not modify the inhibitory response. To study the purinergic component of the relaxation, different concentrations of the $P2Y_1$ receptors antagonists (MRS2279 and MRS2500 were tested in strips previously incubated with L-NNA (1 mmol L^{-1}, 20 min). In the presence of L-NNA, the purinergic antagonists caused a concentration-dependent inhibition of the non-nitrergic relaxation induced by EFS, the IC_{50} was 0.26 μmol L^{-1} ($n = 7$) for MRS2279 and 0.088 μmol L^{-1} ($n = 5$) for MRS2500 (Fig. 1). Table 1 summarizes these results. A comparison with the effect of MRS2179 (Gallego *et al.*, 2006)[3] is shown.

Effect of $P2Y_1$ antagonists on the inhibitory junction potential

Increasing the voltage of stimulation caused a progressive increase in the amplitude of the IJP (Fig. 2). MRS2279 and MRS2500 concentration-dependently reduced the IJP amplitude (ANOVA $P < 0.0001$; $n = 5$ each drug) (Fig. 2). To calculate the IC_{50} of MRS2279 and MRS2500, a protocol using supramaximal IJPs was performed (using 30 V stimuli). The IC_{50} was 0.28 μmol L^{-1} ($n = 5$) for MRS2279 and 0.071 μmol L^{-1} ($n = 5$) for MRS2500 (Fig. 2). Table 1 summarizes these results. A comparison with the effect of MRS2179 (Gallego *et al.*, 2006)[3] is shown.

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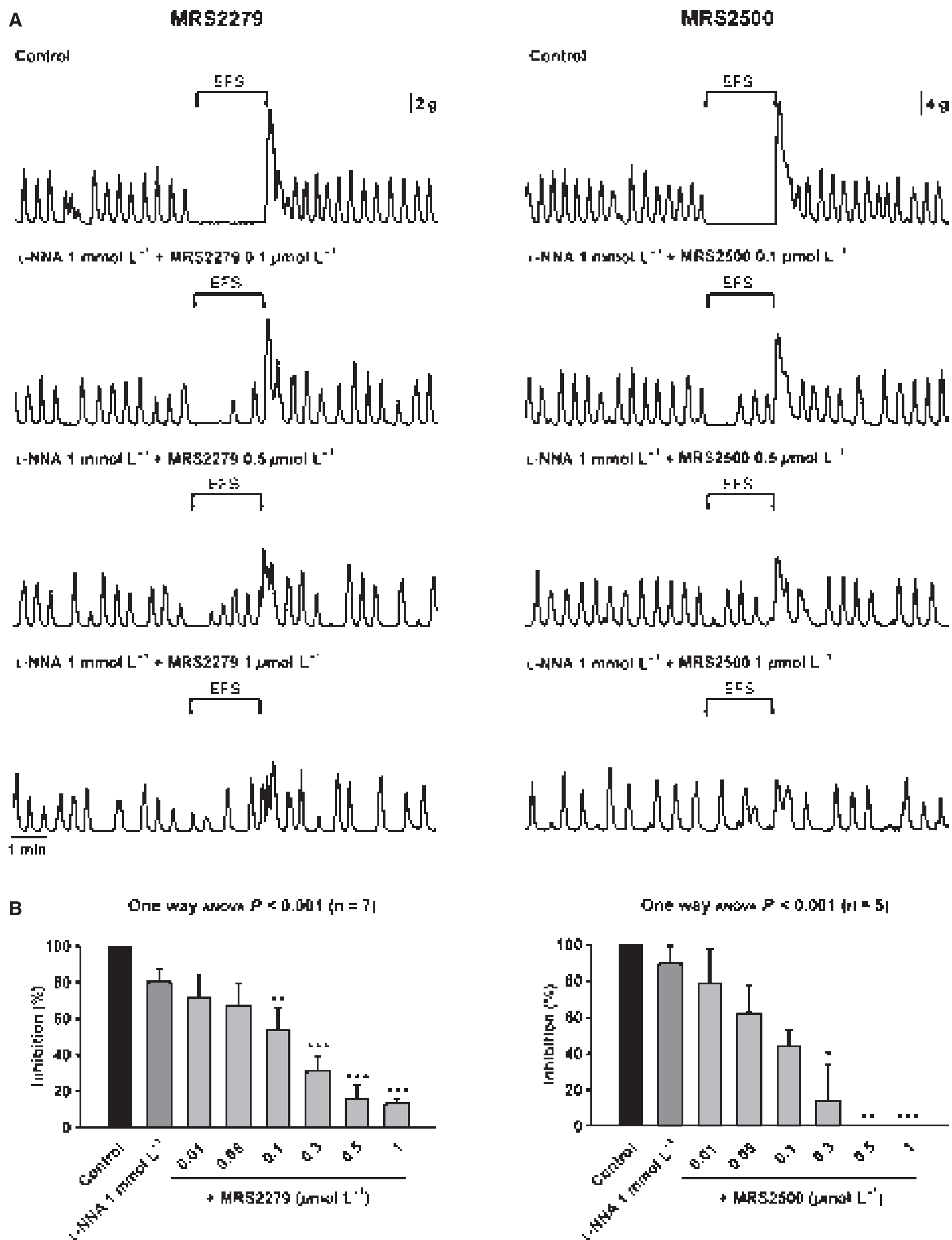

Figure 1 Mechanical recordings (A) and histograms (B) showing the effect of MRS2279 (0.01–1 μmol L^{-1}), n = 5 (left), and MRS2500 (0.01–1 μmol L^{-1}) n = 7, (right), on the inhibition of the spontaneous activity induced by electrical field stimulation (EFS) in the presence of L-NNA (1 mmol L^{-1}). All values are expressed as mean ± SEM. Significant differences were assessed using one-way analysis of variance followed by Bonferroni's Multiple Comparison Test. *P < 0.05; **P < 0.01, ***P < 0.001.

Effect of other P2Y antagonists on the IJP and inhibition of spontaneous motility induced by EFS

The following antagonists were tested in the presence of L-NNA (1 mmol L^{-1}) to isolate the purinergic component of the inhibitory response. AR-C66096, a $P2Y_{12}$ receptor antagonist, was tested at 0.1, 1 and 5 μmol L^{-1}, but neither the IJP (n = 4 each concentration) nor the inhibition of spontaneous motility induced by EFS (n = 4 each concentration) was affected.

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Table 1 Table summarizing the IC_{50} (μmol L^{-1}) of the purinergic antagonists obtained *vs* the endogenous inhibitory transmitter, released by EFS and assessed on mechanical activity (relaxation) and membrane potential (IJP), and *vs* the exogenous agonist ADPβS, assessed in HEK cells

$P2Y_1$ antagonists	Organ bath	Microelectrodes	Calcium image
Response	Relaxation induced by EFS	IJP induced by EFS	Calcium transients induced by ADPβS 1 μmol L^{-1}
MRS2179	0.87*	1.23*	0.13 (0.099–0.179)
MRS2279	0.26 (0.19–0.36)	0.28 (0.21–0.36)	0.0046 (0.0039–0.0055)
MRS2500	0.088 (0.058–0.133)	0.071 (0.064–0.078)	0.0029 (0.0025–0.0033)

IJP, inhibitory junction potential; EFS, electrical field stimulation.
AR-C66096 did not affect the IJP and relaxation induced by EFS, data are expressed as mean (95% confidence interval).
*Data previously published[3].

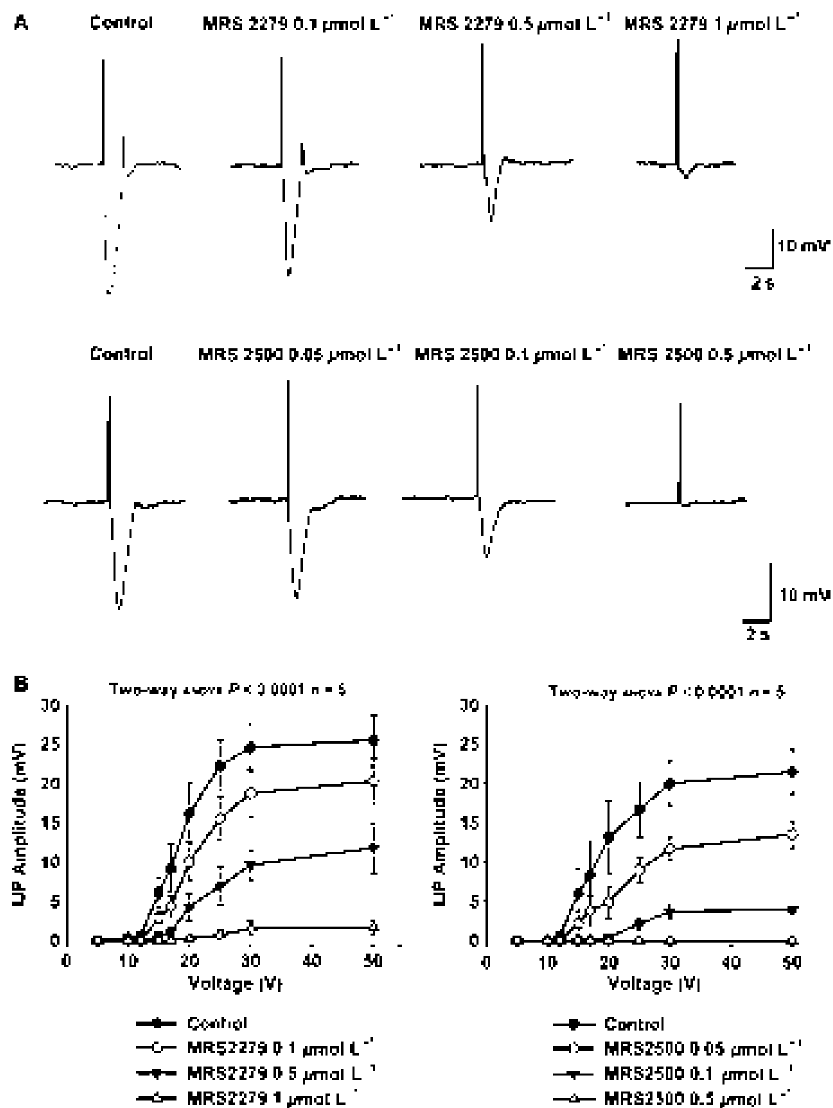

Figure 2 Intracellular microelectrode recordings (A) showing the effect of MRS2279 (0.1, 0.5 and 1 μmol L^{-1}) (top) and MRS2500 (0.05, 0.1 and 0.5 μmol L^{-1}) (bottom) on the IJP induced by a supramaximal EFS of 30 V. Plot graph (B) showing the effect of MRS2279 and MRS2500 on the IJP at increasing voltages of stimulation (5,10,12,15,17,20,25,30, and 50 V). Data are expressed as mean ± SEM. Significant differences were assessed using two-way analysis of variance (n = 5; P < 0.0001 for each drug).

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Effect of exogenous addition of ADPβS and MRS2365 on spontaneous motility

Human colonic strips spontaneously developed rhythmic phasic contractions. In the presence of TTX (1 μmol L^{-1}), the purinergic agonist ADPβS (10 μmol L^{-1}) inhibited the spontaneous contractions displayed by circular muscle strips by 71.3 ± 8.2% (n = 10). After 10-min preincubation with MRS2279 (1 μmol L^{-1}) or MRS2500 (1 μmol L^{-1}), the inhibition caused by ADPβS (10 μmol L^{-1}) was reduced to 29.7 ± 9.0% (n = 5; P < 0.01) and 13.2 ± 4.3% (n = 5, P < 0.001), respectively (Fig. 3A). MRS2365, a selective P2Y$_1$ agonist, caused a concentration-dependent inhibition of spontaneous mechanical activity (0.1 μmol L^{-1}: 1.8 ± 8.8%, n = 3; 1 μmol L^{-1}: 16.9 ± 6.2%, n = 8; and 10 μmol L^{-1}: 51.5 ± 5.5%, n = 11). Previous incubation with MRS2500 1 μmol L^{-1} blocked the inhibitory effect of MRS2365 10 μmol L^{-1} (9.4 ± 4.5%, n = 4, P < 0.001). MRS2279 (10 μmol L^{-1}) completely blocked the inhibitory effect induced by MRS2365 10 μmol L^{-1} (7.1 ± 2.4%, n = 3, P < 0.001) (Fig. 3B).

Effect of exogenous addition of ADPβS and MRS2365 on smooth muscle membrane potential

Transient superfusion of the tissue with ADPβS (1 μmol L^{-1}) hyperpolarized the smooth muscle by −10.7 ± 1.7 mV (n = 8). This hyperpolarization was reduced by MRS2279 (1 μmol L^{-1}) to −2.2 ± 2.2 mV (P < 0.05, n = 3) and MRS2500 (0.5 μmol L^{-1}) to −2.4 ± 1.3 mV (P < 0.05, n = 3). The selective P2Y$_1$ agonist MRS2365 caused a concentration-dependent hyperpolarization (0.1 μmol L^{-1}: −4.2 ± 1.9 mV, n = 5; 1 μmol L^{-1}: −5.4 ± 1.4 mV, n = 6 and 10 μmol L^{-1}: −9.5 ± 2.1 mV, n = 8) (Fig. 4). The hyperpolarization induced by MRS2365 at 0.1 and 1 μmol L^{-1} was completely antagonized by MRS2500 1 μmol L^{-1} and at 10 μmol L^{-1}, the hyperpolarization induced by MRS2365 was reduced to −5.7 ± 4.7 mV by MRS 2500 1 μmol L^{-1} (n = 3). AR-C66096 5 μmol L^{-1} (n = 2) did not modify the hyperpolarization induced by 10 μmol L^{-1} ADPβS.

Effect of ADPβS on calcium transients in HEK293 cells

ADPβS (1 μmol L^{-1}) elicited a calcium increase (ratio 4.09, n = 179) in HEK 293 cells. This calcium increase was concentration-dependently inhibited by MRS2179 IC$_{50}$ = 0.13 μmol L^{-1} (n = 30), MRS2279 IC$_{50}$ = 4.6 nmol L^{-1} (n = 30) and MRS2500 IC$_{50}$ = 2.89 nmol L^{-1} (n = 30). Table 1 summarizes these data.

Effect of β-NAD on spontaneous motility, resting membrane potential, and calcium transient induced in HEK 293 cells

At high concentrations, β-NAD induced a concentration-dependent inhibition of spontaneous motility (IC$_{50}$ = 3.3 mmol L^{-1}; 95% confidence interval 2.4–4.4 mmol L^{-1}; log IC$_{50}$ = −2.49 ± 0.06; n = 8), which was not antagonized by MRS2279 or MRS2500 (1 μmol L^{-1}) (Fig. 3C). Higher concentrations of MRS2279 and MRS2500 (5 μmol L^{-1}) did not revert the inhibition caused by β-NAD (data not shown). β-Nicotinamide adenine dinucleotide caused a slight hyperpolarization of −3.2 ± 0.6 mV at 1 mmol L^{-1} (n = 6) and −4.1 ± 1.1 mV at 10 mmol L^{-1} (n = 2) (Fig. 4). The hyperpolarization caused by β-NAD at 10 mmol L^{-1} was abolished by MRS2500 1 μmol L^{-1} (n = 2). In HEK 293 cells, β-NAD did not induce a significant calcium increase at concentrations up to 5 mmol L^{-1}. At 10 mmol L^{-1}, β-NAD induced a calcium increase in about 50% of the ADPβS responding cells (n = 58) and the amplitude of the response was a third of those induced by ADPβS (ratio 1.37; n = 28 responding cells; P < 0.0001).

DISCUSSION

The aim of the present study has been to characterize the purinergic receptor(s) that participate in the inhibitory effect at the neuromuscular junction in the human colon and to find out the nature of the neurotransmitter(s) involved in the purinergic inhibition. Despite the evidence that purinergic receptors play a key role in purinergic inhibitory neuromuscular transmission in the GI tract,[26] there are very few articles characterizing this neurotransmission in human tissues. In the human colon, NO and a purine are two of the main neuromuscular inhibitory transmitters.[27] Both in the human small intestine and colon repetitive electrical field stimulation causes an IJP with two phases, a fast component followed by an L-NNA-sensitive sustained component.[28,29] The fast component is partially inhibited by apamin, suramine and it is desensitized by ADPβS agonist suggesting an involvement of P2 receptors.[30] Moreover, PPADS and suramine partially inhibited the non-nitrergic relaxation induced by field stimulation both in the human small intestine and colon.[31,32] All these results suggest a putative role of P2 receptors in purinergic inhibitory neuromuscular transmission.

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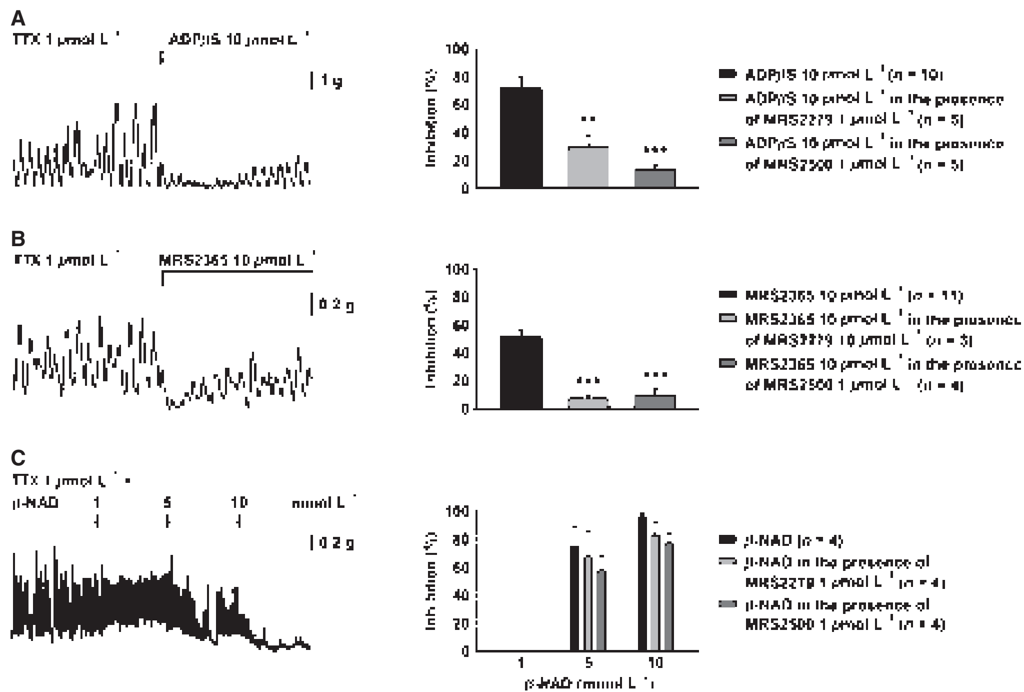

Figure 3 Mechanical recordings (left) and histograms (right) showing the inhibitory effect of ADPβS (A), MRS2365 (B), and β-NAD (C) on the spontaneous motility in the presence of TTX 1 μmol L^{-1}. The effect of MRS2279 (1–10 μmol L^{-1}) and MRS2500 (1 μmol L^{-1}) on the inhibition caused by ADPβS, MRS2365, and β-NAD is plotted in the histograms. All values are expressed as mean ± SEM. Significant differences were assessed using one-way analysis of variance (for ADPβS and MRS2365) or two-way analysis of variance (for β-NAD), followed by Bonferroni's Multiple Comparison Test. $^{**}P < 0.01$, $^{***}P < 0.001$.

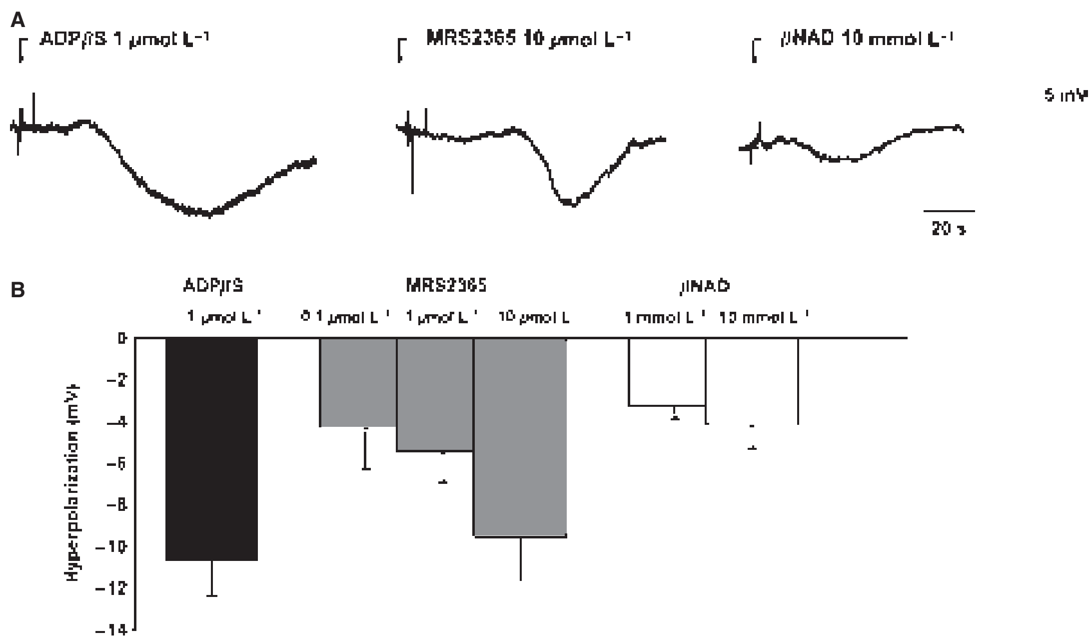

Figure 4 Intracellular microelectrode recordings (A) showing the effect of ADPβS 1 μmol L^{-1} MRS2365 10 μmol L^{-1} and β-NAD 10 mmol L^{-1} on the smooth muscle membrane potential. Histogram (B) showing the effect of ADPβS 1 μmol L^{-1} (n = 8), MRS2365 0.1 μmol L^{-1} (n = 5), 1 μmol L^{-1} (n = 6), 10 μmol L^{-1} (n = 8) and β-NAD 1 mmol L^{-1} (n = 6), 10 mmol L^{-1} (n = 2). Data are expressed as mean ± SEM.

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Recently, we and other research groups have shown that MRS2179, a selective $P2Y_1$ antagonist, inhibits the inhibitory neurotransmission in the human GI tract. In the colon: (i) Both the IJP and the non-nitrergic relaxation are concentration-dependently inhibited by MRS2179 with an IC_{50} of about 1 μmol L^{-1} [3], (ii) when long pulses of 5Hz are applied, the fast component is sensitive to MRS2179, whereas the slow component is sensitive to L-NNA[33] and, (iii) non-nitrergic neural-mediated relaxations induced by stimulation of nicotinic receptors are blocked by MRS2179.[34] In the human small intestine, non-nitrergic relaxation is sensitive to MRS2179 at concentration between 3 and 10 μmol L^{-1}.[35] All these data suggest an involvement of $P2Y_1$ receptors in different areas of the human GI tract. However, data from these articles show that a concentration of 10 μmol L^{-1} of MRS2179 is needed to inhibit purinergic responses and it is possible that at this concentration the antagonist might partially loose selectivity. Data from the present article show that: (i) the fast component of IJP and the non-nitrergic relaxation are inhibited in a concentration-dependent manner by MRS2279 and MRS2500, (ii) 2 the rank of potency of these drugs is MRS2500 > MRS2279 > MRS2179 (see Table 1), (iii) data from the antagonists obtained with organ bath and microelectrode are similar showing that the underlying mechanism responsible of the non-nitrergic relaxation is the purinergic IJP mediated by $P2Y_1$ receptors (iv) concentrations of 1 μmol L^{-1} MRS2279 and 0.5 μmol L^{-1} MRS2500 are able to completely block the inhibitory response, and (v) $P2Y_1$ antagonists alone without NOS inhibition are not able to inhibit the relaxation induced by EFS. The nitrergic slow component of the IJP is probably able to cause a sustained hyperpolarization underlying the mechanical relaxation.[33] It is important to note that AR-C66096, a $P2Y_{12}$ receptor antagonist, did not modify the neural-mediated inhibitory responses. $P2Y_{12}$ receptor antagonists are in development as antithrombotic agents.[19] In the present article, we show for the first time that they do not inhibit inhibitory neuromuscular transmission in the human colon.

We strongly believe that it is mandatory to translate data obtained in animals to humans as important differences between species have been reported. In rodents, MRS2179 is comparatively less potent inhibiting the IJP[8,9,36] than in humans,[3,33] pigs [7] or guinea-pigs.[6] However, when MRS2279 and MRS2500 were tested, the fast component of the IJP in the rat colon was completely inhibited by very low concentrations of these antagonists.[8] The rank of potency of the antagonists is similar in both rats and humans (MRS2500 > MRS2279 > MRS2179). MRS2179 is comparatively more potent in humans (IC_{50} 1.2 μmol L^{-1})[3] than in rats (IC_{50} 13.1 μmol L^{-1}),[8] but both MRS2279 and MRS2500 are more potent in rats (17.8 nmol L^{-1} and 14 nmol L^{-1} respectively)[8] than in humans (280 nmol L^{-1} and 71 nmol L^{-1}, respectively) (present study). Differences in the structure of the receptor between species might be responsible for these findings. An important difference between these antagonists is that MRS2179 is reversed on washout (after 30 min washing with Krebs solution) but MRS2279 and MRS2500 are not washable. This might be due to varying sensitivity to ectonucleotidases.[37]

To test the efficacy of MRS antagonists on $P2Y_1$ receptors we used HEK-293 cells that endogenously express $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors.[38] It has been previously reported that calcium increase, using fura-2 microfluometry, induced by ADPβS was blocked by 30 μmol L^{-1} MRS2179. This effect is probably due to $P2Y_1$ receptor activation. In contrast, UTP, a preferential $P2Y_{2,4}$, agonist, induced a calcium increase that was not reduced by high concentrations (30 μmol L^{-1}) of MRS2179.[25] MRS2179 was a competitive antagonist in HEK-293 cells transfected with the $P2Y_1$ guinea-pig receptor.[39] In line with these results, our data show that calcium increase induced by ADPβS in HEK-293 cells was concentration-dependently decreased by $P2Y_1$ antagonists. The IC_{50} was 2.9 nmol L^{-1} (MRS2500), 4.6 nmol L^{-1} (MRS2279) and 0.12 μmol L^{-1} (MRS2179). Due to the high selectivity and potency of these antagonists,[40] it seems clear that $P2Y_1$ receptors are implicated in the response. It is important to notice that the range of concentration varies greatly when the antagonist is used in experiments with dispersed cells (calcium transients elicited with ADPβS 1 μmol L^{-1}) and experiments, where tissue is used and the response induced by the endogenous neurotransmitter is measured. In this case, data from microelectrode and muscle bath are quite similar.

As we previously reported, ADPβS causes an inhibition of spontaneous motility and a transient hyperpolarization.[3] Both effects were inhibited by MRS2179[3] and in the present study; we have demonstrated that they are also inhibited with MRS2279 and MRS2500. $P2Y_1$ antagonists tested in the present study are selective for $P2Y_1$ receptors.[2] However, the orphan receptor GPR17 is also blocked by low concentrations of MRS2179.[41] GPR17 is coupled with Gi proteins leading to both adenylyl cyclase inhibition and calcium increase. However, GPR17 is not activated by either ATP or ADP. Moreover, GPR17 agonists such as UDP-glucose, which is also an agonist of $P2Y_{14}$ receptors, cause gastric smooth muscle contractions, which are not recorded in $P2Y_{14}$ KO mice.[42] Altogether

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these results suggest that GPR17 is probably not involved in smooth muscle hyperpolarization and relaxation although further experiments are needed to characterize its effect on smooth muscle. In the present study, we demonstrate that MRS2365, a preferential $P2Y_1$ agonist, hyperpolarises and transiently inhibits spontaneous motility, mimicking endogenous IJP and the corresponding mechanical inhibition. This effect is antagonised by $P2Y_1$ antagonists confirming the involvement of this receptor in the inhibitory pathways in the human colon.

The nature of the neurotransmitter involved in purinergic response is still unknown and most articles use the term 'ATP or a related purine', which encompasses several putative purinergic neurotransmitters. It has been recently suggested that β-NAD released by enteric nerves and acting postjunctionally on $P2Y_1$ receptors fits the criteria to be considered an inhibitory neurotransmitter.[14,22] Although in the present study we did not measured endogenous release of purines our data with exogenous addition of β-NAD are similar to those recently reported: (i) Exogenous addition of β-NAD causes inhibition of spontaneous motility with an IC_{50} in the mM range, and (ii) β-NAD causes a slight hyperpolarization of human colonic smooth muscle cells (50 mmol L^{-1}: about 6–7 mV[22] *vs* 10 mmol L^{-1} : 3 mV in the present study), and (iii) β-NAD -induced hyperpolarization is sensitive to the $P2Y_1$ antagonist MRS2500. These data suggest that β-NAD might be an endogenous inhibitory mediator in the human colon acting on $P2Y_1$ receptors. However, (i) β-NAD-induced inhibition of spontaneous motility was not antagonized by MRS2279 or MRS2500. This suggests that β-NAD-induced relaxation independently of the membrane potential. Moreover, β-NAD (10 mmol L^{-1}) induced a very low response both in number of responding cells and in amplitude of the response in HEK-293 cells that constitutively express the $P2Y_1$ receptor. This might explain why the hyperpolarization induced by high concentrations of β-NAD is comparatively smaller than those obtained with MRS2365 (1 μmol L^{-1}). Alternatively, activation of non-selective cation channels possibly not located at the neuromuscular junction might counterbalance the hyperpolarization caused by exogenous administration of the compound.[22] However, a contractile response has never been observed in our muscle bath recordings. Further studies comparing endogenous release of purines *vs* activation of $P2Y_1$ receptors are needed to evaluate the relative contribution of ATP/ADP and β-NAD as inhibitory neurotransmitters in the GI tract.

In conclusion, our data support that $P2Y_1$ receptors mediate the fast component of the IJP and the purinergic relaxation. $P2Y_1$ antagonists show a rank of potency: MRS2500 > MRS2279 > MRS2179 in all three techniques tested. Preferential $P2Y_1$ agonists such as ADPβS and MRS2365 mimicked the effect of the endogenous mediator. These antagonists are valuable pharmacological tools to study purinergic neurotransmission in the GI tract and to help in the development of future putative treatments, where purinergic neurotransmission is impaired.

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AUTHOR CONTRIBUTION

DG, VG, JA and MMC, designed the protocol and performed the experiments. DG, VG and MJ wrote the manuscript. PC organized tissue donation and provided important intellectual insight into this study. MJ planned the study and obtained funding.

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Chapter 3

Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon

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Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon

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[1]*Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra;* [2]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberehd), Instituto de Salud Carlos III, Barcelona, Spain, and* [3]*Department of Pharmacology and Physiology, Universidad de Zaragoza, Zaragoza, Spain*

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Gil V, Gallego D, Grasa L, Martín MT, Jiménez M. Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. *Am J Physiol Gastrointest Liver Physiol* 299: G158–G169, 2010. First published April 15, 2010; doi:10.1152/ajpgi.00448.2009.—Nitric oxide (NO) and ATP mediate smooth muscle relaxation in the gastrointestinal tract. However, the involvement of these neurotransmitters in spontaneous neuronal activity is unknown. The aim of the present work was to study spontaneous neuromuscular transmission in the rat midcolon. Microelectrode experiments were performed under constant stretch both in circular and longitudinal directions. Spontaneous inhibitory junction potentials (sIJP) were recorded. Tetrodotoxin (1 μM) and apamin (1 μM) depolarized smooth muscle cells and inhibited sIJP. N^{ω}-nitro-L-arginine (L-NNA, 1 mM) depolarized smooth muscle cells but did not modify sIJP. In contrast, the $P2Y_1$ antagonist MRS-2500 (1 μM) did not modify the resting membrane potential (RMP) but reduced sIJP (IC_{50} = 3.1 nM). Hexamethonium (200 μM), NF-023 (10 μM), and ondansetron (1 μM) did not modify RMP and sIJP. These results correlate with in vitro (muscle bath) and in vivo (strain gauges) data where L-NNA but not MRS-2500 induced a sustained increase of spontaneous motility. We concluded that, in the rat colon, inhibitory neurons regulate smooth muscle RMP and cause sIJP. In vitro, the release of inhibitory neurotransmitters is independent of nicotinic, P2X, and 5-hydroxytryptamine type 3 receptors. Neuronal NO causes a sustained smooth muscle hyperpolarization that is responsible for a constant inhibition of spontaneous motility. In contrast, ATP acting on $P2Y_1$ receptors is responsible for sIJP but does not mediate inhibitory neural tone. ATP and NO have complementary physiological functions in the regulation of gastrointestinal motility.

gastrointestinal; $P2Y_1$ receptors; nitric oxide; spontaneous inhibitory junction potential

JUNCTION POTENTIALS ARE THE electrophysiological base of communication between enteric motor neurons and smooth muscle cells. Excitatory junction potentials (EJPs) cause smooth muscle depolarization and contractions, whereas inhibitory junction potentials (IJPs) cause smooth muscle hyperpolarization and relaxation. Both EJPs and IJPs can be elicited by electrical field stimulation (EFS). Junction potentials evoked by EFS have been a crucial methodology to study the neurotransmitters and receptors involved in the communication between motor neurons and smooth muscle cells. Spontaneous junction potentials were described for the first time in the guinea pig colon (11). Both EJP and IJP of ~8 and 3 mV, respectively, were recorded in a small proportion of smooth muscle cells and showed a similar time course as those evoked by EFS (11).

Reflexes and neural motor patterns involve several structural elements, including different subclasses of enteric neurons, interstitial cells of Cajal, and the coordination of smooth muscle layers. The mechanisms underlying these neural pathways are still poorly characterized. Distention and mucosal stimulation are stimuli usually required to elicit a reflex or a motor pattern. These stimuli are much more "physiological" than EFS to study neuromuscular transmission. For example, balloon distention causes ascending EJPs and descending IJPs in the guinea pig ileum (27), and a constant circumferential stretch causes activation of a motor pattern in the guinea pig colon involving ascending EJPs and descending IJPs (29). Probably both distention and mucosa deformation elicit neural pathways that converge on common motor neurons causing smooth muscle contraction or relaxation and are usually termed "spontaneous" junction potentials.

It is well known that EFS-induced IJP involves a fast followed by a slow component (6, 12, 16, 18, 24). Apamin, a calcium-sensitive K^+ (sK_{Ca}) channel blocker, has been a pharmacological tool used to distinguish between the fast and the slow component of the IJP in several species, including the rat colon (24). Furthermore, the fast component is abolished by $P2Y_1$ receptor antagonist (8, 13, 14, 24, 34), whereas the slow component is N^{ω}-nitro-L-arginine (L-NNA) sensitive and therefore nitric oxide (NO) mediated (18, 24, 33). Accordingly, a cotransmission process between ATP or a related purine and NO has been proposed with different functions for each neurotransmitter (12). Activation of $P2Y_1$ receptor might mediate phasic relaxation, whereas NO might mediate tonic sustained relaxation. Spontaneous and evoked IJPs might have different properties (28) although it is conceivable that the fast component of the IJP might have similar properties to spontaneous IJP because both of them are apamin sensitive (28).

Accordingly, we have studied the ongoing release of inhibitory neuromuscular transmitters in the rat colon that elicits spontaneous IJPs and modulates resting membrane potential (RMP). Briefly, we found that neural-mediated spontaneous IJPs are MRS-2500 sensitive and therefore due to $P2Y_1$ receptor activation; in contrast, L-NNA depolarize smooth muscle cells without a major effect on spontaneous IJP. These data correlate well with mechanical data both in vitro and in vivo where NO but not ATP mediates tonic muscular inhibition. It is concluded that both neurotransmitters are released by inhibitory motor neurons but they have complementary physiological functions.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (8–10 wk old, 300–350 g) were purchased from Charles River (Lyon, France). Animals were housed under controlled conditions: temperature 22 ± 2°C, humidity 55 ± 10%, 12:12-h light-dark cycle, and access to water and food ad libitum. All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Tissue Samples

All animals were immediately rendered unconscious by stunning and were decapitated within 2–3 s afterward. The colon was quickly removed and placed in carbogenated physiological saline solution. Afterward, it was opened along the mesenteric border and pinned to a Sylgard base (mucosa side up). The midcolon was identified accordingly to anatomical criteria previously described (1). The mucosal and submucosal layers were removed, and circular muscle strips were cut 1 cm long and 0.3 cm wide.

Intracellular Microelectrode Recording

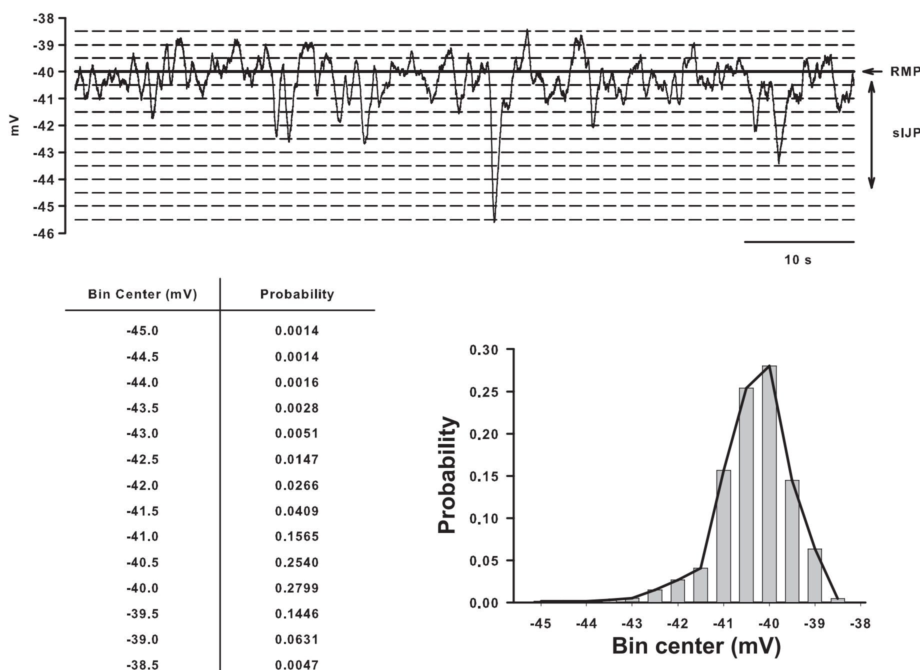
The tissue was pinned with the circular muscle layer facing upward in a Sylgard-coated chamber, and manual stretch (2.1 ± 0.1-fold increase in the length of the circular axis and 1.8 ± 0.1-fold increase in the length of the longitudinal axis, $n = 38$) was applied. Strips were continuously perfused with carbogenated physiological saline solution at 37 ± 1°C and were allowed to equilibrate for 1 h. Circular smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl (30–60 MΩ of resistance). Membrane potential was measured by using the standard electrometer Duo773 (WPI, Sarasota, FL). Tracings were displayed on an oscilloscope 4026 (Racal-Dana, Windsor, England) and simultaneously digitalized (100 Hz) with a PowerLab 4/30 system and Chart 5 software for Windows (both from ADInstruments, Castle Hill, NSW, Australia). To maintain stable impalements, experiments were performed in the presence of nifedipine (1 μM). Spontaneous IJPs (sIJP) were measured by two different methods: *1*) frequency distribution (0.5-mV bins) of the values of the membrane potential (30–60 s) expressed as bin probability from 0 to 1. In this case, when sIJP are recorded, the frequency distribution has a tail toward the most negative values (Fig. 1), and, when sIJP are inhibited, the tail is not present; and *2*) according to this difference in the frequency distribution, the standard deviation (SD; expressed in mV) indicative of the presence of sIJP was measured using the following expression: SD of the recording inside the cell − SD of the recording outside the cell (both of them for a period of at least 30–60 s). Mean ± SD has been previously described as a valid method to measure sIJP because the amplitude of sIJP is correlated ($r^2 = 0.83$) to the SD of the recording (25). Because of the presence of ongoing sIJP, the RMP cannot be estimated measuring the mean of the recording. Accordingly, the RMP (expressed in mV) was calculated as the most probable bin of the frequency distribution (0.1-mV bins; 30-60-s recordings) (Fig. 1).

Muscle Bath Studies

Muscle strips were mounted in a 10-ml organ bath containing carbogenated physiological saline solution maintained at 37 ± 1°C. Motility was measured using an isometric force transducer (Harvard VF-1 Harvard Apparatus, Holliston, MA) connected to a computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. A tension of 1 g was applied, and tissues were allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. The area under the curve (AUC) of contractions from the baseline was measured to estimate the mechanical activity, and the result was expressed in grams per minute (g/min).

In Vivo Motility Studies

Animal preparation. After a fasting period of 6 h, animals were anesthetized by inhalation of isofluorane to allow cannulation of the right jugular vein as previously described (31). Level III of anesthesia was maintained with intravenous thiopental sodium as required, and rats were tracheotomized to facilitate spontaneous breathing. Body temperature was maintained at 37°C by placing the animal on a heating pad. A laparatomy was performed to suture a strain-gauge (3 × 5 mm; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) to the wall of the colon (2 cm from de cecum) to record circular muscle activity. The strain gauge was connected to



Bin Center (mV)	Probability
-45.0	0.0014
-44.5	0.0014
-44.0	0.0016
-43.5	0.0028
-43.0	0.0051
-42.5	0.0147
-42.0	0.0266
-41.5	0.0409
-41.0	0.1565
-40.5	0.2540
-40.0	0.2799
-39.5	0.1446
-39.0	0.0631
-38.5	0.0047

Fig. 1. *Top*: example of microelectrode recording with spontaneous inhibitory junction potentials (sIJP). Intervals of 0.5 mV (*top*) were used to calculate the probability of data in each interval (Table). The value of probability of each interval was plotted vs. the bin center. sIJP were estimated using the SD (mV) of the original recording (see MATERIALS AND METHODS), and the resting membrane potential (RMP) was considered the value of membrane potential with the highest probability (0.1-mV bins in this case).

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a high-gain amplifier (MT8P; Lectromed, Herts, UK), and signals were sent to a recording unit connected to a computer (PowerLab/800; ADInstruments).

Evaluation of motor parameters. The effects of both MRS-2500 (0.2 μmol/kg) and L-NNA (10 μmol/kg) were evaluated in a separate set of animals. After an equilibration period of 30 min, the amplitude and frequency of contractions were determined before and after intravenous administration of drugs. Amplitude of a single contraction was determined by measuring the AUC delimited by the tracing, and the result was expressed in square millimeters (mm^2). To test the effects of MRS-2500 and L-NNA, the mean amplitude of the five consecutive contractions appearing just before the drug administration was compared with the mean amplitude of the five consecutive contractions appearing after the drug administration. To determine the effects of both MRS-2500 and L-NNA in the frequency, contractions recorded during the 10 min before and after drug administration were counted, and the results were expressed as number of contractions per hour.

Solutions and Drugs

The composition of the physiological saline solution was (in mM) 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 1.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3–7.4). In all of the experiments, physiological saline solution contained atropine, phentolamine, and propanolol (1 μM each one) and was continuously bubbled with carbogen (95% O_2 and 5% CO_2). The following drugs were used: apamin, hexamethonium, nifedipine, L-NNA, and phentolamine (Sigma Chemicals, St. Louis, MO); atropine sulfate (Merck, Darmstadt, Germany); and (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 (MRS-2500), 8,8′-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulfonic acid, hexasodium salt (NF-023), ondansetron hydrochloride, propanolol (Tocris, Bristol, UK). Stock solutions were made by dissolving drugs in distilled water except for nifedipine, which was dissolved in 96% ethanol, and L-NNA, which was dissolved in physiological saline solution by sonication.

