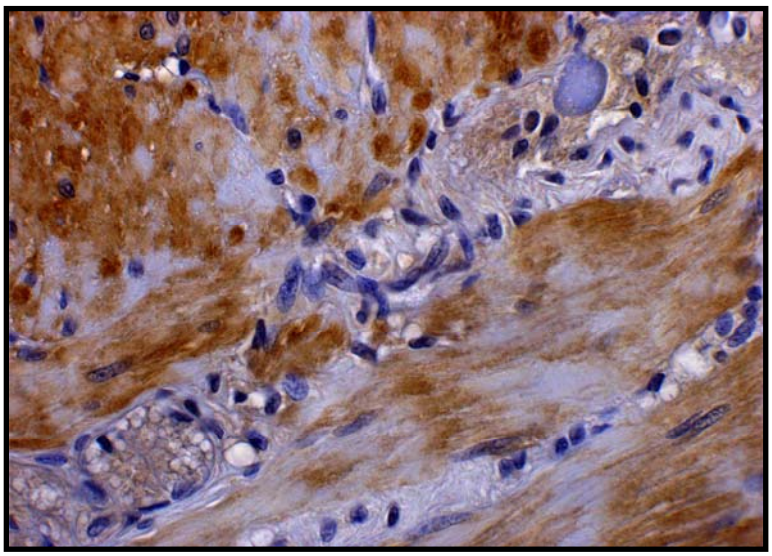
Universitat Autònoma de Barcelona

# Caracterización de la transmisión inhibitoria neuromuscular en el intestino delgado y el colon



**Memoria presentada por Diana Gallego Pérez**
**para optar al grado de Doctor**

**Programa de Doctorado en Neurociencias**

Bellaterra, enero de 2008

**FACULTAD DE VETERINARIA**
**Departamento de Biología Celular, Fisiología e Inmunología**
**Universidad Autónoma de Barcelona**

**Tesis Doctoral dirigida por Marcel Jiménez Farrerons**

Universitat Autònoma de Barcelona

MARCEL JIMÉNEZ FARRERONS, Profesor titular de Fisiología del Departamento de Biología Celular, de Fisiología e Inmunología de la Universidad Autónoma de Barcelona

HAGO CONSTAR

Que la memoria titulada *"Caracterización de la transmisión inhibitoria neuromuscular en el intestino delgado y el colon"* presentada por DIANA GALLEGO PÉREZ para optar al grado de Doctor se ha realizado bajo mi dirección, y al considerarla concluida, autorizo su presentación para ser juzgada por el Tribunal correspondiente.

Y para que conste a los efectos firmo la presente.

Bellaterra, enero de 2008

Dr. Marcel Jiménez Farrerons

Director de la tesis

La imagen de portada es una microfotografía de ganglios del plexo mientérico del íleon porcino, en marrón están marcados los receptores purinérgicos $P2Y_1$.

## Agradecimientos

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Diana Gallego Pérez

Bellatera, enero de 2008

“***Si he hecho descubrimientos invaluables ha sido más por tener paciencia que cualquier otro talento."***

**Isaac Newton**

## Abreviaturas

**ACh** acetilcolina

**ADP** adenosina difosfato

**ADPβS** adenosina 5´-O-2-tiodifosfato

**AH** *"afterhyperpolarization"*

**ATP** adenosina 5'-trifosfato

**AUC** area bajo la curva o "*area under curve*"

**βNAD** beta-nicotinamida adenina dinucleotido

**cAMP** adenosina monofosfato cíclico

**CBS** cistationina γ- liasa

**cGMP** guanosina monofosfato cíclico

**CO** monóxido de Carbono

**CSE** cistationina β-sintasa

**DAG** diacilglicerol

**DMP** plexo muscular profundo o "*deep muscular plexus"*

**DMSO** dimetilsulfóxido

**DPCPX** 8-ciclopentil-1,3-dipropilxantina

**EFS** estimulación eléctrica de campo o "*electrical field stimulation*"

**eNOS** sintasa de óxido nítrico endotelial

**fEPSP** potenciales post sinápticos excitatorios rápidos o "f*ast excitatory postsinaptic potencial"*

**HAPC** actividad propulsiva de alta amplitud

**$H_2S$** sulfhídrico

**$IC_{50}$** concentración inhibitoria al cincuenta por ciento

**ICC** célula intersticial de cajal

**ICC-DMP** célula intersticial de cajal del plexo muscular profundo

**ICC-IM** célula intersticial de cajal que se encuentran intramuscularmente

**ICC-MY** célula intersticial de cajal de la capa intermuscular

**ICC-SM** célula intersticial de cajal de la capa submucosa

**IFAN** neurona aferente intestinofugale

**IJP** potencial postunión inhibitorio o "*inhibitory junction potential*"

**IJPf** potencial postunión inhibitorio rápido o "*inhibitory junction potential fast* "

**IJPs** potencial postunión inhibitorio lento o "*inhibitory junction potential slow* "

**iNOS** sintasa de óxido nítrico inducible

**$IP_3$** inositol 1,4,5-trifosfato

**IPAN** neurona intrínseca aferentes primaria o "*intrinsic primary afferent neuronas"*

**K (ATP)** canales de potasio sensibles a ATP

**LAPC** actividad propulsiva de baja amplitud

**LES** esfínter essofágico inferior o *"lower esophageal sphincter"*

**L-NNA** Nω-nitro-L-arginina

**2-MeSADP** 2-metiltio adenosina difosfato

**MC** complejo motor o *"motor complex"*

**MMC** complejo motor migratorio o "*migrating motor complex*"

**MP** plexo mientérico o de Auerbach

**MRS 2179** $N^6$-metil 2'-deoxiadenosina 3',5'-bisfosfato

**NADPH** nicotiamida-adenina dinucleotido fosfato

**NANC** no adrenérgico no colinérgico

**NaNP** sodio nitroprusiato

**NF 023** ácido 8'-(carbonil bis (imino-3,1-fenilenecarbonilimino) bis (1, 3, naftalenetrisulfonico)

**NKA** neuroquinina A

**nNOS** sintasa de óxido nítrico neuronal

**NO** óxido nítrico

**NOS** sintasa del óxido nítrico

**PACAP** polipéptido activador de la adenilato ciclasa pituitaria o "*pituitary adeylate cyclase activating polypeptide*"

**PPADS** ácido piridoxalfosfate-6-azoprenil-2´,4´-disulfonico

**RMP** potencial de membrana en reposo o *"resting membrane potencial "*

**ROIs** regiones de interés o "*regions of interest*"

**sEPSP** potenciales post sinápticos excitatorios lentos o "slow *excitatory postsinaptic potencial"*

**sKCa** canales de potasio activados por calcio de baja conductancia

**SMP** plexo submucoso o de Meissner

**SNC** sistema nervioso central

**SNE** sistema nervioso entérico

**SP** sustancia P

**TEA** tetraetilamonio

**TRPV "***transient receptor potential vanilloid-responsive"*

**TTX** tetrodotoxina

**UDP** uridina difosfato

**UTP** uridina trifosfato

**VIP** polipéptido vasoactivo intestinal o "*vasoactive intestinal polypeptide*"

Los estudios de esta tesis se han publicado o están pendientes de publicarse en los siguientes artículos:

**$P2Y_1$ receptors mediate inhibitory purinergic neuromuscular transmission in the human colon.**

D. Gallego, P. Hernandez, P. Clave, and M. Jimenez. *Am.J.Physiol Gastrointest.Liver Physiol*, 2006; **291** Pag.G584-G594.

**$P2Y_1$ receptors mediate inhibitory neuromuscular transmission and enteric neuronal activation in small intestine.**

D.Gallego, P.Vanden Berghe, R.Farre, J.Tack and M.Jimenez. Neurogastroenterology and Motility,2008 ; **20** Pag.159-168.

**Purinergic and nitrergic junction potential in the human colon.**

D.Gallego, V.Gil, J.Aleu, M.Auli, P.Clave, and M.Jimenez. *Am.J.Physiol Gastrointest.Liver Physiol. En fase de revision.*

**The novel gaseous mediator, hydrogen sulphide, inhibits motility in the human, rat and mouse colon and jejunum.**

D.Gallego, P.Clave, J.Shaw, R.Rahmati, D.Grundy, M.Jimenez and M J. Beyak. *En manuscrito.*

Y en forma de abstract:

**"$P2Y_1$ receptor mediates inhibitory purinergic neuromuscular transmission in the human sigmoid colon".** D. Gallego, P. Hernàndez, J. Martí-Ragué, P. Clavé and M.Jiménez .Gastroenterology April 2005 Vol.128 Num.4 Pag.A13

**"Inhibitory neuromuscular transmission in the human sigmoid colon involves ATP or a related purine through $P2Y_1$ receptors and nitric oxide".** D. Gallego, P.Hernandez, J.Martí-Ragué, P.Clavé and M. Jiménez. Neurogastroenterology and Motility August 2005 Vol.17 Num.4 Pag.13

**"Purinergic inhibitory neuromuscular transmission is mediated through $P2Y_1$ receptors in the porcine ileum".** R.Farré, D.Gallego, A.Domènech, P.Clavé and M Jiménez .Neurogastroenterology and Motility August 2005 Vol.17 Num.4 Pag .69

**"NO y ATP: Mecanismos complementarios de inhibición neuromuscular en el colon humano**." D.Gallego, A.Doménech, M. Pumarola, P. Clavé, M. Jiménez Revista Española de Enfermedades Digestivas: 98 (Supl 1), 29-105

**"$H_2S$ mediates smooth muscle relaxation through K channels in human and rat colon".** D.Gallego, M.Beyak, P.Clave, D.Grundy and M.Jimenez. Neurogastroenterology and Motility August 2006 Vol.18 Num.8 Pag.673

**"Nitrergic and purinergic co-transmission : complementary mechanisms of relaxations in the human colon."** D. Gallego, J. Aleu, M. Aulí , P. Clavé and M. Jiménez. Neurogastroenterology and Motility August 2006 Vol.18 Num.8 Pag.778

**Premios recibidos:**

Premio a la mejor comunicación póster en motilidad por el trabajo: **"NO y ATP: Mecanismos complementarios de inhibición neuromuscular en el colon humano**." En la semana de las enfermedades digestivas celebrada en Granada en Junio de 2006.

Premio como joven investigadora "Young Investigator Award" por la presentación oral: **"$H_2S$ mediates smooth muscle relaxation through K channels in human and rat colon."** En el 2006 Joint International Neurogastroenterology and Motility Meeting Celebrado en Boston en Septiembre de 2006.