Data Analysis and Statistics

Differences in the RMP, sIJP, and motility (in vitro and in vivo) before and after drug infusion were compared by a paired Student's *t*-test. One-way ANOVA was used *1*) to evaluate the effect of MRS-2500 at different concentrations on IJPs; and *2*) to test the effect of L-NNA and MRS-2500 on the amplitude of contractions in in vivo experiments.

Data are expressed as means ± SE. A $P < 0.05$ was considered statistically significant; "*n*" values indicate the number of samples. Statistical analysis was performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

RESULTS

Characterization of sIJP and RMP

sIJP were recorded without any kind of EFS (Fig. 1). It is important to note that, in electrophysiological experiments, nifedipine (1 μM) was added to the Krebs solution to abolish spontaneous cyclic depolarizations and contractions (see below). Spontaneous IJP had variable amplitude (from <1 mV and up to 20 mV); therefore, the amplitude of individual sIJP was very difficult to measure. To quantify sIJP, two different methodologies were used (25). When the recordings displayed spontaneous inhibitory junction potential (sIJP), we observed: *1*) the presence of a tail toward the most negative values in the frequency distribution of the membrane potential; and *2*) a higher SD of the values of membrane potential. Figure 1 shows the frequency distribution of the membrane potential. To evaluate the "resting" membrane potential when sIJP were present in the recordings, we calculated the value (in mV) with a highest probability. In control conditions, circular smooth muscle cells had a mean RMP of −41.8 ± 0.7 mV ($n = 38$), and the SD was 0.77 ± 0.05 ($n = 38$).

Neural Regulation of sIJP and RMP

Tetrodotoxin (TTX, 1 μM) significantly depolarized 4.7 ± 0.4 mV smooth muscle cells and inhibited sIJP (Table 1 and Fig. 2*A*). Figure 2*B* shows the depolarization and inhibition of sIJP. Notice that, in the presence of TTX: *1*) the value of maximum probability is shifted to the right, indicative of the depolarization of the tracing; *2*) the tail observed in the negative values (sIJP arrow in control) is absent when the tissue is incubated with TTX; *3*) the SD is lower compared with control; and *4*) the value of maximum probability reaches the highest values when sIJP are absent, indicative of a more constant recording. These data demonstrate that both RMP and sIJP are regulated by inhibitory myenteric neurons.

Role of NO and Purinergic Neurotransmission on the Regulation of RMP and sIJP

The NO synthase inhibitor L-NNA (1 mM) depolarized 5.3 ± 0.7 mV smooth muscle cells but did not modify sIJP (Table 1 and Fig. 3*A*). In contrast, the selective $P2Y_1$ antagonist MRS-2500 (1 μM) did not modify the RMP membrane potential but inhibited sIJP (Table 1 and Fig. 3*A*). Notice that, in the presence of L-NNA, the value of maximum probability is shifted to the right, indicative of the depolarization without a

Table 1. *Effect of drug addition on resting membrane potential and spontaneous IJPs*

	n	RMP, mV			SD, mV		
		Control	Drug	*P* Value	Control	Drug	*P* Value
TTX	5	−41.8 ± 1.9	−37.2 ± 2.0	<0.001	0.97 ± 0.12	0.14 ± 0.03	<0.01
L-NNA	14	−40.8 ± 1.3	−35.4 ± 1.3	<0.001	0.71 ± 0.08	0.70 ± 0.09	NS
MR-S2500	5	−41.5 ± 1.9	−39.7 ± 2.0	NS	0.92 ± 0.17	0.21 ± 0.06	<0.01
Hexamethonium	5	−40.9 ± 2.0	−39.7 ± 2.4	NS	0.72 ± 0.14	0.68 ± 0.14	NS
Hex + NF023 + Ond	5	−41.2 ± 2.8	−40.8 ± 2.9	NS	0.75 ± 0.12	0.75 ± 0.08	NS
Apamin	6	−42.1 ± 1.3	−37.4 ± 1.3	<0.05	0.66 ± 0.11	0.22 ± 0.06	<0.01

Values are means ± SE; *n*, no. of samples. IJP, inhibitory junction potential; Hex, hexamethonium; Ond, ondansetron; RMP, resting membrane potential; SD, SD of the membrane potential; TTX, tetrodotoxin; L-NNA, N^{ω}-nitro-L-arginine. The statistical significance of differences was assessed by using paired Student's *t*-test. NS, not significant.

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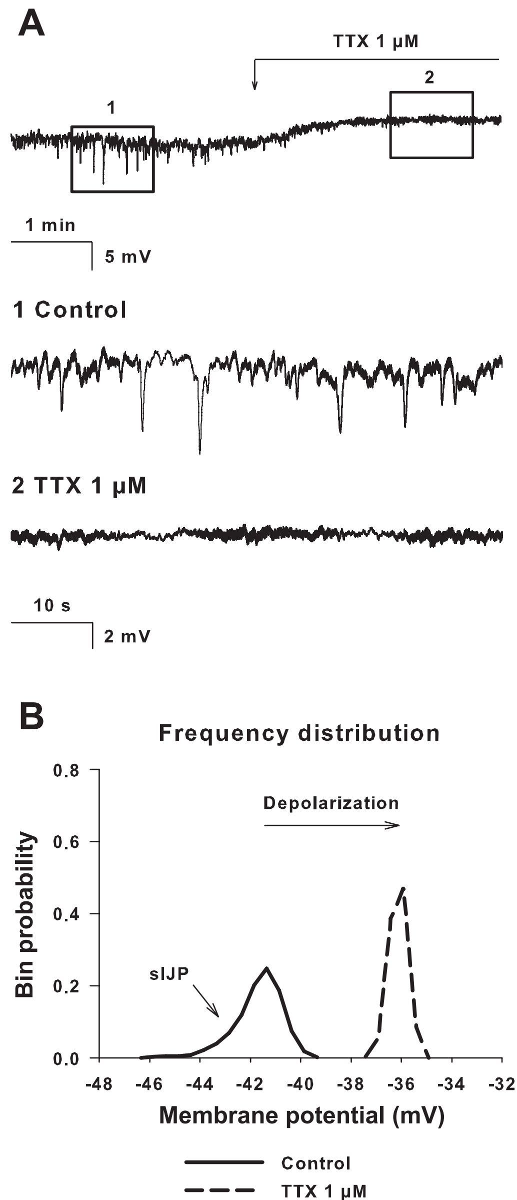

Fig. 2. *A*: intracellular microelectrode recordings showing the effect of tetrodotoxin (TTX, 1 μM) on RMP and sIJP. Expanded traces represent sIJP in control conditions (1) and after TTX (1 μM) administration (2). *B*: frequency distribution (0.5-mV bins) of the membrane potential (60-s recording) from a circular smooth muscle cell in control conditions (solid line) and after TTX (1 μM) administration (short broken line).

major modification of the frequency distribution of the membrane potential (Fig. 3*B*). In contrast, MRS-2500 mainly changes the frequency of distribution of the membrane potential, indicative of inhibition of sIJP without a major shift of the value of maximum probability (Fig. 3*B*). These data demonstrate that the RMP is mainly NO regulated, whereas sIJP are purinergic in origin.

Role of Nicotinic, P2X, and 5-Hydroxytryptamine Type 3 Receptors on the Regulation of RMP and sIJP

Hexamethonium (200 μM) did not depolarize smooth muscle cells and did not modify sIJP (Table 1 and Fig. 4). Hexamethonium (200 μM), NF-023 (10 μM), and ondansetron (1 μM) were used together to block nicotinic, P2X, and 5-hydroxytryptamine type 3 (5-HT_3) receptors, respectively. Combination of these drugs did not change either the RMP or sIJP (Table 1 and Fig. 4). These data demonstrate a minor role of nicotinic, P2X, and 5-HT_3 receptors in the regulation of the RMP and sIJP.

Neural Regulation of Spontaneous Motility In Vitro

Circular muscle strips of the rat midcolon displayed spontaneous rhythmic contractions with a mean AUC of 13.5 ± 1.2 g/min (n = 40). TTX (1 μM) induced an important increase of the spontaneous motility (Table 2 and Fig. 5*A*). A similar increase was observed when the tissue was incubated with L-NNA (1 mM) (Table 2 and Fig. 5*B*). In contrast, MRS-2500 produced a slight decrease of mechanical activity (Table 2 and Fig. 5*C*). It is important to note that preliminary experiments were performed with different degrees of stretch: 1, 2, 3, and 4 g in the circular direction. Data (L-NNA, n = 5 each and MRS-2500, n = 5 each, data not shown) did not show major differences between the different degrees of stretch, and, accordingly, data obtained at 1 g of stretch are reported in the present work. Hexamethonium (200 μM) slightly increased the motility (Table 2 and Fig. 5*D*). Addition of NF-023 (10 μM), ondansetron (1 μM), and hexamethonium (200 μM) also produced a slight increase of the motility (Table 2 and Fig. 5*E*).

Interaction Between Nitrergic and Purinergic Neurotransmission in RMP, sIJP, and Spontaneous Contractions

To evaluate the putative interaction between P2Y_1 receptors and NO, the effect of L-NNA was tested in the presence of MRS-2500. The depolarization induced by L-NNA (1 mM) was not affected by pretreating the tissue with MRS-2500 (1 μM) (8.1 ± 1.4 mV depolarization, MRS-2500: −39.8 ± 4.7 vs. L-NNA + MRS-2500: −31.7 ± 3.5 mV, $P < 0.01$, n = 6; Fig. 6*A*). Notice that MRS-2500 abolished spontaneous IJP, and shift to the right in the frequency distribution of the membrane potential (indicative of depolarization) is observed after L-NNA (Fig. 6*B*). Moreover, L-NNA (1 mM) induced an important increase in the spontaneous motility when it was added to the muscle bath in the presence of MRS-2500 (1 μM) [MRS-2500: 11.4 ± 2.3 vs. L-NNA + MRS-2500: 40.2 ± 9.3 g/min (AUC), $P < 0.05$, n = 7; Fig. 6*C*].

MRS-2500 (1 μM) in the presence of L-NNA (1 mM) produced a similar response to that observed with MRS-2500 (1 μM) alone. A slight depolarization was observed (0.9 ± 0.1 mV, L-NNA: −34.6 ± 1.3 vs. MRS-2500 + L-NNA: −33.7 ± 1.3 mV, $P < 0.01$, n = 4), but the more prominent effect was the inhibition of sIJP [L-NNA: 0.76 ± 0.11 vs. MRS-2500 + L-NNA: 0.12 ± 0.06 (SD) mV, $P < 0.05$, n = 4; Fig. 6*A*]. Notice the change in the frequency distribution of the membrane potential without a major change in RMP (Fig. 6*B*). To characterize the effect of MRS-2500 on sIJP, a concentration-response curve was performed in the presence of L-NNA. As shown in Fig. 7, the MRS-2500 concentration-response inhibited sIJP (IC_{50} = 3.1 nM, 95% confidence interval 1.6–5.9 nM, log IC_{50} = −8.5 ± 0.1, n = 5). Furthermore, MRS-2500 (1 μM) caused a slight decrease in spontaneous contractions in

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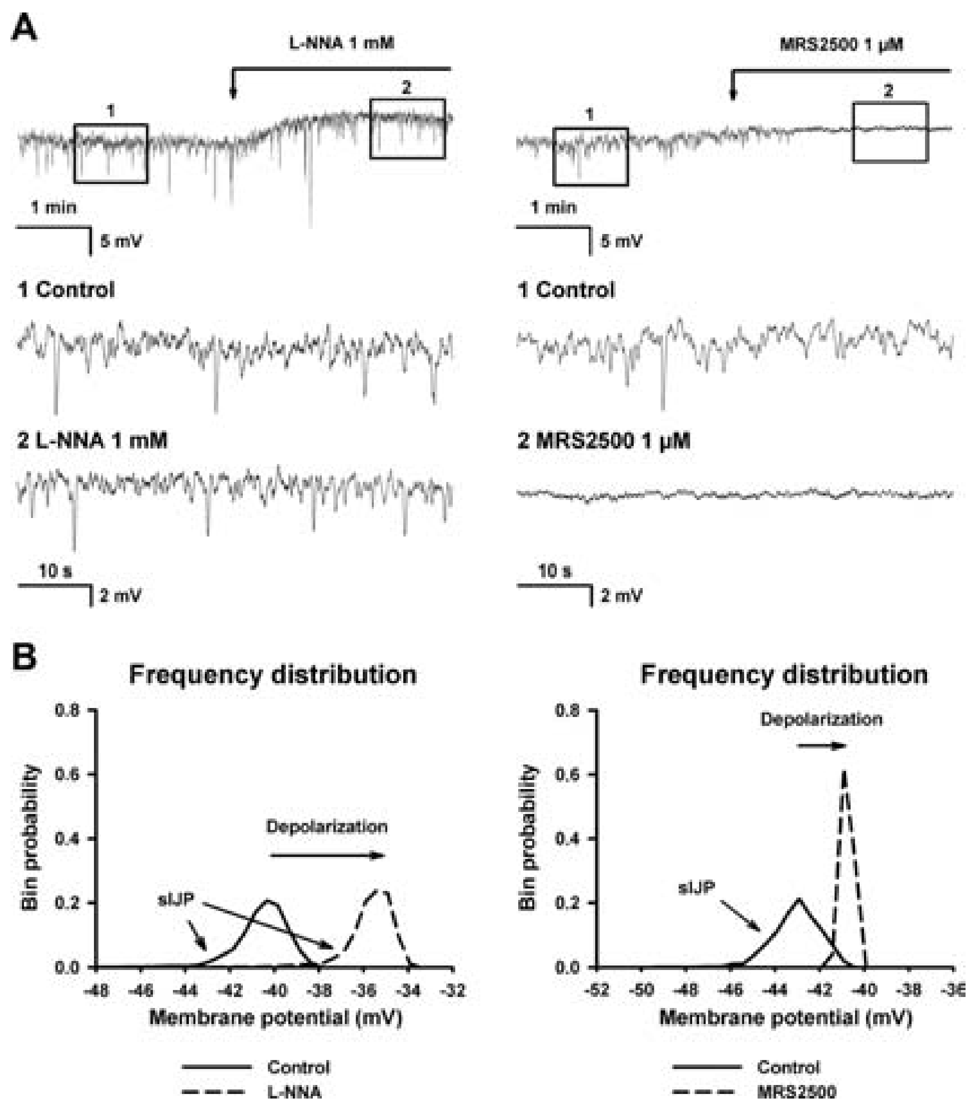

Fig. 3. *Top*: intracellular microelectrode recordings showing the effect of N^{ω}-nitro-L-arginine (L-NNA) (1 mM) (*left*) and MRS-2500 (1 μM) (*right*) on RMP and sIJP. Expanded traces represent sIJP in control conditions (1) and after L-NNA (1 mM) (*left*) and MRS-2500 (1 μM) (*right*) administration (2). *Bottom*: frequency distribution (0.5-mV bins) of the membrane potential (60-s recording) from a circular smooth muscle cell in control conditions (solid line) and after L-NNA (1 mM) administration (short broken line) (*left*) and in control conditions (solid line) and after MRS-2500 (1 μM) administration (short broken line) (*right*).

the presence of L-NNA (1 mM) [L-NNA: 28.8 ± 7.6 vs. MRS-2500 + L-NNA: 26.4 ± 7.1 g/min (AUC), $P > 0.05$, $n = 4$, Fig. 6*C*].

Effect of Apamin on RMP, sIJP, and Spontaneous Mechanical Activity

Apamin, an sK_{Ca} channel blocker, has been a pharmacological tool to distinguish between the fast and the slow component of the IJP. Apamin (1 μM) inhibited sIJP and caused smooth muscle depolarization (Table 1 and Fig. 8). Moreover, apamin increased the spontaneous motility (Table 2 and Fig. 8).

Role of NO and $P2Y_1$ Receptors on In Vivo Spontaneous Motility

Spontaneous contractions were measured in vivo with strain gauge transducers. Spontaneous motor activity was characterized by isolated phasic contractions with a regular frequency of 25.5 ± 6.5 contractions/h ($n = 8$). L-NNA (10 μmol/kg) dramatically increased the frequency of spontaneous contractions (frequency of contractions, control: 22.5 ± 8.6 vs. L-NNA: 60.0 ± 8.8 h^{-1}, $P < 0.05$, $n = 4$) and increased the basal tone (0.5 ± 0.1 g, $P < 0.05$, $n = 4$) but did not modify the amplitude of spontaneous contractions. The $P2Y_1$ antagonist MRS-2500 (0.2 μmol/kg) did not modify either the basal tone or the frequency of spontaneous contractions measured in vivo with the strain gauge transducer. In contrast, a single contraction with high amplitude ($n = 4$, ANOVA, $P < 0.05$) was measured in all of the recordings, but the following contractions had the same amplitude and frequency as those recorded in the basal state (Fig. 9).

DISCUSSION

In the present paper, we demonstrate that spontaneous neuronal activity causes the release of ATP or a related purine and NO with complementary physiological functions: *1*) $P2Y_1$ receptors are responsible for the spontaneous IJP with a minor effect on the regulation of the membrane potential; *2*) tonically released NO regulates the membrane potential; and *3*) NO inhibition but not $P2Y_1$ blockade increases spontaneous motility both in vitro and in vivo. Furthermore, in vitro, neuronal

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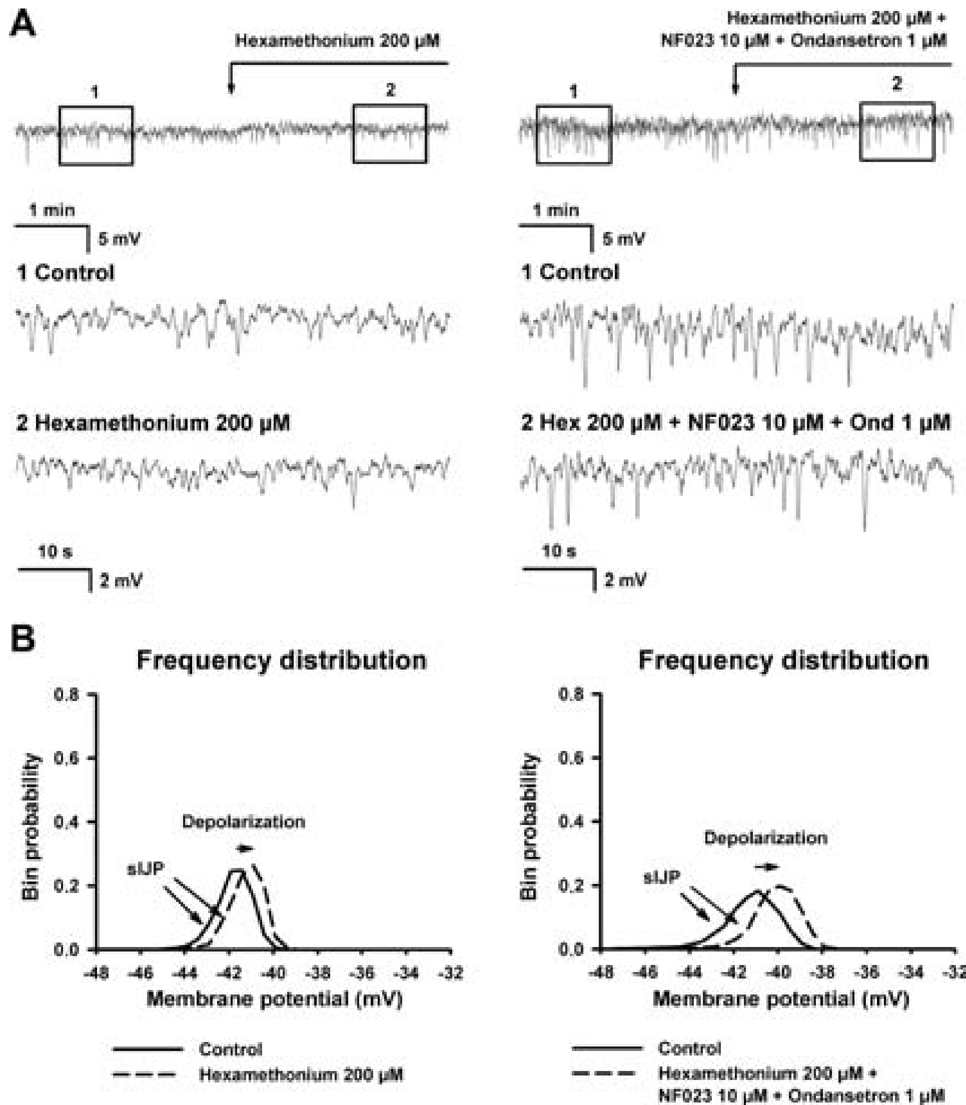

Fig. 4. *Top*: intracellular microelectrode tracings showing the effect of hexamethonium (200 μM) (*left*) and hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) (*right*) on RMP and sIJP. Expanded recordings represent sIJP in control conditions (1) and after hexamethonium (200 μM) (*left*) and hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) (*right*) administration (2). *Bottom*: frequency distribution (0.5-mV bins) of the membrane potential (60-s recording) from a circular muscle cell in control conditions (solid line) and after hexamethonium (200 μM) administration (short broken line) (*left*) and in control conditions (solid line) and after hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) administration (short broken line) (*right*).

activation is partially independent of synaptic inputs involving nicotinic, P2X, and 5-HT_3 receptors.

Quantification of Spontaneous IJP and RMP

The amplitude of sIJP was quite variable, ranging from <1 to ~20 mV; therefore, the amplitude of individual sIJP was difficult to measure. To illustrate and quantify spontaneous IJP, we used two methodologies previously reported (25): *1*) the presence of a tail in the frequency distribution of the membrane potential; and *2*) the SD of the membrane potential. When spontaneous IJP were recorded, the frequency distribution of the values of the tracing had a tail toward the most negative values, and, accordingly, an increase in the SD of the points is obtained. The presence of this tail is illustrative of the sIJP, and it disappears when sIJP are inhibited (see Figs. 1 and 2). SD was measured to quantify sIJP and used for statistical purposes.

Table 2. *Effect of drug addition on spontaneous motility*

		Spontaneous Motility (AUC), g/min			Drug Effect*	
	n	Control	Drug	*P* Value	AUC, g/min	Percentage
TTX	8	10.9 ± 2.5	25.6 ± 5.6	<0.05	14.7 ± 4.2	172.9 ± 36.9
L-NNA	16	10.2 ± 1.4	27.3 ± 3.5	<0.001	17.2 ± 2.4	199.0 ± 33.0
MRS-2500	16	16.1 ± 1.6	13.1 ± 1.5	<0.001	−3.1 ± 0.5	−19.6 ± 2.3
Hexamethonium	7	19.1 ± 4.7	20.7 ± 4.7	<0.05	1.6 ± 0.5	11.6 ± 4.2
Hexamethonium + NF-023 + ondansetron	7	15.2 ± 4.6	19.4 ± 5.0	<0.001	4.2 ± 0.6	35.7 ± 7.8
Apamin	5	21.7 ± 3.5	41.1 ± 4.1	<0.001	19.4 ± 2.0	97.9 ± 16.8

Values are means ± SE; *n*, no. of samples. AUC, area under the curve. The statistical significance of differences was assessed by using paired Student's *t*-test. *Drug effect on spontaneous mechanical activity was expressed as net effect (AUC after drug administration − AUC in control) and percentage [(AUC after drug administration − AUC in control)/(AUC in control)·100].

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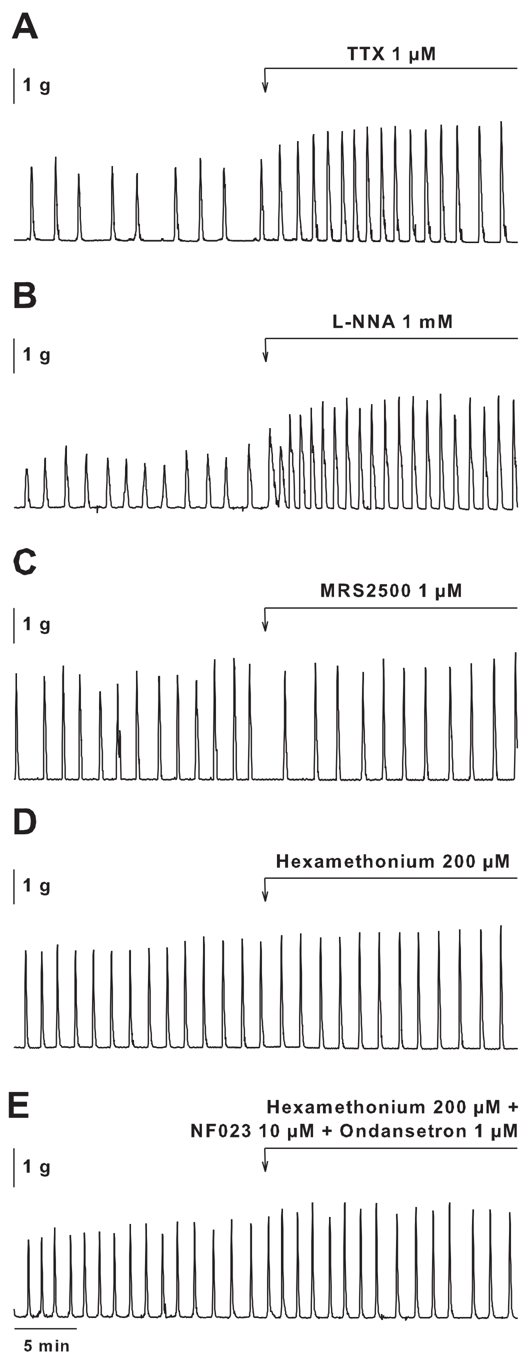

Fig. 5. Mechanical recordings showing the effect of TTX (1 μM) (*A*), L-NNA (1 mM) (*B*), MRS-2500 (1 μM) (*C*), hexamethonium (200 μM) (*D*), and hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) (*E*) on spontaneous motility in vitro.

It has been previously shown that SD correlates well with the amplitude of the sIJP, and therefore, when spontaneous sIJP are inhibited, SD decreases (25). Another difficulty has been to calculate the RMP during ongoing release of inhibitory neurotransmitters. A possible approach was to measure the mean of the values of the recordings. However, this would have been inappropriate because, when sIJP were recorded, the value of RMP would have been lower than those found in the absence of sIJP. To avoid this difficulty, we calculated the value with maximum probability of the frequency distribution (0.1 mV), and we assume that this was the RMP of the cell. The difference in the estimation of the RMP and the way to stretch the tissue might contribute to the difference between the value of RMP found in the present study and our previous published data (14, 24).

Spontaneous Junction Potentials

The first consideration that should be taken into account is what should be considered as spontaneous IJP? Because of the fact that EFS is the most common methodology to elicit IJP, the term spontaneous IJP has been often employed in the literature in opposition to those IJPs that were elicited by EFS. In our recordings, spontaneous IJPs were possibly stretch-dependent because we could record them when a significant stretch was applied to the preparation. However, this was hard to demonstrate because, to impale smooth muscle cells with a microelectrode and to obtain a stable recording, a certain degree of stretch is always necessary. Nonstretched tissues are impossible to impale. Accordingly, it is very hard to demonstrate a putative origin of stretch in spontaneous IJP. Monitorized tissue stretcher is an excellent approach to demonstrate stretch-induced responses, since it has been demonstrated in the guinea pig ileum (3). In this tissue, the major response to stretch are EJPs rather than IJPs (3). It is possible that the responses found in the present study are similar to those described in the guinea pig colon (28) because *1*) sIJP are present with a constant stretch; *2*) they can be recorded in the presence of nifedipine; and *3*) they are TTX sensitive, showing that they are originated by neural-mediated activity.

Origin of Spontaneous IJP

The aim of the present work has been to study the neuromuscular interaction. However, we also investigated the putative origin of the mechanisms that elicit spontaneous IJP. The majority of the studies investigating the electrophysiological properties, structure, and function of enteric neurons have been done in the guinea pig. It is difficult to establish the basis of the neural activity underlying the mechanism that generates spontaneous IJP in the rat colon. However, an important study demonstrates the presence of S neurons and AH neurons with some similarities to those described in the guinea pig (4). An important difference between both species is the absence of muscarinic slow (s) excitatory postsynaptic potential (EPSP) in the rat colon, which should be taken into account since our experiments were performed in the presence of atropine because we wanted to characterize the inhibitory neurotransmission. In the rat colon, AH neurons drive action potentials that are TTX insensitive (4). Moreover, AH neurons are sensitive to L-type calcium blockers (19, 30). Accordingly, AH neurons are unlikely the origin of the neural pattern. In the guinea pig colon, S neurons are stretch dependent (30), and the vast majority of neuronal transmission in the rat colon is due to fast EPSP driven by nicotinic receptors (2, 4). Accordingly, if a neural circuit was activated, then hexamethonium should block the spontaneous IJP and/or modify RMP. Unexpectedly, we found that hexamethonium did not have a major effect on membrane potential (maybe 1 mV that do not reach statistical significance, see below) and spontane-

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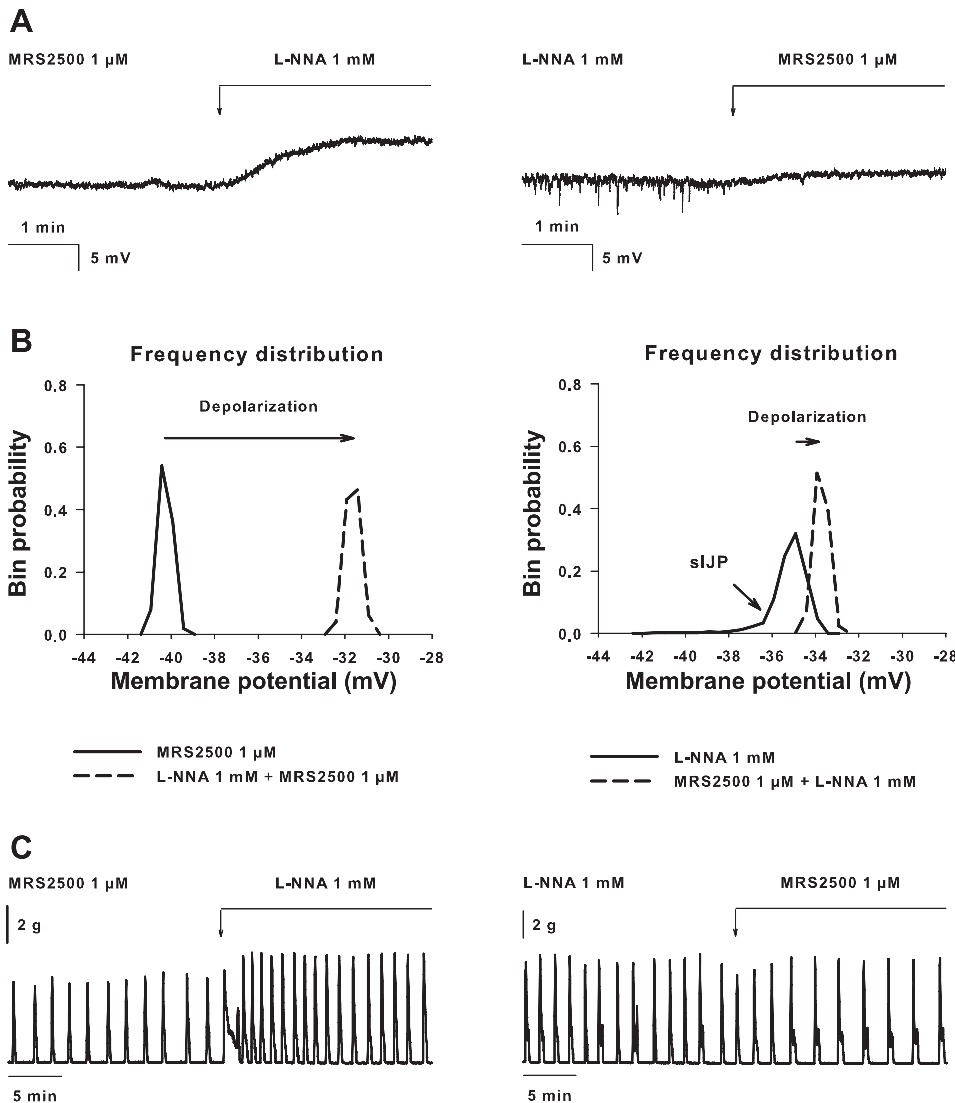

Fig. 6. *A*: intracellular microelectrode recordings showing the effect of L-NNA (1 mM) in the presence of MRS-2500 (1 μM) (*left*) and MRS-2500 (1 μM) in the presence of L-NNA (1 mM) (*right*) on RMP and sIJP. *B*: frequency distribution (0.5-mV bins) of the membrane potential (60-s recording) from a circular muscle cell in control conditions (solid line) and after L-NNA (1 mM) administration in the presence of MRS-2500 (1 μM) (short broken line) (*left*) and in control conditions (solid line) and after MRS-2500 (1 μM) administration in the presence of L-NNA (1 mM) (short broken line) (*right*). *C*: muscle bath recordings showing the effect of L-NNA (1 mM) in the presence of MRS-2500 (1 μM) (*left*) and MRS-2500 (1 μM) in presence of L-NNA (1 mM) (*right*) on spontaneous motility in vitro.

ous IJP. In the mouse colon, hexamethonium abolishes the neurogenic motor complex (5, 22). However, hexamethonium did not modify the RMP (5) or it caused a marked depolarization (22). The discrepancies between these two studies are unknown, but they might be the result of different stretch applied during the pinning to the preparation (22). It is important to notice that hexamethonium did not cause a major effect on spontaneous IJP (22). However, in another study, ~70% of spontaneous IJP were still recorded in the presence of hexamethonium, and no major change in RMP was observed in mucosa-free preparations of the mouse colon (25). These data suggest that the major source of inputs to inhibitory motor neurons is nonnicotinic. ATP acting on P2X receptors and 5-hydroxytryptamine acting on 5-HT_3 receptors mediate fast synaptic transmission in myenteric neurons of the guinea pig ileum (20, 35). However, combination of hexamethonium with P2X and 5-HT_3 antagonists (NF-023 and ondansetron) did not modify the result obtained with hexamethonium alone. All of these data might suggest that, in the rat colon, sIJP are neurally mediated but mainly independent of synaptic inputs. Mechanosensitivity in both excitatory and inhibitory motor neurons have been recently described in the guinea pig ileum. However, in these cases, motor neurons rapidly adapted to the stimuli (23). Neural-mediated relaxation, caused by circular stretch, independent of synaptic inputs has also been reported recently in the mouse lower esophageal sphincter (17). Alternatively, other neurotransmitters not investigated in the present study might be responsible for the neural inhibitory tone. It is important to note that TTX caused: *1*) an inhibition of spontaneous IJPs and *2*) a marked depolarization of the membrane potential. Because a cotransmission process (ATP and NO) is present at the neuromuscular junction (14, 24), we analyzed which neurotransmitter is involved in each response and if the effect might be correlated with the mechanical activity.

Role of $P2Y_1$ Receptors and NO on Spontaneous IJP and RMP

In the present study, we used MRS-2500 as a $P2Y_1$ antagonist and we found that MRS-2500 reduced sIJP without a major change in RMP. To our knowledge, this is the first report to analyze the effect of selective $P2Y_1$ antago-

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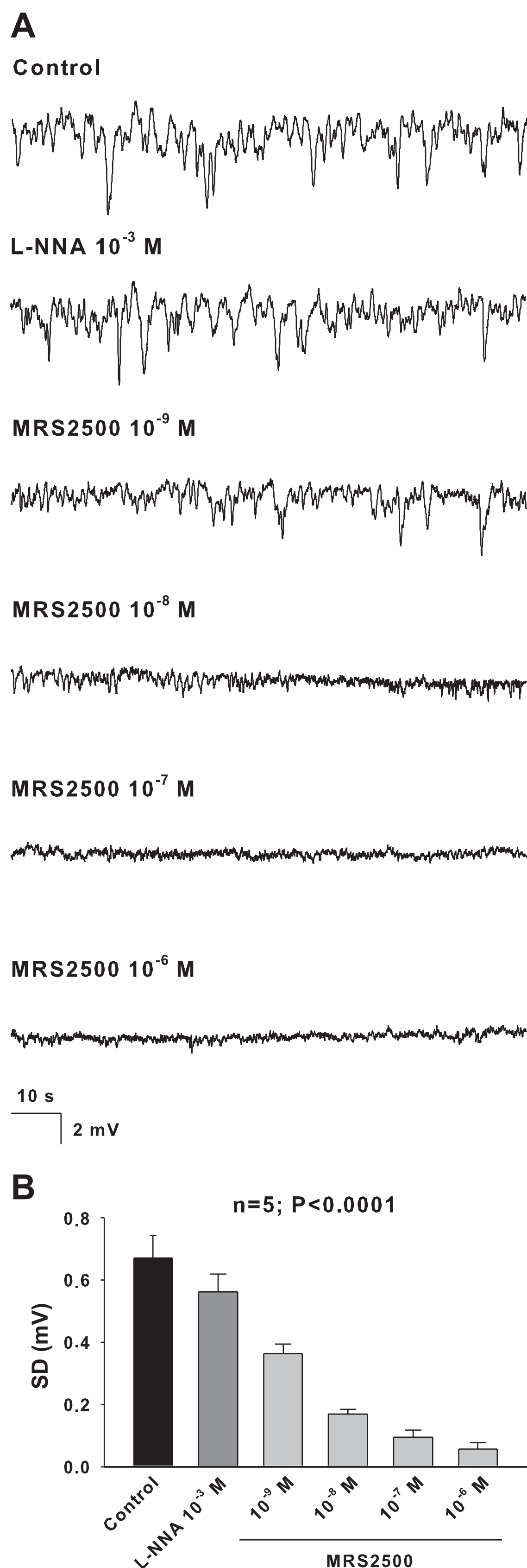

Fig. 7. *A*: intracellular microelectrode tracings showing the concentration-dependent inhibition of sIJP produced by MRS-2500 in the presence of L-NNA (1 mM). *B*: histogram showing the concentration-dependent reduction of the SD exerted by MRS-2500. *n*, No. of experiments.

nists on spontaneous IJPs. Previous data from our laboratory showed that $P2Y_1$ antagonists such as MRS-2179, MRS-2279, and MRS-2500 are useful pharmacological tools to inhibit the fast component of the IJP induced by EFS in the rat colon (14). MRS-2500 was the most potent antagonist (IC_{50} = 16.5 nM); therefore, we used MRS-2500 to antagonize sIJP (present work: IC_{50} = 3.1 nM). This result demonstrates that $P2Y_1$ receptors are responsible for spontaneous IJP probably acting at the postjunctional level. It is important to notice that MRS-2500 did not modify the RMP. This result demonstrates that *1*) prejunctional $P2Y_1$ receptors are not involved in NO release (see below) and *2*) ATP or a related purine is not tonically released from inhibitory motor neurons, causing sustained smooth muscle hyperpolarization. It is conceivable that a quanta release of ATP causes transient sIJP.

In contrast to the effect of MRS-2500, NO inhibition with L-NNA caused a depolarization of the RMP without a major effect on sIJP. These data demonstrate that *1*) NO is tonically released from enteric motor neurons causing a sustained hyperpolarization of smooth muscle cells and *2*) spontaneous IJPs are not NO mediated. Our results are in agreement with those reported in the mouse colon where sIJP were TTX and apamin sensitive and insensitive to NO synthase inhibitors. It was suggested that NO does not mediate sIJP and that probably another mediator involving sK_{Ca} channels might participate in this mechanism (28). In the present paper, we demonstrate that ATP or a related purine acting on $P2Y_1$ receptor is probably the transmitter involved in this response. It is important to note that, in the presence of MRS-2500, L-NNA was still able to cause smooth muscle depolarization, suggesting that prejunctional $P2Y_1$ receptors that might mediate sEPSP (15) are not involved in NO release.

Role of $P2Y_1$ Receptors and NO on Mechanical Activity

To correlate electrophysiological data with mechanical activity, we performed muscle bath studies and in vivo studies where colonic contractions were recorded with strain gauges. A direct correlation cannot be completely performed because *1*) electrophysiological studies are always performed under a certain degree of stretch and therefore a strict parallelism between electrophysiological and mechanical recordings cannot be established; *2*) electrophysiological but not mechanical recordings were performed in the presence of nifedipine; *3*) the neural circuitry that might be activated in vivo in intact tissue is probably different from the circuitry present in a small strip both in mechanical and electrophysiological experiments; and *4*) stretch in muscle bath studies can activate neuronal activity but also L-type calcium channels that are also stretch dependent (7, 21). Taking into account all these limitations, some data are coincident and other show slight differences: *1*) both hexamethonium and the combination of hexamethonium, NF-

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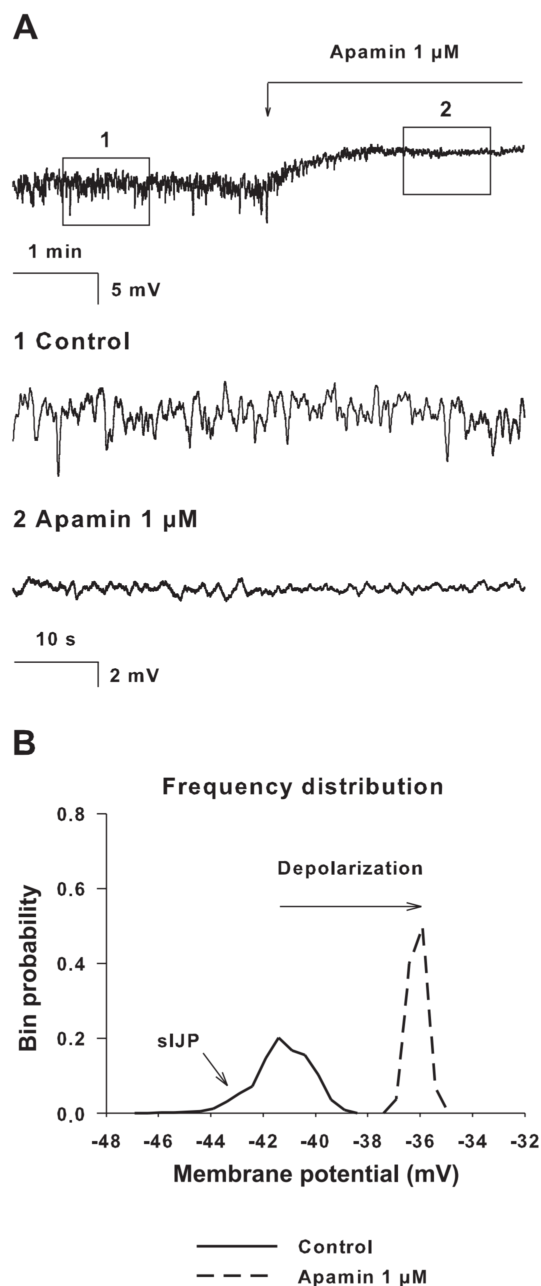
023, and ondansetron caused a slight increase in spontaneous motility without a major effect on RMP and sIJP. It is possible that a slight effect on the RMP (~1 mV) that does not reach statistical significance might explain this result.



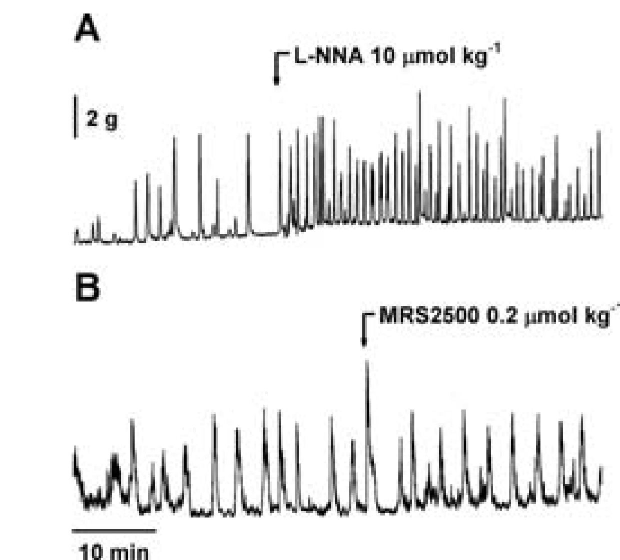


Fig. 9. Mechanical recordings showing the effect of L-NNA (10 μmol/kg) (*A*) and MRS-2500 (0.2 μmol/kg) (*B*) on spontaneous motility in vivo.

However, it is important to note that both the depolarization and increase of spontaneous motility is not comparable to the result obtained with TTX, suggesting that the vast majority of the neural inhibitory tone in vitro is independent of nicotinic, 5-HT_3, and P2X receptors; and *2*) both in vitro and in vivo NO inhibition cause an important increase in spontaneous motility. The underlying mechanism responsible for the increase in spontaneous motility is probably caused by the depolarization observed in the electrophysiological recordings. Mechanical recordings obtained both in vitro and in vivo had an increase in the frequency of spontaneous contractions. However, it is important to note that tone (measured at the baseline level) is increased in vivo but not in vitro (data not shown), and the amplitude of the phasic contraction is increased in vitro but not in vivo. Accordingly, the increase in AUC caused by L-NNA varies according to different experimental conditions that might contribute to different ongoing release of neurotransmitters. In contrast, $P2Y_1$ inhibition with MRS-2500 did not cause a sustained and constant increase in motility (in fact, in vitro, a decrease of ~20% of the spontaneous motility is observed), suggesting that inhibition of $P2Y_1$ receptors and therefore inhibition of sIJP are not translated to an increase in spontaneous motility.

Apamin, a blocker of sK_{Ca} channels, has been widely used to inhibit the fast IJP induced by EFS. Both MRS-2500 and apamin inhibited sIJP, showing that sK_{Ca} channels are involved in spontaneous transient hyperpolarizations (28). However, apamin but not MRS-2500 caused a smooth muscle depolarization and increased motility. The discrep-

Fig. 8. *A*: intracellular microelectrode recordings showing the effect of apamin (1 μM) on RMP and sIJP. Expanded traces represent sIJP in control conditions (1) and after apamin (1 μM) administration (2). *B*: frequency distribution (0.5-mV bins) of the membrane potential (60-s recording) from a circular smooth muscle cell in control conditions (solid line) and after apamin (1 μM) administration (short broken line). *C*: mechanical recording showing the effect of apamin (1 μM) on spontaneous motility in vitro.

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ancy between the effect of MRS-2500 and apamin might be attributable to the different properties of both drugs. Ongoing calcium entry during cyclic contractions might activate sK_{Ca}. A complex distribution of sK_{Ca} channels in different structures in the gastrointestinal tract such as enteric neurons, interstitial cells of Cajal, and fibroblast-like cells might contribute to these discrepancies (9, 10, 32). Alternatively, a partial interaction between NO and sK_{Ca} channels has also been reported (26). At the moment, we cannot explain the inhibitory effect of MRS-2500 on the spontaneous motility found in vitro, and further studies are needed to investigate this effect. In vivo, a single high-amplitude contraction was recorded after MRS-2500 infusion. Probably, prejunctional and/or postjunctional $P2Y_1$ receptors might participate in this effect. The subsequent contractions did not differ from those recorded in control and therefore suggest that $P2Y_1$ receptors are not involved in the inhibitory tone present in vivo.

In the present paper, we propose that $P2Y_1$ antagonists in combination with NO synthase inhibitors are valuable pharmacological tools to investigate the cotransmission process causing smooth muscle relaxation in the gastrointestinal tract. Both neurotransmitters might have complementary physiological functions that need further investigations, and its relative role causing smooth muscle relaxation might vary depending on the activation of a neural pattern and subsequent smooth muscle relaxation. Further studies will be required to study the effect of these antagonists in well-defined motor patterns such as, for example, the peristaltic reflex.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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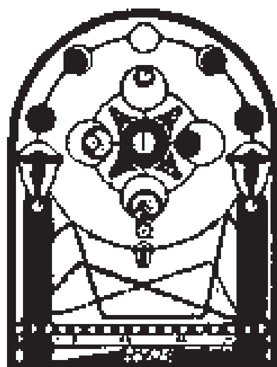

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Chapter 4

Relative contribution of SK_{Ca} and TREK1 channels in purinergic and nitrergic neuromuscular transmission in the rat colon

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Relative contribution of SK_{Ca} and TREK1 channels in purinergic and nitrergic neuromuscular transmission in the rat colon

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[1]*Department of Cell Biology, Physiology and Immunology and Neuroscience Institute, Universitat Autònoma de Barcelona, Bellaterra, Spain;* [2]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas Instituto de Salud Carlos III, Barcelona, Spain; and* [3]*Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Nice Sophia Antipolis, Valbonne, France*

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Gil V, Gallego D, Moha Ou Maati H, Peyronnet R, Martínez-Cutillas M, Heurteaux C, Borsotto M, Jiménez M. Relative contribution of SK_{Ca} and TREK1 channels in purinergic and nitrergic neuromuscular transmission in the rat colon. *Am J Physiol Gastrointest Liver Physiol* 303: G412–G423, 2012. First published May 24, 2012; doi:10.1152/ajpgi.00040.2012.—Purinergic and nitrergic neurotransmission predominantly mediate inhibitory neuromuscular transmission in the rat colon. We studied the sensitivity of both purinergic and nitrergic pathways to spadin, a TWIK-related potassium channel 1 (TREK1) inhibitor, apamin, a small-conductance calcium-activated potassium channel blocker and 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), a specific inhibitor of soluble guanylate cyclase. TREK1 expression was detected by RT-PCR in the rat colon. Patch-clamp experiments were performed on cells expressing hTREK1 channels. Spadin (1 μM) reduced currents *1*) in basal conditions *2*) activated by stretch, and *3*) with arachidonic acid (AA; 10 μM). L-Methionine (1 mM) or L-cysteine (1 mM) did not modify currents activated by AA. Microelectrode and muscle bath studies were performed on rat colon samples. L-Methionine (2 mM), apamin (1 μM), ODQ (10 μM), and N^{ω}-nitro-L-arginine (L-NNA; 1 mM) depolarized smooth muscle cells and increased motility. These effects were not observed with spadin (1 μM). Purinergic and nitrergic inhibitory junction potentials (IJP) were studied by incubating the tissue with L-NNA (1 mM) or MRS2500 (1 μM). Both purinergic and nitrergic IJP were unaffected by spadin. Apamin reduced both IJP with a different potency and maximal effect for each. ODQ concentration dependently abolished nitrergic IJP without affecting purinergic IJP. Similar effects were observed in hyperpolarizations induced by sodium nitroprusside (1 μM) and nitrergic relaxations induced by electrical stimulation. We propose a pharmacological approach to characterize the pathways and function of purinergic and nitrergic neurotransmission. Nitrergic neurotransmission, which is mediated by cyclic guanosine monophosphate, is insensitive to spadin, an effective TREK1 channel inhibitor. Both purinergic and nitrergic neurotransmission are inhibited by apamin but with different relative sensitivity.

apamin; spadin; ODQ; smooth muscle; gastrointestinal

INHIBITORY NEUROMUSCULAR TRANSMISSION in the gastrointestinal tract is predominantly mediated by ATP (or a related purine) and nitric oxide (NO). Neural release of these inhibitory neurotransmitters causes an inhibitory junction potential (IJP) with two phases: a fast (IJPf) followed by a slow (IJPs) component (6, 17). The purinergic pathway involves the activation of $P2Y_1$ receptors, which are responsible for the IJPf (12). $P2Y_1$ antagonists MRS2179, MRS2279, and MRS2500 have helped in the characterization of purinergic IJP (10, 12, 15), recently confirmed with $P2Y_1$ knocked-out mice (11, 19). In contrast, the IJPs is NO mediated and inhibited by L-NNA (23, 34). Using this pharmacological approach, we have recently characterized inhibitory neural tone in the rat colon. In this tissue, ongoing neural release of NO maintains a certain degree of smooth muscle hyperpolarization and consequently tissue incubation with N^{ω}-nitro-L-arginine (L-NNA) causes smooth muscle depolarization and increases spontaneous motility. In contrast, neural release of purines causes "spontaneous" IJP that are sensitive to $P2Y_1$ antagonists (13).

Apamin is a pharmacological tool widely used to discriminate between the fast and the slow component of the IJP and even nowadays the term "apamin sensitive" vs. "apamin insensitive" IJP is often used in the literature. Apamin reduces the IJPf in several areas of the gastrointestinal tract and in several species (22, 38, 39, 43, 46, 49). However, the slow component is also partially reduced by apamin in the human colon (23) and small-conductance calcium-activated potassium channel (SK_{Ca}) channels might participate in nitrergic neurotransmission in the ileum of the hamster (28) and in the esophagus of the opossum where the junction potential is mainly nitrergic (5). These data suggest that SK_{Ca} channels might be involved in both purinergic and nitrergic neurotransmission, but the relative sensitivity to apamin in purinergic and nitrergic pathways is unknown.

Stretch-dependent potassium (SDK) channels have been described in murine colonic smooth muscle cells (25). SDK channels are activated by NO donors and cGMP analogs, suggesting that they can transduce nitrergic signals (25). SDK channels have similar properties to TWIK-related potassium (TREK) channels, a two-pore-domain potassium channel (K2P or KCNK) subgroup (37). TREK channels (TREK1, TREK2, and TRAAK) might be important in the regulation of smooth muscle functions. In particular, TREK1 channels are expressed in murine antrum, fundus, jejunum, and colon (24). TREK1 channels are activated by stretch and might participate in the adaptation of the gastrointestinal tract to content by inducing smooth muscle hyperpolarization and relaxation in regions with reservoir functions. Moreover, it has been shown that sodium nitroprusside (SNP) and 8-Br-cGMP (an analog of cGMP) increased TREK1 currents in COS cells (24). These findings suggest that TREK1 channels might mediate nitrergic neurotransmission in smooth muscle cells, but this is a controversial issue. The NO/cGMP/PKG pathway did not activate K2P channels in vascular smooth muscle (27), and L-methionine, a putative TREK1 channel blocker, did not modify the nitrergic IJP in the lower esophageal sphincter, suggesting that TREK1 channels do not mediate nitrergic neurotransmission (47).

The lack of specific agonists and antagonists has complicated the investigation of the role of TREK1 channels. Sulfur-containing

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amino acids, particularly L-methionine, inhibit SDK channels and reduce IJPs in the murine colon (33). High concentrations (mM range) of L-methionine, however, are needed to inhibit TREK1 channels expressed in COS cells (3). Moreover, nonspecific effects are also possible since sulfur-containing amino acids are involved in the transsulfuration pathway (41) as well as in the synthesis of hydrogen sulfide (14, 21). Recently, spadin, a sortilin-derived peptide, was shown to inhibit murine and human TREK1 channels expressed in COS cells at nanomolar concentrations (29, 32). This compound binds specifically to murine TREK1 channels with an affinity of ~10 nM (29) and does not block TREK2, TRAAK, TASK1, TRESK, IKr, or IKS currents (32). Furthermore, spadin acts as an antidepressant in mice, and this effect is not observed in TREK1-deficient mice (kcnk2$^{-/-}$) showing that it is specifically mediated by TREK1 channel inhibition (29). Spadin is thus an interesting pharmacological tool to investigate the putative involvement of TREK1 channels in the regulation of colonic motility.

In the present study we characterized the nitrergic and purinergic neurotransmission to study the mechanisms responsible for each pathway with special relevance to the putative effects of spadin, a specific TREK1 channel inhibitor (29, 31, 32).

MATERIALS AND METHODS

Animals and Tissue Samples

Male Sprague-Dawley rats (8–10 wk old, 300–350 g) were purchased from Charles River (Lyon, France). Animals were housed under controlled conditions: temperature 22 ± 2°C, humidity 55 ± 10%, 12:12-h light-dark cycle and access to water and food ad libitum. Animals were stunned and killed by decapitation and exsanguination 2–3 s afterward. The colon was quickly removed and placed in carbogenated (95% O_2-5% CO_2), ice-cold physiological saline solution. Then it was opened along the mesenteric border and pinned to a Sylgard base (mucosa side up). The mid colon was identified accordingly to anatomical criteria previously described (2). The mucosal and submucosal layers were removed and muscle strips were cut (1 cm × 0.3 cm) for the intracellular microelectrode and organ bath experiments. All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Real-Time Quantitative PCR Analysis

Total RNA (5 μg) from rat brain and colon was used for reverse transcription reaction carried out with the Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France) according to the protocol of the supplier. Real-time PCR analysis (SYBR Green Mastermix, Roche, Meylan, France) was performed to estimate the level of expression of TREK1 in both tissues. The results were normalized with the endogenous reference cyclophilin D (CycloD). Real-time PCR assays (triplicate for each target gene tested) were performed in 96 well-plates on a Roche 480 Lightcycler apparatus. Data were analyzed by the comparative Cp method where the amount of target was normalized to the endogenous reference (User Bulletin no. 2, Applied Biosystems). Primers used for the different amplicons were as follows: TREK1 forward TTTTCCTGGTGGTCGTCCTC; TREK1 reverse GCTGCTCCAATGCCTTGAAC; CycloD forward GGCTCTTGAAATGGACCCTTC; CycloD reverse CAGCCAATGCTTGATCATATTCTT.

Cell Culture

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum in an atmosphere of 95% air-5% CO_2. The culture medium was completed with 1% (vol/vol) of penicillin-streptomycin and GlutaMAX × 1 (both from Invitrogen, Cergy Pontoise, France). hTREK1/HEK cells were cultured in DMEM, 1% penicillin-streptomycin, 1% GlutaMAX, 0.5 mg/ml G418, FBS 10%.

Patch-Clamp Studies in Cells Expressing hTREK1 Channels

Electrophysiological experiments were performed on COS-7 cells seeded at a density of 20,000 cells/35-mm dish, 24 h before transfection. Cells were transfected by the JetPEI method as described by the manufacturer's protocol (Ozyme, Saint-Quentin-en-Yvelines, France). COS-7 cells were transfected with 25 ng of hTREK1 pIRES-2-eGFP vector and cells were measured 48–72 h after transfection. The whole cell patch-clamp technique was used to evaluate TREK1 potassium currents. Each current was recorded using a RK 400 patch-clamp amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 3 kHz, and digitized at 10 kHz by use of a 12-bit analog-to-digital converter (Digidata 1322 series, Axon Instruments). Patch-clamp pipettes were pulled by use of a vertical puller (PC-10, Narishige, Tokyo, Japan) from borosilicate glass capillaries and had resistances of 3–5 MΩ. All experiments were performed at room temperature (21–22°C). Except for excised patch-clamp experiments in the stretch protocol, TREK1 currents were recorded in the presence of a cocktail of potassium channel inhibitors [K^+ blockers: 3 mM 4-aminopyridine (4-AP), 10 mM tetraethylammonium (TEA), 10 μM glibenclamide, 100 nM apamin, and 50 nM charybdotoxin]. Stimulation protocols and data acquisition were carried out via a microcomputer (Dell Pentium) that used commercial software and hardware (pClamp 8.2). TREK1 currents were recorded from −100 to +60 mV membrane potentials in 20-mV steps applied from a holding potential of −80 mV. Duration of depolarization pulses were 0.825 ms and the pulse cycling rate was 5 s. TREK1 current amplitudes were evaluated at the end of the stimulation pulses. Cells were continuously superfused with microperfusion system. L-Cysteine 1 mM, L-methionine 1 mM, and spadin 1 μM were tested on hTREK1 current. All current amplitudes are expressed in current densities (pA/pF).

For stretch experiments, patch-clamp measurements were performed on hTREK1/HEK cells constitutively expressing hTREK1 (31). Cells were used 2 to 4 days after plating with 50 000 cells per dish. All electrophysiological recordings were performed at room temperature in inside-out mode. Patch pipettes of ~1.5 MΩ were used. Membrane patches were stimulated with negative pressure pulses, from 0 to −40 mmHg in −10 mmHg increments during 300 ms each 3 s, through the recording electrode using a pressure-clamp device (ALA High Speed Pressure Clamp-1 system; ALA Scientific Instruments, Farmingdale, NY). Holding potential was maintained at 0 mV. Spadin was used at 1 μM. For all experiments, currents were filtered at 1 kHz, digitized at 20 kHz, and analyzed with pCLAMP 9.2 and ORIGIN 6.0 (Rithm Informatique, Paris, France) software.

Intracellular Microelectrode Recording in the Rat Colon

The tissue was pinned with the circular muscle layer facing upward in a Sylgard-coated chamber, continuously perfused with carbogenated physiological saline solution at 37 ± 1°C, and allowed to equilibrate for 1 h. Circular smooth muscle cells 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, Sarasota, FL). Tracings were displayed on an oscilloscope 4026 (Racal-Dana, Windsor, UK) and simultaneously digitalized (100 Hz) with PowerLab 4/30 system and Chart 5 software for Windows (both from AD Instruments, Castle Hill, NSW, Australia). Experiments were performed in the presence of nifedipine (1 μM) to stabilize impalements.

Estimation of RMP and sIJP. The spontaneous inhibitory neural tone was characterized as we have described previously (13). Briefly, the resting membrane potential (RMP; expressed in mV) was estimated as the most probable bin of the frequency distribution of the membrane potential (0.1-mV bins; 30–60 s recordings). Spontaneous inhibitory junction potentials (sIJP) were evaluated by calculating the

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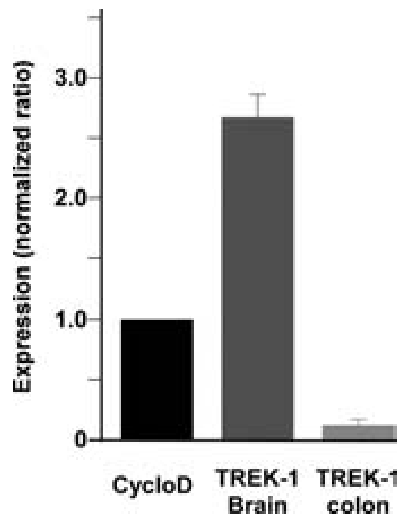

Fig. 1. Relative expression of TREK1 channels in brain and colon tissues from rat analyzed by real-time PCR. Data were normalized to cyclophilin D (cycloD) expression. All values are means ± SE (n = 3).

mean standard deviation (SD) of the distribution of the membrane potential: SD of the recording inside the cell minus SD of the recording outside the cell (expressed in mV).

EFS elicited IJP. As previously described (9), IJP were also elicited by electrical field stimulation (EFS) by using the following parameters: *1*) Single pulses, pulse duration 0.3 ms, and increasing amplitude voltage (8, 12, 16, 20, 24, 28, 32, 36 and 40 V). *2*) In addition, train stimuli of 5-s duration were also performed at 1 Hz (5 pulses) and 5 Hz (25 pulses) at supramaximal voltages (32 V) and pulse duration 0.3 ms.

Purinergic IJP were evaluated in the presence of L-NNA (1 mM) by use of single pulses elicited at supramaximal voltages. In contrast, nitrergic IJP were evaluated in the presence of MRS2500 (1 μM) by using train pulses of 5 Hz elicited at supramaximal voltages. The amplitude of these IJP was measured from the RMP to the most hyperpolarized value and expressed in mV.

Muscle Bath Studies in the Rat Colon

Muscle strips were mounted in a 10-ml organ bath containing carbogenated physiological saline solution maintained at 37 ± 1°C. Motility was measured via an isometric force transducer (Harvard VF-1 Harvard Apparatus, Holliston, MA) connected to a computer through an amplifier. Data were digitized (25 Hz) by using Data 2001 software (Panlab, Barcelona, Spain) coupled to an analog-to-digital 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. To test the stretch sensitivity of the mechanism, different stretch (from 1 to 4 g) were applied in steps of 1 g. EFS was applied after 30 min for each step of stretch. The release of inhibitory neurotransmitters was studied by using EFS applied for 4 min, pulse duration 0.3 ms, frequency 5 Hz, amplitude 30 V. The area under the curve (AUC) of contractions from the baseline was measured to estimate the mechanical activity and the result was expressed in grams per minute (g/min). The basal tone, expressed in grams, was measured in stretch experiments.

Solutions and Drugs

The composition of the physiological saline solution was (in mM) 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3–7.4). In all the experiments, phentolamine, atropine, and propranolol (1 μM) were added to the physiological saline solution to block α- and β-adrenoceptors and muscarinic receptors. For patch-clamp experiments performed in COS-7 cells, the bath solution contained (in mM) 150 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES/NaOH (pH 7.4) and the pipette solution contained (in mM) 155 KCl, 3 $MgCl_2$, 5 EGTA, and 10 HEPES/KOH (pH 7.2). For patch-clamp experiments performed in hTREK1 cells, the bath solution contained (in mM) 155 KCl, 3 $MgCl_2$, 5 EGTA, and 10 HEPES (pH 7.2 with KOH) and the pipette solution contained (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, and 10 HEPES (pH 7.4 with NaOH). The following drugs were used: tetrodotoxin (Latoxan, Valence, France), 4-AP, apamin, arachidonic acid (AA), atropine sulfate, charybdotoxin, glibenclamide, L-cysteine, L-methionine, nifedipine, L-NNA, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), phentolamine, SNP, TEA (Sigma Chemical,

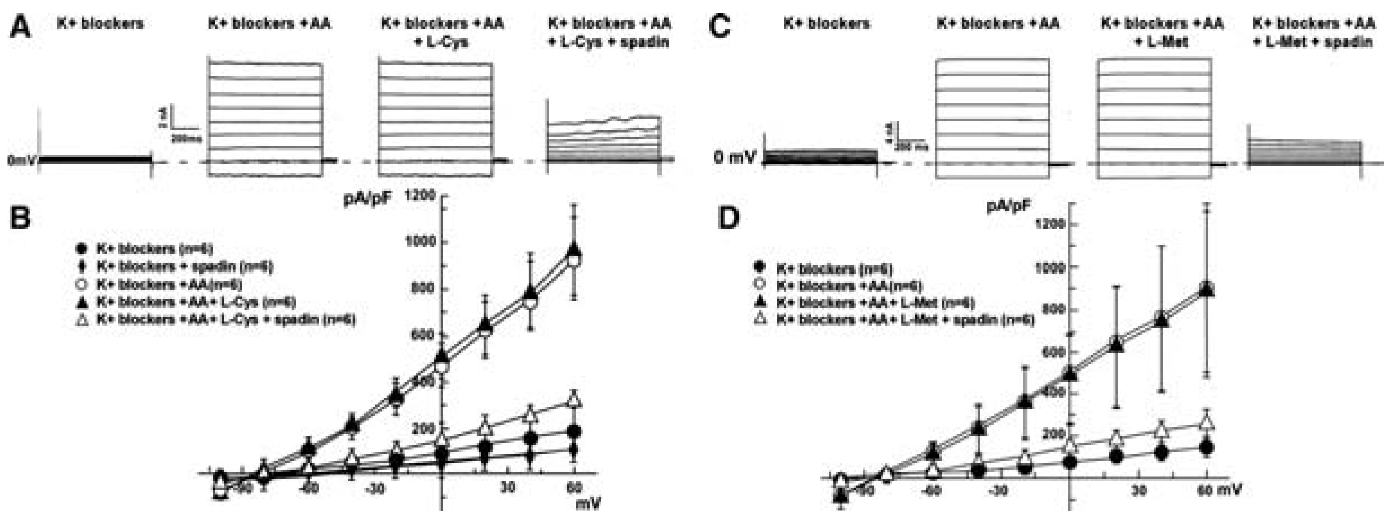

Fig. 2. Effect of L-methionine and L-cysteine on human TREK1 channel activity. All currents were measured in the presence of potassium channel inhibitory cocktail (K^+ blockers). Maximum of the activated hTREK1 current inhibition was obtained by application of 1 μM spadin. *A*: typical traces of human whole cell TREK1 current recorded in the absence (K^+ blockers, n = 6) or presence of 10 μM of arachidonic acid (AA) (K^+ blockers + AA, n = 6). Effects of L-cysteine 1 mM (L-Cys) were recorded on AA activated channels (K^+ blockers + AA + L-Cys, n = 6). *Right*, spadin inhibition (n = 6). *B*: comparison of current-voltage (*I–V*) curves obtained in the different experimental conditions. Notice that spadin was able to block basal and AA-activated currents. *C*: typical traces of human whole cell TREK1 current recorded in the absence (K^+ blockers, n = 6) or presence of 10 μM of AA (K^+ blockers + AA, n = 6). Effects of L-methionine 1 mM (L-Met) were recorded on AA activated channels (K^+ blockers + AA + L-Met, n = 6). *Right*, spadin inhibition (n = 6). *D*: comparison of *I–V* curves obtained in the different experimental conditions described in *C*. All values are means ± SE.

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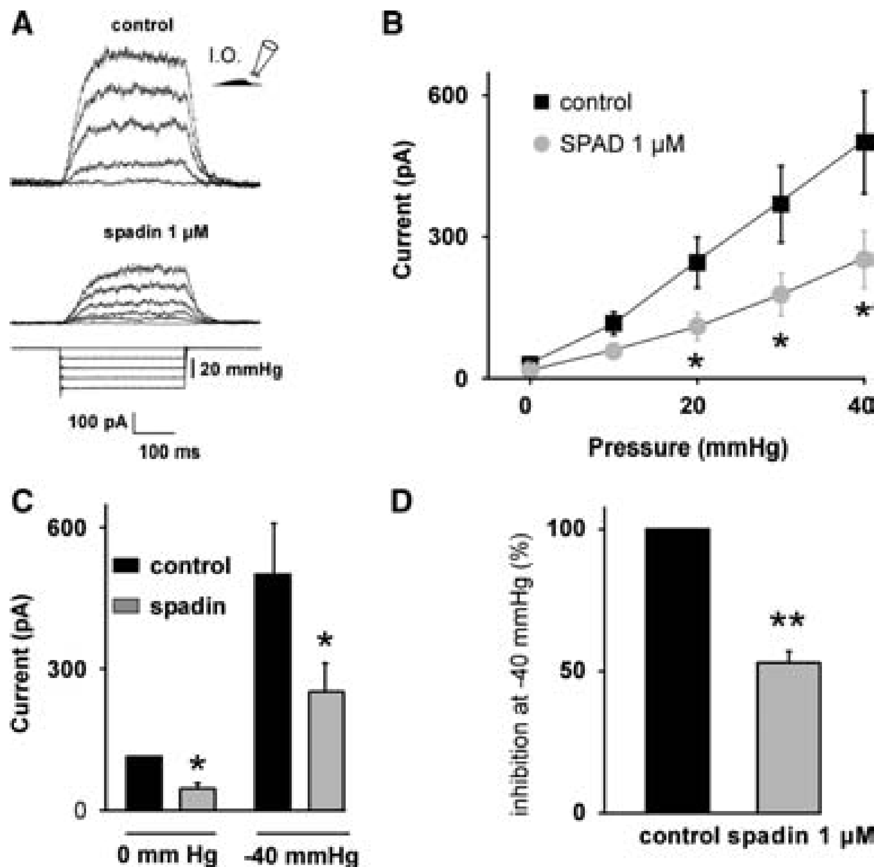

Fig. 3. Effect of spadin on stretch-activated hTREK1 currents. Currents were elicited by negative pressure pulses, from 0 to −40 mmHg in −10 mmHg increments during 300 ms each 3 s in hTREK1/HEK cells constitutively expressing hTREK1 channels. *A*: representative recording of stretch-activated currents inhibited by spadin (1 μM) in inside-out (I.O.) mode. *Top* recording represents a stretch-activated current in control condition and *bottom* trace shows spadin (1 μM) inhibition after 1-min perfusion. At the bottom pressure, the protocol is shown from 0 to −40 mmHg. *B*: corresponding histogram showing the effect of spadin (1 μM) on stretch-activated currents for each pressure. *C*: spadin (1 μM) inhibited both spontaneous (without pressure) and stretch-induced (with pressure) activity of hTREK1. *D*: percentage of spadin inhibition at −40 mmHg. All values are means ± SE (n = 12). Significance was tested with a permutation test (R Development Core Team: http://www.r-project.org/) for $n < 30$. *$P < 0.05$; **$P < 0.01$, significant difference from control.

St. Louis, MO), (1R,2S,4S,5S)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS2500), and propranolol (Tocris, Bristol, UK). Spadin was provided by the Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique (Université de Nice Sophia Antipolis, Valbonne, France). Stock solutions were made by dissolving drugs in distilled water except for nifedipine, ODQ, and AA, which were dissolved in 96% ethanol, and L-NNA, which was dissolved in physiological saline solution by sonication.

Data Analysis and Statistics

To normalize mechanical data, EFS-induced relaxation was calculated as percentage of inhibition by use of the following formula: 1 −[AUC during EFS/AUC previous EFS] × 100. Note that a complete cessation of spontaneous motility means 100% inhibition while 0% is a complete blockade of the inhibitory response observed during EFS. Negative data indicate a larger contractile activity during EFS than the one observed prior EFS.

For electrophysiological experiments on COS-7 cells, statistical analysis of differences between groups was performed by using unpaired t-test. For electrophysiological experiments on hTREK1/HEK cells, significance was tested with a permutation test (R Development Core Team: http://www.r-project.org/) for $n < 30$. Differences in the resting membrane potential, sIJP, purinergic and nitrergic IJP, spontaneous motility (AUC), and hyperpolarizations induced by SNP were compared before and after drug addition by a paired Student's t-test. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to compare the effect of the different drugs on EFS-induced relaxation. Two-way ANOVA followed by Bonferroni's multiple comparison test was performed to evaluate the effect of spadin on EFS-induced relaxation at different levels of tension. IC_{50} were calculated by using a conventional sigmoid concentration-response curve with variable slope.

Data are expressed as means ± SE; n values indicate the number of samples. A $P < 0.05$ was considered statistically significant. Statis-

Table 1. *Effect of L-methionine 2 mM, spadin 1 μM, apamin 1 μM, ODQ 10 μM, and L-NNA 1 mM on membrane potential and spontaneous motility*

	Membrane Potential, mV				Motility, AUC; g/min			
	n	Control	Drug	P value	n	Control	Drug	P value
Spadin 1 μM	4	−44.6 ± 2.2	−44.4 ± 2.2	n.s.	4	19.5 ± 2.7	19.2 ± 2.9	n.s.
Apamin 1 μM	5	−41.4 ± 1.2	−37.9 ± 1.4	< 0.01	4	19.9 ± 3.9	39.8 ± 5.1	< 0.01
ODQ 10 μM	5	−46.5 ± 1.7	−42.4 ± 1.8	< 0.05	4	19.8 ± 2.7	28.7 ± 4.0	< 0.01
L-NNA 1 mM	6	−46.7 ± 1.7	−40.7 ± 1.6	< 0.001	4	13.4 ± 6.6	31.5 ± 9.7	< 0.05
L-Methionine 1 mM	4	−48.1 ± 2.3	−40.9 ± 2.5	< 0.001	4	11.7 ± 2.5	21.2 ± 3.3	< 0.001

Values are means ± SE n, number of samples. AUC, area under the curve; ODQ, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; L-NNA, N^{ω}-nitro-L-arginine. Statistical significance of differences was assessed by paired Student's t-test; n.s., not significant.

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tical analysis and curve fit were performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

RESULTS

TREK1 Channel Expression in the Rat Colon

Real-time quantitative RT-PCR analysis revealed that TREK1 channel mRNA is expressed in the rat colon, although mRNA expression in the colon was ~27-fold lower than in the brain (Fig. 1).

Effect of L-Methionine, L-Cysteine, and Spadin on TREK1 Channel Activity

Human TREK1 channels expressed in COS-7 cells displayed a relatively weak current. The basal current was reduced by spadin (1 μM) (Fig. 2*B*). AA (10 μM) was used to amplify the current. Note that AA (10 μM) did not cause any effect when tested in nontransfected COS-7 cells (data not shown). The contribution of other potassium channels was blocked with the use of a K^+ channel inhibitory cocktail (K^+ blockers). In these experimental conditions, neither L-Cys (1 mM) (Fig. 2, *A* and *B*) nor L-methionine (1 mM) (Fig. 2, *C* and *D*) affected the AA (10 μM)-activated hTREK1 currents that, as previously demonstrated, were inhibited by spadin (1 μM) (29) (Fig. 2, *A–D*). Stretch applied by increasing negative pressure pulses increased currents in hTREK1/HEK cells constitutively expressing hTREK1 channels. Spadin (1 μM) inhibited ~50% stretch-induced currents (n = 12; Fig. 3). Therefore, spadin is a suitable TREK1 channel inhibitor for further studies concerning the implication of these channels in the purinergic and nitrergic neurotransmission process.

Resting Membrane Potential and Spontaneous Motility

Despite the fact that TREK1 channel expression was detected in the rat colon, spadin (1 μM) did not modify smooth muscle membrane potential or mechanical activity (Table 1; Fig. 4*A*). However, apamin (1 μM), ODQ (10 μM), L-NNA (1 mM), and L-methionine (1 mM) depolarized the smooth muscle cells and increased mechanical activity (Table 1; Fig. 4, *B–E*).

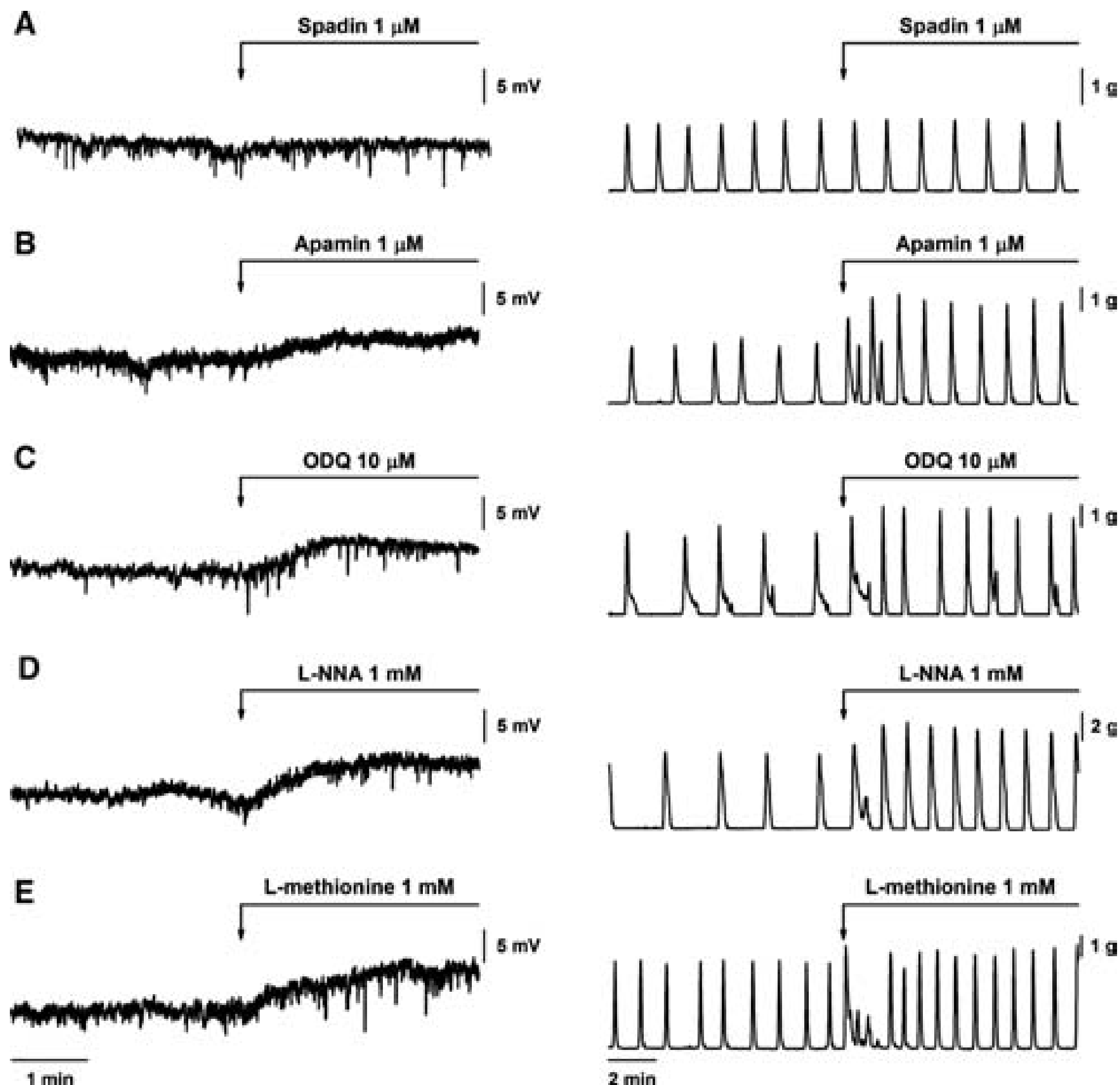

Fig. 4. Intracellular microelectrode tracings (*left*) and mechanical recordings (*right*) showing the effect of spadin (1 μM; *A*), apamin (1 μM; *B*), 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ; 10 μM; *C*), N^{ω}-nitro-L-arginine (L-NNA; 1 mM; *D*), and L-methionine (1 mM; *E*) on membrane potential and spontaneous motility.

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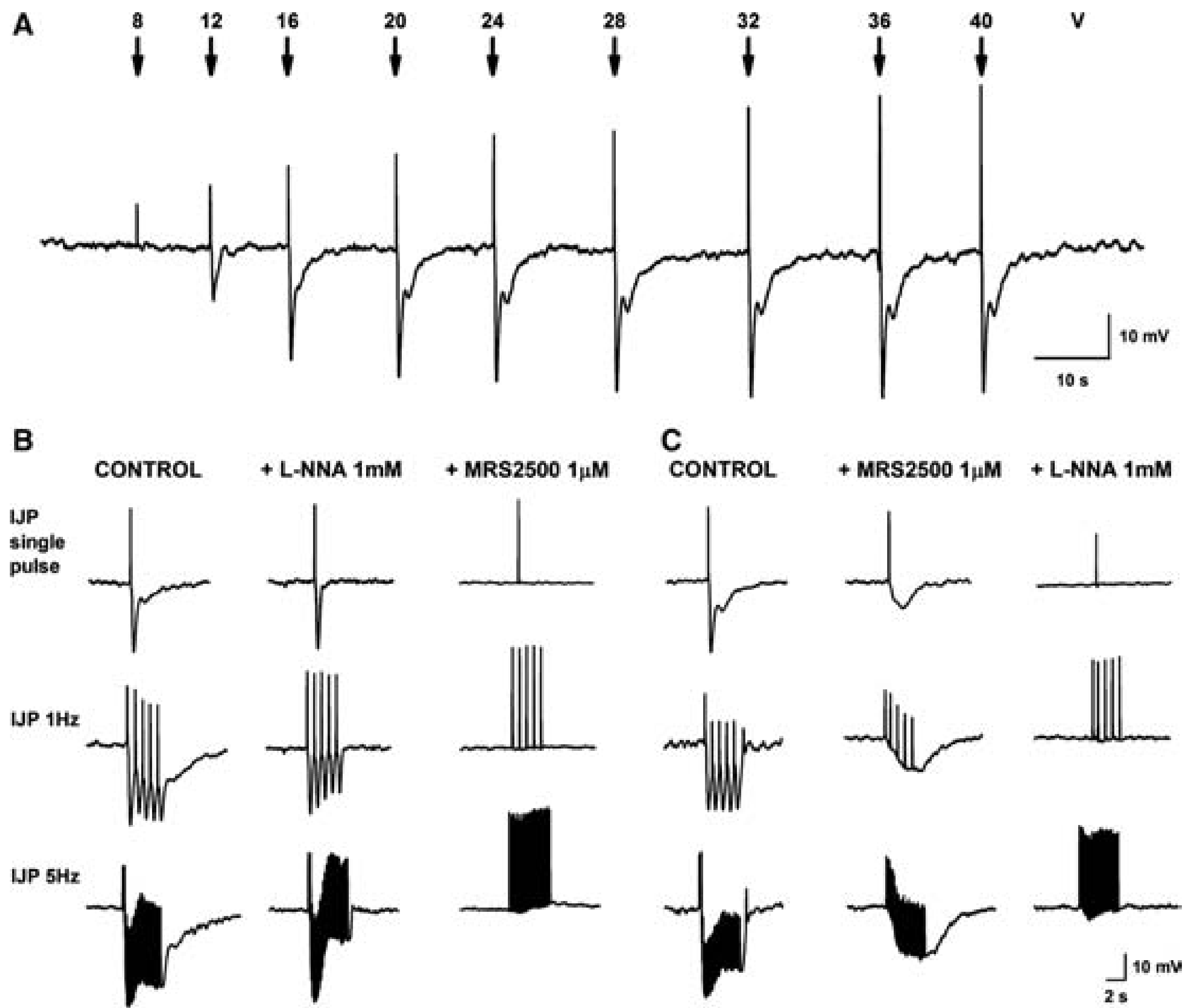

Fig. 5. *A*: intracellular microelectrode recording showing representative electrical field stimulation (EFS)-induced inhibitory junction potentials (IJP) elicited by single pulses increasing the voltage of stimulation (8, 12, 16, 20, 24, 28, 32, 36, and 40 V). *B*: representative EFS-induced IJP in control conditions and after the sequential addition of L-NNA (1 mM) and MRS2500 (1 μM) (from *left* to *right*) obtained with a single pulse (*top*), train stimuli at 1 Hz during 5 s (5 pulses) (*middle*), and train pulses at 5 Hz and 5-s stimulation (25 pulses) (*bottom*) performed at supramaximal voltage (32 V) and pulse duration 0.3 ms. *C*: representative EFS-induced IJP in control conditions and after the sequential addition of MRS2500 (1 μM) and L-NNA (1 mM) (from *left* to *right*) obtained with a single stimulation (*top*), train stimuli at 1 Hz during 5 s (5 pulses) (*middle*), and train pulses at 5 Hz and 5-s stimulation (25 pulses) (*bottom*) elicited at supramaximal voltage (32 V) and pulse duration 0.3 ms.