# Introducción

El Aparato Digestivo está formado por el tubo digestivo o tracto gastrointestinal y las glándulas anexas. El tracto gastrointestinal se divide anatómicamente en esófago, estómago, intestino delgado (que está estrechamente relacionado con la vesícula biliar y el páncreas), intestino grueso o colon y recto. El intestino delgado se subdivide en duodeno, yeyuno e íleon proximal, medio y distal. El colon consta de colon ascendente, transverso, descendente y colon sigma.

Histológicamente el tracto gastrointestinal está formado por capas dispuestas de forma concéntrica. De la luz hacia fuera encontramos:

La mucosa, que está recubierta por células epiteliales responsables de la secreción de electrolitos, mucus y enzimas. Además también es responsable de la absorción de nutrientes, fluidos y de la función neuroinmune. En este epitelio se encuentran células enteroendocrinas. Justo debajo del epitelio se encuentran la lamina propia y la muscularis mucosae. Esta es la capa muscular responsable de los movimientos de los villi.

La siguiente capa es la submucosa, una matriz de colágeno que contiene vasos sanguíneos y linfáticos. A este nivel se encuentra también el plexo submucoso o de Meissner (SMP), uno de los dos plexos ganglionados que forman el sistema nervioso entérico (SNE), que contiene neuronas que inervan la mucosa y sus vasos sanguíneos.

Por debajo de esta capa se encuentra una capa de células musculares lisas circunferencialmente orientadas, la capa muscular circular, que es la responsable de los movimientos anulares del intestino. En la mayoría de las especies, en el intestino delgado, esta capa muscular, se puede dividir en dos, la circular interna y externa, separadas por un plexo aganglionado: el plexo muscular profundo o *deep muscular plexus* (DMP).

La capa muscular longitudinal está formada por fibras musculares orientadas perpendicularmente a la capa circular. Entre la capa circular y la longitudinal se encuentra el plexo mientérico o de Auerbach (MP), que es el segundo de los plexos ganglionados del sistema nervioso entérico. Éste contiene la mayor parte de las interneuronas y motoneuronas excitatorias e inhibitorias (ambos plexos contienen por igual neuronas sensitivas intrínsecas).

La última capa es la serosa, que es la capa más externa (Olsson & Holmgren, 2001; Furness, 2006; Kunze & Furness, 1999; Bertrand, 2003; Guyton & Hall, 1996) (Figura 1).

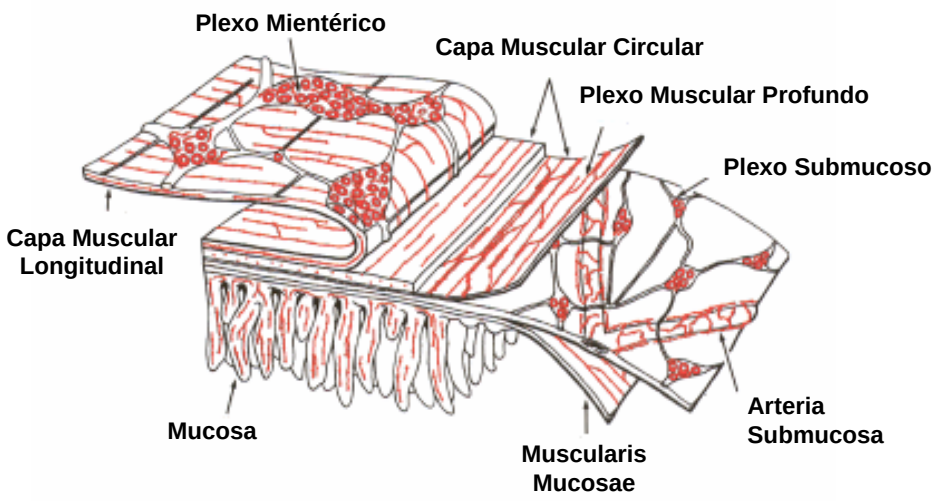



*Figura 1. Estructura por capas del intestino delgado de cobayo. Adaptado de (Furness & Costa, 1980).*

# *1 Motilidad Gastrointestinal*

Una de las funciones más importantes del tracto gastrointestinal es la de transportar los alimentos ingeridos a una frecuencia óptima que permita el mezclado y la correcta exposición a los enzimas digestivos para facilitar su posterior absorción. Los patrones de motilidad del tracto gastrointestinal se podrían definir como movimientos de mezclado y propulsión, más o menos confinados en una determinada región y que están organizados.

En general los movimientos de mezclado y propulsión se generan localmente en el sistema nervioso entérico. Sin embargo, éstos son modificados por el sistema nervioso central y por otras áreas del tubo digestivo (Kunze & Furness, 1999; Olsson & Holmgren, 2001).

## 1.1 Patrones motores del intestino delgado

En el intestino delgado se puede observar principalmente dos tipos de patrones o fases; la fase interdigestiva que se da en ayunas y la fase digestiva o postpandrial.

**En ayuno o en fase interdigestiva,** el patrón de motilidad es el **complejo motor migratorio (MMC)** que es el fenómeno motor más destacable y el más estudiado. El MMC es un patrón de actividad eléctrica y mecánica que se inicia en estómago y duodeno. Éste se propaga atravesando la mayor parte del intestino delgado limpiando residuos digestivos.

El MMC se inicia en la región gastroduodenal a una frecuencia de un MMC por cada 90-150 minutos en el hombre y en el perro y tarda aproximadamente 90 minutos en migrar toda la longitud del intestino delgado.

Por convención posee tres fases o componentes principales. No obstante, se ha propuesto también la presencia de una cuarta fase aunque actualmente se descarta la presencia de ésta (Telford & Sarna, 1991).

La fase I es una fase de quiescencia motora. La fase II consiste en una actividad contráctil irregular que sigue a la fase I, y es importante para la propulsión. Un patrón contráctil particular de la fase II son *clusters* o agrupaciones de contracciones fásicas, que se puede dar en algunos individuos de forma patológica aunque es frecuente en ancianos. La fase III es la más distintiva, es una secuencia de contracciones de propulsión intensa que comienza en la región gastroduodenal.

El MMC se genera en el SNE con la modulación del SNC, hormonas como la motilina (inductora de fases III) y neurotransmisores como la serotonina modulan estas fases de forma endocrina y paracrina. El óxido nítrico podría ser responsable de la fase I mientras que la inhibición de la neurotransmisión nitrérgica podría ser responsable de la fase III (Ruckebusch & Bueno, 1977; Telford & Sarna, 1991; Husebye, 1999; Vantrappen *et al.*, 1977; Rodriguez-Membrilla *et al.*, 1995) (Figura 2).

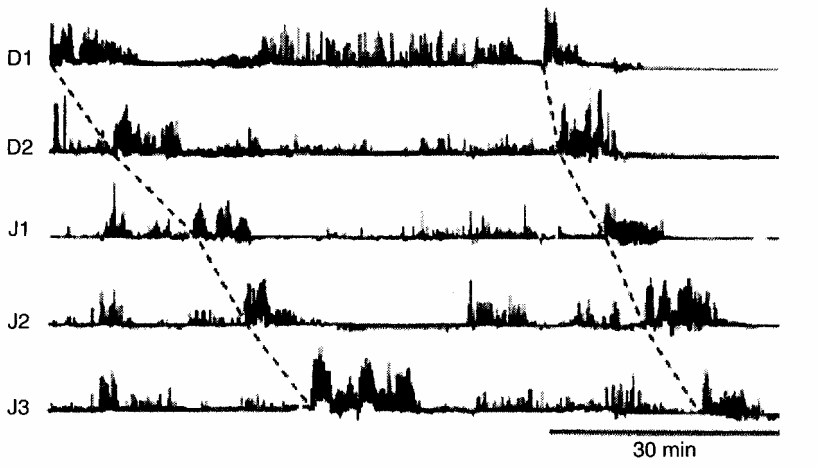



*Figura 2. Registro manométrico de las diferentes fases del complejo motor migratorio (MMC) a medida que progresa del duodeno (D1 y D2) al yeyuno (J1, J2 y J3). Las cuatro fases están indicadas (I, II, III, IV). Reproducido de (Soffer et al., 1998).*

**En fase postpandial o digestiva,** hay un cambio inmediato del patrón motor que se denomina convencionalmente patrón postpandrial. Este patrón parece ser iniciado por un reflejo vagal y se mantiene por influencias endocrinas y paracrinas. Consiste en contracciones fásicas de frecuencia (en el intestino humano oscilan entre

12-15 por minuto), amplitud y propagación variables que permiten el mezclado y la absorción del contenido luminar (Figura 3); y movimientos de propulsión o peristálticos que permiten el transporte del bolo alimentario en dirección aboral.

La duración de este patrón está relacionada con diversos factores como el vaciado gástrico, el contenido calórico de la dieta, la distribución de macronutrientes de la comida o la viscosidad del quimo, y no se ve afectada por factores como el horario de las comidas (Kunze & Furness, 1999; Olsson & Holmgren, 2001).

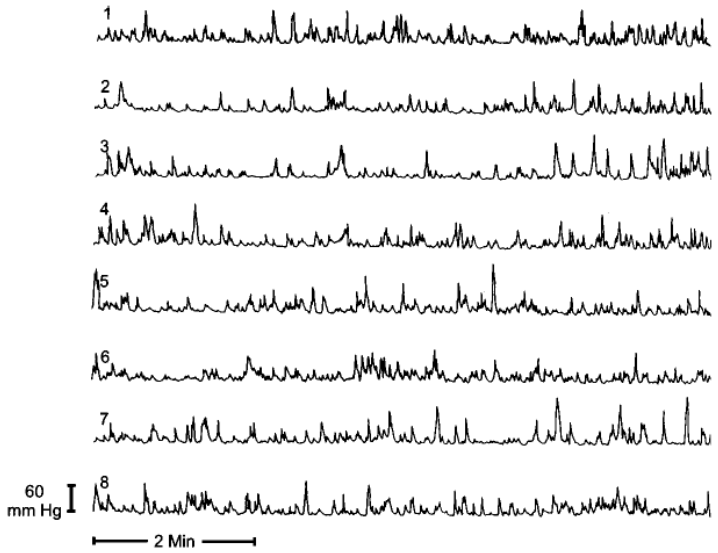



*Figura 3. Típico patrón de la fase digestiva o postpandrial del intestino humano. Registros de la presión intraluminal registrada in vivo en ocho puntos diferentes separados 3 cm. Reproducido de (Summers et al., 1983).*

La peristalsis, que se da a lo largo de todo el tubo digestivo y no sólo en el intestino delgado para propulsar el bolo en dirección aboral, ocurre como resultado de contracciones del músculo circular en dirección oral al bolo alimentario y relajaciones en dirección anal, además de darse una contracción del músculo longitudinal en el sentido anal y una relajación oral. Este reflejo se desencadena porque los contenidos

luminales activan de forma química o por estiramiento las células enterocromafines que se encuentran en la mucosa. La activación de estas células hace que liberen sustancias como la serotonina, (que activa los terminales de las neuronas aferentes intrínsecas o IPANs), que a su vez activan interneuronas de vías ascendentes y descendentes desencadenando la relajación oral y la contracción aboral (Kunze & Furness, 1999; Olsson & Holmgren, 2001; Grider, 2003a; Grider, 2003b; Grider & Jin, 1994; Hansen, 2003a).