Spontaneous Inhibitory Junction Potentials

Spontaneous IJP (sIJP) were recorded in samples of rat mid colon (0.77 ± 0.09 mV SD; n = 13). Spadin (1 μM) did not modify sIJP (control: 0.67 ± 0.04 mV vs. spadin 1 μM: 0.65 ± 0.07 mV SD; n = 4; not significant). Despite causing smooth muscle depolarization, neither ODQ (10 μM) nor L-methionine (1 mM) produced significant changes in sIJP (for ODQ, control: 0.71 ± 0.18 mV vs. ODQ 10 μM: 0.65 ± 0.10 mV SD; n = 5; not significant; and for

Table 2. *Effect of spadin 1 μM, apamin 1 μM, and ODQ 10 μM on the purinergic and nitrergic inhibitory junction potentials*

	IJP Amplitude, mV							
	Purinergic IJP				Nitrergic IJP			
	n	Control	Drug	*P* value	*n*	Control	Drug	*P* value
Spadin 3 μM	3	17.8 ± 3.8	18.0 ± 4.2	n.s.	4	14.6 ± 2.8	14.0 ± 3.0	n.s.
Apamin 1 μM	4	21.4 ± 4.7	4.1 ± 2.0	< 0.05	10	17.5 ± 1.6	10.0 ± 1.2	< 0.001
ODQ 10 μM	3	17.4 ± 4.9	18.0 ± 4.1	n.s.	4	12.8 ± 1.6	0.3 ± 0.2	< 0.001
L-Methionine 2 mM	4	19.6 ± 3.5	23.1 ± 3.7	n.s.	5	22.7 ± 2.9	20.8 ± 3.0	n.s.

Purinergic inhibitory junction potential (IJP) was evaluated using single stimulation at supramaximal voltage (32 V) in the presence of L-NNA (1 mM). Nitrergic IJP was evaluated by using a train stimulation at supramaximal voltage (32V) in the presence of MRS2500 (1 μM). Values are means ± SE; *n*, number of samples. The statistical significance of differences was assessed by paired Student's *t*-test.

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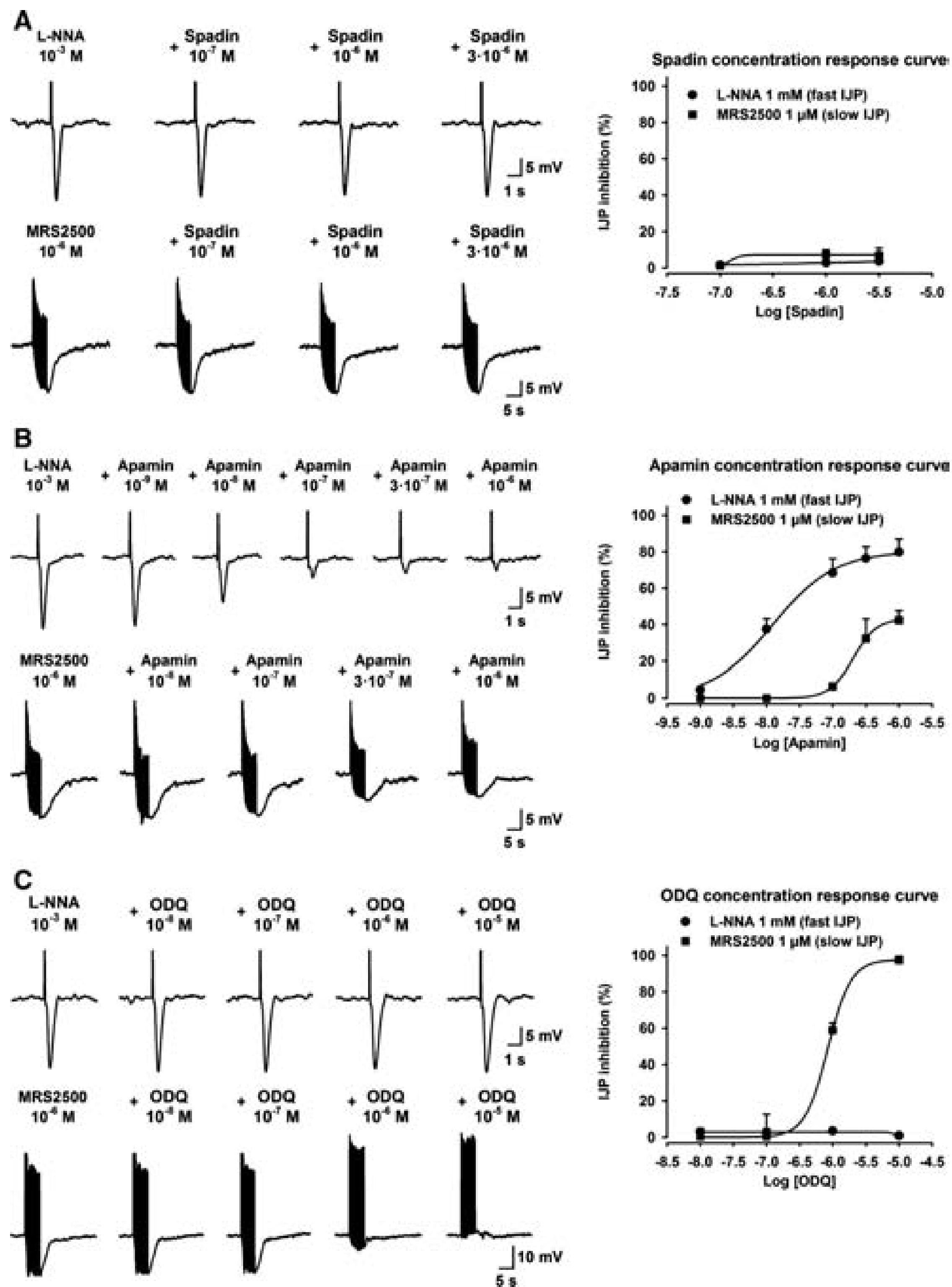

Fig. 6. Intracellular microelectrode recordings (*left*) and concentration response curves (*right*) showing the effect of spadin (*A*), apamin (*B*), and ODQ (*C*) on EFS-induced purinergic and nitrergic IJP. Note that the purinergic IJP was evaluated in the presence of L-NNA (1 mM) and with use of a single-pulse stimulation protocol. In contrast, nitrergic IJP was evaluated by incubating the tissue with MRS2500 (1 μM) and eliciting train pulse hyperpolarizations at 5 Hz during 5 s (25 pulses). In both cases EFS was performed at supramaximal voltages (32 V) and pulse duration 0.3 ms. The number of samples was $n = 3–10$ (see Table 2).

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L-methionine, control: 0.95 ± 0.20 mV vs. L-methionine 1 mM: 0.88 ± 0.10 mV SD; $n = 4$; not significant).

Effect of Spadin, Apamin, ODQ, and L-Methionine on EFS-Induced IJP

Single-pulse stimulation elicited an IJPf followed by an IJPs (Fig. 5, *A–C*). Train pulses at 1 Hz (supramaximal voltage during 5 s) induced five consecutive hyperpolarizations whereas at 5 Hz they elicited a hyperpolarization characterized by a fast component followed by a more sustained one (Fig. 5, *B* and *C*). Similar protocols have been used to characterize the junction potential in human colon (9).

L-NNA (1 mM) and MRS2500 (1 μM) were used to isolate purinergic and nitrergic IJP, respectively. In the presence of L-NNA, the IJP was a pure purinergic IJP because it was completely abolished by the subsequent addition of MRS2500 (1 μM) (Fig. 5*B*). In the presence of MRS2500 (1 μM), the IJP was a pure nitrergic IJP because it was completely abolished by the subsequent addition of L-NNA (1 mM) (Fig. 5*C*). Single-pulse stimulation and trains of 5 Hz were used to evaluate the effect of spadin, apamin, ODQ, and L-methionine on pure purinergic and nitrergic IJP, respectively.

Neither purinergic nor nitrergic IJP were affected by spadin (Table 2, Fig. 6*A*) but both were reduced by apamin (Table 2, Fig. 6*B*). Pure purinergic IJP was more sensitive to apamin (E_{max}: 80.2 ± 6.4%; $IC_{50} = 1.26 \cdot 10^{-8}$ M) than pure nitrergic IJP (E_{max}: 43.2 ± 5.2%; $IC_{50} = 2.05 \cdot 10^{-7}$ M) (Fig. 6*B*). ODQ concentration dependently inhibited nitrergic neuromuscular transmission (E_{max}: 98.5 ± 4.7%; $IC_{50} = 8.5 \cdot 10^{-7}$ M; Table 2; Fig. 6*C*) without affecting purinergic IJP (Table 2; Fig. 6*C*). Finally, concentrations up to 2 mM of L-methionine did not modify purinergic or nitrergic IJP (Table 2).

Effect of Spadin, Apamin, ODQ, and L-Methionine on EFS-Induced Nitrergic Relaxation

In organ bath experiments, blockade of $P2Y_1$ receptors did not modify the relaxation induced by EFS (relaxation: 99.9 ± 0.1%; $n = 30$; Fig. 7). In the presence of MRS2500 (1 μM), EFS-induced relaxation was not reduced by spadin (1 μM) (Fig. 7, *A* and *E*) but was decreased by relatively high concentrations of apamin (1 μM) (Fig. 7, *B* and *E*). ODQ (10 μM) completely abolished EFS-induced relaxation and a contractile response compared with control was observed (Fig. 7, *C* and *E*). As previously described (15), EFS induced a clear contractile response in the presence of MRS2500 (1 μM) and L-NNA (1 mM) compared with control (Fig. 7, *D* and *E*). Moreover, total relaxation was observed in the presence of MRS2500 (1 μM) and L-methionine (2 mM) (not shown).

Because TREK1 channels are stretch dependent, the level of stretch applied to the preparation might change their contribution to nitrergic neuromuscular transmission. To test this hypothesis, relaxations induced by EFS in the presence of MRS2500 (1 μM) were evaluated at different levels of stretch (from 1 to 4 *g*). Stretch induced a sharp increase in tone that slowly decreased, reaching a steady state after 30 min. After each applied stretch, the tone of the preparation measured at the baseline of spontaneous contractions was higher (Fig. 8, *A* and *B*). Spadin (1 μM) did not modify this increase in tone (Fig. 8, *A* and *B*). Furthermore, spadin (1 μM) did not reduce EFS-induced relaxation at different levels of tension (Fig. 8, *A* and *C*).

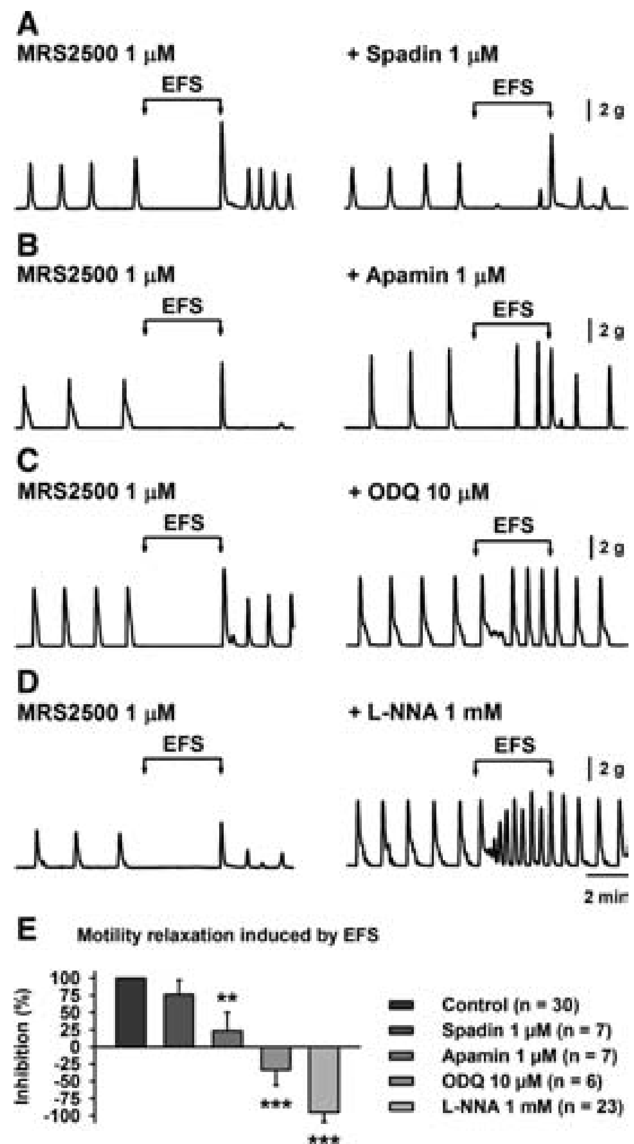

Fig. 7. Muscle bath recordings showing the effect of spadin (1 μM; *A*), apamin (1 μM; *B*), ODQ (10 μM; *C*), and L-NNA (1 mM; *D*) on the relaxation induced by EFS in the presence of MRS2500 (1 μM). *E*: histogram showing the EFS-induced relaxation (expressed as percentage of inhibition) in the presence of MRS2500 (1 μM) alone (control conditions), or combined with spadin (1 μM), apamin (1 μM), ODQ (10 μM), or L-NNA (1 mM). Data were calculated by the following formula: 1 −[area under the curve (AUC) during EFS/AUC previous EFS] × 100. Note that 100% is no drug effect (total inhibition of spontaneous motility) and 0% is a complete blockade of the inhibitory response. Negative data indicate a larger contractile activity during EFS than that observed prior to EFS. All values are means ± SE. Significant differences were assessed by 1-way ANOVA, followed by Bonferroni's multiple comparison test. $^{**}P < 0.01$; $^{***}P < 0.001$, significant difference from control.

Effect of Spadin, Apamin, and ODQ on Exogenous Addition of Sodium Nitroprusside

Exogenous addition of SNP was used to evaluate the involvement of SK_{Ca} and TREK1 channels on nitrergic signaling. Exogenous addition of SNP (1 μM) hyperpolarized the smooth muscle cells (10.8 ± 0.7 mV; $n = 12$). In addition,

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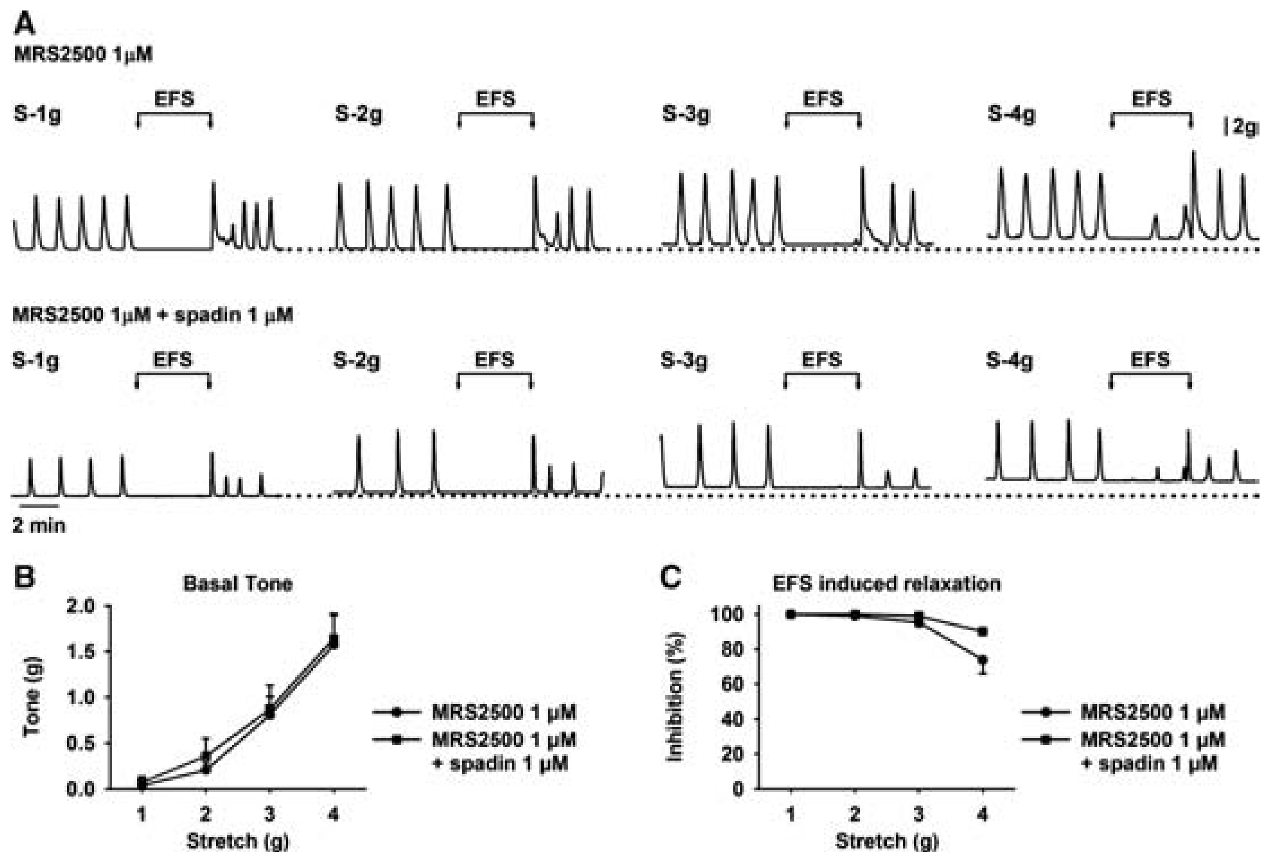

Fig. 8. Muscle bath recordings showing the effect of spadin (1 μM) on the relaxation induced by EFS at different levels of stretch (1–4 g; *A*). Dotted line indicates the increase in tone reached 30 min after each stretch (depicted in *B*). Graph plots showing the effect of spadin (1 μM) on basal tone (*B*) and relaxations induced by EFS (*C*) at different levels of stretch (1–4 g). These experiments were performed in the presence of MRS2500 1 μM and the response induced by EFS was L-NNA sensitive. All values are means ± SE ($n = 5$). Significant differences were assessed using 2-way ANOVA, followed by Bonferroni's multiple comparison test.

SNP concentration dependently inhibited spontaneous mechanical activity in the presence of TTX (1 μM) ($IC_{50} = 25.9$ nM; $n = 5$). SNP-induced hyperpolarization was not modified by spadin (1 μM) (control: 10.8 ± 2.0 mV vs. spadin 1 μM: 9.5 ± 2.0 mV; $n = 4$; not significant; Fig. 9*A*) but was reduced in the presence of apamin (1 μM) (control: 10.4 ± 0.7 mV vs. apamin 1 μM: 6.9 ± 0.7 mV; $n = 4$; $P < 0.05$; Fig. 9*B*) and was blocked by ODQ (10 μM) (control: 11.2 ± 0.8 mV vs. ODQ 10 μM: 0.9 ± 0.5 mV; $n = 4$; $P < 0.01$; Fig. 9*C*). However, neither spadin (1 μM) nor apamin (1 μM) was able to reduce the inhibition of the motility produced by SNP, which was inhibited by ODQ (10 μM) (data not shown).

DISCUSSION

Based on our data from the rat, mice, and human colon (9, 11–13, 15, 34), criteria can be established to consider whether a drug is acting in nitrergic vs. purinergic pathways. To simplify the discussion, Table 3 summarizes these criteria based on our previous work. In the present study we have used MRS2500 (a $P2Y_1$ antagonist) to isolate the nitrergic component, to characterize the nitrergic responses, and to compare them with pure purinergic responses isolated by previous incubation with L-NNA. Two possible hypotheses describe neuromuscular transmission: *1*) a direct effect of neurotransmitters on smooth muscle cells supported by the fact that direct communication between nerve and muscle has been ultrastructurally described (30), and *2*) the "intercalation" hypothesis in which another cell, an interstitial cell of Cajal (ICC) or a PDGFRα$^+$ cell (platelet-derived growth factor receptor α-positive cell or fibroblast-like cell), is intercalated between inhibitory neurons and smooth muscle (4, 20, 26). This is a controversial issue (18, 36). ICC may mediate nitrergic neurotransmission (4, 20) and PDGFRα$^+$ cells, which express $P2Y_1$ receptors and SK3 channels, may contribute to purinergic neurotransmission (26). Taking all this into consideration, we pose the following questions: What is the effect of each drug in terms of motility? Where are the drugs acting? And what is the pathway that explains each mechanism?

ODQ depolarizes smooth muscle cells, increases motility, and blocks the slow IJP and nitrergic relaxation without a major effect on the fast IJP, sIJP, or purinergic relaxation. Therefore, ODQ effects are consistent with an inhibition of the nitrergic neuromuscular transmission (Table 3) that is fully dependent on cGMP pathways. Nitrergic mechanical responses have been found to be totally absent in animals with β1 deletion of NO-sensitive guanylate cyclase (GC) used to generate complete GCKO mice (16). In contrast, mice with selective deletion of smooth muscle GC have functional nitrergic neurotransmission, suggesting that GC located in other cell types (ICC or PDGFRα$^+$ cells) might mediate nitrergic responses (16).

In the present study we detected the presence of TREK1 mRNA in the rat colon although the levels were comparatively lower than those detected in the brain. TREK1 channels are

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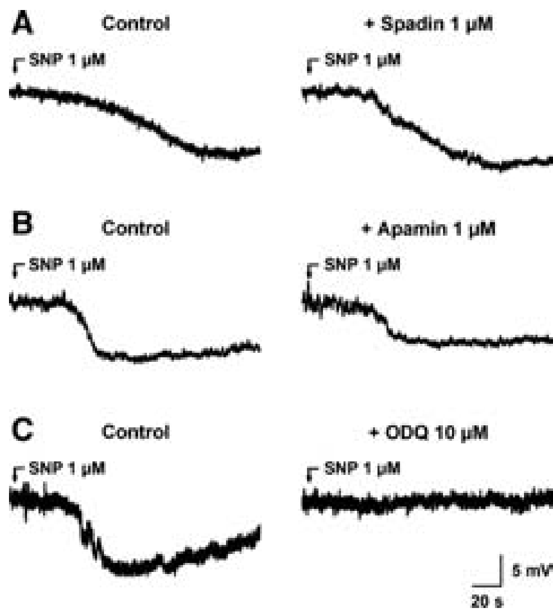

Fig. 9. Intracellular microelectrode recordings showing the effect of spadin (*A*), apamin (*B*), and ODQ (*C*) on the hyperpolarization induce by sodium nitroprusside (SNP; 1 μM).

expressed in smooth muscle cells and might participate in nitrergic neurotransmission (24). However, data to the contrary have been obtained in vascular smooth muscle where TREK1 channels do not participate in NO/cGMP pathways (27), and in the lower esophageal sphincter (LES) where putative TREK1 channel inhibitors do not modify nitrergic neurotransmission (47). One of the main difficulties in studying the function of TREK1 channels has been the lack of available specific antagonists. Patch-clamp experiments show that spadin is a potent and specific inhibitor of the human and mouse TREK1 channel (Refs. 29, 31, 32, and present study). Spadin does not block TREK2, TRAAK, TASK1, TRESK, IKr, or IKS currents (32). Human and rat TREK1 channels exhibit high levels of homology both at the nucleic level (90%) and at the protein level (96%). Furthermore, antidepressant effects had been described both in mice and rats (29). This work demonstrates that spadin reduces *1*) basal currents, *2*) AA-amplified currents, and *3*) stretch-activated currents at the micromolar range in cells expressing hTREK1 receptors. All these data point out that spadin is a valuable pharmacological tool to test mechanisms in which TREK1 channels might be involved both at cellular level or even in vivo. To our knowledge this is the first time that spadin has been tested in the gastrointestinal tract. Spadin neither increased spontaneous motility nor depolarized smooth muscle cells. Furthermore, in the presence of MRS2500, spadin did not inhibit the slow IJP or EFS-induced relaxation. In the latter, no effect was observed even when different levels of stretch were applied to the strips. These results suggest that the relative contribution of TREK1 channels in nitrergic neurotransmission is minor (Table 3). Moreover, they are not involved in purinergic neurotransmission because neither spontaneous IJP nor EFS-induced purinergic IJP is affected by spadin.

Interestingly, L-methionine and L-cysteine are not able to inhibit TREK1 channels expressed in COS-7 cells at the millimolar range, showing that these sulfur-containing amino acids are not suitable blockers of TREK1 channels. It is important to note that L-methionine increases motility and depolarizes smooth muscle cells as has been previously reported (33, 47); therefore, the effect of these compounds on gastrointestinal motility is probably not related to TREK1 channel inhibition. In addition, L-methionine do not modify purinergic or nitrergic IJP, showing that the biological effects caused by this compound are not related to a modification of the inhibitory neuromuscular transmission.

Apamin has been used as a pharmacological tool to distinguish between purinergic and nitrergic neurotransmission and the terminology "apamin sensitive" to define the purinergic component vs. "apamin insensitive" to define the nitrergic is often still used in the literature. The effect of apamin suggests that SK_{Ca} are involved in both pathways (Table 3) but different sensitivities were estimated. Apamin reduces the occurrence of spontaneous IJP (13, 40) and reduces the fast component of the IJP (34), suggesting that SK_{Ca} are involved in purinergic neurotransmission. In the present article we confirm the sensitivity of the purinergic IJP (MRS2500 sensitive) to apamin, suggesting that activation of $P2Y_1$ receptors (11) causes an increase in calcium that binds to calmodulin and opens SK_{Ca} (1). Moreover, at the micromolar range, apamin increases spontaneous motility, depolarizes smooth muscle cells, and partially inhibits the nitrergic junction potential and nitrergic mechanical relaxation. However, it is important to notice that apamin inhibits ~40% of the nitrergic IJP, suggesting that SK_{Ca} channels might partially mediate nitrergic responses. The mechanism that links cGMP elevation with SK_{Ca} activation is unknown. Thus both purinergic and nitrergic pathways partially converge in one mechanism although not necessarily in the same cell. SK_{Ca} channels are expressed in smooth muscle (35), ICC (8), and PDGFRα^+ cells (7, 42), and all three cell types also express GC, suggesting the presence of redundant mechanisms in the gastrointestinal tract (16). In addition, two subtypes of SK_{Ca} channels (i.e., SK2 and SK3) might participate in the responses. Apamin is a poor subtype-specific SK_{Ca} channel blocker (for review, see Refs. 1, 45) and it is hard to discriminate between SK2 and SK3 subtypes. However, SK2 are slightly more sensitive to apamin than SK3 [rat SK1 channels are insensitive to apamin (44)]. Subtype-specific SK_{Ca} blockers are needed to verify the relative contribution of each subtype in

Table 3. *Criteria to identify the effect of a drug in nitrergic and purinergic pathways*

	Inhibition of	
	Nitrergic neurotransmission	Purinergic neurotransmission*
Membrane potential†	Depolarization	No effect
Spontaneous motility†	Increase	No effect/Decrease‡
Spontaneous IJP†	No effect	Inhibition
EFS-induced IJP	Inhibition of the slow component	Inhibition of the fast component
EFS-induced relaxation§	Partial reversion	No effect/Partial reversion

*Based on previous data using inhibitors of $P2Y_1$ receptors (13, 15) and $P2Y_1$ knockout mice (11). †These criteria should be used if an inhibitory neural tone is present in the preparation (13). ‡A decrease in spontaneous motility might be expected if ATP is limiting pre/post junctional NO effect. §Electrical field stimulation (EFS)-induced relaxations might be reversed by $P2Y_1$ antagonists/NO synthase inhibitors depending on the frequency of EFS (9).

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purinergic and nitrergic responses. It is important to note that calcium-activated chloride channels (Cl_{Ca}) might also be involved in nitrergic smooth muscle hyperpolarization and relaxation (48). The contribution of these channels in each cell subtype needs further studying.

It is important to note that apamin was able to reduce the hyperpolarization induced by exogenous addition of SNP by ~30% whereas spadin did not modify the response. However, neither apamin nor spadin was able to reverse the inhibitory effect induced by SNP in mechanical relaxation, suggesting that the residual hyperpolarizing response (~70%) was enough to maintain smooth muscle cells hyperpolarized and that voltage-dependent calcium channels could not open. It is important to note that, if specialized areas to transduce signals are present (i.e., muscle, ICC, or PDGFRα^+ cells), the exogenous addition of the neurotransmitter might not mimic exactly the endogenous release from enteric inhibitory neurons.

We conclude that, in the rat colon, isolation of the nitrergic pathway by use of a $P2Y_1$ antagonist allows proper characterization of nitrergic neurotransmission, which is mediated by cGMP-pathway activation. Apamin inhibited both purinergic and nitrergic IJP with a different relative sensitivity. Thus the term and pharmacological approach "apamin sensitive vs. apamin insensitive" to distinguish purinergic vs. nitrergic pathways should not be used in the cotransmission process without previous studies of sensitivity to apamin in each pathway. Spadin, a TREK1 channel inhibitor, was used for the first time in the gastrointestinal tract and demonstrated the lack of TREK1 channel involvement in both purinergic and nitrergic neurotransmission. L-Methionine effects on motility did not appear to be related to TREK1 channel inhibition.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

V.G., D.G., H.M., R.P., and M.M.C. designed the protocols, performed the experiments, and participated in the preparation of the manuscript. C.H. and M.B. participated in the design of patch-clamp experiments. M.B. and M.J. edited and revised the manuscript.

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Chapter 5

Effects of inhibitors of hydrogen sulphide synthesis on rat colonic motility

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RESEARCH PAPER

Effects of inhibitors of hydrogen sulphide synthesis on rat colonic motility

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BACKGROUND AND PURPOSE
The role of hydrogen sulphide (H_2S) as a putative endogenous signalling molecule in the gastrointestinal tract has not yet been established. We investigated the effect of D,L-propargylglycine (PAG), an inhibitor of cystathionine γ-lyase (CSE), amino-oxyacetic acid (AOAA) and hydroxylamine (HA), inhibitors of cystathionine β-synthase (CBS) on rat colonic motility.

EXPERIMENTAL APPROACH
Immunohistochemistry, H_2S production, microelectrode and organ bath recordings were performed on rat colonic samples without mucosa and submucosa to investigate the role of endogenous H_2S in motility.

KEY RESULTS
CSE and CBS were immunolocalized in the colon. H_2S was endogenously produced (15.6 ± 0.7 nmol·min^{-1}·g^{-1} tissue) and its production was strongly inhibited by PAG (2 mM) and AOAA (2 mM). PAG (2 mM) caused smooth muscle depolarization and increased spontaneous motility. The effect was still recorded after incubation with tetrodotoxin (TTX, 1 μM) or N$^{\omega}$-nitro-L-arginine (L-NNA, 1 mM). AOAA (2 mM) caused a transient (10 min) increase in motility. In contrast, HA (10 μM) caused a 'nitric oxide-like effect', smooth muscle hyperpolarization and relaxation, which were antagonized by 1H-[1,2,4] oxadiazolo[4,3-α]quinoxalin-1-one (ODQ, 10 μM). Neither spontaneous nor induced inhibitory junction potentials were modified by AOAA or PAG.

CONCLUSIONS AND IMPLICATIONS
We demonstrated that H_2S is endogenously produced in the rat colon. PAG and AOAA effectively blocked H_2S production. Our data suggest that enzymatic production of H_2S regulates colonic motility and therefore H_2S might be a third gaseous inhibitory signalling molecule in the gastrointestinal tract. However, possible non-specific effects of the inhibitors should be considered.

Abbreviations
AOAA, amino-oxyacetic acid; AUC, area under the curve; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; D-AP5, D-(-)-2-amino-5-phosphonopentanoic acid; EFS, electrical field stimulation; GI, gastrointestinal; HA, hydroxylamine; ICC, interstitial cells of Cajal; IJP, inhibitory junction potential; IR, immunoreactivity; L-NNA, N$^{\omega}$-nitro-L-arginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; PAG, D,L-propargylglycine; RMP, resting membrane potential; TTX, tetrodotoxin

Introduction

Hydrogen sulphide (H_2S) is an endogenous gaseous signalling molecule similar to NO and carbon monoxide (CO). The biology of H_2S is an emerging topic of research and H_2S potentially plays important roles in several areas including the CNS, cardiovascular, renal, respiratory and digestive systems (Fiorucci *et al*., 2006). In mammals, two pyridoxal

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phosphate-dependent enzymes are responsible for H_2S synthesis: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). These two enzymes are expressed in a great variety of organs and tissues including the liver, kidney, vascular and central nervous system. Both enzymes use L-cysteine as a substrate to produce H_2S (review by Szabo, 2007). A third route of H_2S synthesis involves 3-mercaptopyruvate sulphurtransferase in combination with cysteine aminotransferase (Shibuya *et al.*, 2009a; Shibuya *et al.*, 2009b). Three inhibitors of these enzymes are commonly used to inhibit H_2S synthesis: D,L-propargylglycine (PAG), an inhibitor of CSE and both amino-oxyacetic acid (AOAA) and hydroxylamine (HA), inhibitors of CBS. These inhibitors are important pharmacological tools for investigating the effects of endogenous production of hydrogen sulphide (reviewed by Szabo, 2007).

Little is known as yet about the physiological function of H_2S in the gastrointestinal (GI) tract (Linden *et al.*, 2010; Wallace, 2010) but it might play a role as a 'gasotransmitter' and the criteria to consider it as such have recently been reviewed: 'The gasotransmitter must: 1. be small molecules of gas, 2. be freely permeable to membranes, 3. be endogenously and enzymatically generated in a regulated manner, 4. have well-defined specific functions at physiologically relevant concentrations, and 5. act at specific cellular targets' (Linden *et al.*, 2010). H_2S fulfils part of these criteria.

H_2S is endogenously produced in the colon of the mouse (Linden *et al.*, 2008). Smooth muscle homogenates produce H_2S through CBS and CSE enzymes (Hosoki *et al.*, 1997). Both enzymes have been detected immunohistochemically in rat colonic smooth muscle cells (Hennig and Diener, 2009). CSE immunoreactivity (IR) has been found in neurons of the mouse and guinea-pig myenteric plexus and in neurons of the guinea-pig and human submucous plexus as well as in certain subclasses of interstitial cells of Cajal (ICC) in the guinea-pig colon (Schicho *et al.*, 2006; Linden *et al.*, 2008). CBS-IR has also been detected in guinea-pig myenteric and submucous plexus and in human submucous plexus (Schicho *et al.*, 2006). These data suggest that several cell structures are able to synthesize H_2S, which is therefore a potential signalling molecule regulating GI motility.

Sodium hydrosulphide (NaHS) is the source of H_2S usually employed to study H_2S functions. The effects induced by NaHS and its mechanisms of action, however, differ between species and might also depend on the concentration used in the experiments. NaHS exerts a prosecretory effect through a neurally mediated mechanism [tetrodotoxin (TTX)-sensitive] both in the guinea-pig and human colon (Schicho *et al.*, 2006; Krueger *et al.*, 2010). In the rat colon, a TTX-sensitive and also an insensitive response, probably due to direct stimulation of apical as well as basolateral epithelial K^+ channels, have been reported (Hennig and Diener, 2009; Pouokam and Diener, 2011). NaHS causes concentration-dependent relaxation of the guinea-pig and rabbit ileum (Hosoki *et al.*, 1997; Teague *et al.*, 2002). We have recently demonstrated that NaHS inhibits the peristaltic activity in the mouse colon and small intestine and also inhibits the spontaneous contractions in rat and human colon measured in organ bath (Gallego *et al.*, 2008a). NaHS also caused inhibition of precontracted gastric and colonic strips in mice (Dhaese and Lefebvre, 2009; Dhaese *et al.*, 2010). H_2S donors are drugs that could be used to treat several GI disorders such as ulcers and colonic inflammation (Fiorucci *et al.*, 2007; Wallace *et al.*, 2007; Wallace *et al.*, 2009), although their putative beneficial and toxic effects are much debated in the literature (Coffey *et al.*, 2009; Matsunami *et al.*, 2009; Schemann and Grundy, 2009).

Little is known about the enzymatic endogenous production of H_2S and colonic motility. In the present paper, we characterized the effects of H_2S synthesis inhibitors, PAG, AOAA and HA, as well as the effect of L-cysteine, which is a precursor of H_2S synthesis, on rat colonic motility. Briefly, we found that PAG and AOAA effectively blocked H_2S production and increased motility, suggesting that H_2S is an endogenous inhibitory signalling molecule in the GI tract. However, non-specific effects of these compounds should be considered.

Methods

Animals and tissue samples

Male Sprague-Dawley rats (8–10 weeks old, 300–350 g) were purchased from Charles River (Lyon, France). Animals were housed under controlled conditions: temperature 22°C ± 2°C, humidity 55% ± 10%, 12:12 h light–dark cycle and access to water and food *ad libitum*. Animals were stunned and killed by decapitation and exsanguination 2–3 s afterwards. The colon was quickly removed and placed in carbogenated (95% O_2 and 5% CO_2), ice-cold physiological saline solution. Then, it was opened along the mesenteric border and pinned to a Sylgard® base (mucosa side up). The mid colon was identified according to anatomical criteria previously described (Alberti *et al.*, 2005). The mucosal and submucosal layers were removed and muscle strips were cut 1 cm × 2 cm for the endogenous production of H_2S and 1 cm × 0.3 cm for the intracellular microelectrode and organ bath experiments. All procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Immunohistochemistry

Mid colon samples were fixed with paraformaldehyde (4%) in PBS (0.2 M) and embedded in paraffin. Paraffin sections were cut, mounted on glass slides, deparaffinized and rehydrated. To reduce autofluorescence, a 30 min pretreatment with NH_4Cl (50 mM; pH 8.0) was performed. After the sections had been washed in PBS-T (PBS containing; 0.01% Triton X-100; 0.02% Tween 20), they were incubated at 100°C in citrate buffer (10 mM; pH 6.0) for 20 min to retrieve antigens, cooled for 30 min and rinsed again with PBS-T. Non-specific binding was blocked by incubating the samples in PBS-T containing normal goat serum (10%). Mouse monoclonal anti-CSE or mouse polyclonal anti-CBS (both 1:50; ABNOVA, Taipei, Taiwan) diluted in PBS were incubated with the sections overnight at 4°C. These antibodies were co-incubated with a rabbit polyclonal Anti-HuD (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to label enteric neurons. After 24 h, the samples were rinsed in PBS-T and then incubated with the secondary antibodies (both 1:200; Alexa Fluor® 488 goat anti-mouse and Alexa Fluor® 568 goat anti-rabbit from Invitrogen Ltd, Paisley, UK) diluted in PBS for 1 h at room temperature. Negative controls were per-

formed by leaving out the primary antibodies during the staining procedure. Additional controls with pre-absorbed antisera with CSE or CBS recombinant proteins (both 1 μM; ABNOVA, Taipei, Taiwan) were performed as well. The samples were examined on an Axioskop 40 FL fluorescence microscope equipped with a AxioCam MRm camera and the AxioVision Release 4.8.1 software for image acquisition (all of them from Carl Zeiss, Inc., Göttingen, Germany).

Endogenous production of hydrogen sulphide

Production of H_2S was measured as previously described (Linden *et al*., 2008). Briefly, the tissue was placed in a sealed polypropylene vial containing physiological saline solution with L-cysteine (10 mM) and pyridoxal 5′-phosphate (2 mM) which was connected to a second vial containing 0.5 mL of 1% (w v^{-1}) zinc acetate. The zinc acetate solution did not come in contact with the tissue. The first vial was bubbled with a gas mixture of 95% O_2 and 5% CO_2 at a rate of 1–4 mL·min^{-1} in order to minimize the degradation of H_2S (Linden *et al*., 2008). The increase in pressure in the first vial forced the gases to move into the second vial where they bubbled through the zinc acetate solution and H_2S was trapped as zinc sulphide. The incubation mixture was prepared on ice and the reaction was started by transferring the vials to a water bath at 37 ± 1°C. The reaction was stopped at 30 min by injecting 0.5 mL of 50% (w v^{-1}) trichloroacetic acid through a stainless steel needle. Gas flow was allowed to continue for an additional 30 min to ensure complete trapping of H_2S. In some experiments, PAG (2 mM) or AOAA (2 mM) were added to the incubation mixture in order to block CSE and CBS activity, respectively.

H_2S was measured by using a colorimetric method (Siegel, 1965; Stipanuk and Beck, 1982; Abe and Kimura, 1996). The content of the second vial was transferred to test tubes and 3.5 mL of distilled water, 0.4 mL of N,N-dimethyl-p-phenylenediamine sulphate (20 mM) in HCl (7.2 M) and 0.4 mL of $FeCl_3$ (30 mM) in HCl (1.2 M) were added to each tube. After 20 min of incubation at room temperature, the absorbance of the resulting solution at 670 nm was determined with a Ultrospec 2000 spectrophotometer [Pharmacia Biotech (Biochrom) Ltd, Cambrige, UK]. A linear regression was set up with defined concentrations of NaHS and the concentration of H_2S was estimated and expressed in nmol·min^{-1}·g^{-1} tissue.

Intracellular microelectrode recording

The tissue was pinned with the circular muscle layer facing upward in a Sylgard-coated chamber and continuously perfused with carbogenated physiological saline solution at 37°C ± 1°C and was allowed to equilibrate for 1 h. Circular smooth muscle cells 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, UK) and simultaneously digitalized (100 Hz) with PowerLab 4/30 system and Chart 5 software for Windows (both from ADInstruments, Castle Hill, NSW, Australia). In order to stabilize impalements, experiments were performed in the presence of nifedipine (1 μM). The spontaneous inhibitory neural tone was characterized as we have described previously (Gil *et al*., 2010). Briefly, the resting membrane potential (RMP) (expressed in mV) was estimated as the most probable bin of the frequency distribution of the membrane potential (0.1 mV bins; 30–60 s recordings). Spontaneous inhibitory junction potentials (sIJP) were illustrated with the frequency distribution (0.5 mV bins) of the values of the membrane potential (30 to 60 s) expressed as bin probability (from 0 to 1), and for statistical purposes we calculated the mean SD of the distribution of the membrane potential: SD of the recording inside the cell minus SD of the recording outside the cell (expressed in mV). IJP were induced by electrical field stimulation (EFS) using the following parameters: total train duration 100 ms, frequency 20 Hz, pulse duration 0.3 ms and increasing amplitude voltage (5, 10, 12, 15, 17, 20, 25, 30 and 50 V). Both IJP amplitude (from RMP to the most hyperpolarized value) and duration (measured at the baseline) were estimated for each voltage of stimulation.

Muscle bath studies

Muscle strips were mounted in a 10 mL organ bath containing carbogenated physiological saline solution maintained at 37 ± 1°C. Motility was measured using an isometric force transducer (Harvard VF-1, Harvard Apparatus Inc., Holliston, MA, USA) connected to a computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. A tension of 1 g was applied and tissues were allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. The release of inhibitory neurotransmitters was studied by using EFS applied for 4 min; pulse duration 0.3 ms; frequency 5 Hz; amplitude 30 V. The area under the curve (AUC) of contractions from the baseline was measured to estimate the mechanical activity and the result was expressed in g·min^{-1}.

Solutions and drugs

The composition of the physiological saline 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). All the chemicals employed in the H_2S production were purchased from Sigma Chemicals (St. Louis, MO, USA). The following drugs were used: TTX (Latoxan, Valence, France); AOAA, PAG, HA, L-cysteine, nifedipine, N^{ω}-nitro-L-arginine (L-NNA), 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) (Sigma Chemicals, St. Louis, MO, USA); D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) (Tocris, Bristol, UK). Stock solutions were made by dissolving drugs in distilled water except for: (i) nifedipine which was dissolved in 96% ethanol; (ii) L-NNA which was dissolved in physiological saline solution by sonication; and (iii) AOAA which was dissolved in physiological saline solution and the pH was adjusted to 7.4 by using NaOH. Drug and receptor nomenclature conform to the guidelines of the *British Journal of Pharmacology* (Alexander *et al*., 2009).