Los **patrones de defensa** consisten en contracciones altamente propulsivas denominadas "*giant migrating contractions*" o "*prolonged propagated contractions*", que se desencadenan por la presencia de determinadas substancias consideradas como nocivas, como por ejemplo, ácidos grasos de cadena corta. También se dan patrones motores para evitar el reflujo de substancias (Lu *et al.*, 1997; Sarna, 2003; Kellow, 2004).

## 1.2 Patrones de motilidad del colon

La ciclicidad de patrones motores y la clara alternancia de fases que se da en el intestino delgado no se da en el colon, ya que éste no presenta una actividad cíclica y recurrente salvo excepciones como el colon canino y el de ratón, donde se ha descrito la presencia de complejos motores (Spencer, 2001).

Pese a estas limitaciones a la hora de establecer los patrones motores colónicos, mediante técnicas manométricas, se ha establecido que ésta puede ser agrupada en dos tipos principales: **actividad segmentaria y actividad propulsiva**.

La **actividad segmentaria**, también denominada como contracciones fásicas rítmicas (Sarna, 2006), es la mayor parte de la actividad colónica. Consiste en contracciones de baja amplitud (que oscilan entre 5-50 mm de Hg). Aunque ocasionalmente se pueden dar contracciones de mayor amplitud, que pueden ser

aisladas o agrupadas, estas agrupaciones no presentan usualmente una ritmicidad. Sin embargo, un porcentaje muy pequeño del tiempo, estas contracciones, pueden presentar una frecuencia rítmica de unos tres ciclos por minuto; este patrón rítmico se observa principalmente en el colon distal (en el colon descendente y colon sigma).

Esta actividad contráctil tiene como finalidad el mezclar y propulsar la materia fecal propulsándola lentamente hacia el recto, permitiendo una absorción óptima de agua, electrolitos, ácidos grasos de cadena corta y metabolitos bacterianos. (Figura 4)

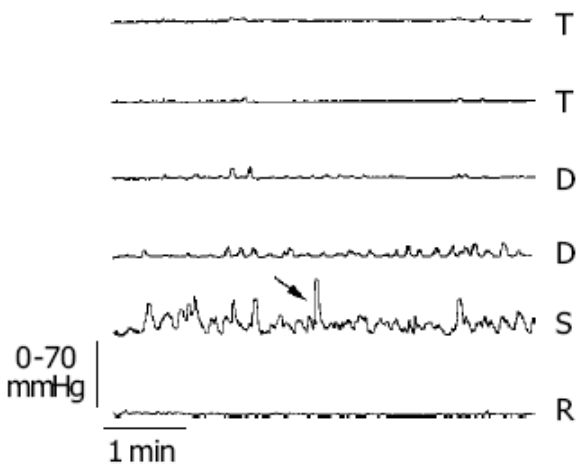



*Figura 4. Registro manométrico representativo de la actividad colónica segmentaria. La mayoría de las contracciones son de baja amplitud aunque excepcionalmente (Flecha) pueden exceder los 50 mm de Hg. Los puntos de registro están separados 12 cm y proceden de colon transverso (T), distal (D), Sigma (S) y recto (R). Reproducido de (Bassotti et al., 2005).*

La **actividad propulsiva** se subdivide en dos subtipos según su amplitud. Existen actividades propulsiva de baja amplitud o **LAPC** (*Low-Amplitude Propagated Contractions)* y de alta amplitud o **HAPC** (*High-Amplitude propagated contractions)*. Las LAPC son relativamente frecuentes, poseen una amplitud de unos 100 mm de Hg y se dan unas 100 al día. Probablemente la función principal de estas contracciones es la de transportar fluidos o gases. (Figura 5)

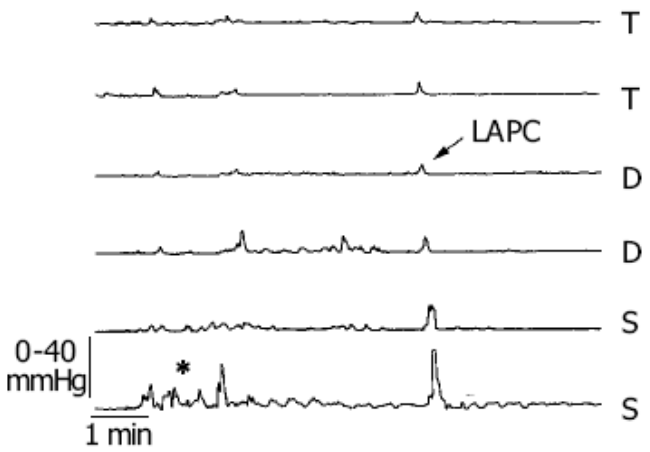



*Figura 5. Registro manométrico representativo de la actividad propulsiva de baja amplitud LAPC (Flecha). En el último trazado se puede observar la presencia (asterisco) de actividad segmentaria en el colon sigma. Los puntos de registro están separados 12 cm y proceden de colon transverso (T), distal (D) y Sigma (S). Reproducido de (Bassotti et al., 2005).*

Las segundas, o HAPC, tienen una amplitud media de más de 100 mm de Hg y son mucho más infrecuentes, unas 6 al día, aunque en especies como la rata, la frecuencia de estas contracciones se incrementa considerablemente (a aproximadamente 40 por hora) (Gonzalez & Sarna, 2001). Las HAPC fueron descritas a principios de siglo XIX mediante técnicas radiográficas y se corresponden con los **movimientos de masa**. También son denominadas contracciones **gigantes migratorias** (giant migrating contractions) (Sarna, 2006). Las HAPC son capaces de propulsar gran cantidad de contenido fecal a largas distancias aunque en el caso de la rata, la distancia de migración es menor. La mayoría de estudios realizados sobre motilidad colónica se han centrado en este patrón (Malcolm & Camilleri, 2000; Rao *et al.*, 2001) (Figura 6).

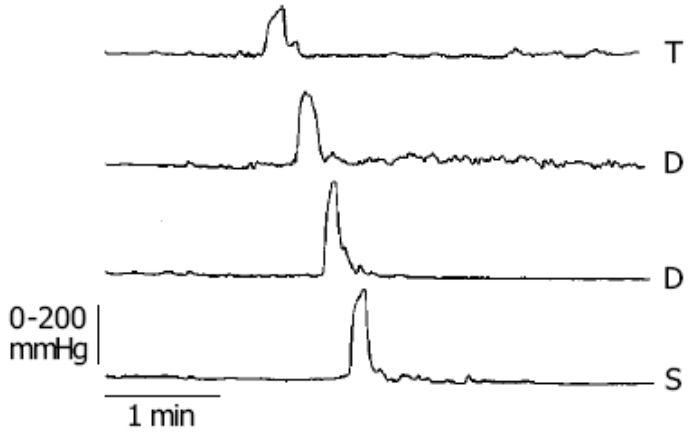



*Figura 6. Registro manométrico representativo de la actividad propulsiva de alta amplitud HAPC donde se puede observar la diferencia respecto a la actividad segmentaria que se observa de fondo. Los puntos de registro están separados 12 cm y proceden de colon transverso (T), distal (D) y Sigma (S). Reproducido de (Bassotti et al., 2005).*

Las HAPC están fuertemente relacionadas con la defecación. Estudios recientes han asociado la presencia de estas contracciones en el colon descendente con la relajación del esfínter anal interno (Bassotti *et al.*, 2005; Malcolm & Camilleri, 2000).

También se ha propuesto un tercer tipo de actividad contráctil, el **tono**. Gracias al tono y la reducción de la luz intestinal que éste genera los movimientos de mezclado y propulsión se ven favorecidos (Sarna, 2006).

Se ha descrito que la motilidad colónica podría verse influenciada por factores hormonales, y que podría seguir un ritmo circadiano, ya que hay estudios que han mostrado que la motilidad colónica en mujeres es inferior a la de los hombres, y que ésta, es inferior en las horas de sueño (Rao *et al.*, 2001).

# *2 Control de la motilidad gastrointestinal*

## 2.1 Control no neural

### Actividad eléctrica de las células musculares y actividad marcapasos

El potencial de membrana de las células musculares lisas no es estable sino que presenta oscilaciones espontáneas. Estas oscilaciones se denominan ondas lentas. El potencial de membrana, que oscila entre -40 y -80 mV, está determinado por la actividad de la bomba sodio potasio y por canales de potasio de difusión pasiva. Además de los canales de difusión pasiva, las células musculares poseen canales selectivos para determinados iones que pueden ser regulados por diferencia de potencial y por otros mediadores. Especialmente los canales de calcio dependientes de voltaje y los canales de potasio activados por calcio, son fundamentales para esta ritmicidad de las células musculares lisas (Boeckxstaens 2002; Hansen, 2003a). Esta ritmicidad eléctrica determina la máxima frecuencia de contractibilidad del músculo liso. Los potenciales de acción tienen lugar en la parte de mayor despolarización de estas ondas lentas. Los *inputs* excitatorios de las neuronas entéricas provocan despolarización de la célula muscular, abertura de canales de calcio operados por voltaje (tipo L) de la célula muscular y una contracción (Figura 7).