Data analysis and statistics

Differences in the RMP, sIJP, spontaneous motility (AUC) and EFS-induced relaxation were compared before and after drug addition by Student's paired *t*-test. One-way ANOVA followed

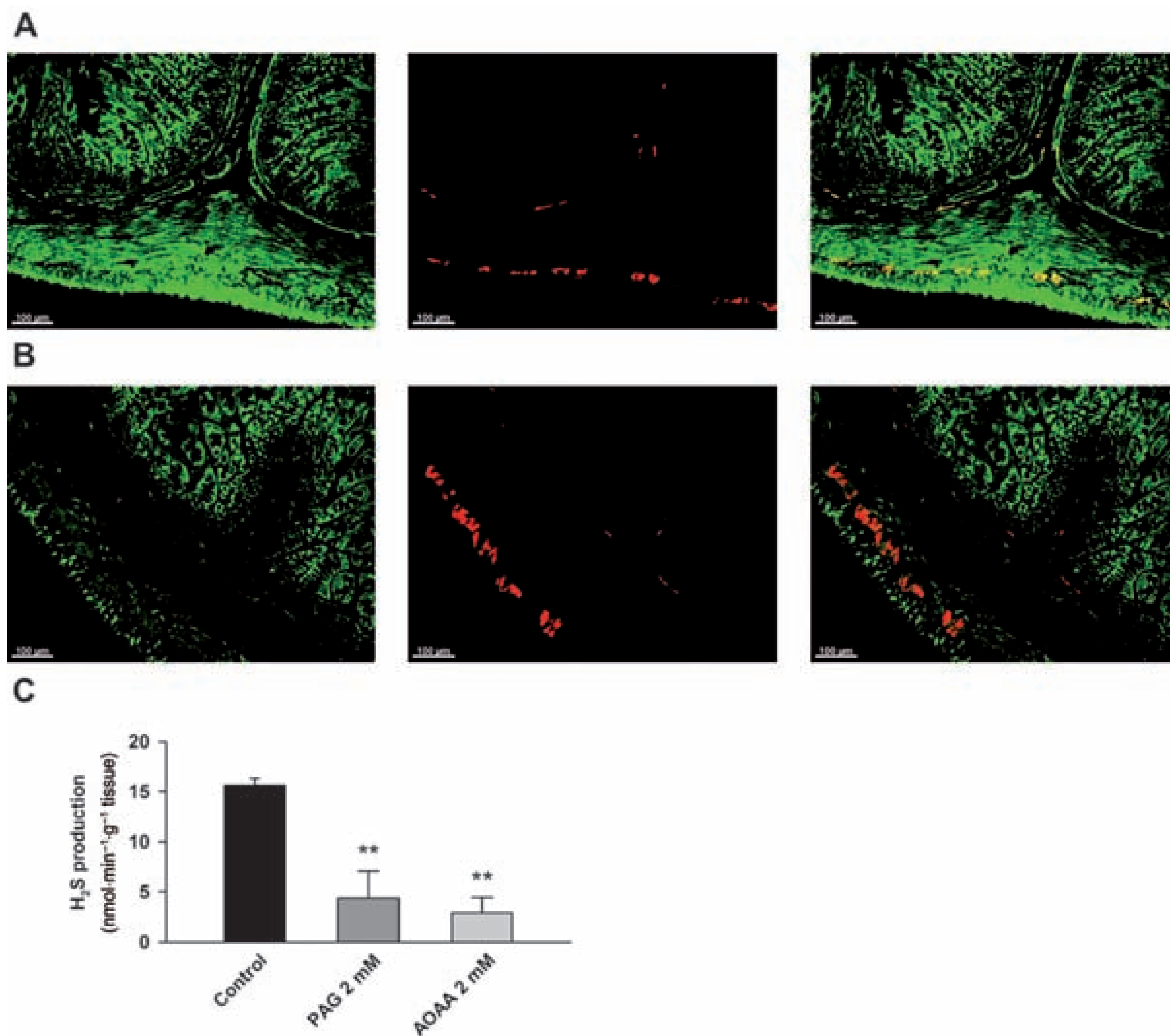

Figure 1

Distribution of cystathionine γ-lyase (CSE) (A) and cystathionine β-synthase (CBS) (B) in the rat mid colon. Left: CSE/CBS-immunoreactivity (IR); middle: HuD-IR; right: merged. Scale bar = 100 μm. Histogram showing the production of H_2S in rat colonic samples devoid of mucosa and submucosa in control conditions and in the presence of PAG (2 mM) and AOAA (2 mM) (C). All values are mean ± SEM. Significant differences were assessed using one-way ANOVA, followed by Bonferroni *post hoc* test. $^{**}P < 0.01$; significant difference from control.

by Bonferroni *post hoc* test was used to evaluate the effect of PAG and AOAA on the endogenous H_2S production. Differences between the amplitude and duration of the electrically-elicited IJPs before and after drug infusion were compared by two-way ANOVA (drug and voltage). IC_{50} values were calculated using a conventional sigmoid concentration–response curve with variable slope.

Data are expressed as mean ± SEM. A $P < 0.05$ was considered statistically significant; *n* values indicate the number of samples. Statistical analysis and curve fit were performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

Results

CSE and CBS expression in the rat mid colon and endogenous production of H_2S

CSE-IR was mainly observed in the circular and longitudinal smooth muscle layers. Double-labelling with the neuronal marker anti-HuD showed that CSE was expressed in neurons of the enteric nervous system as well. Furthermore, diffuse CSE-IR was also present in the mucosa and submucosa layers (Figure 1A). A completely different pattern was found for CBS. Positive IR for this enzyme was mainly localized in the

Table 1

Effects of hydrogen sulphide synthesis inhibitors (PAG, AOAA and HA) and the precursor of its synthesis (L-cysteine), in the absence and presence of TTX, L-NNA or ODQ, on smooth muscle membrane potential and motility

Drug	Control[a] In the presence of:	Membrane potential (mV) n	Control[a]	Drug	P-value	Motility (g·min^{-1}) n	Control*	Drug	P-value
PAG	–	9	–47.4 ± 2.8	–39.2 ± 2.8	<0.001	9	10.3 ± 0.9	17.7 ± 1.6	<0.001
PAG	TTX	5	–43.8 ± 0.6	–37.6 ± 1.5	<0.01	8	18.6 ± 4.0	25.2 ± 4.9	<0.05
PAG	L-NNA	4	–42.7 ± 1.1	–37.0 ± 1.7	<0.01	6	32.3 ± 6.8	41.4 ± 6.6	<0.001
AOAA	–	5	–44.5 ± 1.5	–42.4 ± 1.7	n.s.	11	12.8 ± 2.3	18.2 ± 3.2	<0.05
AOAA	TTX	5	–45.7 ± 3.0	–43.9 ± 3.1	n.s.	14	27.3 ± 4.3	15.6 ± 2.4	<0.01
AOAA	L-NNA	4	–39.8 ± 3.6	–39.0 ± 3.9	n.s.	4	48.0 ± 9.8	21.0 ± 4.2	<0.05
HA	–	4	–45.8 ± 2.2	–51.5 ± 1.6	<0.05	5	22.0 ± 4.0	0.2 ± 0.2	<0.01
HA	TTX	4	–44.0 ± 2.6	–53.1 ± 3.4	<0.05	6	34.0 ± 10.2	0.0 ± 0.0	<0.05
HA	ODQ	4	–41.9 ± 1.3	–42.8 ± 1.4	n.s.	5	32.0 ± 6.8	28.4 ± 6.0	<0.05
L-cys	–	4	–52.5 ± 1.7	–42.8 ± 1.3	<0.01	9	11.4 ± 2.1	21.3 ± 3.7	<0.001
L-cys	TTX	5	–40.9 ± 2.6	–36.0 ± 2.3	<0.05	7	23.9 ± 5.0	38.1 ± 6.8	<0.001

[a]Control are values of the membrane potential or motility before drug addition but in the presence of TTX, L-NNA, L-cysteine, etc.
Values are means ± SE. n, no. of samples; PAG, D,L-propargylglycine (2 mM); TTX, tetrodotoxin (1 μM); L-NNA, N$^{\omega}$-nitro-L-arginine (1 mM); L-cys, L-cysteine (1 mM); AOAA, amino-oxyacetic acid (2 mM); HA, hydroxylamine (10 μM); ODQ, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (10 μM). The statistical significance of differences was assessed by using Student's paired t-test. n.s., not significant.

colonic epithelium, although a diffuse pattern was also observed in the muscular layers. Colocalization between CBS-IR and HuD-IR was not observed showing that CBS was not expressed in neurons (Figure 1B). No CSE-IR or CBS-IR was detected when primary antibodies were left out or pre-absorption with recombinant proteins was performed (data not shown).

Colonic tissue in which mucosa and submucosa had been removed was able to produce H_2S (15.6 ± 0.7 nmol·min^{-1}·g^{-1} tissue; n = 4; Figure 1C). H_2S production was reduced when the experiments were performed in the presence of PAG (2 mM) (4.4 ± 2.7 nmol·min^{-1}·g^{-1} tissue; n = 4; $P < 0.001$; Figure 1C) and AOAA (2 mM) (2.9 ± 1.5 nmol·min^{-1}·g^{-1} tissue; n = 3; $P < 0.01$; Figure 1C), showing that it was due to CSE and CBS activity. We did not test HA on H_2S production due to the 'NO-like effects' described below.

Effect of PAG on RMP and spontaneous mechanical activity

Effect of PAG was evaluated on the RMP and mechanical activity. PAG induced a concentration-dependent increase in motility (IC_{50} = 1.55 mM; 95% confidence interval 1.26–1.90 mM; log IC_{50} = –2.81 ± 0.09; n = 4; Figure 2A). A time-dependent control was performed and the spontaneous motility remained stable during the experiment (not shown). Furthermore, administration of PAG (2 mM) depolarized smooth muscle cells and increased mechanical activity (Table 1 and Figure 2B,C). In order to test whether the depolarization and increase in motility were due to a neural effect, we performed experiments with the tissue pre-incubated with TTX (1 μM) and L-NNA (1 mM). As previously reported (Gil *et al.*, 2010), both TTX and L-NNA caused depolarization (Control: –48.2 ± 1.2 mV vs. TTX: –43.6 ± 1.2 mV; n = 19; $P < 0.001$ and Control: –47.1 ± 1.8 mV vs. L-NNA: –41.2 ± 1.8 mV; n = 8; $P < 0.001$) and an increase in spontaneous motility (Control: 9.5 ± 1.1 g·min^{-1} vs. TTX: 24.1 ± 2.5 g·min^{-1} AUC; n = 39; $P < 0.001$ and Control: 15.2 ± 4.3 g·min^{-1} vs. L-NNA: 39.7 ± 7.1 g·min^{-1} AUC; n = 8; $P < 0.01$). In the presence of TTX (1 μM), PAG (2 mM) induced smooth muscle depolarization and an increase in spontaneous motility (Table 1 and Figure 2D,E). In the presence of L-NNA (1 mM), PAG (2 mM) also caused smooth muscle depolarization and increased spontaneous mechanical activity (Table 1 and Figure 2F,G). The depolarization induced by PAG (2 mM) was repetitive. Four consecutive transient (5 min) incubations with PAG (2 mM) produced the same depolarization (1st addition: 8.0 ± 0.4 mV, 2nd addition: 9.6 ± 1.4 mV, 3rd addition: 9.2 ± 0.4 mV, 4th addition: 9.0 ± 0.5 mV; n = 3; not significant; Figure 2H). Note that after each incubation, the membrane potential of smooth muscle cells returned to a value similar to control levels (Control: –43.5 ± 5.3 mV; 1st addition –43.9 ± 5.6 mV, 2nd addition –44.1 ± 5.0 mV, 3rd addition –44.5 ± 5.1 mV and 4th addition –45.0 ± 4.9 mV n = 3; not significant; Figure 2H).

Effect of AOAA on RMP and motility

AOAA is a CBS inhibitor. The effect of AOAA was tested on membrane potential and spontaneous motility. AOAA (2 mM) did not depolarize the smooth muscle cells and produced a transient increase in motility, which lasted for 10 min (Table 1 and Figure 3A,B). After 10 min, the spontaneous motility returned to basal values. In order to investigate the putative neural effects of AOAA, experiments were performed in the presence of the neural blocker TTX or the NOS inhibitor L-NNA. Surprisingly, AOAA (2 mM) induced a

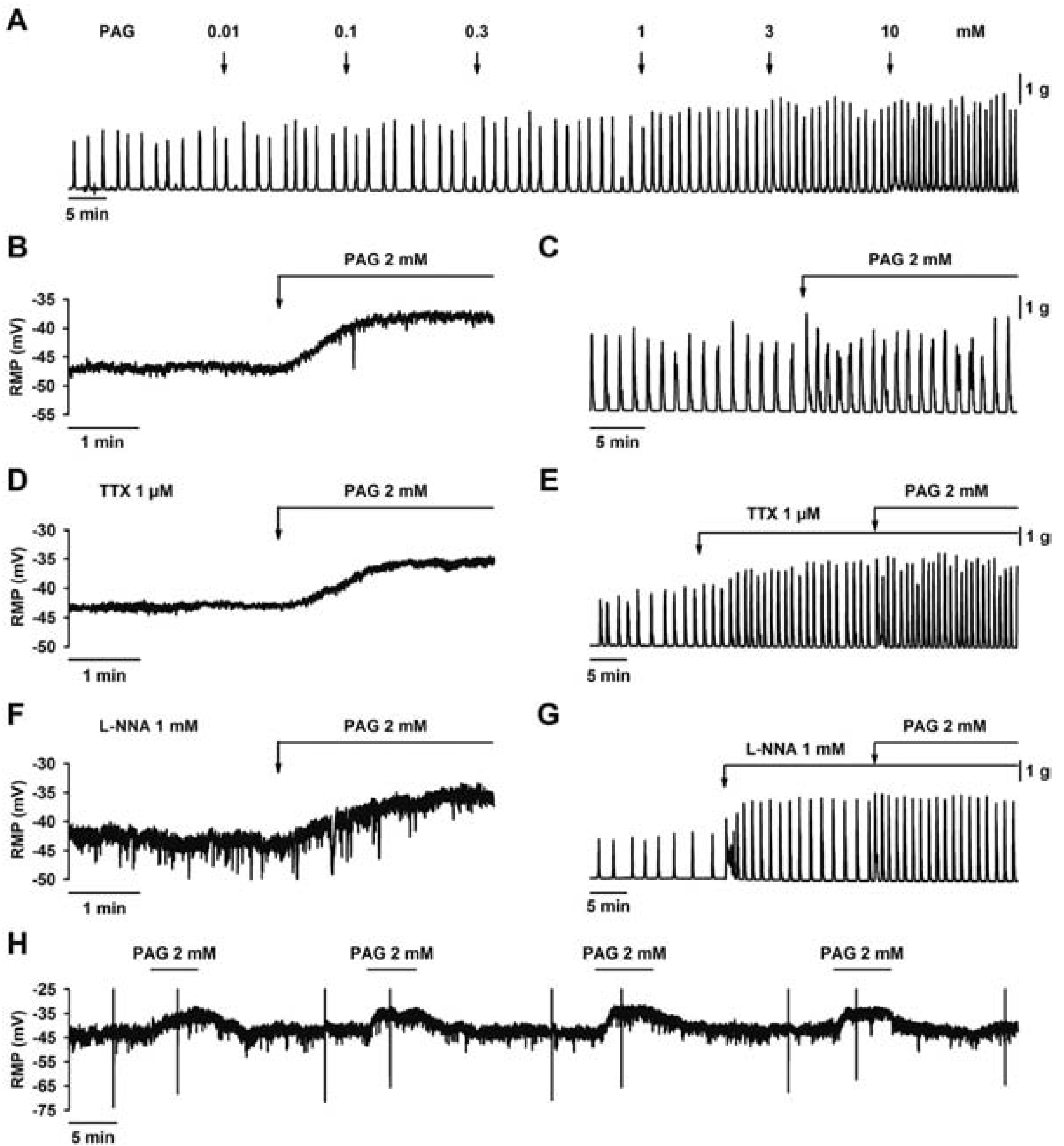

Figure 2

Effect of D,L-propargylglycine (PAG) on smooth muscle resting membrane potential and spontaneous mechanical activity. (A) Mechanical recording showing that PAG increased spontaneous motility in a concentration-dependent manner. PAG (2 mM) induced smooth muscle depolarization (B) and an increase in spontaneous motility (C) which was still recorded in the presence of TTX (1 µM) (D, E) and L-NNA (1 mM) (F, G). Four consecutive incubations with PAG (2 mM) produced four consecutive depolarizations (H). For statistics, see Table 1.

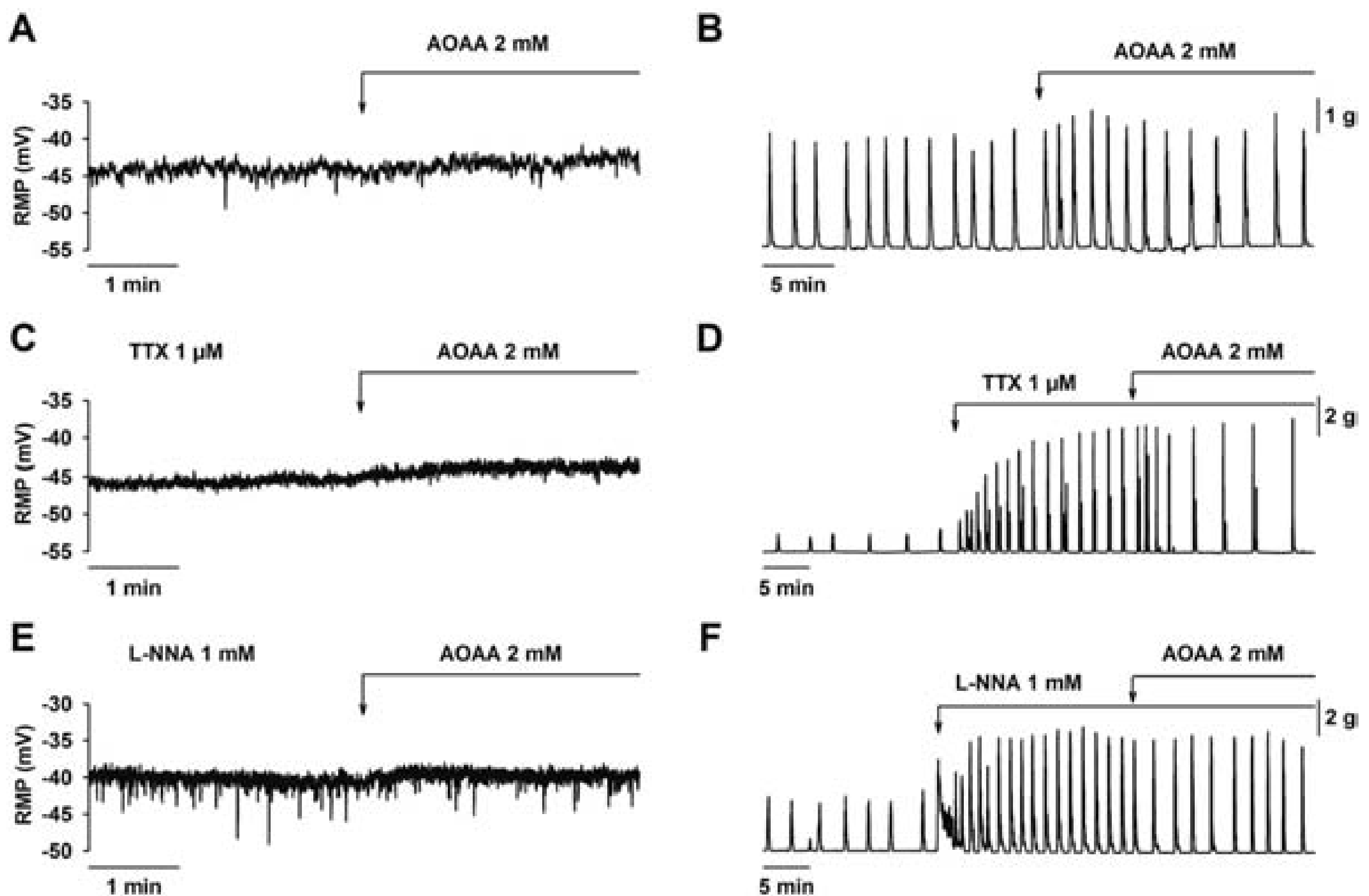

Figure 3

Effect of aminooxyacetic acid (AOAA) on smooth muscle resting membrane potential and spontaneous mechanical activity. AOAA (2 mM) did not modify the resting membrane potential in control (A), in the presence of TTX (1 μM) (C) or L-NNA (1 mM) (E). AOAA (2 mM) caused a transient increase in spontaneous motility (B). In the presence of TTX (1 μM) (D) or L-NNA (1 mM) (F), AOAA (2 mM) caused an inhibition of spontaneous motility. For statistics, see Table 1.

significant reduction of spontaneous motility in the presence of TTX (1 μM) or L-NNA (1 mM) (Table 1 and Figure 3D,F). No change in the RMP was observed in the presence of TTX (1 μM) or L-NNA (1 mM) (Table 1 and Figure 3C,E).

Effect of HA on RMP and motility

HA inhibited the mechanical spontaneous activity in a concentration-dependent manner (IC_{50} = 0.31 μM; 95% confidence interval 0.21–0.45 μM; log IC_{50} = –6.51 ± 0.08; n = 4. Figure 4A). HA (10 μM) hyperpolarized the smooth muscle cells and almost abolished spontaneous mechanical activity (Table 1 and Figure 4B,C). In the presence of TTX (1 μM), HA (10 μM) also hyperpolarized smooth muscle cells and abolished the spontaneous mechanical activity (Table 1 and Figure 4D,E). HA might act as an NO donor (Southam and Garthwaite, 1991; Correia *et al.*, 2000) and so we tested HA in the presence of ODQ, a cGMP inhibitor. ODQ (10 μM) prevented both the hyperpolarization [HA (10 μM): 5.7 ± 1.1 mV vs. ODQ (10 μM) + HA (10 μM): 0.9 ± 0.5 mV hyperpolarization; n = 4; $P < 0.05$; Table 1 and Figure 4F] and the inhibition of spontaneous motility [HA (10 μM): 98.9 ± 1.1 % vs. ODQ (10 μM) + HA (10 μM): 10.9 ± 1.5 % of inhibition; n = 5; $P < 0.001$; Table 1 and Figure 4G].

Effect of AOAA and PAG on neural-mediated responses

sIJPs were recorded in samples of rat mid colon (0.72 ± 0.06 mV SD; n = 14). AOAA (2 mM) did not modify these sIJPs [Control: 0.65 ± 0.08 mV vs. AOAA (2 mM): 0.66 ± 0.07 mV SD; n = 5; not significant; Figure 5A]. Similar results were obtained with PAG (2 mM) (Control: 0.76 ± 0.08 mV vs. PAG (2 mM): 0.75 ± 0.09 mV SD; n = 9; not significant; Figure 5B). It is important to note that the shift to the right in the frequency distribution is indicative of smooth muscle depolarization. The effect of AOAA and PAG was tested on the IJP and relaxation induced by EFS. AOAA (2 mM) did not modify the IJP or the inhibition of motility induced by EFS (Figure 6A,D). PAG (2 mM) slightly increased IJP amplitude at the highest voltages but did not modify IJP duration or EFS-induced relaxation (Figure 6B,E). Combined addition of

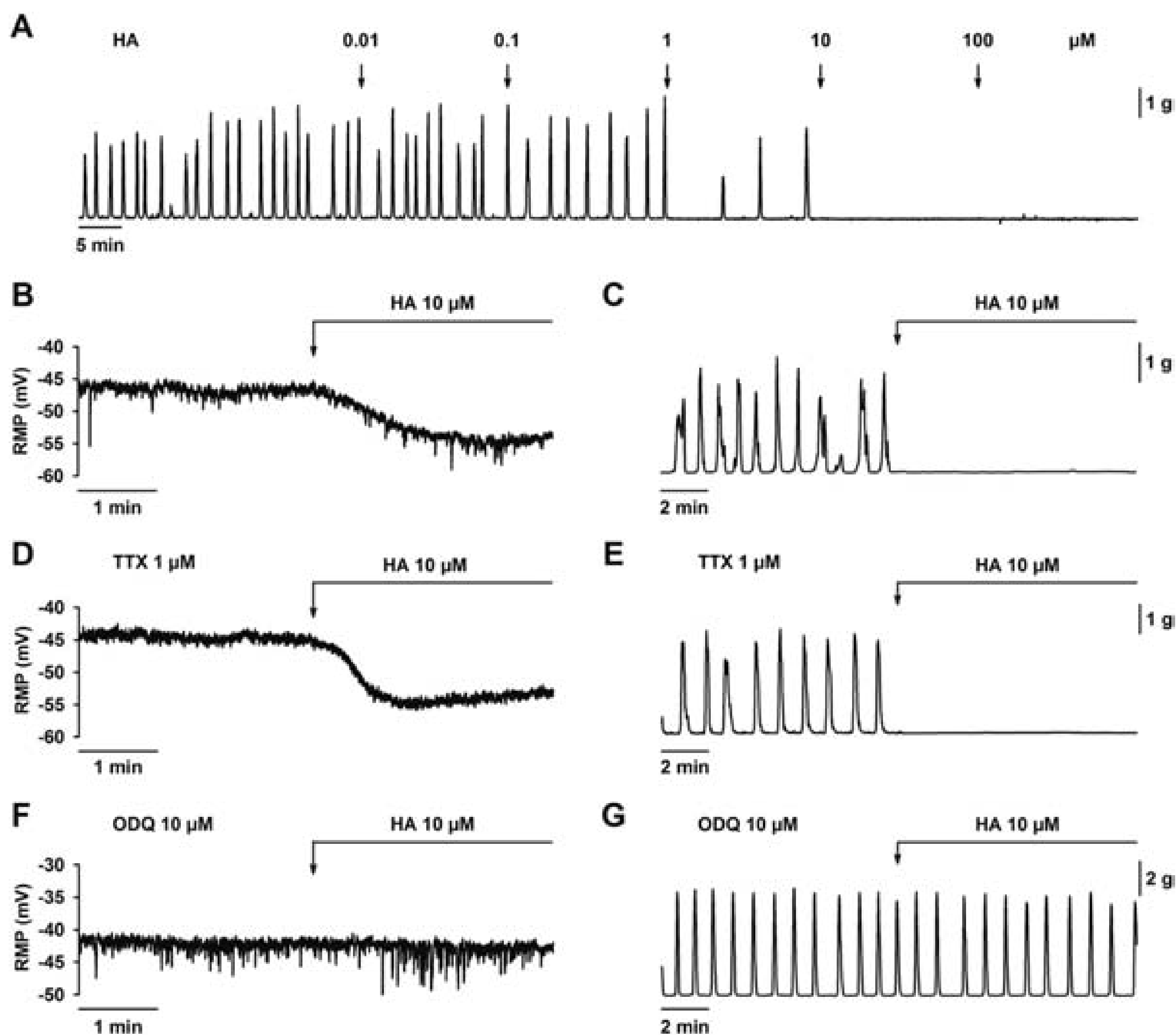

Figure 4

Effect of hydroxylamine (HA) on smooth muscle resting membrane potential and spontaneous mechanical activity. (A) Mechanical recording showing that HA decreased spontaneous motility in a concentration-dependent manner. HA (10 μM) induced smooth muscle hyperpolarization (B) and abolished spontaneous motility (C). Both effects were still recorded in the presence of TTX (1 μM) (D, E) but ODQ (10 μM) inhibited HA (10 μM) induced hyperpolarization (F) and inhibition of spontaneous motility (G). For statistics, see Table 1.

AOAA (2 mM) and PAG (2 mM) produced very similar effects to those observed when PAG (2 mM) was administered alone (Figure 6C,F).

Effect of L-cysteine on spontaneous motility and RMP

L-cysteine is the substrate of both CBS and CSE enzymes so we wanted to test its effect on membrane potential and spontaneous motility. L-cysteine increased spontaneous motility in a concentration-dependent manner (IC_{50} = 0.72 mM; 95% confidence interval 0.55–0.93 mM; log IC_{50} = −3.14 ± 0.11; *n* = 4. Figure 7A). Addition of L-cysteine (1 mM) depolarized smooth muscle cells and increased spontaneous motility (Table 1 and Figure 7B,C). It is important to note that the effect of L-cysteine was immediately observed after its addition and it was not affected by neural blocking by TTX (1 μM) (Table 1 and Figure 7D,E). L-Cysteine is an agonist of NMDA receptors (Schicho *et al.*, 2006). Therefore, to evaluate this putative pathway, organ bath experiments were performed using D-AP5, a NMDA receptor antagonist. However, pre-incubation with D-AP5 (100 μM) did not modify the effect of

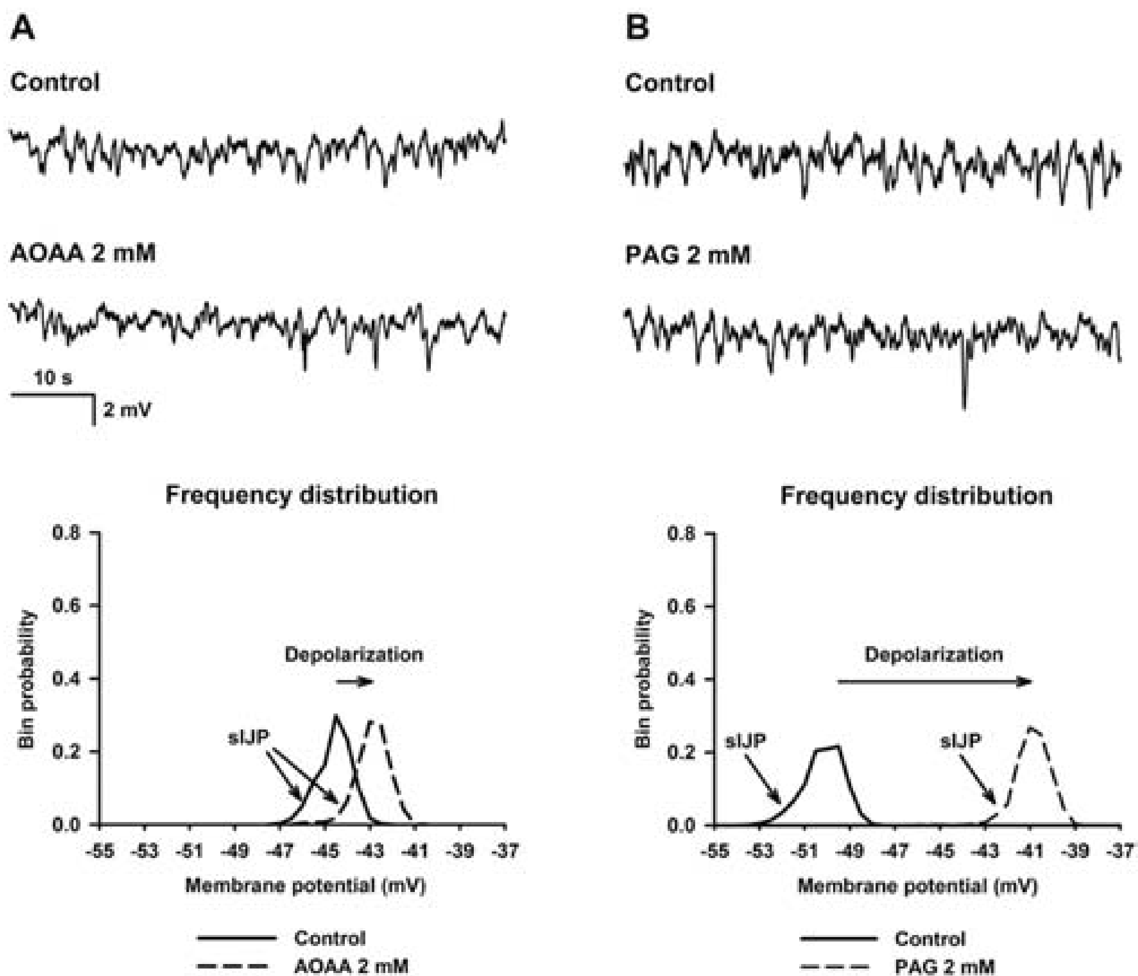

Figure 5

Effect of amino-oxyacetic acid (AOAA, 2 mM) (A) and D,L-propargylglycine (PAG, 2 mM) (B) on resting membrane potential and spontaneous inhibitory junction potentials (sIJP). Top: representative microelectrode recordings. Bottom: representative frequency distribution (0.5 mV bins) of the membrane potential in control and after drug addition.

L-cysteine on spontaneous motility (Control: 26.2 ± 6.1 g·min^{-1} vs. L-cysteine (1 mM): 38.9 ± 7.1 g·min^{-1} AUC; $n = 4$; $P < 0.01$), showing that NMDA receptors are not involved in the response to L-cysteine.

Discussion

H_2S is a gaseous mediator that is endogenously produced in several systems of the body including the GI tract (Linden *et al.*, 2008; Martin *et al.*, 2010). Although its physiological function is still unknown (Linden *et al.*, 2010), it has been shown that H_2S donors cause colonic secretion (Schicho *et al.*, 2006; Hennig and Diener, 2009; Krueger *et al.*, 2010; Pouokam and Diener, 2011) and regulate motility, causing inhibition of peristalsis and smooth muscle relaxation in several areas of the GI tract (Hosoki *et al.*, 1997; Teague *et al.*, 2002; Gallego *et al.*, 2008a; Dhaese and Lefebvre, 2009; Dhaese *et al.*, 2010). H_2S donors have been proposed as potential therapeutic agents to treat colonic inflammation and improve ulcer healing (Fiorucci *et al.*, 2007; Wallace *et al.*, 2007), so H_2S might have important therapeutic potential (Szabo, 2007; Bannenberg and Vieira, 2009). However, high concentrations of H_2S are known to be toxic, causing inhibition of mitochondrial cytochrome C oxidase (Li and Moore, 2007). To be able to distinguish between physiological, pharmacological and toxicological effects, one possible experimental approach is to block the endogenous synthesis of H_2S. PAG has been commonly used as a putative inhibitor of CSE and both AOAA and HA have been employed as putative inhibitors of CBS. These inhibitors are usually employed to inhibit H_2S production in cell homogenates at the tissue level and also in some *in vivo* experiments using animal models of disease (see for review Szabo, 2007).

In the present study, we indentified strong CSE-IR in smooth muscle cells of the lamina propia in the rat colon whereas CBS-IR was less intense but still positive in muscle layers. A similar staining was described in the rat colon (Hennig and Diener, 2009). Note that major differences between species might exist. For example, a 'diffuse staining'

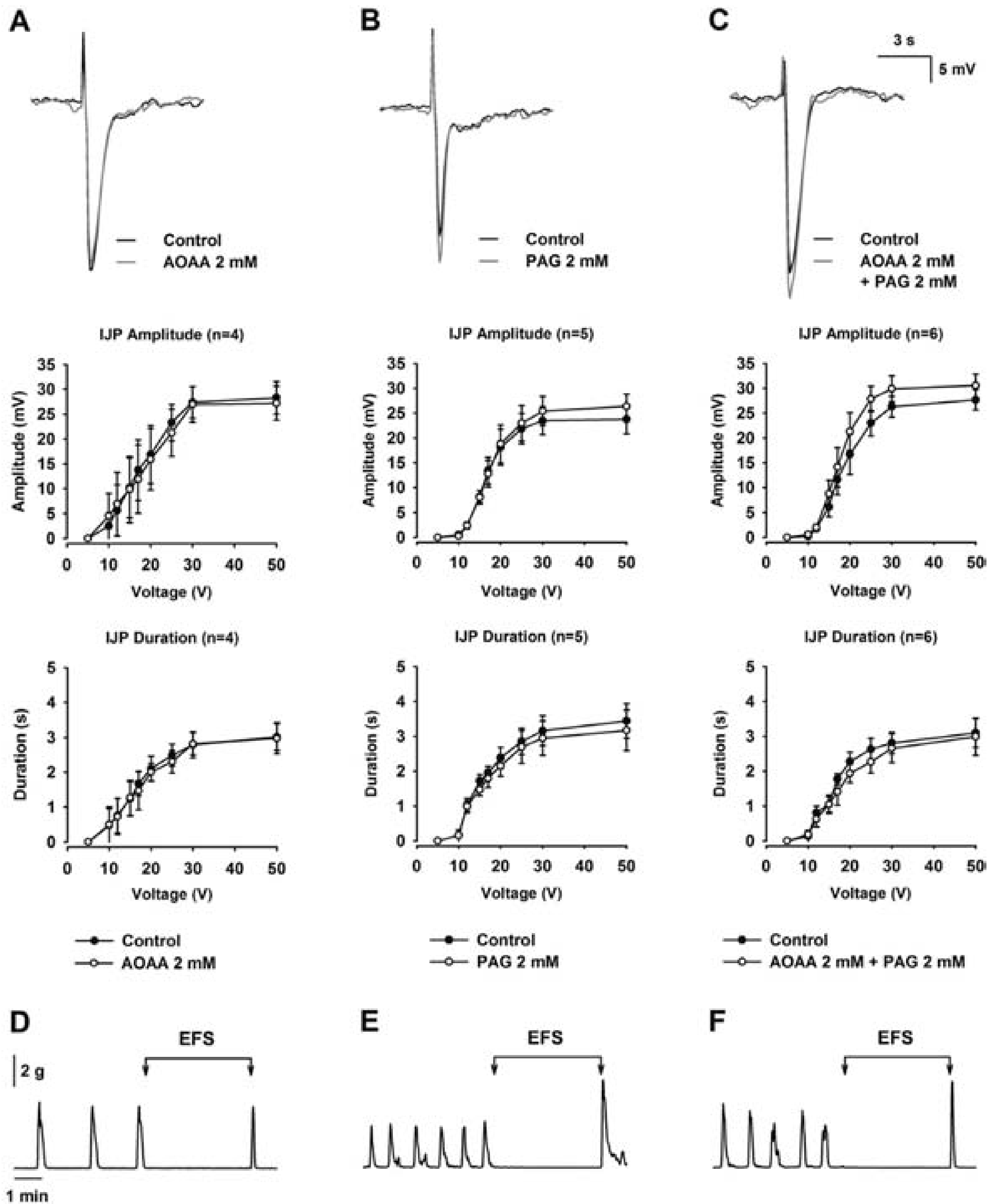

Figure 6

Effect of amino-oxyacetic acid (AOAA) and D,L-propargylglycine (PAG) on EFS-induced IJP and mechanical relaxation. (A, D): AOAA (2 mM); (B, E): PAG (2 mM); (C, F): AOAA (2 mM) + PAG (2 mM). Note that none of the treatments modified the amplitude or duration of the IJP (A, B and C) or the mechanical relaxation induced by EFS (D, E and F). IJPs shown in the figure were elicited with a pulse of 30 V.

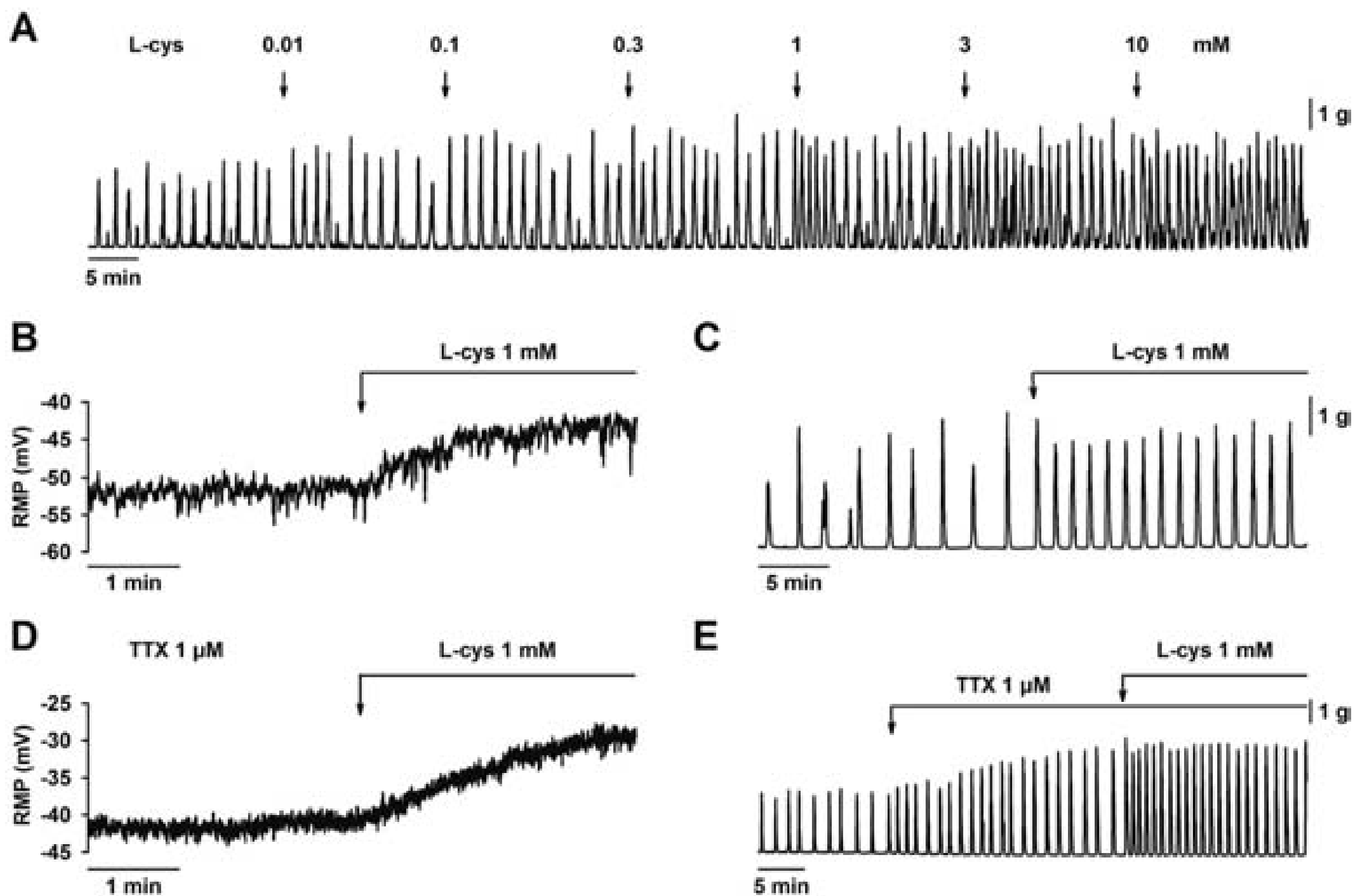

Figure 7

Effect of L-cysteine on smooth muscle resting membrane potential and spontaneous mechanical activity. (A) Mechanical recording showing that L-cysteine increased spontaneous motility in a concentration-dependent manner. L-cysteine (1 mM) induced smooth muscle depolarization (B) and an increase in spontaneous motility (C) which were both still recorded in the presence of TTX (1 µM) (D, E). For statistics, see Table 1.

in the lamina propia was detected in the mouse colon (Linden *et al.*, 2008) and positive staining was detected in neurons of the mouse, guinea-pig and human colon, but the precise neurons expressing each particular enzyme might vary between species (Schicho *et al.*, 2006; Linden *et al.*, 2008). Moreover, in the present study we also found positive CSE-IR in neurons of both the myenteric and submucous plexus. Whether these major differences in the distribution of the enzymes are due to different technical approaches or are real species differences needs further study. In the current study, we used the methodology developed by Linden *et al.*, (2008) to measure endogenous production of H_2S in several mouse tissues. Note that the measurements of H_2S production were performed in colonic samples where the mucosa and submucosa had been previously removed and consequently H_2S production was not due to the activity of the epithelium. Effective inhibition of H_2S production was obtained with PAG (2 mM) or AOAA (2 mM) suggesting that both CSE and CBS participate in the endogenous production of H_2S synthesis in the rat colon. Effective blockade of H_2S production in intact tissue was obtained using a combination of PAG (2 mM) and HA (2 mM) in the mouse colon (Linden *et al.*, 2008). However, due to the NO-like effects of HA (see below), we did not test this inhibitor on H_2S production. Note that AOAA was the only inhibitor that was able to block H_2S production in rat colon homogenates (Martin *et al.*, 2010). However, under our experimental conditions – the strip devoid of mucosa and submucosa – (this is a major difference between the present and other studies), the tissue was able to endogenously produce H_2S and both AOAA and PAG were effective inhibitors of this H_2S production.