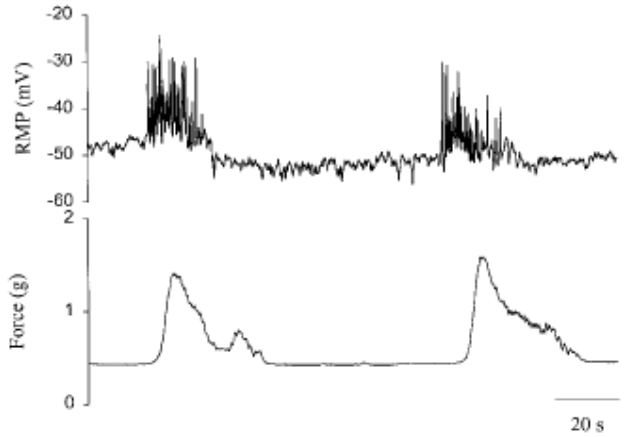



*Figura 7.Registro intracelular y mecánico del colon de rata donde se puede observar la correlación entre actividad mecánica (ondas lentas) arriba y las contracciones mecánicas abajo. Adaptado de (Pluja et al., 2001).*

En un principio se pensaba que las ondas lentas eran resultado de las propiedades del músculo liso y se denominaron “miogénicas”. Actualmente, se ha demostrado que las ondas lentas se generan en células especializadas de origen mesenquimático, las Células intersticiales de Cajal o (ICCs), descritas por Santiago Ramón y Cajal a principios del siglo XX (Ramón y Cajal, 1904) Se ha propuesto que las ICCs poseen tres funciones principales: La de generar este mecanismo marcapasos, la facilitación de la propagación de fenómenos eléctricos, ya que se encuentran unidas por uniones abiertas “gap junctions” a las células musculares, y también se ha descrito que podrían mediar la neurotransmisión, ya que poseen receptores para determinados neurotransmisores. Algunos investigadores han propuesto una cuarta función que sería la de actuar como mecanoreceptores (Sanders, 1996; Sanders *et al.*, 2006).

Según su distribución, las ICCs se pueden clasificar en:

Las ICCs de la capa submucosa **ICC-SM**, que se encuentran en la superficie de la capa muscular circular en contacto con la capa submucosa del colon.

Las ICCs de la capa intermuscular **ICC-MY**, que se encuentran en la región mientérica del estómago, intestino delgado y colon coexistiendo con el plexo neural mientérico.

Las ICCs del plexo muscular profundo o “*deep muscular plexus”* **ICC-DMP**, que se encuentran en la región del deep muscular plexus en el intestino delgado.

Finalmente las ICCs que se encuentran intramuscularmente o **ICC-IM** (Horowitz *et al.*, 1999) (Figura 8).

Recientemente se ha propuesto una clasificación que simplifica la anterior donde las ICC-SM serían **ICC submucosas**, las ICC-MY (intermusculares) serían **ICCs mientéricas** y las ICC-DMP y ICC-IM serían englobadas como **ICCs**

**intramusculares** (Takaki, 2003). La tabla I muestra la distribución de las ICCs en función de los distintas áreas del tracto gastrointestinal.

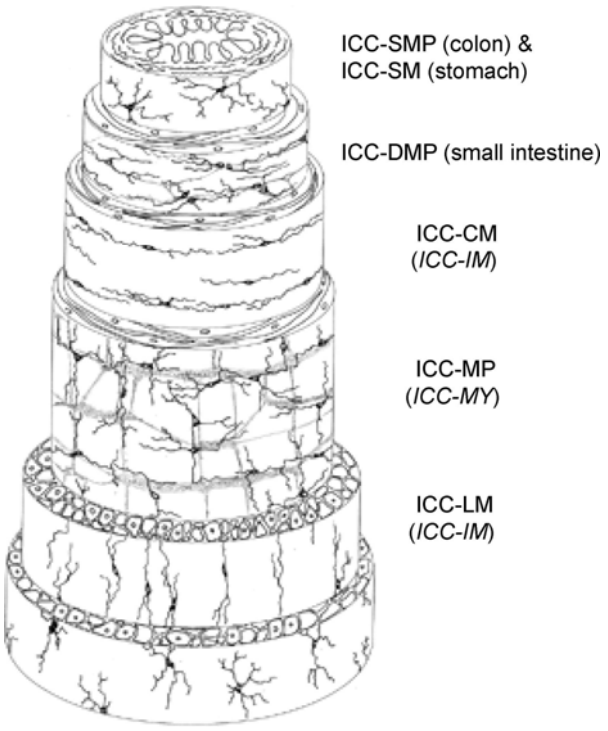



*Figura 8.Clasificación de las ICCs según su distribución adaptado de (Komuro, 2006).*

| Región del tracto Gastrointestinal | | ICC-MY/MP | ICC-DMP | ICC-SMP | ICC-IM |
|---|---|---|---|---|---|
| Estómago | Antro | X | | | X |
| | Corpus | X | | | X |
| | Fundus | | | | X |
| Intestino delgado | | X | X | | |
| Colon | | X | | X | X |

*Tabla I. resumen de la localización de las ICCs en las diferentes capas del tracto gastrointestinal.*

En el intestino delgado la principal área marcapasos es la del plexo mientérico ICCs-MP. Este hecho se ha demostrado en diversas especies incluyendo el cerdo y el hombre, ya que las ondas lentas registradas mediante microelectrodos intracelulares cerca de este plexo, poseen una mayor amplitud que zonas más lejanas (Jimenez *et al.*, 1999; Rumessen & Thuneberg, 1991). Otros estudios han puesto de manifiesto diferencias entre especies como es el caso del íleon canino, que presenta otra zona marcapasos capaz de generar ondas lentas, la del DMP (*Deep Muscular Plexus*) (Jimenez *et al.*, 1996).

En cambio, en el colon el mecanismo marcapasos se origina en la red de ICCs cercana a la zona submucosa o ICCs-SM. Este hecho se ha demostrado en el colon canino, porcino y humano (Liu & Huizinga, 1993; Smith *et al.*, 1987; Serio *et al.*, 1991;Rae *et al.*, 1998). En este caso las ondas lentas decrecen en amplitud al alejarse de esta zona. Aunque las ICCs del plexo mientérico o ICCs-MP generan unas contracciones cíclicas de frecuencia superior denominadas "*Myenteric Potential Oscillations"* (MPOs), la actividad eléctrica final es consecuencia de las ondas lentas generadas en ambas regiones.

En el colon del ratón se ha puesto de manifiesto que el mecanismo marcapasos es de origen neural. Éste se ve inhibido por fármacos como la tetrodotoxina y el hexamentonio. Se ha propuesto que una población de neuronas intrínsecas del sistema nervioso entérico, con actividad rítmica, actúan como células marcapasos (Spencer, 2001).

Otra excepción es el colon de rata, donde se ha descrito la presencia de dos marcapasos que dan lugar a dos tipos diferentes de actividad motora *in Vitro*. Las contracciones de baja amplitud y de alta frecuencia generadas por las ICCs de la zona submucosa ICC-SMP, y las contracciones de alta amplitud y baja frecuencia generadas por las ICCs del Plexo mientérico ICC-MY (Pluja *et al.*, 2001). Ambas contracciones se encuentran en todo el colon de la rata, aunque pueden existir

diferencias importantes entre la densidad de ICCs y el patrón motor entre el colon proximal, medio y distal (Alberti *et al.*, 2005). Por otra parte, alteraciones en el correcto desarrollo de las ICCs, como por ejemplo en las ratas Ws/Ws, altera el marcapasos colónico (Alberti *et al.*, 2007).

## *2.2 Control neural*

### 2.2.1 Intrínseco

El Sistema Nervioso Entérico (SNE) se encuentra en las paredes de todo el tracto gastrointestinal desde el esófago hasta el ano, incluyendo las glándulas asociadas como las glándulas salivales, el páncreas o la vesícula biliar. Es una parte del Sistema Nervioso Autónomo que integra motilidad, secreciones flujo sanguíneo y respuestas inmunes (Hansen, 2003d).

El SNE humano consta del Plexo Mientérico (Plexo de Auerbach) y del Plexo Submucoso, que se divide en tres plexos separados: el Plexo submucoso interno (Plexo de Meissner) que está justo debajo de la muscularis mucosae, el Plexo submucoso externo (Plexo de Schabadasch o plexo de Henle), que es adyacente a la capa muscular circular y un Plexo submucoso intermedio que se sitúa entre ambos. En analogía con otras especies, esta distribución anatómica tiene una relevancia funcional, ya que el plexo mientérico regula la actividad muscular, mientras que el plexo submucoso está involucrado en la regulación de las funciones de la mucosa. Esta distribución funcional no es estricta, puesto que algunas neuronas del plexo submucoso externo inervan el músculo circular, y neuronas del plexo mientérico en el íleon humano proyectan a la mucosa (Schemann & Neunlist, 2004).

Las neuronas del SNE son, principalmente, las responsables de los patrones motores intestinales. Sin embargo, las diferentes técnicas experimentales basadas en la morfología (forma de las neuronas), electrofisiología (propiedades electrofisiológicas

y neurotransmisión), inumohistoquímica (presencia de neurotransmisores o de su vía de síntesis) y baño de órganos (actividad motora “in Vitro”) no son suficientes para explicar la compleja interacción entre red neural y patrón motor. Por tanto, muchas veces, la información está segmentada y es realmente difícil poder establecer la base fisiológica y estructural responsable de un determinado mecanismo motor. Por otro lado, uno de los animales de experimentación más estudiado ha sido el cobayo, puesto que constituye un modelo experimental único debido a la facilidad de acceso a los distintos plexos nerviosos. Sin embargo, la extrapolación al hombre de los resultados obtenidos en el cobayo no siempre es fácil y es necesario ampliar el conocimiento de los mecanismos de regulación en el tracto gastrointestinal humano.

Las neuronas entéricas se pueden clasificar por su morfología como Dogiel tipo I, tipo II, tipo III y de forma muy minoritaria también se encuentran neuronas entéricas tipo IV, V, VI y VII. Las neuronas Dogiel tipo I poseen numerosas dendritas y un axón. Las neuronas Dogiel tipo II, poseen diversos axones que se pueden o no ramificar a poca distancia del cuerpo celular pasando a denominarse en este caso neuronas pseudounipolares. Este tipo de neuronas son muy numerosas en intestino delgado y colon de cobayo. Las neuronas Dogiel tipo III poseen pocas dendritas, que se van estrechando a medida que se alejan del cuerpo celular se han identificado en el intestino delgado de cobayo. Los otros tipos son poco frecuentes en el tracto gastrointestinal (Brehmer *et al.*, 1999; Furness, 2006).

Las neuronas del SNE generalmente expresan diferentes combinaciones de neurotransmisores; esto se conoce como codificación. Esta **codificación química** permite clasificar las neuronas entéricas según los neurotransmisores que exprese.