In the present study, we analysed the effect of PAG, AOAA and HA on GI motility. Our first report using NaHS as a source of H_2S showed that NaHS inhibited human and rat colonic motility (Gallego *et al.*, 2008a). However, little is known about the blockade of the endogenous production of H_2S on GI motility. Our results demonstrated that PAG was able to cause a smooth muscle depolarization and increase spontaneous motility. Accordingly PAG was mimicking the effect of NO inhibition with L-NNA (Gil *et al.*, 2010). AOAA caused a minor and transient effect on membrane potential and spontaneous motility. These results suggest that H_2S might be a third inhibitory signalling molecule in the GI tract, regulating GI motility. However, it is important to distinguish

between a neural and a non-neural origin of endogenous H_2S. In the first case, H_2S should be considered a 'gasotransmitter', whereas in the second H_2S might be an endogenous signalling molecule produced in non-neural cell types (i.e. smooth muscles and/or ICC) able to regulate motility.

Inhibitory motor neurons are predominant in human colonic circular muscle (Gallego *et al.*, 2006; Gallego *et al.*, 2008b). In the rat colon, inhibitory neurons spontaneously release NO and a purine acting on $P2Y_1$ receptors. NO regulates the membrane potential whereas the purine acting on $P2Y_1$ receptors is responsible for the spontaneous IJP (Gil *et al.*, 2010). EFS-evoked IJP show a fast component followed by a slow one, being blocked by $P2Y_1$ receptor antagonists and NOS inhibitors respectively (Grasa *et al.*, 2009). For this reason we wanted to investigate the effect of AOAA and PAG on neuromuscular interaction. AOAA, PAG or a combination of both did not modify the spontaneous IJP or the EFS-induced IJP either (neither the fast nor the slow component were affected). Complete mechanical relaxation was still present after incubation with both antagonists. These results suggest that H_2S does not participate in neurally mediated relaxation or alternatively we have not been able to use the appropriate parameters of stimulation to elicit H_2S release. Further studies using EFS and H_2S detection are needed to evaluate the possible neural release of H_2S.

Note that depolarization and increase in motility induced by PAG occur in the presence of TTX and L-NNA. This result suggests that the effect is not related to NO production and to TTX-sensitive neurally mediated responses. According to the distribution of the enzyme, ongoing production of H_2S in smooth muscle cells (and/or ICC) may keep the membrane potential hyperpolarized and therefore the inhibition of H_2S production would depolarize smooth muscle cells, increasing the motility. Our experiments showed that this effect was more consistent with PAG than AOAA, suggesting that the endogenous source of H_2S was due to CSE with a minor contribution of CBS.

At least part of the observed effects could be unrelated to inhibition of H_2S production and other 'side' effects of the drugs might explain part of our results. This is not new and researchers working in the field are aware that, for example, AOAA has been used as a pharmacological tool to investigate multiple pharmacological actions including several pyridoxal phosphate-dependent enzymes such as aspartate transaminase, 4-aminobutyrate transaminase or dopa-decarboxylase (John and Charteris, 1978). Regarding colonic motility, possible side effects of the drugs might be due to the following findings:

(1) In the rat colon, L-cysteine, the precursor of H_2S synthesis, depolarizes smooth muscles cells and increases motility, not mimicking the effect of exogenous NaHS (Gallego *et al.*, 2008a). Moreover, the effect of L-cysteine was probably not due to activation of the NMDA receptor because pre-incubation with D-AP5, an NMDA receptor antagonist, did not modify the motor response (Schicho *et al.*, 2006). It has been demonstrated that L-cysteine at the concentrations used in the present study blocks stretch-dependent potassium channels in the murine colon (Park *et al.*, 2005). These stretch-dependent potassium channels participate in background potassium conductance and are expressed in a variety of excitable cells including smooth muscle (see for review: Sanders and Koh, 2006). It might be possible that the smooth muscle depolarization and the increase in spontaneous motility in the rat colon might be due to a direct effect of L-cysteine on these channels. Note, however, that L-cysteine mimics the effects of exogenous H_2S donors in other studies, acting like a prosecretory molecule in the guinea-pig and human colon (Schicho *et al.*, 2006), enhancing ulcer healing in the rat stomach (Wallace *et al.*, 2007) and exerting antinociceptive effects in the rat colon (Distrutti *et al.*, 2006). In addition, in these studies its effects are reversed by AOAA and/or PAG.

(2) HA, another CBS inhibitor, is causing opposite effects to those found with AOAA. HA is usually used at high concentrations, up to 1 mM, to inhibit the CBS enzyme. Our preliminary results showed that at 1 mM, HA completely inhibited spontaneous motility. Therefore, we decided to perform a concentration–response curve to investigate its effects and the IC_{50} was close to 1 μM. Furthermore, the hyperpolarization and inhibition of spontaneous motility was antagonized by ODQ, showing that HA was probably causing its effects through cGMP activation. HA is an NO-generating compound that relaxes smooth muscle (Iversen *et al.*, 1994) and it has been previously demonstrated in the rat duodenum that HA mimics the NO effect (Correia *et al.*, 2000). Our results confirm these results which means HA is unlikely to be a selective H_2S inhibitor.

(3) In the presence of TTX, PAG and AOAA caused an opposite effect. AOAA alone caused a slight but consistent increase in motility but, in the presence of TTX or L-NNA, AOAA inhibited spontaneous motility. We still do not know why this dual effect occurs. This result suggests that in the absence of NO, which is 'spontaneously' released from inhibitory neurons (Gil *et al.*, 2010), AOAA causes smooth muscle inhibition. AOAA is a pharmacological tool widely used to inhibit NADH shuttles (see e.g.: Eto *et al.*, 1999; Casimir *et al.*, 2009). These shuttles participate in the transfer of reducing equivalents between the cytosolic compartment and the mitochondrial matrix. In vascular smooth muscle, AOAA caused inhibition of O_2 consumption, inhibition of the tricarboxylic acid cycle, increase in lactate production and reduction of contractility (Barron *et al.*, 1998; Barron *et al.*, 1999). The reduction in spontaneous motility without a change in membrane potential might be due to an effect on a shuttle. Unfortunately, octanoate, which partially reverses the effect of AOAA on malate-aspartate shuttle (Barron *et al.*, 1998), did not reverse the inhibitory effect of AOAA (not shown).

(4) Finally, it is important to note that PAG exerts a covalent and irreversible inhibition of CSE (Sun *et al.*, 2009). However, repetitive depolarizations observed with PAG are not consistent with an irreversible effect on CSE.

In the present study, we demonstrated that rat colonic strips devoid of mucosa and submucosa are able to enzymatically produce H_2S, in which both CBS and CSE enzymes are involved. Effects of PAG, AOAA, HA and L-cysteine on rat colonic RMP and motility were reported as well. Both PAG and AOAA increased motility, suggesting that H_2S is an endogenous inhibitory signalling molecule in the GI tract. However, it is important for future investigations that possible side effects of the drugs are presented that might (or might not) be related to H_2S production, so as to encourage further research into the design of novel inhibitors with higher selectivity, potency and cell-membrane permeability.

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Conflict of interest

None.

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Chapter 6

Effects of hydrogen sulphide on motility patterns in the rat colon

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RESEARCH PAPER

Effects of hydrogen sulphide on motility patterns in the rat colon

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BACKGROUND AND PURPOSE
Hydrogen sulphide (H_2S) is an endogenous gaseous signalling molecule with putative functions in gastrointestinal motility regulation. Characterization of H_2S effects on colonic motility is crucial to establish its potential use as therapeutic agent in the treatment of colonic disorders.

EXPERIMENTAL APPROACH
H_2S effects on colonic motility were characterized using video recordings and construction of spatio-temporal maps. Microelectrode and muscle bath studies were performed to investigate the mechanisms underlying H_2S effects. NaHS was used as the source of H_2S.

KEY RESULTS
Rhythmic propulsive motor complexes (RPMCs) and ripples were observed in colonic spatio-temporal maps. Serosal addition of NaHS concentration-dependently inhibited RPMCs. In contrast, NaHS increased amplitude of the ripples without changing their frequency. Therefore, ripples became the predominant motor pattern. Neuronal blockade with lidocaine inhibited RPMCs, which were restored after administration of carbachol. Subsequent addition of NaHS inhibited RPMCs. Luminal addition of NaHS did not modify motility patterns. NaHS inhibited cholinergic excitatory junction potentials, carbachol-induced contractions and hyperpolarized smooth muscle cells, but did not modify slow wave activity.

CONCLUSIONS AND IMPLICATIONS
H_2S modulated colonic motility inhibiting propulsive contractile activity and enhancing the amplitude of ripples, promoting mixing. Muscle hyperpolarization and inhibition of neurally mediated cholinergic responses contributed to the inhibitory effect on propulsive activity. H_2S effects were not related to changes in the frequency of slow wave activity originating in the network of interstitial cells of Cajal located near the submuscular plexus. Luminal H_2S did not modify colonic motility probably because of epithelial detoxification.

Abbreviations
CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; EFS, electrical field stimulation; EJP, excitatory junction potential; ICC, interstitial cells of Cajal; ICC-SMP, ICC associated with the submuscular plexus; ICC-MP, ICC associated with the myenteric plexus; IJP, inhibitory junction potential; K_{ATP}, ATP-sensitive potassium channels; L-NNA, N^{ω}-nitro-L-arginine; MRS2500, (1R,2S,4S,5S)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo [3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt; ODQ, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; RPMCs, rhythmic propulsive motor complexes; RMP, resting membrane potential; SK_{Ca}, small conductance calcium-activated potassium channels; S-T maps, spatio-temporal maps; TTX, tetrodotoxin

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Introduction

Gasotransmitters are gas molecules endogenously synthesized in a regulated manner, causing well-defined physiological and/or pathophysiological effects, acting at specific cellular and molecular targets and employing specific mechanism(s) of inactivation (Wang, 2002; Li and Moore, 2007; Linden *et al.*, 2010). Hydrogen sulphide (H_2S) fulfils, at least in part, the criteria to be considered as an endogenous gaseous signalling molecule in the gastrointestinal tract with putative functions regulating motility (Jimenez, 2010; Linden *et al.*, 2010).

Endogenous production of H_2S in the gastrointestinal tract has been demonstrated in tissue homogenates (Hosoki *et al.*, 1997; Martin *et al.*, 2010) and colonic strips (Linden *et al.*, 2008; Gil *et al.*, 2011). Two pyridoxal-dependent enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1), are mainly responsible for H_2S synthesis. A third route of H_2S synthesis involves 3-mercaptopyruvate sulphurtransferase in combination with cysteine aminotransferase (Shibuya *et al.*, 2009a,b). CBS and CSE have been found along the entire gastrointestinal tract (Martin *et al.*, 2010). Both enzymes are detected in several cell types including smooth muscle cells, enteric neurons, interstitial cells of Cajal (ICC) and epithelial cells, varying between species and regions of the gastrointestinal tract and suggesting that several cell types have the capacity to produce H_2S (Schicho *et al.*, 2006; Linden *et al.*, 2008; Hennig and Diener, 2009; Martin *et al.*, 2010; Gil *et al.*, 2011). Sulphide quinone reductase is responsible for H_2S catabolism in the muscularis externa of the colon and might be the main enzyme involved in the termination of H_2S-mediated signals (Linden *et al.*, 2012).

In the large intestine, luminal bacteria also represent a source of H_2S (Blachier *et al.*, 2010). However, although high concentrations of H_2S are present in the colon (mM range), the vast majority of H_2S is bound to luminal content (Jorgensen and Mortensen, 2001; Levitt *et al.*, 2002). Thus, low levels of free H_2S are available in the colonic lumen, being quickly metabolized in the colonic mucosa (Furne *et al.*, 2001; Blachier *et al.*, 2010).

Sodium hydrosulphide (NaHS) is commonly used as a source of H_2S in '*in vitro*' experiments. In the gastrointestinal tract, both excitatory and inhibitory effects on smooth muscle have been reported. For example, in the guinea pig and mouse stomach, H_2S causes dual effect, that is, a contraction is observed at low concentrations whereas at high concentrations H_2S causes relaxation (Zhao *et al.*, 2009; Han *et al.*, 2011). NaHS concentration-dependently relaxed prostaglandin $F_{2\alpha}$-contracted circular muscle strips of mouse fundus and distal colon (Dhaese and Lefebvre, 2009; Dhaese *et al.*, 2010). NaHS also exerted relaxant effects on guinea pig, rabbit and rat ileum and jejunum preparations (Hosoki *et al.*, 1997; Teague *et al.*, 2002; Nagao *et al.*, 2011; 2012; Kasparek *et al.*, 2012). Furthermore, NaHS inhibits peristaltic activity in the mouse small intestine and colon (Gallego *et al.*, 2008). Spontaneous circular smooth muscle contractions observed in rat and human colonic strips are also concentration-dependently inhibited by NaHS (Gallego *et al.*, 2008). However, the mechanisms underlying these relaxant effects are unclear and might include activation of myosin light chain phosphatase (Dhaese and Lefebvre, 2009; Nagao *et al.*, 2012), ATP-sensitive potassium channels (K_{ATP}) (Gallego *et al.*, 2008; Zhao *et al.*, 2009; Nagao *et al.*, 2012), small conductance calcium-activated potassium channels (SK_{Ca}) (Gallego *et al.*, 2008) and even sodium channel activation (Strege *et al.*, 2011; channel nomenclature follows Alexander *et al.*, 2011.). In addition, a direct effect on ICC, responsible for pacemaker activity, has also been reported (Parajuli *et al.*, 2010). All these data suggest that the potential mechanisms underlying motility changes are variable, which is consistent with the various effects of NaHS on different targets.

In the colon, different motor patterns cause propulsion and/or mixing of luminal contents, allowing absorption of water and electrolytes, storage of food residues and defecation. In a recent study, video recording of rat colonic motility and construction of spatio-temporal maps (S-T maps) have revealed two main motor patterns: (i) rhythmic propulsive motor complexes (RPMCs) and (ii) rhythmic propagating ripples (Huizinga *et al.*, 2011). The first pattern is characterized by large, propulsive contractions, propagating aborally at low frequency (1.2 cpm in the proximal colon and 0.5 cpm in the mid colon). The second one showed a higher frequency (about 10 cpm) with smaller amplitude contractions propagating both orally and aborally in the proximal colon (Huizinga *et al.*, 2011). Similar motility patterns have been described in experiments using strain-gauge transducers (Li *et al.*, 2002). It has been hypothesized that these patterns depend on both neural modulation and ICC-mediated pacemaker activity (Huizinga *et al.*, 2011). *In vitro*, electrophysiological experiments in rat colon have revealed that ICC associated with the submuscular plexus (ICC-SMP) mediate slow wave activity, which is related to high frequency contractions, whereas ICC associated with the myenteric plexus (ICC-MP) underlie cyclic depolarizations, related to low frequencycontractions (Pluja *et al.*, 2001).

In the present study, we characterized the effects of H_2S on motility patterns in colon segments and on muscle contractions in muscle strips and investigated a possible effect on pacemaker activity in the rat colon. Briefly, H_2S modulated colonic motility reducing RPMCs and enhancing the amplitude of ripples without modifying slow wave activity. Mechanisms involved in H_2S-induced relaxant effects included both hyperpolarization of smooth muscle cells caused by potassium channel activation and reduction of cholinergic excitatory neuromuscular transmission. It is important to characterize H_2S effects on motility for its therapeutic potential in the treatment of colonic inflammation (Fiorucci *et al.*, 2007; Wallace *et al.*, 2009).

Methods

Animals

All animal care and experimental procedures complied with and were approved by the local Animal Ethics Boards. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 60 animals were used in the experiments described here. Male Sprague-Dawley rats (8–10 weeks old, 300–350 g) were purchased from Charles River (Lyon, France for experiments

performed in Spain, and Saint Constant, QC, Canada for experiments carried out in Canada). Both in Spain and in Canada, animals were housed under controlled conditions: temperature 22°C ± 2°C, humidity 55% ± 10%, 12:12 h light–dark cycle and access to water and food *ad libitum*. For motility studies in colonic segments, animals were anaesthetized using isoflurane and killed by cervical dislocation (procedure approved by the McMaster's Animal Research Ethics Board, McMaster University, Hamilton, ON, Canada). For microelectrodes and organ bath experiments, animals were stunned and killed by decapitation and exsanguination 2–3 s afterward (procedure approved by the Ethics Committee of the Universitat Autònoma de Barcelona, Bellaterra, Spain).

Tissue samples

After opening the abdominal cavity, the colon was removed and placed in carbogenated (95% O_2 and 5% CO_2), ice-cold physiological saline solution. Colonic segments for motility studies of about 10–12 cm in length were prepared by flushing out the content with physiological saline solution. For electrophysiological and mechanical studies carried out in muscle strips, the colon was opened along the mesenteric border and pinned to a Sylgard® base (Dow Corning Corporation, Midland, MI, USA) (mucosa side up). The proximal and the mid colon were identified accordingly to anatomical criteria previously described (Alberti *et al.*, 2005). Three different types of samples were prepared: (i) whole colon preparations to evaluate motor patterns in organ baths; (ii) samples prepared by peeling off the mucosal layer for electrophysiological studies to evaluate slow wave activity; and (iii) preparations removing both the mucosal and submucosal layers for electrophysiological studies to evaluate the membrane potential and the inhibitory and excitatory neuromuscular transmission. Circular muscle strips were cut 1 cm long and 0.3 cm wide.

Motility studies performed in colonic segments

Colonic segments were placed into an organ bath containing warmed carbogenated physiological saline solution (37 ± 1°C). Both the proximal and distal ends were cannulated and fixed to the bottom of the organ bath to prevent shortening of the preparation. The samples were perfused with physiological saline solution keeping the inflow pressure at 6 cm H_2O. The outflow was measured using a COBE pressure transducer (Sorin Biomedical Inc., Irvine, CA, USA) placed at the bottom of a collecting container (1 cm diameter). Then, the signal was amplified using a Grass LP 122 amplifier (Astro-Med, Brossard, QC, Canada) and was digitized using a MiniDigi 1A A-D converter and Axoscope 9 software (Pclamp 9 software, Molecular Devices, Toronto, ON, Canada). After a period of 30 min of equilibration, colonic segments showed spontaneous mechanical activity. Motility was recorded using a Sony HDR-SR11 Digital HD Video Camera (Sony Corporation, Tokyo, Japan) placed above the preparation. Using 7 min duration (25 frames s^{-1}) video recordings, S-T maps were calculated with an ImageJ plug in (Gut trace) with a Java algorithm loosely based on Hennig *et al*. (1999). The edges of the colon were identified, and the diameter of the colon was calculated for every pixel along the preparation and for each video frame. S-T maps were obtained by plotting the diameter of the colon as image intensity in greyscale over time (X-axis) for the whole length of the colon (Y-axis). Thus, the reduction of the diameter of the colon (contraction) was showed in black colour, whereas the increase in diameter (relaxation) was depicted in white. A proportional level of grey was associated to intermediate diameters. Pixels were calibrated using a metric scale ruler, and lengths and diameters were expressed in centimetres (cm). Time-plots were prepared by representing the changes in diameter over time at a particular point of the colon. In these plots, the X-axis (diameter) was inverted and, therefore, a reduction of the diameter (contraction) was observed as a positive inflexion whereas an increase of the diameter (relaxation) was plotted as a negative inflexion. Thus, the recordings obtained were oriented similarly to those obtained with muscle bath experiments. For the motility patterns observed, the frequency (contractions per minute – cpm), the amplitude (cm) and the duration (s) were measured using Clampfit software (Pclamp 9 software, Molecular Devices). The velocity of propagation (cm min^{-1}.) was measured using ImageJ software. The outflow was expressed as cm H_2O (pressure).

Intracellular microelectrode recording

Samples were pinned with the submucosal or circular muscle layer facing upward in a Sylgard-coated chamber, continuously perfused with carbogenated physiological saline solution at 37°C ± 1°C. Tissue was allowed to equilibrate for 1 h before starting the experiment. Circular smooth muscle cells 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. Inhibitory junction potentials (IJP) were elicited by electrical field stimulation (EFS) using single pulses (0.3 ms duration) and increasing amplitude voltage (8, 12, 16, 20, 24, 28, 32, 36 and 40 V). Excitatory junction potentials (EJP) were 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. The amplitude (mV) and duration (s) of the EFS-induced IJP and EJP were measured before and after drug infusion. In samples in which submucosal layer was removed, resting membrane potential (RMP), expressed in mV, was estimated as the most probable bin of the frequency distribution of the membrane potential (0.1 mV bins; 30–60 s recordings) (Gil *et al.*, 2010). Slow waves were recorded in samples including the submucosal layer and their frequency (cycles per minute – cpm), amplitude (mV) and duration (s) measured. In these samples, RMP was established as the average of the membrane potential between each slow wave (in mV).

Muscle bath studies

Strips (1 × 0.3 cm) including all colon layers were mounted in a 10 mL organ bath containing carbogenated physiological saline solution maintained at 37 ± 1°C. Contractions from the circular muscle layer were measured using an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc., Holliston, MA, USA) connected to a computer through an ampli-

fier. 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. The frequency (cpm), the amplitude (g) and the duration (s) of contractions were measured to estimate the mechanical activity.

Data analysis and statistics

Data are expressed as mean ± SEM. Differences in the motility (in colonic segments and strips), RMP and slow waves induced by a single addition of NaHS (1 mM) were compared using a paired Student's *t*-test. Motility patterns in colonic segments observed in the proximal and the mid colon and the effect observed with lidocaine and lidocaine + carbachol on motility patterns were compared using a paired Student's *t*-test as well. An unpaired Student's *t*-test was used to compare the hyperpolarizations caused by NaHS (1 mM) in samples including or devoid of the submucosal layer. One-way ANOVA followed by Bonferroni's multiple comparison test was used to evaluate the effect of serosal addition of NaHS (0.3 and 1 mM) on motility patterns observed in colonic segments and to compare the hyperpolarizations caused by NaHS (0.3 mM) in the presence of ODQ and potassium channel inhibitors to control values. The differences between the amplitude and duration of the EJP before and after NaHS infusion and the effect of L-NNA and ODQ on NaHS concentration response curves were compared by two-way ANOVA followed by Bonferroni's multiple comparison test. IC_{50} values were calculated using a conventional sigmoid concentration–response curve with variable slope. $P < 0.05$ was considered to indicate statistical significance. '*n*' values indicate the number of animals. Statistical analysis and curve fitting were performed with GraphPad Prism version 4.00, (GraphPad Software, San Diego, CA, USA).

Materials

The composition of the physiological saline solution was (in mM) glucose 10.10, NaCl 115.48, $NaHCO_3$ 21.90, KCl 4.61, NaH_2PO4 1.14, $CaCl_2$ 2.50 and $MgSO_4$ 1.16 (pH 7.3–7.4). The following drugs were used: tetrodotoxin (TTX) (Latoxan, Valence, France); apamin, carbachol (carbamoylcholine chloride), charybdotoxin, glibenclamide, lidocaine, nifedipine, N^{ω}-nitro-L-arginine (L-NNA), 1H-[1,2,4]oxadiazolo[4,3-α] quinoxalin-1-one (ODQ), sodium hydrogen sulphide (NaHS) (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), 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) (Tocris, Bristol, UK). Stock solutions were made by dissolving drugs in distilled water except for nifedipine, ODQ and TRAM-34, which were dissolved in 96% ethanol, and L-NNA, which was dissolved in physiological saline solution by sonication.

Results

Motility patterns in colonic S-T maps

As previously described, two different motor patterns were observed in S-T maps obtained from video recordings from colonic segments of the rat: RPMCs and rhythmic propagating ripples (Huizinga *et al.*, 2011). RPMCs were observed in the proximal colon at a frequency of 1.3 ± 0.1 cpm ($n = 16$; Figure 1) and about 40% of them propagated to the mid colon (0.5 ± 0.1 cpm; $n = 16$; Figure 1). The amplitude of the contraction was lower in the proximal (2.2 ± 0.2 mm) compared to the mid colon (3.8 ± 0.2 mm $P < 0.001$; $n = 16$; Figure 1). The duration was higher in the mid than in the

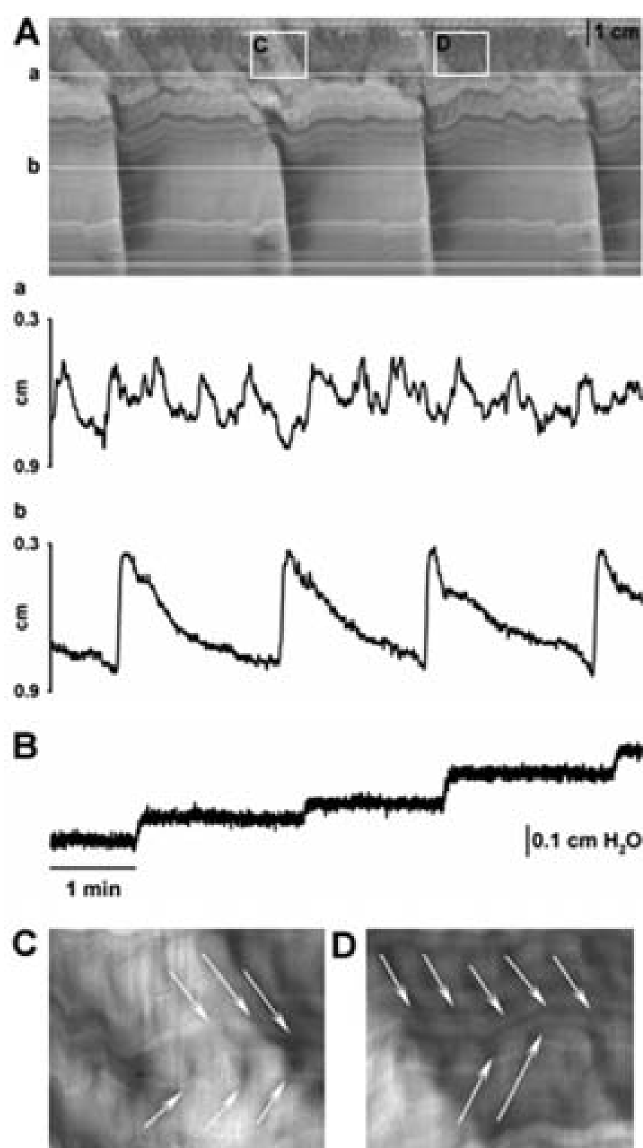

Figure 1

S-T map showing motor patterns observed in colonic segments (A). Note that black is narrowing of the lumen (contraction) and white is widening of the lumen (relaxation). RPMCs (large contractions) with superimposed ripples (small contractions) are observed in the proximal colon. RPMCs propagate aborally but only some of these contractions reached the mid colon. Detail of the diameter of the colon in the proximal (Aa) and mid (Ab) regions (dotted lines in S-T map). RPMCs in the mid colon are associated with outflow of content (B). Detail of ripples (arrows) observed in the proximal colon (C,D). Note that ripples propagate both orally and aborally.

Table 1

Effect of serosal and intraluminal addition of NaHS on RPMCs in the proximal and mid colon and ripples

		Serosal NaHS		Intraluminal NaHS	
RPMCs (proximal)	**Control**	**0.3 mM**	**1 mM**	**Control**	**1 mM**
Frequency (cpm)	1.4 ± 0.4	0.5 ± 0.3	0.0 ± 0.0[a]	1.1 ± 0.2	1.2 ± 0.2
Amplitude (mm)	2.0 ± 0.5	1.4 ± 0.5	0.0 ± 0.0[b]	1.9 ± 0.2	2.3 ± 0.5
Duration (s)	30.9 ± 4.9	35.1 ± 15.2	0.0 ± 0.0	37.9 ± 5.1	31.6 ± 5.1
Propagation (cm min^{-1})	5.8 ± 1.2	3.8 ± 1.3	0.0 ± 0.0[a]	5.2 ± 0.7	7.3 ± 2.5
RPMCs (mid)	**Control**	**0.3 mM**	**1 mM**	**Control**	**100 μM**
Frequency (cpm)	0.6 ± 0.1	0.1 ± 0.1[c]	0.0 ± 0.0[c]	0.5 ± 0.1	0.5 ± 0.1
Amplitude (mm)	3.0 ± 0.3	0.9 ± 0.9[a]	0.0 ± 0.0[b]	3.5 ± 0.6	3.4 ± 0.7
Duration (s)	60.9 ± 12.2	34.2 ± 34.2	0.0 ± 0.0	65.3 ± 8.9	66.1 ± 15.1
Propagation (cm min^{-1})	14.8 ± 9.2	0.2 ± 0.2	0.0 ± 0.0	11.6 ± 2.5	23.6 ± 6.0[a]
Ripples	**Control**	**0.3 mM**	**1 mM**	**Control**	**100 μM**
Frequency (cpm)	9.5 ± 0.5	9.2 ± 0.2	9.6 ± 0.4	9.6 ± 1.0	8.9 ± 0.3
Amplitude (mm)	0.4 ± 0.1	0.7 ± 0.1[a]	0.9 ± 0.1[c]	0.5 ± 0.1	0.6 ± 0.1
Duration (s)	4.6 ± 0.2	5.1 ± 0.4	5.9 ± 0.3	4.9 ± 0.5	5.4 ± 0.4
Presence (cm)*	3.5 ± 0.2	4.3 ± 0.6	6.4 ± 0.8[a]	3.5 ± 0.3	3.8 ± 0.6
Propagation (cm min^{-1})					
Aboral (anterograde)	6.8 ± 2.0	7.9 ± 1.6	7.0 ± 1.2	9.9 ± 2.6	6.6 ± 1.4
Oral (retrograde)	5.8 ± 0.8	5.5 ± 0.5	6.1 ± 0.8	7.5 ± 2.1	7.0 ± 1.1

Values are means ± SEM. For each experiment $n = 4$. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, significant difference from control; one-way ANOVA, followed by Bonferroni's multiple comparison test (for serosal NaHS experiment) and paired Student's *t*-test (for intraluminal NaHS experiment). *Distance in which ripples were observed from the proximal edge of the colon.

proximal colon (proximal: 34.2 ± 2.0 s vs. mid: 57.5 ± 5.1 s; $P < 0.001$; $n = 16$; Figure 1). In all cases, RPMCs propagated in aboral direction (velocity of propagation in proximal colon: 5.2 ± 0.3 cm min^{-1}; and in mid colon: 21.3 ± 3.5 cm min^{-1}; $P < 0.001$; $n = 16$; Figure 1) and were associated with outflow (Figure 1). In contrast, ripples were mainly observed in the proximal colon (they were present in the proximal 36 ± 2% of the total length of the colon used in the experiment; $n = 16$). They propagated both in oral and aboral direction at similar velocity (oral: 6.2 ± 0.6 cm min^{-1} vs. aboral: 7.3 ± 0.8 cm min^{-1}; n.s.; $n = 16$; Figure 1). This pattern was characterized by shallow contractions (amplitude: 0.5 ± 0.1 mm and duration: 4.8 ± 0.1 s; $n = 16$) measured at a frequency of 10.4 ± 0.1 cpm ($n = 16$). Ripples were not associated with outflow but were superimposed on RPMCs (Figure 1).

NaHS, administered at the serosal side, concentration-dependently inhibited RPMCs causing a complete cessation of this activity at 1 mM ($n = 4$; Table 1; Figure 2). In contrast, the amplitude of the ripples was enhanced but no changes in their frequency, duration or velocity of propagation were observed ($n = 4$; Table 1; Figure 2). Furthermore, ripples were recorded in the 70 ± 7% of the total length of the colon reaching 6.4 ± 0.8 cm from the proximal edge at 1 mM ($n = 4$; Table 1; Figure 2). In contrast, luminal administration of NaHS ($n = 4$; 1 mM; 30 min) did not modify motility patterns ($n = 4$; Table 1; Figure 3).

In order to distinguish between putative myogenic and neurogenic effects of hydrogen sulphide, NaHS was tested in the presence of the neuronal sodium channel blocker lidocaine. Lidocaine (100 μM) *per se* reduced RPMCs in the proximal colon and completely abolished RPMCs in the mid colon ($n = 4$; Table 2; Figure 4). In contrast, ripples were still recorded in the presence of lidocaine (100 μM) ($n = 4$; Table 2; Figure 4). In the presence of lidocaine (100 μM), NaHS abolished the remaining RPMCs in the proximal colon ($n = 4$; Figure 4) and concentration-dependently increased the amplitude of ripples ($n = 4$; Table 2; Figure 4).

As we previously described (Huizinga *et al.*, 2011), carbachol (10 μM) restored RPMCs after the blockade produced by lidocaine (100 μM) ($n = 4$; Table 3; Figure 4) showing that RPMCs were independent of neuronal cyclic activity but dependent on cholinergic neuronal input. Moreover, ripples were still recorded with characteristics similar to those observed in control ($n = 4$; Table 3; Figure 4). In the presence of lidocaine (100 μM) and carbachol (10 μM), NaHS caused effects similar to those observed in control conditions ($n = 4$; Table 3, Figure 4).

Mechanical activity in strips using muscle bath

Low frequency and high frequency contractions are observed in muscle bath recordings using isolated colonic strips with

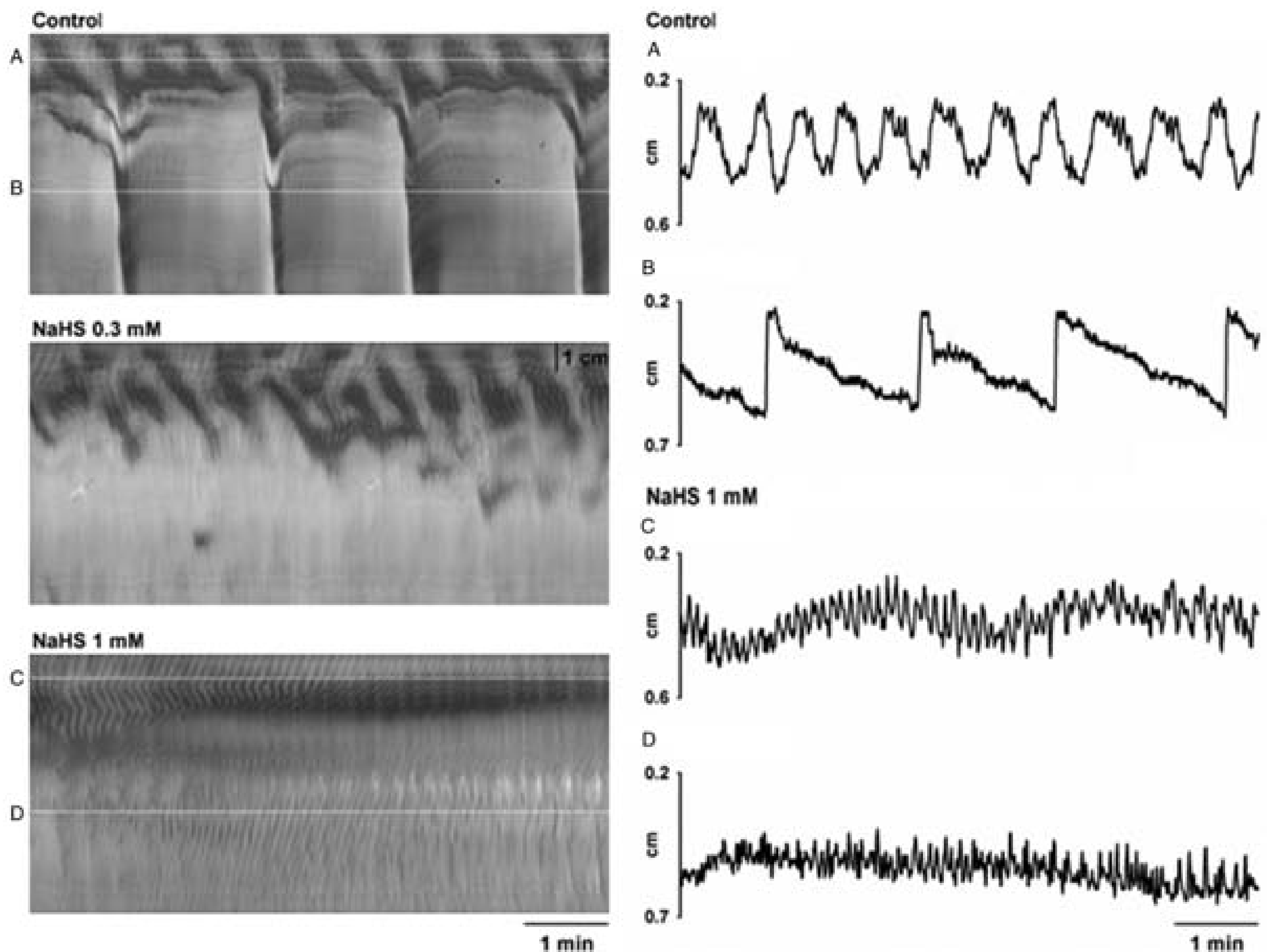

Figure 2

S-T maps (on the left) showing the effect produced by the serosal addition of NaHS (0.3 and 1 mM) on motor patterns observed in colonic segments. Detail of the diameter of the colon in the proximal (A) and mid (B) regions in control conditions and in the proximal (C) and mid (D) regions in the presence of NaHS (1 mM) (dotted lines in S-T maps). Note, on the right, that NaHS concentration-dependently inhibited RPMCs (large contractions) and increased the amplitude of ripples.

intact submucosal and myenteric plexuses (Pluja *et al.*, 2001; Alberti *et al.*, 2005). Both patterns are correlated with the activity observed in colonic segments; however, in isolated strips both activities were still recorded in the presence of TTX (1 μM). In mid colon strips, NaHS (1 mM) inhibited low frequency contractions, whereas high frequency contractions were still recorded (n = 4; Figure 5). It is important to note that the frequency of high frequency contractions was not modified by NaHS (n = 4; Figure 5). Furthermore, the amplitude of these contractions was not increased, as observed with ripples in colonic segments (n = 4; Figure 5). NaHS caused similar effects in the proximal colon and in the presence of the neuronal blocker TTX (1 μM) (data not shown).

RMP and slow wave activity

In order to properly evaluate the effect of NaHS on RMP, circular smooth muscle cells in strips devoid of the ICC network near the submuscular plexus (ICC-SMP), were impaled for electrophysiological recording. In this preparation, NaHS concentration-dependently hyperpolarized the smooth muscle cells (IC_{50} = 105.6 μM; log IC_{50} = −3.98 ± 0.33; n = 4; Figure 6A,B). ODQ (10 μM) slightly decreased NaHS (0.3 mM)-induced hyperpolarization. Glibenclamide (10 μM; n = 4), a K_{ATP} blocker, and apamin (1 μM; n = 4), a SK_{Ca} inhibitor, significantly reduced the effect of NaHS (0.3 mM) on smooth muscle membrane potential (Table 4; Figure 6C,D). A reduction of the NaHS (0.3 mM)-induced hyperpolarization was also observed when the tissue was pre-incubated with a cocktail of potassium channel blockers including apamin (1 μM), TRAM-34 (1 μM), glibenclamide (10 μM) and charybdotoxin (0.1 μM) (n = 3; Figure 6C,D).

To evaluate the effect of NaHS on slow wave activity, strips with an intact ICC-SMP network were studied. Slow waves were recorded in the presence of nifedipine (1 μM) at a frequency of 11.1 ± 0.8 cpm (n = 4; Figure 7). NaHS (1 mM) induced a smooth muscle hyperpolarization of −7.1 ± 1.8 mV

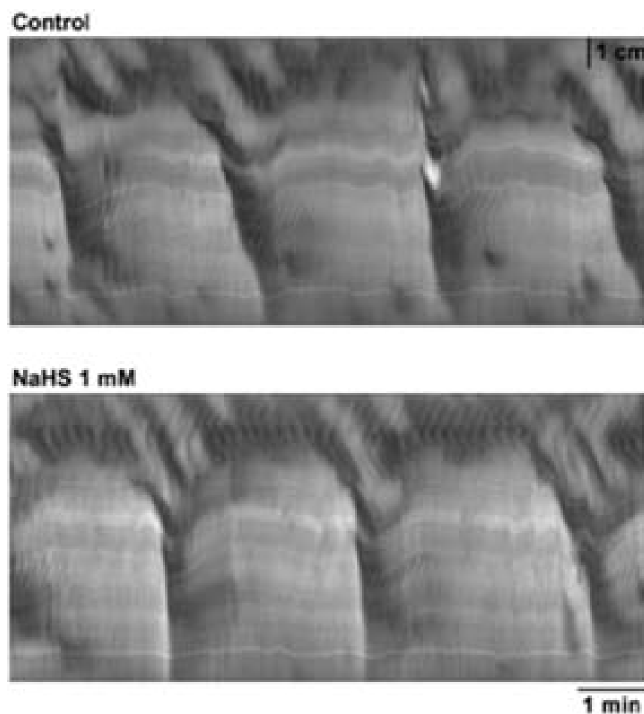

Figure 3

(A) S-T maps showing motor patterns observed in colonic segments under control conditions (top) and after the intraluminal addition of NaHS (1 mM) (bottom). No effect was observed.

(n = 4; Figure 7A). Neither the frequency nor the duration nor the amplitude of slow waves was affected by NaHS (1 mM) infusion (n = 4; Figure 7B).

Interaction between NaHS, NO and guanylate cyclase (GC)

To evaluate a putative interaction between NaHS, NO and GC, rat colonic strips devoid of submucous plexus were studied. In the presence of TTX (1 µM), NaHS caused a concentration-dependent inhibition of mechanical activity (IC_{50} = 96.2 µM; log IC_{50} = –4.02 ± 0.04; n = 9; Figure 8A,B). Interestingly, when tissues were incubated with the GC inhibitor, ODQ (10 µM), the inhibitory effect of NaHS was reduced (IC_{50} = 252.5 µM; log IC_{50} = –3.60 ± 0.04; $P < 0.001$; n = 7; Figure 8A,B). However, in the presence of L-NNA (1 mM), NaHS was as potent an inhibitor as it was under control conditions (IC_{50} = 100.2 µM; log IC_{50} = –4.00 ± 0.05; n.s.; n = 5). Similar experiments had been previously performed with apamin and glibenclamide (Gallego *et al*., 2008) and consequently were not repeated in the present study.