La codificación depende de muchos factores como la especie y el segmento del tracto gastrointestinal. Así por ejemplo, las neuronas submucosas del intestino del cerdo tienen una mayor co-relación inmunocitoquímica con el colon que con el intestino humano (Timmermans *et al.*, 1997).

En general, los neurotransmisores y los receptores del SNE parecen estar bastante conservados y los del SNE humano poseen grandes similitudes con los de otras especies. No obstante, algunas de las colocalizaciones (codificación química para más de un neurotransmisor) y la expresión de los receptores difieren; un ejemplo, es la colocalización del VIP y la Acetilcolintransferasa en el plexo submucoso humano, que no coincide con las neuronas secretomotoras identificadas en el tracto gastrointestinal de cobayo (Schemann & Neunlist, 2004).

El **potencial de membrana** en reposo de las neuronas entéricas es normalmente menos negativo que el de las neuronas del sistema nervioso central (-40 a -70 mV), y está muy determinado por canales de potasio. Los potenciales de acción son principalmente llevados a cabo por calcio y sodio (Vanden Berghe *et al.*, 2001).

Según sus propiedades electrofisiológicas, las neuronas entéricas se pueden clasificar en dos tipos: las de Tipo S, que son neuronas que reciben potenciales post sinápticos excitatorios rápidos o fEPSP (f*ast Excitatory PostSinaptic Potencial*) y las Tipo AH (*Afterhyperpolarization*) que poseen una hiperpolarización de larga duración. Se ha descrito en el intestino de cobayo que las neuronas tipo S son Dogiel tipo I y las neuronas tipo AH suelen ser Dogiel tipo II (Furness, 2000).

La gran mayoría de las neuronas en el SNE humano tienen propiedades eléctricas similares a las de tipo S y reciben fEPSP. Las neuronas AH son difíciles de encontrar en el Plexo mientérico y no se han detectado en el plexo submucoso. Aunque las neuronas Dogiel tipo II se han identificado en el SNE humano, la fuerte correlación entre morfología Dogiel tipo II y características electrofisiológicas AH observadas en el cobayo no se puede aplicar al SNE humano (Hansen, 2003d; Schemann & Neunlist, 2004).

Aunque las neuronas entéricas se pueden clasificar según las propiedades mencionadas anteriormente, la clasificación más común de las neuronas del SNE es según sus características funcionales.

Éstas se pueden clasificar como neuronas sensitivas, motoneuronas e interneuronas (Kunze & Furness, 1999; Furness, 2000;Costa *et al.*, 2000;Brookes, 2001).

Las **neuronas sensitivas** se pueden clasificar en dos grupos:

Las **neuronas intrínsecas aferentes primarias** denominadas **IPANs** (*Intrinsic Primary Afferent Neurones)* o EPANs (*Enteric Primary Afferent Neurones),* tienen sus cuerpos celulares tanto en los ganglios del plexo mientérico como en los del plexo submucoso y proyectan sus terminales hacia la mucosa intestinal. Responden a estímulos químicos, deformación mecánica de la mucosa y otros estímulos como el estiramiento radial y la tensión muscular. Las células enterocromafines liberan mediadores como la serotonina y el ATP (Bertrand & Bornstein, 2002), y responden a estímulos químicos y mecánicos que a su vez activan los terminales de las IPANs. En el cobayo estas neuronas son Dogiel tipo II y poseen características electofisiológicas AH; se han descrito neuronas con la misma morfología y características electrofisiológicas en otras en otras especies como el cerdo (Cornelissen *et al.*, 2000) y el ratón (Nurgali *et al.*, 2004), por lo que las IPANs podrían poseer características comunes independientemente de la especie a la que pertenezcan (Furness *et al.*, 2004; Costa *et al.*, 2000). Las IPANs están interconectadas formando redes y reciben *inputs* de otras IPANs, además conectan con interneuronas y con motonenuronas.

Las **neuronas aferentes intestinofugales** o **IFANs**, son un grupo de neuronas cuyos cuerpos neuronales se encuentran en los ganglios del plexo mientérico y sus axones migran de la pared del tubo digestivo para contactar con los ganglios simpáticos prevertebrales (mesentérico inferior, superior y celiaco). Estas neuronas

poseen mecanoreceptores y responden a estiramiento de la capa muscular circular (Szurszewski *et al.*, 2002).

Las **motoneuronas** representan la conexión final motora con las células musculares lisas de las capas circular y longitudinal. Se pueden subclasificar en **motoneuronas excitatorias** e **inhibitorias**, según los neurotransmisores que codifican (Furness, 2000), y también se pueden subclasificar según la capa muscular que inervan (Costa *et al.*, 2000). Se ha establecido que los neurotransmisores excitatorios que codifican mayoritariamente las motoneuronas excitatorias son la acetilcolina (ACh), las taquiquininas, (principalmente la NKA y la Substancia P). También se ha descrito el ATP como neurotransmisor excitatorio actuando a través de receptores $P2X_1$. Como principales neurotransmisores inhibitorios codificados por motoneuronas se han propuesto el óxido nítrico (NO), el ATP, el VIP, el PACAP y el monóxido de carbono (CO) (Lecci *et al.*, 2002) (Figura 8). En el cobayo, es probable que éstas neuronas sean Dogiel tipo I y tengan propiedades electrofisiológicas de tipo S.

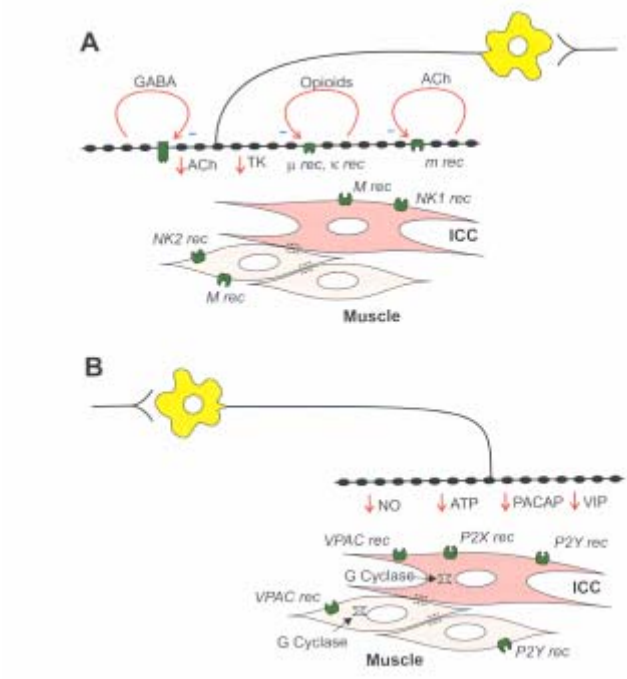



*Figura 9. Transmisión de las motoneuronas (A) excitatorias e (B) inhibitorias al músculo liso gastrointestinal. Imagen de (Furness, 2006).*

Dentro de las motoneuronas podemos encontrar un pequeño subgrupo, las neuronas **secretomotoras y vasomotoras**, que son neuronas que proyectan a mucosa y a vasos sanguíneos locales.

Las **interneuronas** han sido identificadas en todas las regiones del tracto gastrointestinal y, posiblemente, son las neuronas que más varían entre regiones, más que los otros dos tipos neuronales. Las interneuronas forman cadenas en dirección oral y aboral. Se pueden diferenciar, por tanto, interneuronas **ascendentes** y **descendentes**. En el intestino de cobayo hay una clase de neuronas ascendentes y de tres a cuatro tipos de descendentes según su codificación química. Las interneuronas ascendentes parecen estar implicadas en reflejos locales así como dos de los tipos de interneuronas descendentes (Bornstein *et al.*, 2004; Kunze & Furness, 1999) (Figura 9).

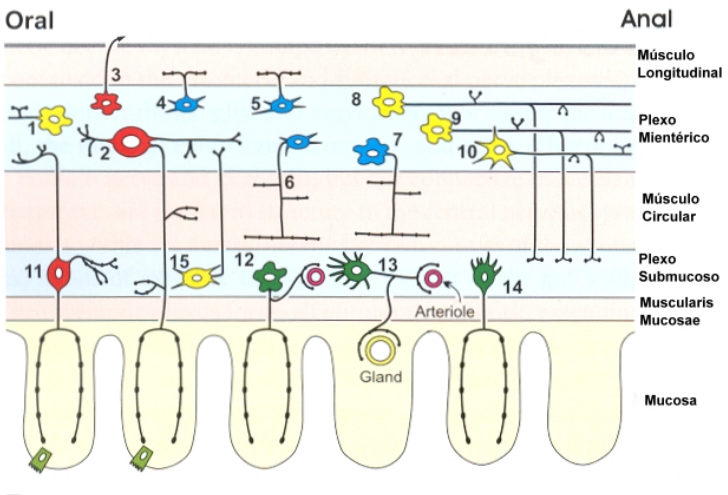



*Figura 10. Tipos de neuronas del intestino de cobayo (1) interneurona ascendente, (2) IPAN mientérica, (3) IFAN (4) motoneurona longitudinal excitatoria e inhibitoria (5), motoneurona circular excitatoria (6) e inhibitoria (7), interneurona descendente (8 ,9 y 10), IPAN submucosa (11), neuronas secretomotoras y vasodilatadores (12,13 y 14), neuronas que proyectan al plexo mientérico (15). Adaptado de (Timmermans et al., 2001).*

Además de Neuronas, en el SNE encontramos la glia entérica, que fue en un principio descrita por Dogiel. Actualmente se sabe que superan a las neuronas entéricas en un número de 4 a 1.

En un principio se pensaba que la glia entérica tenía básicamente un papel de soporte y de aporte de nutrientes, pero recientemente se ha demostrado que la glia entérica puede tener varias funciones asociadas a la neurotransmisión, participando a su vez, en diversas funciones motoras y de barrera. Diversas evidencias experimentales apoyan dichas hipótesis, auque en la actualidad, la función de la glia es aún muy desconocida a nivel del SNE. Las células gliales forman una red en el SNE y probablemente están unidas por gap junctions, igual que las células musculares lisas, formando un sincitio. Por otra parte, puesto que la glia entérica presenta receptores para neurotransmisores, ésta podría ejercer como intermediaria en la neurotransmisión, pudiendo servir por tanto como comunicación entre el SNE y el sistema inmune. Además de las funciones anteriormente mencionadas, la glia entérica participa en la síntesis e inactivación de neurotransmisores, ya que por ejemplo, expresan glutamina sintetasa (Kato *et al.*, 1990). Además estudios recientes inmunohistoquímicos y ultraestructurales, han revelado que la reactividad para la L-arginina precursor del Óxido Nítrico (NO) se encuentra en células gliales pero no en neuronas, lo que indicaría que poseen un papel importante en la síntesis de este neurotransmisor inhibitorio (Nagahama *et al.*, 2001). Utilizando algunos modelos de Knock out para glia, se ha observado que la pérdida de ésta provoca pérdida de la función barrera e inflamación intestinal (Bush *et al.*, 1998).

Por tanto, la glia entérica está activamente involucrada en la regulación neural en el SNE y tiene un papel fundamental para su mantenimiento y funcionalidad (Ruhl *et al.*, 2004; Ruhl, 2005).

### 2.2.2 Extrínseco

El sistema nervioso central es capaz de modular, pero no de ejercer un control total, sobre la motilidad a través de los dos componentes del **sistema nervioso autónomo**: el sistema nervioso **simpático** y el **parasimpático** (Hansen, 2003c; Guyton & Hall, 1996). En ambos casos existe información aferente (sensitiva) y eferente (motora), que es la base de la regulación extrínseca de la motilidad intestinal.

Las **fibras eferentes** simpáticas y parasimpáticas, representan la ruta de regulación del Sistema Nervioso Entérico por parte del sistema nervioso central. El SNC, a través de las fibras eferentes parasimpáticas, regula por ejemplo, las fases digestivas e interdigestivas de la motilidad intestinal regulando los patrones motores, la secreción, el flujo sanguíneo, mientras que la vía simpática conduce la información que media la respuesta a estrés, entre otras.

Las fibras eferentes **parasimpáticas** (colinérgicas), son preganglionares y terminan en neuronas colinérgicas del plexo mientérico, para llevar a cabo un control sobre la actividad motora o bien en neuronas colinérgicas del plexo submucoso, para regular la actividad secretora o la circulación visceral. Estas fibras eferentes preganglionares se localizan en los nervios vagales y pélvicos, y las neuronas postganglionares parasimpáticas se encuentran en los ganglios del sistema nervioso entérico.

Las fibras eferentes **simpáticas** (principalmente noradrenérgicas aunque también se ha descrito que liberan adrenalina, somatostatina y neuropéptido Y (Hansen, 2003c)), son postganglionares y algunas de ellas terminan en neuronas colinérgicas del SNE para inhibir la liberación de acetilcolina vía activación de sus receptores $\alpha 2$ pre-sinápticos, o bien, directamente sobre células musculares lisas inhibiendo a la motilidad. Puede actuar también sobre vasos sanguíneos regulando la circulación visceral.

Las fibras simpáticas eferentes preganglionares del tubo digestivo se originan en la médula espinal entre los segmentos T5 y T11, y convergen en ganglios simpáticos mesentéricos, celíaco y pélvico (inervación del recto) de donde emergen las fibras postganglionares, que viajan a través de los nervios esplácnicos que inervan el tracto gastrointestinal (Aziz & Thompson, 1998; Konturek *et al.*, 2004).

Las **fibras aferentes** transmiten al sistema nervioso central información sensitiva de los diversos estímulos que tienen lugar en el tracto gastrointestinal como son la distensión mecánica por estiramiento o distorsión, estímulos químicos como la composición de los nutrientes del lumen, hormonas, neurotransmisores así como citoquinas o mediadores inflamatorios (Grundy, 2006).

Las fibras aferentes **parasimpáticas**, transmiten la información de la región superior del tracto gastrointestinal a través del nervio vago, y la información de la región colorectal a través de los nervios pélvicos.

El 70-90% de las fibras vagales son aferentes amielínicas que tienen sus cuerpos neuronales en el ganglio nodoso y que transportan la información hacia el núcleo del tracto solitario. Estas aferentes vagales poseen un umbral de respuesta bajo a estimulación mecánica y se saturan en un rango fisiológico, con lo cual, se piensa que median las sensaciones no nocivas como la saciedad o la náusea, aunque diversos estudios han demostrado que poseen un papel importante en la nocicepción.

Las fibras aferentes **simpáticas** transmiten la información de todo el tracto gastrointestinal a través de los nervios esplácnicos.

Usualmente, estas fibras aferentes simpáticas no se suelen denominan como tal, sino como afererentes viscerales espinales. Estas fibras aferentes viscerales espinales, constituyen de un 5-10% de de las fibras de la raíz dorsal torácica y lumbar, y llegan a la médula espinal vía ganglios prevertebrales y paravertebrales; las primeras participan en la mediación de reflejos autonómicos locales. Estas fibras aferentes son amielínicas C y A$\delta$ que poseen sensibilidad para estímulos químicos y mecánicos. A

pesar de que se piensa que estas fibras transmiten exclusivamente información nociceptiva, cubren también rangos de fisiológicos de estimulación (Berthoud *et al.*, 2004; Grundy, 2002).

### 2.2.3 Mediadores hormonales

Estímulos específicos en el lumen como la hiperosmolaridad, los carbohidratos, la distensión mecánica, productos bacterianos, etc., activan células enteroendocrinas que se encuentran en la mucosa y liberan sus mediadores (que principalmente son Colecistoquinina (CCK), somatostatina, serotonina (5-HT) y corticotropin-releasing factor (CRF), que activan terminales nerviosos que se encuentran en la lámina propia : aferentes primarias intrínsecas (IPANs) y neuronas extrínsecas (p.e vagales), que contactan con motoneuronas inhibitorias y excitatorias, regulando así el control de la función del tracto gastrointestinal (Furness, 2000;Hansen, 2003c;Hansen, 2003b).

### 2.2.4 Mediadores Inmunitarios

Diversos tipos celulares del sistema inmune, como leucocitos polimorfonucleares, linfocitos, macrofagos, dendrocitos, mastocitos etc., se encuentran en la mucosa intestinal, en la lamina propia y en las capas musculares histológicamente muy asociados al sistema nervioso entérico , a las fibras vagales y a nervios espinales.

Se ha descrito que mediadores inmunes liberados pueden, de forma paracrina, alterar el comportamiento eléctrico y sináptico de las neuronas entéricas (Wang *et al.*, 2004). También se ha descrito que mediadores inmunes liberados por los mastocitos, activan aferentes vagales poniendo en marcha diversos de mecanismos secretores y motores (Hansen, 2003d).

Las respuestas motoras y secretoras responden a antígenos específicos como toxinas bacterianas y por tanto hay una comunicación directa con el SNE.

De la misma forma, estas células del sistema inmune también responden a mediadores liberados por el sistema nervioso entérico y por el sistema nervioso extrínseco. Se ha descrito que los linfocitos, los neutrófilos, los eosinófilos y los mastocitos humanos expresan receptores para neuropéptidos como el VIP, la somatostatina, el CGRP (calcitonin gene related peptide) o el GRP (gastrin-released peptide). (Genton & Kudsk, 2003).

## *3 Neurotransmisores inhibitorios no adrenérgicos no colinérgicos (NANC)*

En un principio, en los años 50 y comienzos de los 60, se pensaba que el sistema nervioso autónomo poseía sólo dos neurotransmisores, la acetilcolina y la noradrenalina; y puesto que la acetilcolina era el principal neurotransmisor excitatorio en el tracto gastrointestinal, la noradrenalina debía ser el principal neurotransmisor inhibitorio. Posteriormente, cuando los bloqueadores adrenérgicos no tuvieron efecto sobre la neurotransmisión inhibitoria en el músculo liso intestinal, se pasó a hablar de neurotransmisión inhibitoria no adrenérgica no colinérgica. Por lo tanto, los neurotransmisores que no eran bloqueados por antagonistas colinérgicos ni adrenérgicos pasaron a denominarse como “NANC”.

Para el estudio de los neurotransmisores “NANC”, dos de las técnicas más utilizadas son la de baño de órganos y las técnicas electrofisiológicas, como la técnica de microelectrodos.

La **técnica de baño de órganos**, permite el estudio de la actividad motora, tono y respuesta a estimulación eléctrica de campo o EFS (*Electrical Field Stimulation*) de segmentos intestinales, y permite evaluar por adición exógena el efecto de los posibles neurotransmisores y la presencia de estos neurotransmisores, utilizando antagonistas y evaluando las respuestas sobre EFS, tono y motilidad.

La **técnica de microelectrodos**, permite medir el potencial de membrana de las células musculares lisas del tracto gastrointestinal, colocando un electrodo intracelular (o microelectrodo) y un electrodo extracelular de referencia. En esta técnica se pueden estimular selectivamente las neuronas inhibitorias y observar la hiperpolarización del potencial de membrana o RMP (*Resting Membrane Potential*) de las células musculares. Estas hiperpolarizaciones se denominan potenciales postunión inhibitorios o IJPs (*Inhibitory Junction Potentials)*. Además, esta técnica permite

evaluar el efecto por adición exógena de los posibles neurotransmisores sobre el RMP (hiperpolarización el caso de los NANC inhibitorios) y el efecto de diferentes antagonistas sobre los IJPs (Figura 11).