Effect of NaHS on neuromuscular transmission

Inhibitory neurotransmission was evaluated by measuring the amplitude and the duration of the IJP. None of these parameters was affected by NaHS (1 mM) (data not shown). In the presence of L-NNA (1 mM) and MRS2500 (1 µM), an atropine-sensitive EJP could be recorded (Figure 9A). The EJP amplitude was 3.0 ± 0.6 mV and the EJP duration was

Table 2

Effect of NaHS on RPMCs and ripples in the presence of lidocaine

RPMCs (proximal)	Control	Lidocaine 100 µM	Serosal NaHS 0.3 mM	Serosal NaHS 1 mM
Frequency (cpm)	1.3 ± 0.3	0.5 ± 0.1[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Amplitude (mm)	2.0 ± 0.2	2.3 ± 0.4	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Duration (s)	32.2 ± 3.3	21.7 ± 2.0	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Propagation (cm min^{-1})	4.6 ± 0.2	5.8 ± 0.3[a]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Ripples	**Control**	**Lidocaine 100 µM**	**Serosal NaHS 0.3 mM**	**Serosal NaHS 1 mM**
Frequency (cpm)	11.1 ± 0.9	13.4 ± 0.4	13.4 ± 0.9	11.1 ± 1.1[a]
Amplitude (mm)	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	1.0 ± 0.2[a]
Duration (s)	4.7 ± 0.2	3.8 ± 0.2	4.4 ± 0.4	5.3 ± 0.7[a]
Presence (cm)*	3.5 ± 0.2	4.4 ± 0.6	5.9 ± 0.5	6.1 ± 0.9
Propagation (cm min^{-1})				
Aboral (anterograde)	6.3 ± 0.4	6.6 ± 1.0	5.6 ± 0.4	6.3 ± 0.4
Oral (retrograde)	6.0 ± 0.1	6.3 ± 0.2	6.3 ± 0.6	6.6 ± 0.9

Values are means ± SEM. n = 4. Note that lidocaine (100 µM) abolished RPMCs in the mid colon, therefore these data were not included in this table. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, significant difference from control or lidocaine (100 µM) respectively; paired Student's t-test (lidocaine vs. control) and one-way ANOVA, followed by Bonferroni's multiple comparison test (NaHS vs. lidocaine). *Distance in which ripples were observed from the proximal edge of the colon.

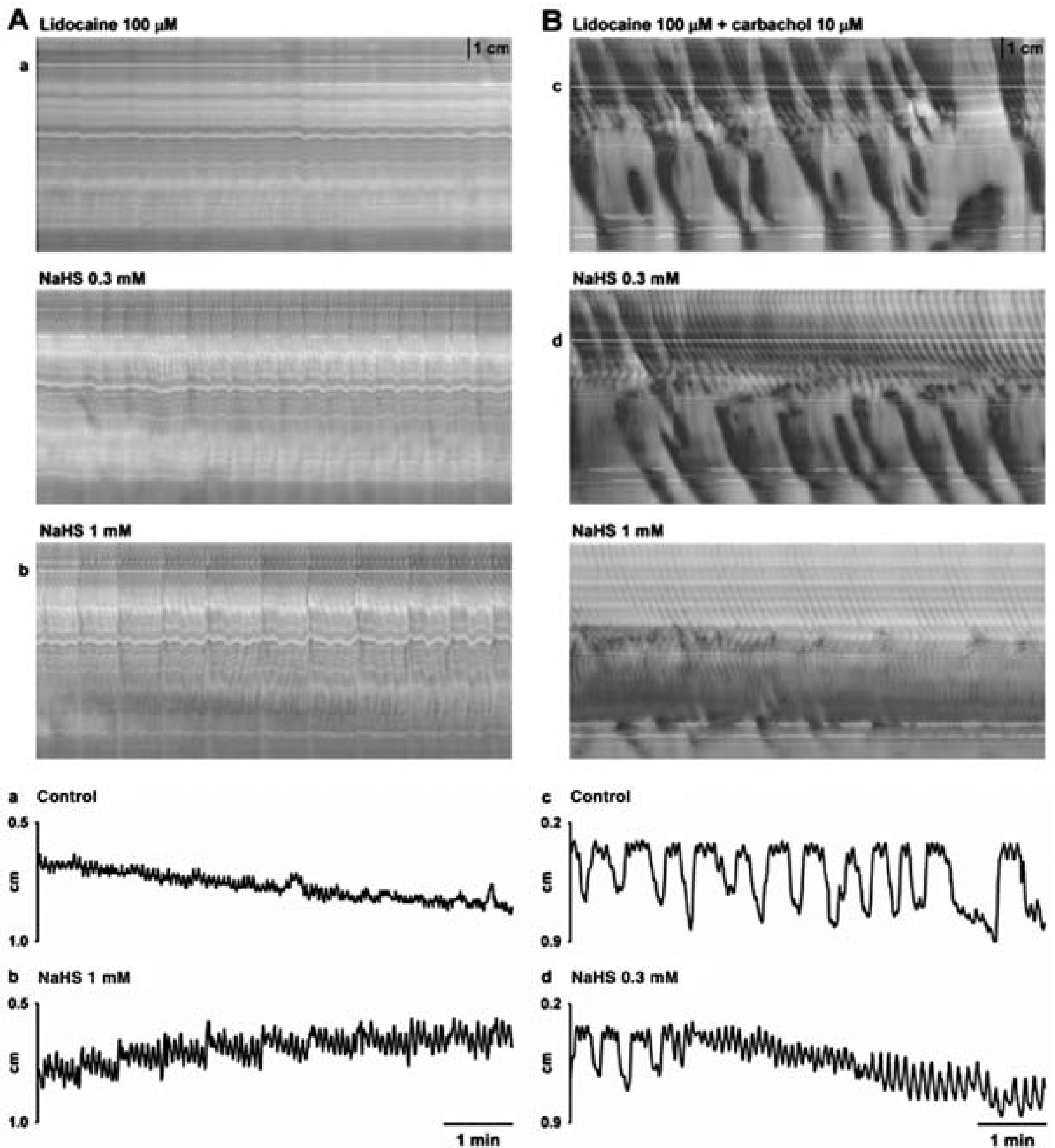

Figure 4

S-T maps showing the effect caused by the serosal addition of NaHS (0.3 and 1 mM) on motor patterns observed in colonic segments in the presence of lidocaine (100 μM) (A) and lidocaine (100 μM) + carbachol (10 μM) (B). Note that in the presence of lidocaine alone, ripples but not RPMCs are observed. Addition of carbachol restored RPMCs. Detail of the diameter of the colon in the proximal region in control conditions (Aa) and after the addition of NaHS (1 mM) (Ab) (dotted lines in S-T maps) in the presence of lidocaine (100 μM). Detail of the diameter of the colon in the proximal region in control conditions (Bc) and after the addition of NaHS (0.3 mM) (Bd) (dotted lines in S-T maps) in the presence of lidocaine (100 μM) + carbachol (10 μM). In both cases, NaHS caused effects similar to those observed in control conditions.

Table 3

Effect of NaHS on RPMCs and ripples in the presence of lidocaine and carbachol

RPMCs (proximal)	Control	Lidocaine (100 μM) + Carbachol (10 μM)	Serosal NaHS 0.3 mM	Serosal NaHS 1 mM
Frequency (cpm)	1.3 ± 0.1	1.8 ± 0.2[a]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Amplitude (mm)	2.9 ± 0.4	3.8 ± 0.2	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Duration (s)	35.6 ± 2.6	26.3 ± 1.0[a]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Propagation (cm min^{-1})	5.2 ± 0.5	8.9 ± 1.0	0.0 ± 0.0[c]	0.0 ± 0.0[c]
RPMCs (mid)	**Control**	**Lidocaine (100 μM) + Carbachol (10 μM)**	**Serosal NaHS 0.3 mM**	**Serosal NaHS 1 mM**
Frequency (cpm)	0.8 ± 0.2	1.0 ± 0.2	0.8 ± 0.5	0.3 ± 0.3
Amplitude (mm)	4.2 ± 0.5	4.6 ± 0.6	2.2 ± 1.3[a]	0.1 ± 0.1[b]
Duration (s)	37.1 ± 8.8	29.9 ± 0.6	15.3 ± 9.4	6.2 ± 6.2
Propagation (cm min^{-1})	25.5 ± 4.0	10.4 ± 1.6	5.0 ± 3.2	3.6 ± 3.6[a]
Ripples	**Control**	**Lidocaine (100 μM) + Carbachol (10 μM)**	**Serosal NaHS 0.3 mM**	**Serosal NaHS 1 mM**
Frequency (cpm)	11.3 ± 0.2	10.1 ± 1.0	8.2 ± 0.4	9.5 ± 0.9
Amplitude (mm)	0.7 ± 0.1	0.8 ± 0.1	1.6 ± 0.1[a]	1.1 ± 0.4
Duration (s)	4.9 ± 0.1	4.9 ± 0.3	7.0 ± 0.3[b]	5.8 ± 0.6
Presence (cm)*	3.4 ± 0.4	5.4 ± 0.8	6.3 ± 0.8	7.3 ± 0.7
Propagation (cm min^{-1})				
Aboral (anterograde)	6.0 ± 0.5	6.5 ± 1.1	7.1 ± 0.8	7.1 ± 0.8
Oral (retrograde)	5.6 ± 0.7	7.8 ± 0.7[a]	7.2 ± 0.7	8.5 ± 1.5

Values are means ± SEM. $n = 4$. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, significant difference from control or lidocaine + carbachol respectively; paired Student's *t*-test (lidocaine + carbachol vs. control) and one-way ANOVA, followed by Bonferroni's multiple comparison test (NaHS vs. Lidocaine + carbachol). *Distance in which ripples were observed from the proximal edge of the colon.

Table 4

Effect of ODQ, glibenclamide, apamin and a combination of potassium channel blockers on smooth muscle membrane potential and hyperpolarization induced by NaHS

Treatment	*n*	Membrane potential (mV) Basal[1]	Membrane potential (mV) Pretreatment[2]	Membrane potential (mV) NaHS 0.3 mM[3]	Net effect on MP (mV) Pretreatment[2]	Net effect on MP (mV) NaHS 0.3 mM[3]
Control	4	−40.8 ± 0.8	–	−50.3 ± 1.3[b]	–	−9.5 ± 0.8
ODQ 10 μM	4	−44.4 ± 2.2	−41.6 ± 2.8[a]	−48.3 ± 2.9[c]	+2.8 ± 0.7	−6.7 ± 0.5
Gliben 10 μM	4	−48.1 ± 2.0	−44.5 ± 2.9[a]	−48.7 ± 2.8[a]	+3.6 ± 1.1	−4.2 ± 0.7
Apamin 1 μM	4	−46.5 ± 3.1	−41.4 ± 2.4[a]	−45.2 ± 1.8[a]	+5.1 ± 1.2	−3.8 ± 0.7
K^+ blockers[4]	3	−40.0 ± 0.4	−36.4 ± 0.7	−39.2 ± 0.9	+3.6 ± 1.0	−2.8 ± 0.8

Values are means ± SEM. *n*, no. of samples. MP, membrane potential; Gliben, glibenclamide.
[1]Smooth muscle cells resting membrane potential (when no drug has been added).
[2]Pretreatment: effect of the drug (treatment column) incubated before NaHS.
[3]Effect of NaHS on smooth muscle membrane potential.
[4]Combination of K + blockers comprised glibenclamide 10 μM, apamin 1 μM, charybdotoxin 0.1 μM and TRAM-34 1 μM.
Note that positive and negative symbols included in the net effect column indicate the depolarization or the hyperpolarization observed by the drug (for pretreatment calculated from basal values and for NaHS 0.3 mM calculated from the values of membrane potential observed after the addition of the pretreatment drugs). [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, significant differences; paired Student's *t*-test (pretreatment vs. basal and NaHS 0.3 mM vs. pretreatment; for control NaHS 0.3 mM was compared to basal).

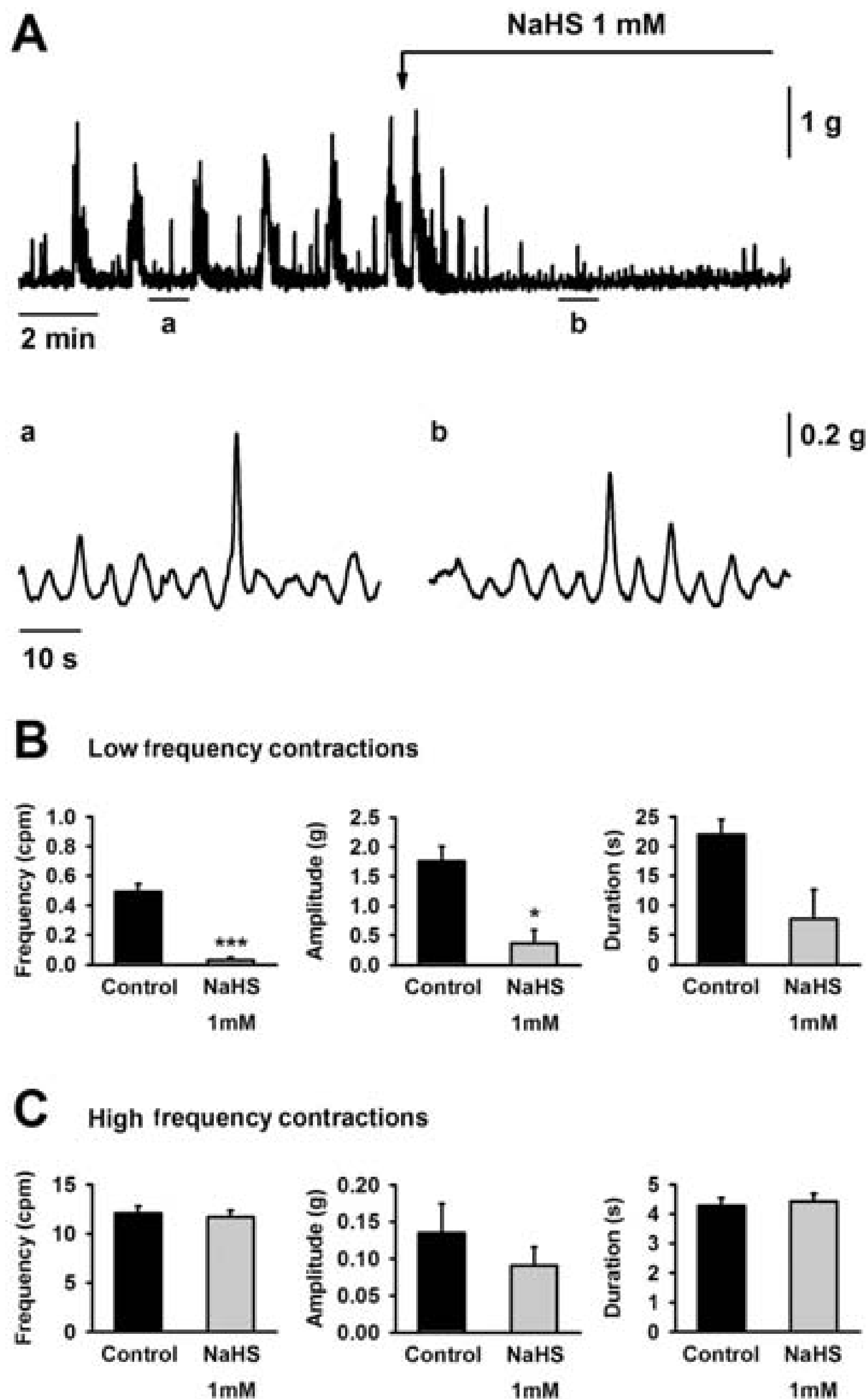

Figure 5

Muscle bath recordings showing the effect of NaHS (1 mM) on spontaneous mechanical activity in the rat mid colon (A). Detail of the high frequency contractions in control conditions (Aa) and in the presence of NaHS (1 mM) (Ab). Effect of NaHS (1 mM) on the frequency (left), amplitude (middle) and duration (right) of low frequency contractions (B) and high frequency contractions (C). All values are mean ± SEM. n = 4. *$P < 0.05$; ***$P < 0.001$, significant difference from control; paired Student's t-test.

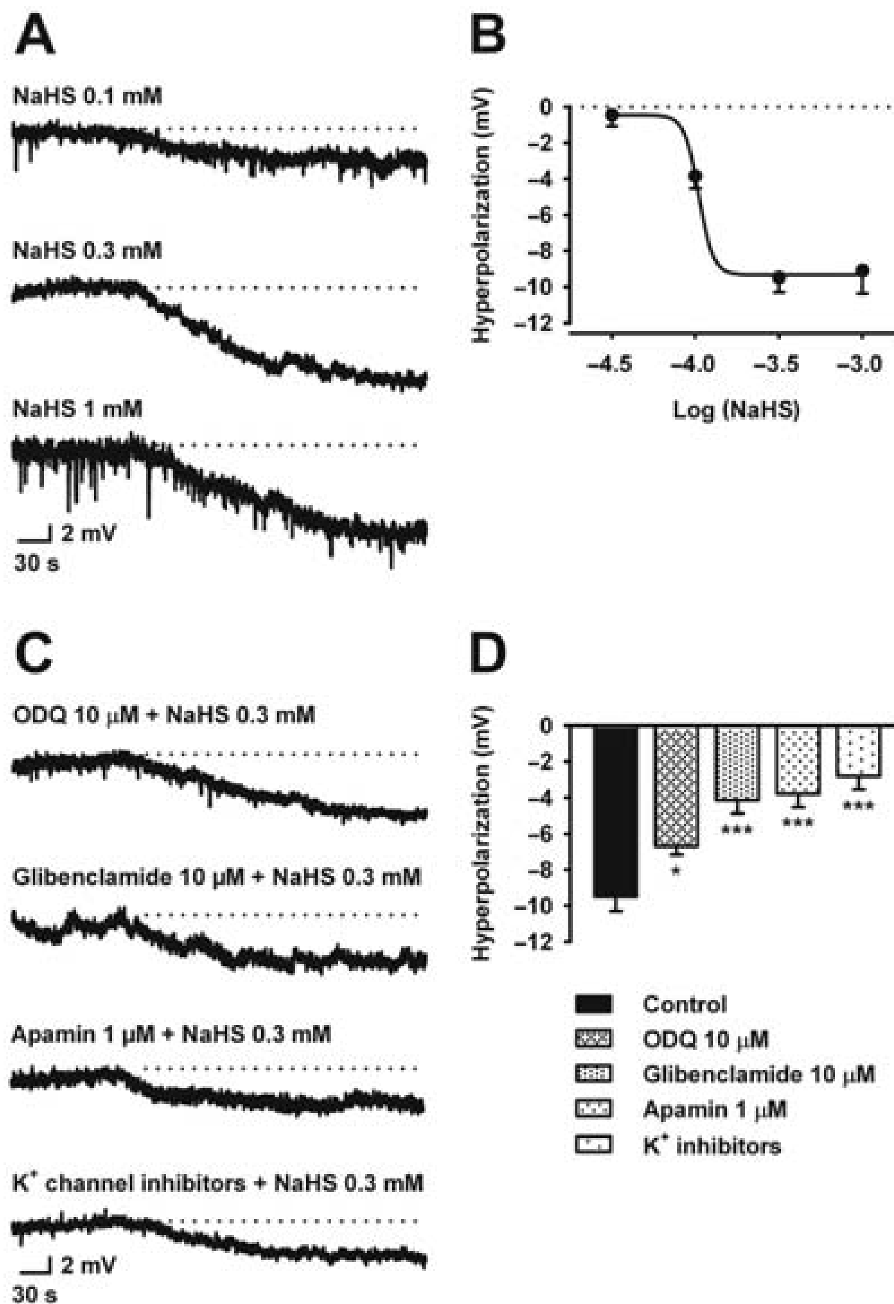

Figure 6

Intracellular microelectrode recordings showing the effect of NaHS (0.1 – 1 mM) on resting membrane potential in the rat mid colon (A). Dotted lines indicate the resting membrane potential. Concentration-response relationship for NaHS (B). Intracellular microelectrode recordings showing the hyperpolarization caused by NaHS (0.3 mM) in the presence of (i) ODQ (10 μM); (ii) glibenclamide (10 μM); (iii) apamin (1 μM); and (iv) a cocktail of potassium channel blockers including apamin (1 μM), TRAM-34 (1 μM), glibenclamide (10 μM) and charybdotoxin (0.1 μM) (from the top) (C). Dotted lines indicate the resting membrane potential. Histogram showing the effect of the different drugs mentioned above on the hyperpolarization produced by NaHS (0.3 mM) (D). All values are mean ± SEM. n = 4 for each experiment. *P < 0.05, ***P < 0.001, significant difference from control; one-way ANOVA, followed by Bonferroni's multiple comparison test.

1.3 ± 0.1 s at 32 V of stimulation (n = 3). The EJP was clearly inhibited by NaHS (1 mM) (n = 3; Figure 9B,C). In order to check that the effect was due to a pre- or post-junctional effect, the response to carbachol was tested in muscle bath experiments. To avoid post-junctional inhibitory effects due to smooth muscle hyperpolarization and consequently inhibition of L-type calcium channels, these experiments were performed in the presence of nifedipine. Under these conditions, carbachol induced a small atropine-sensitive contraction (Figure 9D), probably caused by calcium release from intracellular calcium stores (Oh *et al*., 1997). The contractile responses induced by carbachol were decreased by NaHS

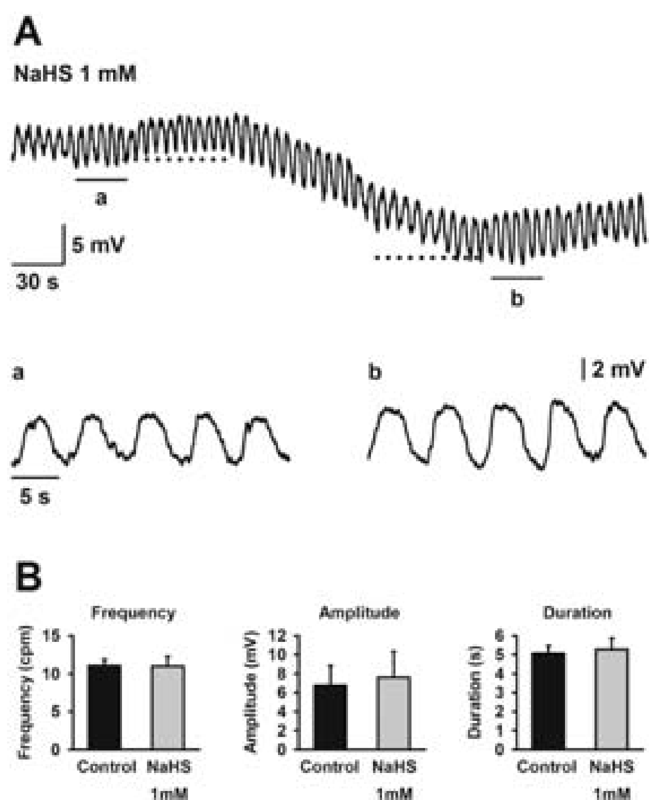

Figure 7

Intracellular microelectrode recordings showing slow waves recorded in samples with intact ICC-SMP network in the presence of nifedipine (1 µM) (A). Dotted lines indicate the resting membrane potential and the maximum hyperpolarization caused by NaHS (1 mM) respectively. Detail of slow waves in control conditions (Aa) and in the presence of NaHS (1 mM) (Ab). Histograms showing that NaHS (1 mM) did not modify frequency, amplitude or duration of slow waves (B). All values are mean ± SEM. $n = 4$. Significant differences were assessed using paired Student's *t*-test.

(1 mM) (carbachol 10 µM: 5.7 ± 0.8 g min^{-1} vs. carbachol 10 µM in the presence of NaHS 1 mM: 2.6 ± 0.2 g min^{-1} AUC; $P < 0.01$; $n = 5$; Figure 9D). Although we cannot completely rule out a pre-junctional effect, these experiments demonstrate that, at least in part, NaHS acted post-junctionally on cholinergic myogenic responses.

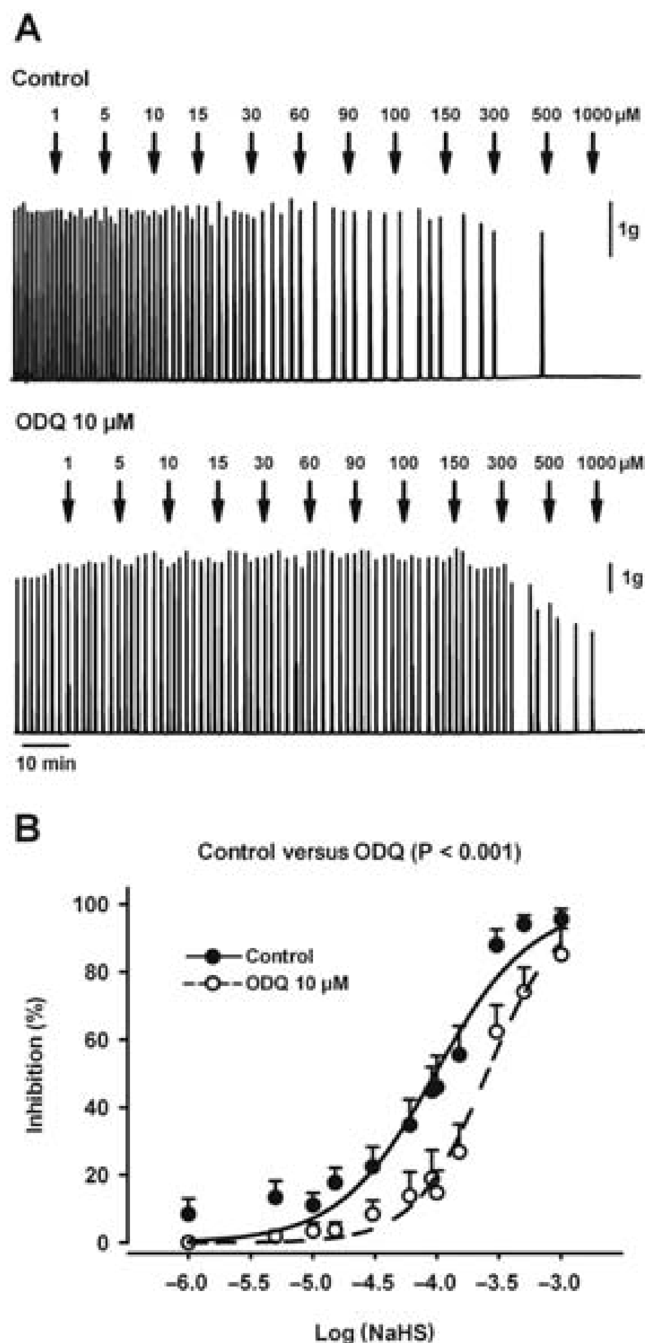

Figure 8

Effect of NaHS on spontaneous motility in muscle strips devoid of mucosa and submucosa. (A) Muscle bath recordings showing the concentration-dependent effect of NaHS (1–1000 µM) on spontaneous mechanical activity in the presence of TTX (1 µM) (upper recording) or TTX (1 µM) and ODQ (10 µM) (lower recording). (B) Concentration–response curves for NaHS in control conditions and in the presence of ODQ (10 µM). All values are mean ± SEM. Control, $n = 9$; ODQ, $n = 7$. Significant differences were assessed using two-way ANOVA, followed by Bonferroni's multiple comparison test.

Discussion

In the present study, we investigated the effect of NaHS on colonic motor patterns in the rat colon. S-T maps of intestinal segments are an interesting methodology to characterize motility patterns *in vitro* and to study responses to different stimuli, that is, luminal distension or luminal and serosal drug application (Hennig *et al*., 1999; 2010; D'Antona *et al*., 2001; Berthoud *et al*., 2002; Janssen *et al*., 2007; Lentle *et al*., 2007; 2008; Dinning *et al*., 2012). Two motor patterns are identified in rat colonic S-T maps (Huizinga *et al*., 2011 and present study). RPMCs were observed in the proximal colon

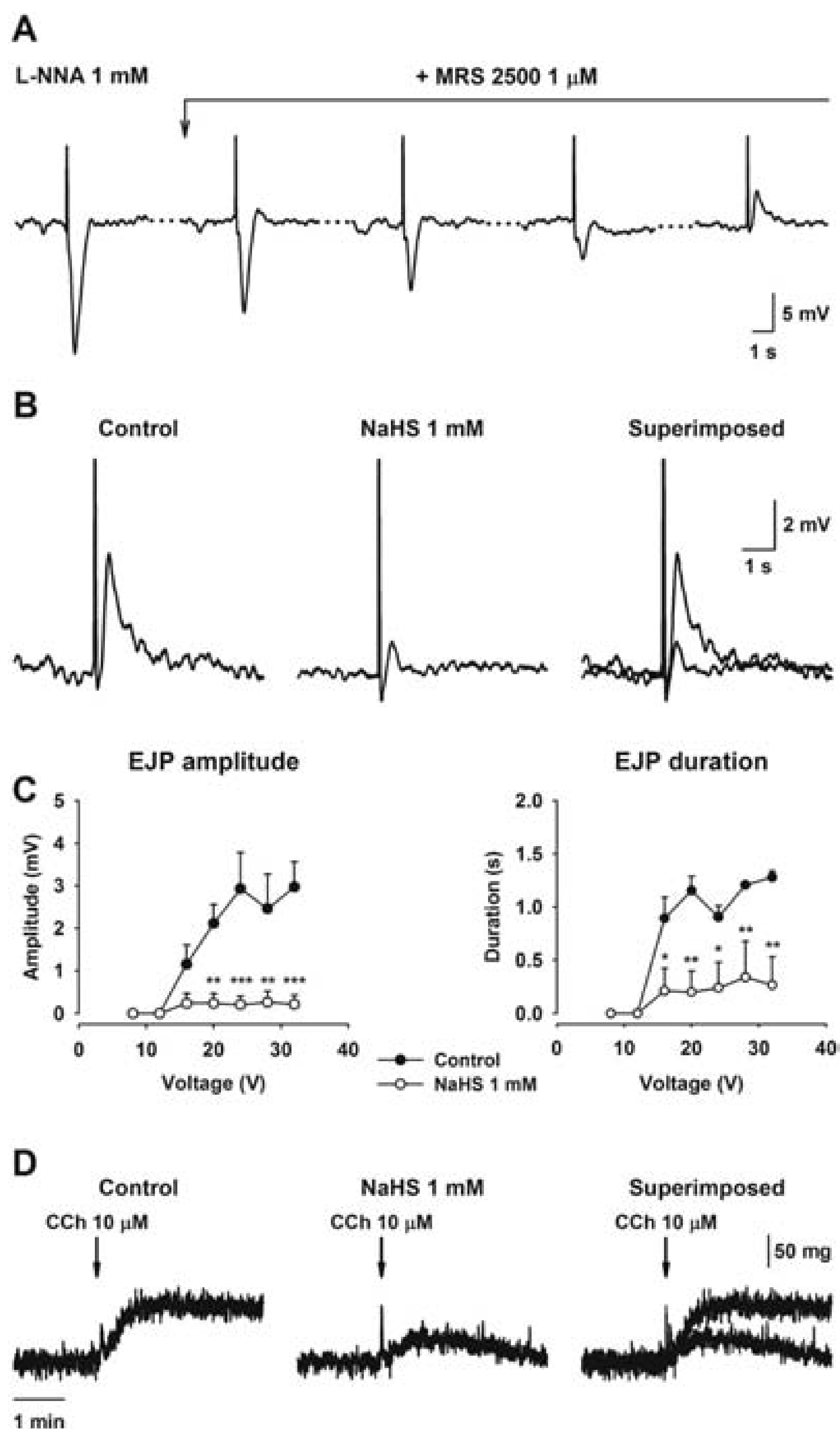

Figure 9

Intracellular microelectrode recordings showing how, in the presence of L-NNA (1 mM), MRS2500 (1 μM) gradually blocked the purinergic IJP revealing a hidden EJP (A). Breaks in the time axis are indicated by dotted lines. The EJP was clearly reduced by NaHS (1 mM) (B). The graphs represent the inhibitory effect of NaHS (1 mM) on both the amplitude and duration of the EFS-induced EJP (C). Muscle bath recordings in the presence of nifedipine (1 μM) showing the effect of NaHS 1 mM on contractile responses induced by carbachol (10 μM) (D). All values are mean ± SEM. Electrophysiological experiments: $n = 3$; muscle bath experiments: $n = 5$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significant difference from control; two-way ANOVA, followed by Bonferroni's post test.

and about a quarter propagated to the mid colon causing a contraction strong enough to cause outflow. The second pattern consisted of high frequency, low-amplitude contractions, ripples, which propagated both in oral and aboral direction. Ripples did not cause outflow and hence were likely to promote mixing. Interestingly, similar motility patterns have been identified *in vivo* in rodents using other techniques such as strain-gauge transducers (Li *et al*., 2002) or colonic manometry (Croci *et al*., 1994). Therefore, construction of S-T maps is an interesting methodology to study motility in colonic segments *in vitro*. In the present work, we found that NaHS caused inhibition of RPMCs and enhancement of the amplitude of ripples without a major effect on their frequency and duration. Hence, ripples were the dominant pattern of motility both in the proximal and mid colon when tissue samples were incubated with NaHS. These results showed that NaHS inhibited propulsive movements in the colon and enhanced mixing movements. It is not known if the overall effect of NaHS will contribute to water absorption because both neuronally mediated and direct prosecretory effects of NaHS have been described in the colon (Schicho *et al*., 2006; Hennig and Diener, 2009; Krueger *et al*., 2010; Pouokam and Diener, 2011). Interestingly, changes in motility were observed when tissue was incubated with NaHS at the serosal side mimicking the location of H_2S as an endogenous mediator. In contrast, no effects were observed when NaHS was perfused in the lumen. This result is consistent with the marked capacity of colonic epithelial cells to metabolized H_2S (Furne *et al*., 2001; Mimoun *et al*., 2012). Thus, it is possible that H_2S produced by the microbiota does not reach the ‘contractile’ apparatus and, therefore, luminal H_2S is unlikely to modify mechanical activity, under physiological conditions. We can hypothesize that only when the intestinal barrier is disrupted (i.e. possibly when permeability is increased) or the inactivation by the epithelium is impaired, then luminal H_2S might reach, possibly by diffusion or via the blood stream, the zone where smooth muscle, neurons and ICC work cooperatively to organize motility.

Strips develop two types of myogenic contractions that we previously defined as low frequency (0.5–2 cpm) and high frequency contractions (10–12 cpm) (Alberti *et al*., 2005). Using selective dissections (Pluja *et al*., 2001) and animals with impaired ICC development (Alberti *et al*., 2007), we have demonstrated that both ICC networks are necessary to record low frequency and high frequency contractions respectively. Both types of contractions are orchestrated by rhythmic oscillations in the RMP of smooth muscle cells: cyclic depolarizations with strong superimposed spiking activity, probably originating from the ICC associated with the myenteric plexus (ICC-MP), underlying low frequency contractions, and slow waves, likely to originate from the ICC associated with the submuscular plexus (ICC-SMP), causing high frequency contractions (Pluja *et al*., 1999; 2001; Martin *et al*., 2004; Alberti *et al*., 2007). Although both experimental conditions are not identical (i.e. strips are stretched and the enteric circuitry is possibly partially disrupted), a correlation in terms of amplitude and frequency of contractions can be established between low frequency contractions recorded in the muscle bath and RPMCs observed in segments and between high frequency contractions and ripples (Pluja *et al*., 2001; Alberti *et al*., 2005; Huizinga *et al*., 2011). Briefly: (i) the frequencies of high frequency contractions and ripples as well as low frequency contractions and RPMCs are similar; (ii) a gradient in frequency (higher in proximal colon) is observed in low frequency contractions, which is similar to the gradient in frequency observed in RPMCs; (iii) both high frequency contractions and ripples have a constant frequency all along the colon; (iv) low frequency contractions are usually of high amplitude, which is similar to the RPMCs, which aborally propagate and cause propulsion and outflow; and (v) the amplitude of high frequency contractions in strips is low, similar to that of ripples. Accordingly, similar motility patterns are observed both in strips and colonic segments despite the important differences in experimental conditions. In strips, NaHS inhibits low frequency contractions without modifying high frequency contractions (Gallego *et al*., 2008 and present study). This suggests that in small strips the mechanism responsible for low frequency contractions is more sensitive to NaHS than the mechanism responsible for high frequency contractions, which is similar in segments and muscle strips. At the moment, we do not know why the amplitude of ripples is enhanced.

An important difference between segments and muscle strips is the sensitivity to neuronal blockade. In muscle bath recordings, low frequency and high frequency contractions are still observed in the presence of TTX showing that the musculature intrinsically has the mechanism to develop both patterns (Pluja *et al*., 2001). In contrast, neuronal blockade selectively inhibits RPMCs without modifying the frequency of ripples [present paper and (Huizinga *et al*., 2011)]. One conclusion might be that the enteric nervous system orchestrates both the pattern and the force of contraction. However this may not be the case because after neuronal blockade, carbachol can re-establish RPMCs and consequently this motility pattern does not depend on cyclic activation of motor neurons (neural pacemaker). Under the experimental conditions of the segment studies, the ICC-MP act as pacemaker cells with neuronal input essential for RPMCs (Huizinga *et al*., 2011). Interestingly NaHS inhibited RPMCs and enhanced ripples after neuronal blockade and subsequent restoration of the motility with carbachol, suggesting that NaHS can also affect muscle activity through non-neuronal pathways.

In colon strips, the circular muscle layer is predominantly innervated by inhibitory motor neurons releasing nitric oxide and ATP (Grasa *et al*., 2009; Gil *et al*., 2010; 2012). In the present study, we used a combination of L-NNA and MRS2500 to block inhibitory neurotransmission. Under these conditions, atropine-sensitive EJPs were recorded. This particular experimental approach allowed us to evaluate the effect of NaHS on the excitatory neuromuscular transmission. Interestingly, NaHS inhibited cholinergic EJPs induced by EFS. In addition, NaHS also inhibited carbachol-mediated contractions in the presence of nifedipine. These experiments were performed in the presence of nifedipine to exclude the possibility that the effect observed with NaHS on carbachol-induced contractions was related to muscle hyperpolarization (see below). Therefore, inhibition of cholinergic neuromuscular transmission at the post-junctional level, is a possible mechanism that might explain the effect of NaHS on RPMCs, that is, NaHS inhibits cholinergic input mimicking the effect of atropine that also blocks RPMCs [present study and

(Huizinga *et al.*, 2011)]. Consistent with these results, it has been demonstrated that H_2S inhibited cholinergic contractions in the rabbit ileum (Teague *et al.*, 2002) and relaxed carbachol pre-contracted preparations of guinea pig taenia caecum (Denizalti *et al.*, 2011).

However, strips develop low frequency contractions even in the presence of atropine (i.e. classical NANC conditions). Consequently, inhibition of cholinergic responses cannot be responsible for the inhibitory effect observed in the muscle strips. Thus, our observed effect of smooth muscle hyperpolarization is a more likely mechanism. It is important to note that, as cyclic depolarizations are nifedipine-sensitive (Pluja *et al.*, 2001), a slight hyperpolarization (i.e. NaHS effect) might be enough to markedly reduce opening probability of L-type calcium channels and contractions will be consequently inhibited. The mechanism responsible for NaHS-induced hyperpolarization and inhibition of spontaneous low frequency contractions is probably quite complex and several mechanisms should be taken into account. The first possibility is that NaHS is causing NO release from inhibitory motor neurons leading smooth muscle hyperpolarization and relaxation. However, in the presence of L-NNA, NaHS caused similar inhibition of spontaneous motility and, therefore, the mechanism was independent of nNOS. Interestingly, ODQ partially inhibited both NaHS-induced hyperpolarization and inhibition of spontaneous motility suggesting that GC is a potential target for NaHS. Recently, it has been demonstrated that NaHS induced release of NO from nitrosothiols in rat brain homogenates (Ondrias *et al.*, 2008). Therefore, it might be possible that the inhibitory effects observed with NaHS were due to NO release from this type of NO donor with subsequent activation of GC (Gil *et al.*, 2012). Another potential mechanism is a direct or indirect effect on potassium channels, which would be consistent with our previous results obtained in muscle bath (Gallego *et al.*, 2008). The present study demonstrates that apamin and glibenclamide partially reduce the hyperpolarization induced by NaHS, confirming a potential role for SK_{Ca} and K_{ATP} channels mediating NaHS effects. However, it is important to note that the 'cocktail' of potassium channel inhibitors was more effective in reducing NaHS hyperpolarization, suggesting that the NaHS effects are unlikely to be mediated by a single potassium channel. Our results suggest that smooth muscle hyperpolarization might be the outcome of different direct and indirect mechanisms.

The question is then, does hyperpolarization affect the slow waves? Recordings in strips with the intact submucosa and ICC-SMP allow the measurement of slow wave activity that occurs at a similar frequency to high frequency contractions and ripples. Interestingly, NaHS causes a 7–8 mV hyperpolarization, which does not affect the frequency of the dominant slow wave driven pacemaker system, showing that slow wave activity, in terms of frequency, is independent of membrane potential (Ohba *et al.*, 1975; Jimenez *et al.*, 1999). Consistent with this finding, the frequency of slow waves was unaffected by long-lasting hyperpolarizations induced by endogenous inhibitory neurotransmitters released by EFS (unpublished data). This is consistent with different innervations of the two pacemaker systems.

We conclude that H_2S modulated colonic motility by inhibiting RPMCs and enhancing the amplitude of ripples, probably promoting mixing. Under physiological conditions, epithelial detoxification is probably able to limit the quantity of luminal H_2S that reaches the contractile apparatus and, therefore, no effects on motility are observed when NaHS is luminally applied. The mechanisms causing the effects on motility are diverse, which is consistent with the capacity of H_2S to affect different targets. In the present work, we demonstrated that cholinergic EJPs are inhibited, which is consistent with muscle bath experiments where the effect of NaHS on carbachol-induced contraction was measured. Inhibition of cholinergic neuronal responses might be consistent with the inhibition of RPMCs observed in S-T maps. However, *in vitro* low frequency contractions that were developed by intrinsic myogenic mechanisms are atropine insensitive. In this case, smooth muscle hyperpolarization might be crucial in the inhibition of these contractions. The frequency of slow waves was independent of cholinergic input and hyperpolarization caused by NaHS. Consequently, ripples, which are related to slow wave activity, were not inhibited by NaHS. Under our experimental conditions, H_2S shows relaxant properties and, therefore, further studies should be carried out to establish its putative use as an antispasmodic molecule (i.e. measurements of muscular tone and compliance), taking into account the potentiality of H_2S in the treatment of colonic inflammation (Fiorucci *et al.*, 2007; Wallace *et al.*, 2009).

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Conflicts of interest

The authors state no conflict of interest.

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Discussion

Coordination and regulation of muscle contractibility create defined motor patterns in the colon in order to accomplish specific functions; i.e. absorption of water and electrolytes, storage of content or defecation (Huizinga & Lammers, 2009; Sarna, 2010). Mechanical contractility in colonic smooth muscle cells is regulated by changes in membrane potential. Depolarizing stimuli cause contraction whereas hyperpolarizing stimuli cause muscular relaxation (Bolton *et al.*, 1999; Somlyo & Somlyo, 1994). Despite the fact that smooth muscle cells have intrinsic mechanisms to regulate its contractibility (i.e. contraction by activation of L-type calcium channels in response to distension (Farrugia *et al.*, 1999), coordinated regulation by enteric neurons and Interstitial Cells of Cajal (ICC) allows a fine regulation of muscle contraction/relaxation to define the above mentioned motor patterns (Alberti *et al.*, 2005; Bayguinov *et al.*, 2010a; Bayguinov *et al.*, 2010b; Huizinga *et al.*, 2011; Chen *et al.*, 2013).