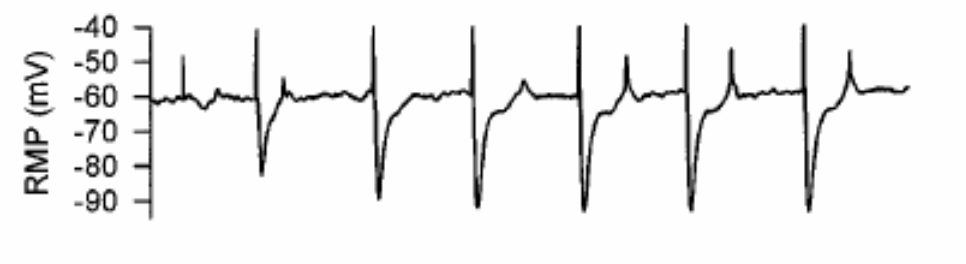



*Figura 11. Registro intracelular del músculo liso circular del colon de rata. En este registro se pueden observar los dos componentes que en la mayoría de los casos presentan los IJPs: uno rápido o IJP fast (IJPf) y uno más sostenido o IJPslow (IJPs) adaptado de (Pluja et al., 1999).*

## 3.1 ATP o purinas relacionadas

En 1970, el ATP o un nucleótido relacionado fue propuesto por Burnstock y colaboradores como neurotransmisor no adrenérgico no colinérgico en diversas especies de mamíferos. Se ha demostrado su liberación por parte de neuronas entéricas, sus efectos relajando el músculo liso por adición exógena y su presencia mediante técnicas inmunocitoquímicas utilizando quinacrina, (un marcador específico de estructuras proteicas unidas a ATP) (Burnstock *et al.*, 1970; Burnstock, 2006). Posteriormente estos resultados fueron corroborados en el intestino humano utilizando una modificación de la técnica de *sucrose gap,* que permite medir cambios en el potencial de membrana y también en la actividad mecánica. Estos estudios demostraron que, el ATP era el neurotransmisor responsable de las hiperpolarizaciones del músculo liso o IJPs (potenciales post-unión inhibitorios) en

condiciones no adrenérgicas. Se propuso que su vía de acción era mediada por canales de potasio activados por calcio sensibles a apamina (Zagorodnyuk *et al.*, 1989). Otros estudios realizados en células musculares aisladas de íleon de ratón ratificaron que los agonistas purinérgicos activan estos canales de potasio sensibles a apamina hiperpolarizando el potencial de membrana del músculo liso intestinal (Vogalis & Goyal, 1997).Sin embargo estudios posteriores en colon de rata e intestino humano pusieron en entredicho la especificidad de este fármaco para la vía purinérgica (Pluja *et al.*, 2000;Xue *et al.*, 1999).Mediante la técnica de microelectrodos, se aportaron datos que apoyaban la teoría de que el ATP o una purina relacionada es la responsable del componente rápido del IJP (IJPf) en el yeyuno humano, y en el colon distal de ratón, mientras que se propuso que el óxido nítrico mediaría la parte lenta o hiperpolarización sostenida del IJP (IJPs) en estas especies (Xue *et al.*, 1999;Serio *et al.*, 2003).Sin embargo, también hay excepciones que no respaldan esta teoría como el yeyuno canino, donde el óxido nítrico (NO) sería el principal responsable de ambas fases del IJP, (aunque una parte del componente rápido parece estar mediada por ATP) o un neurotransmisor sensible a apamina, pero esta purina tendría en este caso una acción local liberando NO (Xue *et al.*, 2000).

Diversos estudios han secundado una posible co-transmisión entre el óxido nítrico y el ATP, siendo el ATP o un componente sensible a apamina el que mediaría el componente rápido del IJP, y el óxido nítrico el componente lento de esta hiperpolarización, como es el caso del colon humano y de rata. (Boeckxstaens *et al.*, 1993; Keef *et al.*, 1993; Pluja *et al.*, 1999).

Diversos factores han dificultado el esclarecimiento de los receptores y vías de acción del ATP y análogos. Entre estos factores se encuentran el hecho de que los antagonistas utilizados como específicos para la vía purinérgica presentaran interferencias con otras vías.

Un ejemplo es el caso de la apamina, que inhibe las hiperpolarizaciones causadas por PACAP además de las causadas por ATP en el colon de rata (Pluja *et*

*al.*, 2000) y que bloquea parte del componente lento del IJP mediado por óxido nítrico en el intestino humano (Xue *et al.*, 1999). Otro ejemplo es el caso de la suramina, que revierte la relajación causada por ATP, pero también de la causada por VIP en colon proximal de cobayo (Briejer *et al.*, 1995) y no permite distinguir entre las diferentes subclases de receptores P2.

Otro factor es el hecho de que diversos fármacos para las vías purinérgicas no permiten diferenciar entre los diferentes receptores que bloquean, como por ejemplo es el caso del PPADS, que no permite diferenciar entre los receptores P2X que bloquea.

El desarrollo de fármacos específicos como por ejemplo el MRS 2179, que bloquea los receptores $P2Y_1$ (Camaioni *et al.*, 1998;Alexander *et al.*, 2005), o inhibidores específicos de la adenilato ciclasa y la fosfolipasa C, ha permitido profundizar en el estudio de estas vías.

Utilizando estas herramientas farmacológicas, se ha demostrado en diversos segmentos del tracto gastrointestinal de ratón que el ATP, o una purina relacionada, actúa a través de receptores P2Y, principalmente $P2Y_1$, situados post-sinápticamente (Giaroni *et al.*, 2002; De Man *et al.*, 2003) y que en el colon de esta especie la vía intracelular está mediada por la activación de la adenilato ciclasa (Zizzo *et al.*, 2006).

**Receptores y vía intracelular**

Hay dos familias de receptores para purinas (adenosina, ATP y ADP) y pirimidinas (UDP y UTP):

Los receptores P1 que son receptores para adenosina, se han descrito cuatro subtipos los $A_1$, $A_{2A}$, $A_{2B}$ y $A_3$. Todos ellos acoplados a proteína G, dando lugar a la activación de segundos mensajeros. Actúan mediante la inhibición ($A_1$ y $A_3$) o la estimulación ($A_{2A}$ y $A_{2B}$) de la adenilato ciclasa.

Los receptores P2 reconocen principalmente ATP, ADP, UDP y UTP. Dentro de esta familia encontramos dos subclases de receptores:

Los P2X, que son ionotrópicos, es decir, son receptores unidos a canales iónicos que median la entrada de cationes de forma selectiva y rápida (10ms). Se han descrito siete receptores $P2X_1$-$P2x_7$ y todos ellos están unidos a canales catiónicos para sodio, potasio y calcio.

Los P2Y, que son metabotrópicos, están unidos a proteínas G y se han descrito ocho subtipos: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ y $P2Y_{14}$, aunque actualmente se han propuesto nuevos subtipos como los $P2Y_{15}$. Su respuesta es más lenta que la de los receptores ionotrópicos (100ms). La mayoría de ellos están unidos a una proteína G que activa la fosfolipasa C dando lugar a diacilglicerol (DAG) y fosfoinositoltrifosfato ($IP_3$), y por tanto, produciendo una movilización del calcio intracelular. También se ha descrito que algunos pueden estar unidos a proteínas G, que activan la adenilato ciclasa, dando lugar a un incremento de AMP cíclico. Incluso se ha llegado a describir una acción mixta, como por ejemplo, la el receptor $P2Y_{11}$ que activa las dos vías (Ralevic *et al.*, 1998; Burnstock & Knight, 2004).

## 3.2 VIP y péptidos relacionados

Poco después se propuso el VIP como neurotransmisor NANC, ya que fue detectado en fibras nerviosas que inervaban el músculo liso intestinal. Diversos estudios respaldaron esta observación, ya que se demostró que este péptido era un potente relajante del músculo liso gastrointestinal (Bitar & Makhlouf, 1982) y que éste era liberado por las neuronas entéricas (Bitar *et al.*, 1980).

En diversas regiones del tracto gastrointestinal el VIP contribuye a la relajación muscular pero en otras su contribución es mínima.

Otro péptido de la familia del VIP, el PACAP, relaja el músculo liso gastrointestinal y se encuentra en neuronas entéricas. Se ha demostrado que tiene un papel importante en la relajación NANC en el colon humano (Schworer *et al.*, 1993) y de rata (Pluja *et al.*, 2000).

**Receptores y vía intracelular**

Se han propuesto tres receptores para VIP y PACAP. Éstos han sido identificados y caracterizados según su afinidad por los diferentes péptidos de la familia del VIP. Los receptores $VPAC_1$ y $VPAC_2$ se unen al VIP y al PACAP con la misma afinidad que el tercer receptor descrito, el $PAC_1$, se une preferencialmente al PACAP en el tracto gastrointestinal.

Todos ellos están unidos a proteina G, que activa la adenilato ciclasa y produce, por tanto, un incremento del AMP cíclico, y éste activa a su vez una proteinkinasa, la PKA, que lleva a cabo una serie de fosforilaciones que desencadenan la relajación del músculo liso (Harmar *et al.*, 1998).

## 3.3 Óxido nítrico (NO)

Posteriormente se propuso el óxido nítrico NO como uno de los neurotransmisores NANC inhibitorios en el tracto gastrointestinal (Bult *et al.*, 1990). Se ha descrito que este neurotransmisor tiene una función importante en la relajación del esfínter esofágico superior (LES) (Gonzalez *et al.*, 2004), que promueve la acomodación y el vaciado gástrico (Andrews *et al.*, 2007), que interviene en la relajación del esfínter anal interno (O'Kelly *et al.*, 1993), y que media también la relajación del músculo liso en las diversas áreas del tubo digestivo (Keef *et al.*, 1993; Stark *et al.*, 1993; Boeckxstaens *et al.*, 1993; Tam & Hillier, 1992; Allescher *et al.*, 1992).

La carencia de óxido nítrico, o de su enzima de síntesis, está implicada en diversas patologías como la acalasia (Mearin *et al.*, 1993), la estenosis hipertrófica pilórica o la disfunción motora causada por la neuropatía diabética.

El oxido nítrico (NO) es una molécula generada por una familia de enzimas denominadas NO sintasas (NOS), que producen óxido nítrico a partir de L-arginina.

Hay tres genes independientes que codifican para la NOS, la neuronal (nNOS), endotelial (eNOS) e inducible (iNOS). Todos ellos producen óxido nítrico a través de mecanismos independientes y con diferentes mecanismos de regulación.

La influencia como neurotransmisor inhibitorio en el tracto gastrointestinal se lleva a cabo mediante la nNOS. En un principio se pensó que la nNOS estaba presente exclusivamente en neuronas, pero posteriormente se ha demostrado que está más ampliamente distribuido. Los efectos sobre la función vascular se llevan a cabo mediante la eNOS y el NO derivado de la iNOS contribuye a la inflamación celular (Shah *et al.*, 2004).

**Receptores y vía intracelular**

El NO es lipofílico, con lo cual, difunde a través de la membrana plasmática. Por tanto, puede señalizar intra e intercelularmente. La vía intracelular más caracterizada para el NO es la mediada por la guanilato ciclasa, que produce GMP cíclico (De Man *et al.*, 2007), que activa una protein kinasa G generando una cascada de fosforilaciones. En algunas condiciones fisiopatológicas y posiblemente fisiológicas puede tener un efecto independiente de la guanilato ciclasa, en ese caso el NO regula directamente la función de canales iónicos (Bolotina *et al.*, 1994), enzimas y otro número de proteínas; efecto mediado en parte a través de la nitrosilación del grupo tiol de cisteínas de las proteínas diana.

## 3.4 Monóxido de carbono (CO)

El monóxido de carbono (**CO**) se ha propuesto recientemente como neurotransmisor NANC. Se ha demostrado que este gas hiperpolariza las células musculares lisas del intestino humano y canino (Farrugia *et al.*, 1998). Se ha descrito también como uno de los neurotransmisores que intervienen en la relajación de

esfínteres como el esfínter anal interno de zarigüeya, de rata o el esfínter esofágico inferior (LES) porcino. (Chakder *et al.*, 2000; Rattan *et al.*, 2004; Farre *et al.*, 2006).