The aim of this experimental work was to contribute to the current knowledge on the mechanisms regulating mechanical activity in the colon. In particular we investigated inhibitory neuromuscular transmission and its potential role regulating colonic motility. The main areas of research were the following: (1) pharmacological characterisation of purinergic neuromuscular transmission; (2) characterization of the inhibitory neural tone (3) study of the nature of purinergic neurotransmission in the colon (4) characterization of cellular mechanisms involved in purinergic and nitrergic neuromuscular transmission; and (5) evaluation of hydrogen sulphide (H_2S) as a putative signalling molecule regulating contractility in the colon.

Pharmacological characterisation of purinergic neuromuscular transmission

In the human colon, the $P2Y_1$ antagonist MRS2179 reduced both electrical (hyperpolarization) and mechanical (relaxation) responses induced by EFS (Gallego *et al.*, 2006; Gallego *et al.*, 2008b) or by stimulation of enteric neurons with nicotine (Auli *et al.*, 2008). Such observations have allowed to conclude that possibly, in the human colon, the non-nitrergic component of the neuromuscular transmission is purinergic in origin and exclusively mediated by activation of the purinergic receptor subtype $P2Y_1$. However, some authors have suggested that other purinoreceptors might also mediate neural purinergic responses. Their hypothesis is based in fact that the fast component of

the IJP is relatively resistant to MRS2179 blockade in some GI regions like the internal anal sphincter or the caecum (McDonnell *et al.*, 2008; Zizzo *et al.*, 2007). In order to verify this hypothesis, we performed a pharmacological study both in the human and rat colon using two new developed $P2Y_1$ antagonists, MRS2279 and MRS2500 (Boyer *et al.*, 2002; Kim *et al.*, 2003). Our results showed that MRS2279 and MRS2500 are between 10 and 100-fold more potent than MRS2179 blocking the purinergic neurotransmission both in human and rat colon (Table 1). Comparatively, relevant differences were observed between both inhibitors and species (human and rat colon). In human colon all three antagonists were able to abolish the purinergic IJP and relaxation, although concentrations up to 10 µM of MRS2179 were needed to achieve a total blockade. However, in the rat colon, MRS2179 at 20 µM just caused a partial inhibition of the non-nitrergic response whereas MRS2279 and MRS2500 produced total inhibition at concentrations about 0.1 µM.

Table 1. Summary of IC_{50} values for $P2Y_1$ antagonists

Antagonists	*Human colon*		*Rat colon*		
	IJP	*Muscle Relaxation*	*Spontaneous IJP*	*IJP*	*Muscle Relaxation*
MRS2179	1.2 µM* *(n = 5)*	0.9 µM* *(n = 4)*	-	13.1 µM *(n = 4)*	3.5 µM *(n = 9)*
MRS2279	0.28 µM *(n = 5)*	0.26 µM *(n = 5)*	-	0.018 µM *(n = 4)*	0.044 µM *(n = 8)*
MRS2500	0.071 µM *(n = 5)*	0.088 µM *(n = 7)*	0.003 µM *(n = 5)*	0.014 µM *(n = 4)*	0.017 µM *(n = 7)*

* Data previously published (Gallego *et al.*, 2006). IJP, inhibitory junction potential; n, number of strips.

Therefore, according to these results purinergic inhibitory neuromuscular transmission is possibly exclusively mediated by $P2Y_1$ receptors. Interestingly, the crucial role of $P2Y_1$ receptors mediating inhibitory neural responses in the colon has recently been confirmed by using knocked out animals, in which the purinergic component of the IJP and the purinergic relaxation are not observed (Gallego *et al.*, 2012; Hwang *et al.*, 2012). In addition, it is important to note that $P2Y_1$ receptors are also the main purinergic subtype receptor mediating purinergic neuromuscular transmission in other regions in the gastrointestinal tract, as it has been demonstrated by using different experimental approaches (Table 2).

Table 2. Summary of studies describing the involvement of $P2Y_1$ receptors in purinergic neuromuscular transmission in other areas of the gastrointestinal tract

Gut region	*Species*	*Approach*	*Publication*
Gastric antrum	Mouse	Pharmacological	Chaudhury *et al.*, 2011
Gastric antrum	Mouse	Genetic model	Gil *et al.*, 2013
Small intestine	Pig	Pharmacological	Gallego *et al.*, 2008c
Small intestine	Guinea pig	Pharmacological	Wang *et al.*, 2007
Caecum	Mouse	Genetic model	Gil *et al.*, 2013
Internal anal sphincter	Mouse	Pharmacological	Opazo *et al.*, 2011
Internal anal sphincter	Mouse	Pharmacological	Duffy *et al.*, 2012

Therefore, our results indicate that $P2Y_1$ receptors exclusively mediate purinergic inhibitory neuromuscular responses in the gastrointestinal tract. Studies carried out in genetic models confirm this observation.

Characterization of the inhibitory neural tone

The pharmacological approach using selective $P2Y_1$ antagonists allowed the evaluation of the co-transmission process responsible for the inhibitory neural tone. In the colon, ongoing release of inhibitory neurotransmitters maintains muscle membrane potential in a sustained state of hyperpolarisation (Bayguinov *et al.*, 2010b; Keef *et al.*, 1997; Lyster *et al.*, 1995; Sha *et al.*, 2010; Spencer *et al.*, 1998b). In the present work, we identified the nature of the pathways involved in ongoing release of neurotransmitters. Two main features of the electrophysiological recordings were observed: 1) spontaneous inhibitory junction potentials (sIJP) and 2) the level of resting membrane potential (RMP). sIJP are usually randomly observed in the recordings without any kind of exogenous electrical or chemical stimuli. When sIJP are recorded 1) a tail to the lower values of the frequency distribution is detected and 2) an increase in the standard deviation of the data corresponding to the tracing is observed. Moreover for this purpose we considered the RMP as the most probable value of the frequency distribution. These three values allowed us to characterise the inhibitory neural tone. We observed that: 1) TTX depolarised smooth muscle cells, inhibited sIJP and increased spontaneous motility; 2) L-NNA caused smooth muscle depolarization and increased motility without affecting sIJP; 3) MRS2500 inhibited sIJP (Table 1) without causing major changes in RMP or motility; and 4) high

concentrations of apamin depolarised smooth muscle cells, increased motility and reduced sIJP. Thus, the pharmacological study demonstrates that the inhibitory neural tone is mediated by endogenous firing of inhibitory neurons due to sodium carried action potentials causing "spontaneous" release of inhibitory neurotransmitters. sIJP are mediated by purine release acting on $P2Y_1$ receptors (sensitive to MRS2500) whereas the RMP is determined by tonic release of NO (L-NNA sensitive). Several studies have established that NO tonically released by enteric neurons participate in maintaining the inhibitory tone (Dickson *et al.*, 2010; Keef *et al.*, 1997; Lyster *et al.*, 1995). Recently, it has also been proposed that neural carbon monoxide (CO) might also contribute in setting such an inhibitory tone in the mouse colon. In the mouse, CO released by submucosal neurons also participates in setting a transwall gradient in RMP (Sha *et al.*, 2010). In our study, a similar depolarization is observed after addition of the neural blocker, TTX, or the nitric oxide synthase (NOS) inhibitor, L-NNA (about 5 mV). Furthermore, both drugs cause a similar increase in mechanical activity. Therefore, at least in the rat colon, NO seems to be the main neurotransmitter mediating the sustained inhibitory neural tone. Further experiments are needed to establish the putative role of CO as an inhibitory neurotransmitter in the rat colon and to define if a transwall gradient in RMP is present in this species. In addition, phasic and transient hyperpolarisations of muscle membrane potential have also been reported in the colon of several mammalian species in which no exogenous stimulation (i.e. chemical or electrical) is applied (Furness, 1969; Powell *et al.*, 2001; Spencer *et al.*, 1998a). These spontaneous IJP are probably mediated by activation of SK_{Ca} channels (Powell *et al.*, 2001; Spencer *et al.*, 1998a). We observed that SK_{Ca} channels are also the final effector in the rat colon as apamin blocked sIJP. However, it is possible that high concentrations of apamin might also reduce nitrergic responses (see below). Consequently, an increase in motility and depolarization was observed. In addition, in this work, we demonstrate for the first time that these phasic spontaneous events (sIJP) are purinergic in origin and mediated by activation of $P2Y_1$ receptors. Based on our approach, similar results have been recently observed in mouse colon and internal anal sphincter (Gallego *et al.*, 2012; Opazo *et al.*, 2011). It is important to note that neither L-NNA (NOS inhibitor), ODQ [guanylate cyclase (GC) inhibitor], D,L-Propargylglycine (PAG) nor Aminoxyacetic acid (AOAA) modified sIJP. Therefore,

this phasic, rapid and transient hyperpolarisations are not mediated by NO or H_2S signalling.

The effect of exogenous agonists/donors of both purinergic and nitrergic pathways were also evaluated in the present work. Both agonists/donors showed different effects on membrane potential and muscle contractibility, mimicking what it was observed with "spontaneous" neural activity ((Gallego *et al.*, 2008b) and present study). On the one hand, when $P2Y_1$ agonists (ADPβS and MRS2365) were exogenously added a fast, intense but transient inhibitory effect was observed both on membrane potential and muscle mechanical activity. On the other hand, the nitric oxide donor, sodium nitroprusside (SNP), caused both sustained hyperpolarization and relaxation. This is consistent with previous studies showing that neural release of purines mediates phasic changes in smooth muscle membrane potential and, therefore, fast and transient relaxations; whereas NO causes sustained muscle hyperpolarisations and persistent relaxations (Gallego *et al.*, 2008b).

Taken all this into account, it can be concluded that inhibitory motor neurons fire action potentials randomly releasing ATP or a related purine and NO. This cotransmission process is responsible for the inhibitory neural tone with complementary physiological functions being NO and ATP (or a related purine) the tonic and the phasic functional neurotransmitters, respectively.

Nature of purinergic neurotransmitter(s) in the colon

Purinergic neurotransmission was first described in the early 70s and a wide number of studies have suggested that ATP is the endogenous inhibitory purinergic neurotransmitter in the colon (Burnstock *et al.*, 1970; Burnstock *et al.*, 1978; Burnstock, 2008; Su *et al.*, 1971). Moreover ATP metabolites such as ADP or adenosine, braked down by ecto-nucleotidases, might also be bioactive and the mechanism might contribute to limit purine diffusion and quickly terminate the process. However, it has been recently proposed that β-nicotinamide adenine dinucleotide (β-NAD) and/or ADP-ribose are the endogenous purines released by enteric neurons in the colon (Durnin *et al.*, 2012; Durnin *et al.*, 2013; Hwang *et al.*, 2011; Hwang *et al.*, 2012; Mutafova-Yambolieva *et al.*, 2007; Mutafova-

Yambolieva, 2012). Consequently the actual identity of the purine involved in the inhibitory post-junctional responses in the gastrointestinal tract is still controversial (Burnstock, 2008; Goyal, 2011; Hwang *et al.*, 2011; Mutafova-Yambolieva, 2012). Thus, the term ATP or a related purine, usually referred to ATP metabolites, is nowadays more valid than ever because β-NAD and/or ADP-ribose might also contribute to purinergic inhibitory neurotransmission. The neurotransmitter(s) is still waiting for the final identification and pre and post-junctional criteria might be considered.

Neurotransmitter identification: pre-junctional criteria

ATP, β-NAD and other metabolites including ADP, AMP and adenosine are detected with reverse-phase gradient HPLC assay after electrical and chemical neural stimulation. Interestingly, release of β-NAD, but not of ATP, is decreased by neurotoxins, including tetrodotoxin (TTX), an inhibitor of voltage-gated sodium channels, and ω-conotoxin GVIA (ω-CTX GVIA), a blocker of N type voltage-dependent calcium channels (VDCC) (Durnin *et al.*, 2013; Hwang *et al.*, 2011; Mutafova-Yambolieva *et al.*, 2007). It is important to note that electrical stimulation at high voltages can directly open VDCC and consequently purine release might be independent of sodium driven action potentials (TTX insensitive purine release) (Pluja *et al.*, 1999). Furthermore, the sensitivity of N type calcium channel blockers on purinergic release is variable among species. Thus, it has been observed that in rodents several types of ω-CTX barely inhibit the IJP elicited by electrical stimulation (Borderies *et al.*, 1996). In the present work we demonstrate that spontaneous inhibitory purinergic neuromuscular transmission in the rat colon is blocked in the presence of TTX. Thus, consistent with the presence of an inhibitory neural tone, TTX should cause a decrease in purinergic release in the basal state. However, release of purines in basal conditions and its sensitivity to neural blockade has not been demonstrated yet.

Neurotransmitter identification: post-junctional criteria

Effect on $P2Y_1$ receptors

Both ATP and ADP have been described as $P2Y_1$ agonists (Abbracchio *et al.*, 2006; Palmer *et al.*, 1998). By now the number of studies regarding the pharmacodynamic

properties of β-NAD is still reduced (even smaller for ADP-ribose). However, β-NAD is a potential agonist of $P2Y_1$ and $P2Y_{11}$ purinoreceptors by (Moreschi *et al.*, 2006; Mutafova-Yambolieva *et al.*, 2007). In this work, we confirm the agonism of β-NAD on $P2Y_1$ receptors using HEK-293 cells which constitutively express this purinoreceptor (Fischer *et al.*, 2005). However it is important to note that high concentrations of β-NAD are needed to reveal a $P2Y_1$ response. Consistent with this result if β-NAD is the endogenous neurotransmitter high concentrations of β-NAD should be locally present at the neuromuscular junction.

Pharmacological characterization in colonic strips

In colonic samples, muscle hyperpolarisation and relaxation has been described to all the above mentioned purines (Durnin *et al.*, 2012; Gallego *et al.*, 2006; Hwang *et al.*, 2011; Mutafova-Yambolieva *et al.*, 2007; Van Crombruggen & Lefebvre, 2004). In our studies, we observed that β-NAD only caused a slight hyperpolarisation on human circular smooth muscle cells (~4 mV at 10 mM). In addition, maximal relaxation of muscle strips was only obtained at concentrations of 10 mM and the IC_{50} obtained in these studies was 3.3 mM. Some studies have shown that the inhibitory responses caused by exogenous addition of ATP and ADP in the colon are not antagonized by $P2Y_1$ inhibitors, whereas the ones induced by β-NAD are reduced by these antagonists (Durnin *et al.*, 2012; Hwang *et al.*, 2011). Nevertheless, experiments reporting the complete opposite findings have also been published. In the human and guinea-pig colon, relaxation induced by ATP is antagonised by MRS2179 (Gallego *et al.*, 2006; King & Townsend-Nicholson, 2008). Furthermore, in the present work, mechanical inhibitory responses induced by β-NAD are not reverted by MRS2279 or MRS2500 used up to 5 µM. In agreement with this finding, it has been recently reported that muscle hyperpolarizing responses to β-NAD are also present in the caecum of $P2Y_1$ knockout mice (Gil *et al.*, 2013). It is important to have in mind that all these purines are potential agonists of other P2 receptors in addition to $P2Y_1$ (Abbracchio *et al.*, 2006; Alexander *et al.*, 2011; Von Kugelgen, I, 2006). In the colon, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{14}$ receptors have been described, being expressed in a wide variety of cells, including enteric neurons, SMCs, ICC, platelet derived growth factor receptor α-positive cells (PDGFRα+ cells) and enteric glial cells (Gallego *et al.*, 2006; Giaroni *et al.*, 2006; King & Townsend-Nicholson, 2008;

Kurahashi *et al.*, 2011; Monaghan *et al.*, 2006; Van Crombruggen & Lefebvre, 2004; Van Nassauw *et al.*, 2006). Activation of these receptors may cause direct or indirect smooth muscle relaxation and, accordingly, pharmacological studies using "exogenous" purinergic agonists usually conclude that more than one purinoreceptor subtype is involved in purinergic relaxation (Giaroni *et al.*, 2002; Van Crombruggen & Lefebvre, 2004). Thus, given that the endogenous purinergic neurotransmission is mediated by $P2Y_1$ receptors, it might be possible that exogenous purines activate purinoreceptors which are "extrajunctionally" located, whereas these receptors are not activated when purines are focally released from enteric neurons. In this case, endogenous purinergic inhibitory neurotransmission would be limited to specific post-junctional areas. Some authors have stated that purinergic neuromuscular transmission might be transduced by a specific population of interstitial cells, the fibroblast-like cells or PDGFRα+ cells. This cellular type abundantly expresses $P2Y_1$ receptors and SK3 channels. In addition, isolated PDGFRα+ cells respond to exogenous addition of ATP with outward currents which are blocked with MRS2500 and apamin (Kurahashi *et al.*, 2011). However, it is important to note that $P2Y_1$ receptors are also widely expressed in the colonic SMCs and, therefore, direct transduction of neural signals to muscle is possible as well (Gallego *et al.*, 2006; King & Townsend-Nicholson, 2008; Kong *et al.*, 2000; Van Crombruggen & Lefebvre, 2004). Further experiments are needed to identify if specific areas of purinergic neuromuscular transmission effectively exist in the colon and the mechanisms by which this type of signalling is exclusively mediated by $P2Y_1$ receptors despite the fact that other P2Y receptors have been observed in this tissue.

Purinergic neuromuscular transmission is more complex than previously thought. Thus, a wide variety of purines including ATP, ADP, β-NAD and even ADPr might be involved in mediating purinergic inhibitory neuromuscular responses. However, further research is needed to clearly identify the nature of the purine/s involved in the inhibitory responses in the colon. We speculate that the endogenous neurotransmitter might not have a single identity and different kinds of purines might be released by enteric neurons and even bioactive compounds might be junctionally and transiently created.

Mechanisms involved in the transduction of purinergic and nitrergic signalling

Based on the results obtained in our studies, we have been able to define criteria to establish if a drug is acting inhibiting purinergic or nitrergic neuromuscular signalling (Table 3). This approach let us to investigate the transduction mechanisms responsible for purinergic and/or nitrergic pathways.

Table 3. Criteria to identify the effect of a drug in nitrergic and purinergic pathways

	Inhibition of	
	Nitrergic neurotransmission	**Purinergic neurotransmission** [1]
Membrane potential [2]	Depolarization	No effect
Spontaneous Motility [2]	Increase	No effect / Decrease [3]
Spontaneous IJP [2]	No effect	Inhibition
EFS-induced IJP	Inhibition of the slow component	Inhibition of the fast component
EFS-induced relaxation [4]	Partial reversion	No effect/Partial reversion

IJP, inhibitory junction potential; EFS, electrical field stimulation. [1] Based on data using inhibitors of $P2Y_1$ receptors (Chapters 1 and 3) and $P2Y_1$ knockout mice (Gallego *et al.*, 2012). [2] These criteria should be used if an inhibitory neural tone is present in the preparation (Chapter 1). [3] A decrease in spontaneous motility might be expected if ATP is limiting pre/post junctional NO effect. [4] EFS-induced relaxation might be reversed by $P2Y_1$ antagonists/NOS inhibitors depending on the frequency of EFS (Gallego *et al.*, 2008).

Our results using ODQ demonstrated that nitrergic responses are completely mediated by activation of guanylate cyclase (GC). Thus, when NO is released from neurons the post-junctional effect of these neurotransmitter is totally dependent in GC activation and production of cGMP. This result is in agreement with previous studies, in which it has been observed that nitrergic responses are absent is knock out animals lacking GC (Groneberg *et al.*, 2011). However, it is still unknown the relative contribution of GC located in SMCs, ICC or even PDGFRα+ cells to transduce nitrergic signals leading relaxation (Groneberg *et al.*, 2013).

It has been previously proposed that TREK1 might participate in the transduction of nitrergic responses to smooth muscle cells in the colon (Koh *et al.*, 2001; Park *et al.*, 2005; Sanders & Koh, 2006). However, the lack of specific antagonists had made difficult the study of these channels in the transduction of nitrergic signals. Spadin has been recently identified as a potent and selective TREK1 channel inhibitor (Mazella *et al.*,

2010; Moha Ou *et al.*, 2011; Moha Ou *et al.*, 2012). Spadin reduced TREK1 currents at the basal state and activated by Arachidonic Acid or pressure (patch clamp technique). However Spadin was not able to inhibit the purinergic or nitrergic component of the electro-mechanical relaxation. Consequently TREK1 channels are not involved in the transduction of either nitrergic or purinergic responses in the colon. As observed in previous studies (Park *et al.*, 2005), exogenous addition of L-methionine and L-cysteine caused muscle depolarization and contraction. However, despite the fact that these sulphur-containing amino acids have been described as inhibitors of TREK1 channels (Baker *et al.*, 2008), under our experimental conditions neither L-methionine nor L-cysteine blocked arachidonic acid induced TREK1 currents. Therefore, their biological effect on smooth muscle cells is not related with the blockade of TREK1 channels. Note that both amino acids are involved in the transsulfuration pathway and the synthesis of hydrogen sulphide (Stipanuk, 2004). Furthermore, it might be possible that L-methionine and L-cysteine could block other types of K_{2P} channels activated by stretch like TREK2 or TRAAK channels (Honore, 2007; Sanders & Koh, 2006). Therefore, these compounds are not reliable pharmacological tools to study the participation of TREK1 channels in biological responses.

Another type of potassium channels involved in mediating inhibitory responses in the colon are the small conductance calcium-activated potassium (SK_{Ca}) channels. The role of these channels in the genesis of the IJP has been widely investigated using the bee venom apamin. A large number of studies have demonstrated that apamin inhibits the fast component of the IJP (Bennett, 1997; Pluja *et al.*, 1999; Serio *et al.*, 2003; Zagorodnyuk & Maggi, 1994; Zagorodnyuk *et al.*, 1996) and, therefore, SK_{Ca} channels participate in the transduction of purinergic responses. Nevertheless, some studies have demonstrated that apamin is also able to inhibit the sustained component of the IJP responses (El-Mahmoudy *et al.*, 2006; Keef *et al.*, 1993; Xue *et al.*, 1999). Accordingly the effect of apamin was investigated, after isolation of the purinergic and nitrergic IJP in the rat colon. These experiments showed that the purinergic response was almost abolished (~ 85 %) by apamin whereas this neurotoxin inhibits ~ 40 % of the nitrergic IJP at micromolar concentrations. Thus, SK_{Ca} channels mediate purinergic responses in the rat colon but there is no window of concentration to appropriately discriminate between purinergic and nitrergic responses with apamin. The terminology *apamin sensitive vs. apamin insensitive*

IJP, widely used in the literature, needs deep revision after the present work. A specific subtype of SK_{Ca} channel or alternatively non-specific effects of apamin in other channels are possible hypothesis to explain apamin effects on nitrergic responses.

We have established mechanical and electrophysiological criteria to identify the effect of a drug in nitrergic and purinergic pathways. In the rat colon, purinergic neurotransmission is mediated by SK_{Ca} channels. Nitrergic neurotransmission is mediated by GC activation. In addition, nitrergic hyperpolarization is at least in part apamin sensitive. According to our pharmacological data TREK-1 channels unlikely mediate nitrergic inputs.

Hydrogen sulphide as signalling molecule in the colon

H_2S might be the third gasotransmitter in the GI tract with putative inhibitory effects on the mechanical activity (Gallego *et al.*, 2008a; Hosoki *et al.*, 1997; Jimenez, 2010; Linden *et al.*, 2010; Teague *et al.*, 2002). In the present work, we investigated if H_2S fulfils the criteria to be considered as a gaseous signalling molecule regulating motility in the rat colon.

Expression of the enzymatic machinery for H_2S synthesis

Immunoreactivity for cystathionine β- synthase (CBS) and cystathionine γ-lyase (CSE) was observed in the rat colon. Both enzymes were widely distributed in the colon and they were expressed in the epithelium and the muscle wall. Furthermore, CSE was also expressed in neurons of both plexuses whereas CBS was not expressed in neural cells. Similar results have been reported in the murine colon with expression of these to enzymes in a wide variety of cellular types (Hennig & Diener, 2009; Linden *et al.*, 2008; Liu *et al.*, 2013; Martin *et al.*, 2010). Nevertheless, differences between species are apreciated as the distribution of these enzimes in the human colon is limited to the neural plexuses and ICC (Schicho *et al.*, 2006). It is important to note that both murine and human colon posses the ezymatic machinary to endogenously produce H_2S.

The enzymatic machinery for H_2S synthesis is present in the rat colon and both CSE and CBS are distributed in different cell types.

Enzymatic production of H_2S in the colonic wall

Previous studies have demonstrated that H_2S is enzymatically produced in the mouse colon (Linden *et al.*, 2008). In the present work, we adapted the methodology developed by Linden and colleagues to measure H_2S production in the rat colon (Linden *et al.*, 2008). Such experiments were performed with samples of colon in which the mucosa and submucosa were removed and with a high concentration of the H_2S precursor, L-cysteine (10 mM). Under these experimental conditions, measurable levels of H_2S were detected. Furthermore, H_2S is enzymatically synthesised as the CSE and CBS inhibitors, PAG and AOAA respectively, significantly reduced its production. Therefore, we demonstrated that H_2S is endogenously produced by well defined enzymatic pathways in the rat colon. In this tissue, as in other tissues, H_2S might be synthesised on demand or released from sulphur stores in response to physiologic signals (Ishigami *et al.*, 2009; Kimura, 2010; Yang *et al.*, 2008). However, further research is needed to establish the specific mechanisms involved in such a regulation.

Our data demonstrate that H_2S is enzymatically produced in the rat colonic wall.

Motor consequences of H_2S synthesis inhibition raised the discussion about the selectivity of the inhibitors of H_2S-producing enzymes

In the present work, the role of the H_2S endogenously produced by colonic tissue has been evaluated by using the CSE inhibitor, D,L-Propargylglycine (PAG), and the CBS inhibitors, Aminooxyacetic acid (AOAA) and Hydroxylamine (HA). These inhibitors have been widely used in experiments performed with tissue homogenates and at cellular level (Hosoki *et al.*, 1997; Linden *et al.*, 2010; Stipanuk & Beck, 1982; Szabo, 2007). However, these compounds are non-selective inhibitors of CSE and CBS and they show several effects on other enzymes and receptors as well (John & Charteris, 1978; Szabo, 2007; Teague *et al.*, 2002; Whiteman *et al.*, 2011). Therefore, the interpretation of the results obtained with these pharmacological tools is difficult and it must be always carried out with the support of other experimental findings (Jimenez, 2010; Szabo, 2007; Whiteman *et al.*, 2011). Note that we immediately ruled out HA for these experiments as we confirmed that this compound acts as a NO donor causing muscle hyperpolarisation and relaxation (Correia *et al.*, 2000; Iversen *et al.*, 1994). Therefore, PAG and AOAA

were used in the rest of experiments. It is noteworthy that PAG clearly produced smooth muscle depolarization and contraction, whereas AOAA only caused a mild increase in muscle contraction. H_2S has been identified as a relaxant molecule in the colon of several species (Dhaese & Lefebvre, 2009; Dhaese *et al.*, 2010; Distrutti *et al.*, 2006; Gallego *et al.*, 2008a; Liu *et al.*, 2013). In the present work we demonstrate that (i) both CSE and CBS were expressed in the colonic wall and (ii) the colonic tissue was able to enzymatically produce H_2S All together these results suggest that H_2S could be endogenously synthesised and released in a tonic manner in the colon. This would also suggest a major role of CSE in the synthesis of H_2S in the rat colon. However, it should be note that these effects might also be related to a non-selective action of these compounds, as mentioned above. Therefore, such a finding should be confirmed in the future with more selective inhibitors of the H_2S synthesising enzymes (when available) and/or genetic models. In spite of the pharmacodynamic features of these inhibitors, they let us evaluate the putative participation of H_2S in the regulation of colon motility as a neurotransmitter. Given the neural distribution of the H_2S producing enzymes (only CSE according to our results), H_2S might be considered as a neurotransmitter in the colon (Jimenez, 2010; Linden *et al.*, 2010). Interestingly, the depolarisation and the increase of motility observed with PAG are still observed after neural blockade with TTX. Furthermore, the spontaneous IJP and the IJP and relaxation induced by EFS are also not modified by PAG and AOAA. All these results suggest that H_2S does not participate in the neuromuscular transmission in the rat colon and, therefore, it should be considered as a modulator / signalling molecule in this organ.

According to our data on synthesis inhibition, H_2S might be considered an endogenously produced inhibitory signalling molecule in the colon. More selective pharmacological tools are needed to confirm this result.

Effect of NaHS on rat colonic motor patterns: results obtained after serosal application

Video recordings of spontaneous active colonic segments revealed two types of movements: 1 – Low frequency aboral propulsive motor movements and 2 – High frequency non-propulsive low amplitude contractions (ripples) ((Huizinga *et al.*, 2011)

and present work). Propulsive movements cause outflow and consequently their most likely function is to propel pellets in aboral direction. In contrast ripples probably participate in segmentation responsible for mixing movements (Huizinga *et al.*, 2011). Both motility patterns are probably related to the presence of two pacemaker systems in the colon (Alberti *et al.*, 2005; Pluja *et al.*, 2001). In order to establish how the H_2S produced in the colonic wall might modify colonic motor patterns, NaHS was infused from the serosal side to mimic endogenous production. NaHS caused a concentration dependent decrease of propulsive contractions. However, an increase in the amplitude of ripples was observed. These results were compared with the ones obtained with muscle bath recording using muscle strips. In this case, NaHS produced a decrease of low frequency contractions without major changes on high frequency contractions. Therefore, it is possible to speculate that the mechanism of action of H_2S (smooth muscle hyperpolarization, see below) might affect differently both types of contractions.

NaHS applied from the serosal side inhibits propulsive movements without affecting segmentation possibly enhancing mixing.

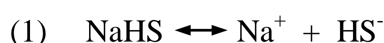
Effect of NaHS on rat colonic motor patterns: results obtained after luminal application

In the colon, H_2S is also produced by the sulphate-reducing bacteria present in the lumen (Blachier *et al.*, 2010; Levitt *et al.*, 1999). However, our results demonstrated that NaHS infused to the lumen is not able to cause motor changes in the colonic mechanical activity. This lack of effect can be explained by the high capacity of colonic epithelial cells to metabolize H_2S (Furne *et al.*, 2001; Mimoun *et al.*, 2012). Therefore, it can be hypothesized that this source of H_2S will not be able to modify colonic functions when the integrity of the barrier is preserved. Further studies are needed to evaluate if under pathological conditions, leading barrier disruption or impairment of epithelium metabolism, the H_2S produced in the lumen can reach the effector cell and consequently modify motility.

NaHS applied from the luminal side do not modify motility patterns suggesting a high capacity of detoxification in epithelial colonocytes.

Mechanism of action of NaHS

In our experiments we obtained an IC_{50} for NaHS of about 100 µM, both in muscle bath and microelectrodes experiments. It is important to note that NaHS is a hydrogen sulphide sodium salt which dissociates to form sodium cation (Na^+) and hydrosulphide anion (HS^-) in solution (1). Under physiological conditions, HS^- associate with protons (H^+) to produce H_2S (2) as the dissociation of HS^- to H^+ and sulphide (S^{2-}) is negligible (pK_a ~ 12). Note that at 37ºC the pK_a of reaction (2) is 6.755 which means that under common experimental conditions (37ºC and pH 7.4) the ratio of $[HS^-]:[H_2S]$ is 4.4 $pH = pK_a + \log_{10}([HS^-]:[H_2S])$, being only an 18.5 % of H_2S as an undissociated form (Dombkowski *et al.*, 2004; Linden *et al.*, 2010).



In addition, evaporation during the experiment (Jimenez, 2010; Kimura *et al.*, 2006), binding to proteins (Ishigami *et al.*, 2009; Kimura, 2010) and degradation (Linden *et al.*, 2012) might reduce the effective concentrations of H_2S over time. Therefore, it is quite difficult to determine real concentrations of H_2S during the experiments and the IC_{50} values for H_2S might be in the range of 1 – 10 µM. Evaporation, binding to proteins and degradation could also explain the differences observed with previous results published by our laboratory in the rat colon in which an IC_{50} of ~ 30 µM was determined (Gallego *et al.*, 2008a).

NaHS inhibitory effects on motility in the colon are not mediated by neurons, as they are still observed when TTX or lidocaine are used in the experiments, confirming previous results from our laboratory (Gallego *et al.*, 2008a). This is an important difference with other effects observed with NaHS in the colon, like pro-secretory effects, which have been described to be mediated, at least in part, by activation of neurons (Krueger *et al.*, 2010; Schicho *et al.*, 2006). Interestingly, two main mechanisms of action for H_2S have been identified in the rat colon: (i) muscle hyperpolarisation by activation of potassium channels, and (ii) inhibition of cholinergic excitatory nuromuscular trasmission at the post-junctional level.

Smooth muscle hyperpolarization. Activation of K_{ATP} channels by H_2S has been described in a wide variety of studies with vascular smooth muscle cells (Cheng *et al.*, 2004; d'Emmanuele di Villa Bianca *et al.*, 2009; Dombkowski *et al.*, 2004; Zhao *et al.*, 2001). Participation of this channel in NaHS relaxant responses in the colon has also been reported (Distrutti *et al.*, 2006; Gallego *et al.*, 2008a; Liu *et al.*, 2013; Nagao *et al.*, 2012; Zhao *et al.*, 2009). However, our results show that other potassium channels are involved in mediating NaHS induced effects in this organ. Among these channels, K_{ATP} and SK_{Ca} channels might be the main channels in the mediation of such effects ((Gallego *et al.*, 2008a) and present work). Therefore, SK_{Ca} channels participate in mediating ATP, NO and H_2S signalling (see above). GC might also participate in the mediation of NaHS responses in the colon. However, it might be possible NaHS indirectly activated GC by release of NO from nitrosothiols as observed in the brain (Ondrias *et al.*, 2008). Nevertheless, this possibility needs further investigation to be proved.

Inhibition of cholinergic responses. In addition to muscle hyperpolarisation, H_2S might also produce inhibitory effects by inhibiting excitatory cholinergic neuromuscular transmission. NaHS is able to inhibit the EJP elicited by EFS in the presence of L-NNA and MRS2500. In addition, this compound also reduces contractile responses to carbachol in the presence of nifedipine (note that under these experimental conditions the possible effect of NaHS on membrane potential is ruled out). The latter finding indicates that NaHS effects on cholinergic neuromuscular transmission are at the post-junctional level.

It is possible that other mechanisms not investigated in the present study might also contribute to the responses of NaHS. Activation of cAMP-dependent pathways (Kimura, 2000), myosin light chain phosphatase (MLCP) (Dhaese & Lefebvre, 2009; Nagao *et al.*, 2012), Na(V)1.5 voltage-dependent sodium channels (Strege *et al.*, 2011), Cav3.2-T-type channels (Matsunami *et al.*, 2009) are examples of potential targets of NaHS possibly leading smooth muscle relaxation.

The mechanism of action of H_2S involves post-junctional inhibition of cholinergic neuronal responses and muscle hyperpolarization involving several types of potassium channels including K_{ATP} and SK_{Ca} channels.

Therefore, H_2S fulfils the criteria to be considered as a signalling molecule in the colon. It might be released by a wide variety of cellular types in a tonic manner and modify mechanical activity modulating smooth muscle membrane potential and excitatory cholinergic neuromuscular transmission.

Comparative view of ATP (or a related purine), nitric oxide and hydrogen sulphide as a potential inhibitory mediators regulating motility in the rat colon

The main pharmacologic characteristics, pathways involved and the effect on motility (caused by endogenous release and exogenous addition) of these three mediators are summarised and compared in table 4.

Table 4. Comparison between ATP (or a related purine), nitric oxide and hydrogen sulphide as potential inhibitory mediators regulating motility in the rat colon

	ATP or a related Purine	Nitric oxide	Hydrogen sulphide
Pathways / pharmacology			
Synthesis	Mitochondria	nNOS	CBS, CSE
Possible NT (donor)	ATP, ADP, β-NAD, ADPr	NO (SNP)	H_2S (NaHS)
Receptor/Pathway	$P2Y_1$ [a] / SK_{Ca} (apamin)	GC (ODQ)	Not determined
Antagonists / Synthesis inhibitors	MRS2179 < MRS2279 < MRS2500	L-NNA	AOAA (CBS)[1], PAG (CSE)[2]
Endogenous responses			
EFS-induced IJP	Yes: Fast component	Yes: slow component	Not involved
EFS-induced relaxation	Yes	Yes	Not involved
Effects or RMP	No effect	Depolarization	[2]Depolarization
Effects on motility	No effect	Increase contractions	[2]Increase contractions
Effects on sIJP	Responsible	Not involved	Not involved
Effect of agonists / donors			
Effect on RMP	Transient hyperpolarization	Hyperpolarization	Hyperpolarization
Other effects			Inhibition of cholinergic neuronal responses
Effects on contractility	Transient decrease	Sustained decrease	Decrease of HF contractions
Effects on motility			Decrease of propulsive contractions

nNOS, nitric oxide synthase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; β-NAD, β-nicotinamide adenine dinucleotide; ADPr, Adenosine 5'-diphosphate-ribose NO, nitric oxide; H_2S, hydrogen sulphide; SK_{Ca}, small conductance calcium-activated potassium channels; GC, guanylate cyclase; AOAA, amino-oxyacetic acid; PAG, D,L-propargylglycine; IJP, inhibitory junction potential; RMP, resting membrane potential; HF, high frequency.
[a] Confirmed with $P2Y_1$ KO. Numbers in superscript correlates the inhibitors with their effect on membrane potential and motility

Comparison between ATP (or a related purine), nitric oxide and hydrogen sulphide reveals that NO and H_2S possibly share common functions as gaseous neurotransmitters such as regulating smooth muscle RMP and therefore smooth muscle excitability. However, it is still unknown if H_2S has a role as a classical inhibitory neurotransmitter.

Mechanisms of action of purines, NO and H_2S: pathophysiological consequences and possible pharmacological approaches. An open door for future studies

NO donors have been used to locally treat hypertonic sphincters such as the internal anal sphincter in anal fissure (Collins & Lund, 2007; Festen *et al.*, 2009). It is possible that H_2S donors can be used to treat this type of disorders if both 1- the antinociceptive effect of H_2S (Linden, 2013) and 2- the relaxant effect in vascular tissue (Yang *et al.*, 2008) are confirmed. Nevertheless, it is quite improbable that purinergic agonists can be valuable therapeutic tools to reduce contractility because, at least in vitro, most of the agonists tested have a transient response causing a quick desensitization.

Increased endogenous production of these mediators can lead to hypomotility as it is observed when iNOS is induced after inflammation (Auli & Fernandez, 2005; Porras *et al.*, 2006). Therefore, it is possible that, in future studies, an increased production of H_2S may also be detected in pathological disorders causing hypomotility (Wang *et al.*, 2012). On the contrary, impaired production of H_2S causes hypermotililty as it has been observed in a model of chronic stress in rats (Liu *et al.*, 2013). The future development of selective inhibitors of both CBS and CSE might be crucial to understand the role of H_2S under pathophysiological circumstances.

According to our findings, it is possible that propulsive movements are highly dependent on smooth muscle resting membrane potential. Therefore, mediators which caused muscle hyperpolarisation such as NO or H_2S might reduce this motor pattern. In addition, atropine blocks propulsive contractions suggesting that cholinergic inputs are needed to develop this pattern “ex vivo” and possibly “in vivo” as well. An enhancement of cholinergic neurotransmission is achieved with prokinetic drugs such as 5-HT_4 agonists (i.e. prucalopride) leading to an increase in motility (Cellek *et al.*, 2006; Priem &

Lefebvre, 2011). In this sense H_2S might be considered an antispasmodic because it hyperpolarises the tissue (reducing L-type calcium channel opening probability) and reduces cholinergic inputs. Similar pharmacological properties have been attributed to the spasmolytic drug otilonium bromide (L-type calcium channel blocker and antimuscarinic) (Gallego *et al.*, 2010; Martin *et al.*, 2004; Strege *et al.*, 2004) and consequently NaHS might share some pharmacological properties with this drug. However, an important side effect of H_2S donors as potential spasmolytic drugs might be the increase in secretion (Hennig & Diener, 2009; Krueger *et al.*, 2010; Pouokam & Diener, 2011; Schicho *et al.*, 2006) which would stimulate propulsion due to an increase of the luminal volume. In fact, guanylate cyclase-C agonists (such as linaclotide), a specific transmembrane GC subtype which is not involved in nitrergic transduction (Brierley, 2012), are currently used to promote intestinal secretion as it is a possible treatment for chronic constipation.

This work has also contributed to the identification of $P2Y_1$ receptors involving at least in part SK_{Ca} pathways as a main target for purinergic neuromuscular transmission. Our work has translational value since it is performed at least in part in human tissue. Genetic modified animals lacking $P2Y_1$ receptors have confirmed our hypothesis (Gallego *et al.*, 2012; Gil *et al.*, 2013; Hwang *et al.*, 2012). These animals have increased colonic transit time (Hwang *et al.*, 2012) suggesting that $P2Y_1$ receptors are also needed to coordinate a proper colonic motor function. In some pathological circumstances the mechanism might also be impaired (Strong *et al.*, 2010). We believe that $P2Y_1$ receptors have crucial physiological relevance but we also believe that future studies will answer the question about the relevance of the underlying mechanism in pathological circumstances. $P2Y_1/SK_{Ca}$ pathways are potential endogenous mechanisms to modulate motility.

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Conclusions

1) Purinergic neuromuscular transmission is more complex than previously thought. Thus, a wide variety of purines including ATP, ADP, β-NAD and even ADPr might be involved in mediating purinergic inhibitory neuromuscular responses. However, further research is needed to clearly identify the nature of the purine/s involved in inhibitory responses in the colon. We speculate that the endogenous neurotransmitter might not have a single identity and different kinds of purines might be released by enteric neurons and even bioactive compounds might be junctionally and transiently created.

2) Our results indicate that $P2Y_1$ receptors exclusively mediate purinergic inhibitory neuromuscular responses in the gastrointestinal tract. Studies carried out in genetic models confirm this observation.

3) Inhibitory motor neurons fire action potentials randomly releasing ATP or a related purine and NO. This cotransmission process is responsible for the inhibitory neural tone with complementary physiological functions being NO and the purine the tonic and the phasic functional neurotransmitters, respectively.

4) We have established mechanical and electrophysiological criteria to identify the effect of a drug involved in nitrergic and purinergic pathways regulating motility.

5) In the rat colon, purinergic neurotransmission is mediated by SK_{Ca} channels. Nitrergic neurotransmission is mediated by guanylate cyclase activation. In addition, nitrergic hyperpolarization is at least in part apamin sensitive. According to our pharmacological data TREK-1 channels unlikely mediate nitrergic inputs.

6) Hydrogen sulphide (H_2S) fulfils the criteria to be considered as a signalling molecule in the rat colon:

    a. The enzymatic machinery for H_2S synthesis is present in this tissue and both CSE and CBS are distributed in different cell types.

b. H_2S is enzymatically produced in the rat colonic wall.

c. With the available pharmacological tools causing synthesis inhibition, H_2S might be considered an inhibitory endogenous molecule.

d. The H_2S donor, sodium hydrosulphide (NaHS) causes smooth muscle relaxation both in muscle strips and in colonic segments when applied from the serosal side.

e. The mechanism of action of H_2S involves post-junctional inhibition of cholinergic neuronal responses and muscle hyperpolarization involving several types of potassium channels including K_{ATP} and SK_{Ca} channels.

f. NaHS applied from the luminal side do not modify motility patterns suggesting a high capacity of detoxification in epithelial colonocytes.

7) Further experiments are needed to evaluate the relevance of the underlying mechanism in pathological circumstances and the potential effect of purinergic nitrergic and sulphidrergic pathways to treat colonic motor disorders.