El CO es producido a partir de protoporfirina, principalmente por dos enzimas la hemooxigenasa 1 y la hemooxigenasa 2.

**Receptores y vía intracelular**

El monóxido de carbono, igual que el óxido nítrico, difunde a través de la membrana plasmática. La vía intracelular también es común a la del NO, ya que activa la guanilato ciclasa y la producción de GMP cíclico (Gibbons & Farrugia, 2004).

## 3.5 Sulfhídrico ($H_2S$)

Actualmente se ha propuesto que este gas podría estar implicado en numerosas funciones fisiológicas, ya que es producido en numerosos tejidos de diversas especies incluyendo el hombre. (Fiorucci *et al.*, 2006).

Se ha descrito que el $H_2S$ podría actuar como:

Neuromodulador se han detectado grandes concentraciones (50-160μmol/L) en el cerebro de numerosas especies de mamíferos como la rata, el ratón o incluso en humanos. Actúa mediando a nivel hipocampal la potenciación a largo plazo (LTP) a través de receptores NMDA (Kamoun, 2004; Abe & Kimura, 1996). Posee funciones antinociceptivas (Distrutti *et al.*, 2006). Se ha descrito que posee también acciones anti-inflamatorias por parte de este gas, aunque el efecto contrario incrementando el daño tisular producido por la inflamación también ha sido observado. El $H_2S$ provoca cambios de tono en el músculo liso vascular provocando vaso dilatación e hipotensión mediada por la activación de canales de potasio sensibles a ATP. En este tejido actúa de forma sinérgica con el óxido nítrico potenciando su liberación (Bhatia, 2005; Tang *et al.*, 2005; Cheng *et al.*, 2004; Zhao *et al.*, 2001).

Puesto que se ha demostrado que este gas produce relajación y disminución del tono en músculo liso intestinal en especies como el cobayo y el conejo, podría ser propuesto como un tercer neurotransmisor gaseoso NANC en el tracto gastrointestinal. Esta teoría se ve reforzada por el hecho de que recientemente, se ha demostrado en el colon humano y de cobayo que este gasotransmisor tiene funciones pro-secretoras (Teague *et al.*, 2002; Hosoki *et al.*, 1997; Schicho *et al.*, 2006).

**Síntesis, receptores y vía intracelular**

Los precursores de síntesis del $H_2S$ son los aminoácidos que contienen sulfuro, especialmente la cisteina, que es el substrato de dos enzimas dependientes de piridoxal fosfato la cistationina-β-sintasa (CBS) y la cistationina-γ-liasa (CSE) (estos dos enzimas producen $H_2S$, amonio y piruvato a partir de la cisteina).

La CBS es el principal productor de síntesis de sulfhídrico en cerebro y sistema nervioso, siendo especialmente abundante en hipocampo y cerebelo, aunque también abunda en hígado y riñón.

La CSE se expresa principalmente en hígado y músculo liso (vascular y no vascular) (Robert *et al.*, 2003; Ishii *et al.*, 2004).

Se ha propuesto un tercer enzima productor de sulfhídrico no dependiente de piridoxal fosfato, el Mercaptopiruvato sulfurtransferasa (Kamoun, 2004).

Este gas podría producirse incluso a través de reacciones no enzimáticas, ya que se ha demostrado que eritrocitos humanos incubados con glucosa y sulfuro elemental producen sulfhídrico a una tasa constante. Como tanto la glucosa como el sulfuro se encuentran disponibles en sangre, esta podría ser una posible vía de producción *In vivo*. (Searcy & Lee, 1998)

Finalmente, se ha descrito que el $H_2S$ produce la activación de la PKA a través de la activación de una proteína G, e induce la formación de cAMP en neuronas cultivadas procedentes de cerebro de rata (Kimura, 2000).

## *Objetivos*

Es fundamental, de cara a poder tratar las enfermedades que afectan al aparato digestivo, conocer, entre otras cosas, los neurotransmisores que están implicados en la relajación de la musculatura lisa intestinal. Actualmente, está bastante aceptado que los principales neurotransmisores inhibitorios implicados en esta relajación en la mayorías de mamíferos son el óxido nítrico y el ATP o un nucleótido relacionado. Sin embargo, el papel de ambos en el tubo digestivo humano y la implicación de cada uno de ellos es todavía desconocido. Además, la mayoría de los estudios previos se han realizado con fármacos poco selectivos lo que imposibilita el conocimiento y la caracterización de los receptores implicados en la relajación de origen purinérgico.

Por tanto los objetivos de esta tesis doctoral han sido:

El estudio de la neurotransmisión inhibitoria no adrenérgica no colinérgica (NANC), no nitrérgica en el colon humano. Caracterización de los receptores implicados en la relajación purinérgica del colon humano.

Estudiar el mismo mecanismo en otras áreas del tracto gastrointestinal y otras especies. Caracterización de la neurotransmisión purinérgica en el intestino delgado de cerdo.

Estudiar la co-transmisión funcional entre NO y ATP en el colon humano y establecer la función de cada uno de estos neurotransmisores.

El estudio de otros posibles neurotransmisores inhibitorios como el sulfhídrico ($H_2S$) y su vía de acción en el colon humano y de otras especies.

Para llevar a cabo estos objetivos se han utilizando, principalmente, las técnicas de baño de órganos y de microelectrodos, que permiten la caracterización in Vitro de las vías inhibitorias, incluyendo la identificación de los neurotransmisores, los

receptores y las vías intracelulares implicadas en la interacción neuromuscular. Además se han utilizado de forma complementaria otras técnicas como la inmunohistoquímica. Además y a modo de colaboración externa hemos incluido el estudio de los receptores purinérgicos en neuronas del plexo mientérico de cobayo (técnica de medida de calcio con Fluo-4) y hemos caracterizado el efecto del sulfhídrico en segmentos intestinales donde se preserva el circuito nervioso responsable de la peristalsis.

# *Bibliografía*

Abe K & Kimura H (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* **16**, 1066-1071.

Alberti E, Mikkelsen HB, Larsen JO, & Jimenez M (2005). Motility patterns and distribution of interstitial cells of Cajal and nitrergic neurons in the proximal, mid- and distal-colon of the rat. *Neurogastroenterol Motil* **17**, 133-147.

Alberti E, Mikkelsen HB, Wang X, Diaz M, Larsen JO, Huizinga JD, & Jimenez M (2007). Pacemaker activity and inhibitory neurotransmission in the colon of Ws/Ws mutant rats. Am J Physiol Gastrointest Liver Physiol. **292**, 1499-510.

Alexander SP, Mathie A, & Peters JA (2005). Guide to receptors and channels, 1st edition (2005 revision). *Br J Pharmacol* **144 Suppl 1**, S1-128.

Allescher HD, Tougas G, Vergara P, Lu S, & Daniel EE (1992). Nitric oxide as a putative nonadrenergic noncholinergic inhibitory transmitter in the canine pylorus in vivo. *Am J Physiol* **262**, G695-G702.

Andrews CN, Bharucha AE, Camilleri M, Low PA, Seide B, Burton D, Baxter K, & Zinsmeister AR (2007). Nitrergic contribution to gastric relaxation induced by glucagon-like peptide-1 (GLP-1) in healthy adults. *Am J Physiol Gastrointest Liver Physiol* **292**, G1359-G1365.

Aziz Q & Thompson DG (1998). Brain-gut axis in health and disease. *Gastroenterology* **114**, 559-578.

Bassotti G, de RG, Castellani D, Sediari L, & Morelli A (2005). Normal aspects of colorectal motility and abnormalities in slow transit constipation. *World J Gastroenterol* **11**, 2691-2696.

Berthoud HR, Blackshaw LA, Brookes SJ, & Grundy D (2004). Neuroanatomy of extrinsic afferents supplying the gastrointestinal tract. *Neurogastroenterol Motil* **16 Suppl 1**, 28-33.

Bertrand PP (2003). ATP and sensory transduction in the enteric nervous system. *Neuroscientist* **9**, 243-260.

Bertrand PP & Bornstein JC (2002). ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. *J Neurosci* **22**, 4767-4775.

Bhatia M (2005). Hydrogen sulfide as a vasodilator. *IUBMB Life* **57**, 603-606.

Bitar KN & Makhlouf GM (1982). Relaxation of isolated gastric smooth muscle cells by vasoactive intestinal peptide. *Science* **216**, 531-533.

Bitar KN, Said SI, Weir GC, Saffouri B, & Makhlouf GM (1980). Neural release of vasoactive intestinal peptide from the gut. *Gastroenterology* **79**, 1288-1294.

Boeckxstaens GE (2002). Understanding and controlling the enteric nervous system. *Best Pract Res Clin Gastroenterol* **16**, 1013-1023.

Boeckxstaens GE, Pelckmans PA, Herman AG, & Van Maercke YM (1993). Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. *Gastroenterology* **104**, 690-697.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, & Cohen RA (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**, 850-853.

Bornstein JC, Costa M, & Grider JR (2004). Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil* **16 Suppl 1**, 34-38.

Brehmer A, Schrodl F, & Neuhuber W (1999). Morphological classifications of enteric neurons--100 years after Dogiel. *Anat Embryol (Berl)* **200**, 125-135.

Briejer MR, Akkermans LM, Meulemans AL, Lefebvre RA, & Schuurkes JA (1995). 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn Schmiedebergs Arch Pharmacol* **351**, 126-135.

Brookes SJ (2001). Classes of enteric nerve cells in the guinea-pig small intestine. *Anat Rec* **262**, 58-70.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, & Herman AG (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345**, 346-347.

Burnstock G (2006). Historical review: ATP as a neurotransmitter. *Trends Pharmacol Sci* **27**, 166-176.

Burnstock G, Campbell G, Satchell D, & Smythe A (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* **40**, 668-688.

Burnstock G & Knight GE (2004). Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* **240**, 31-304.

Bush TG, Savidge TC, Freeman TC, Cox HJ, Campbell EA, Mucke L, Johnson MH, & Sofroniew MV (1998). Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell* **93**, 189-201.

Camaioni E, Boyer JL, Mohanram A, Harden TK, & Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem* **41**, 183-190.

Chakder S, Cao GY, Lynn RB, & Rattan S (2000). Heme oxygenase activity in the internal anal sphincter: effects of nonadrenergic, noncholinergic nerve stimulation. *Gastroenterology* **118**, 477-486.

Cheng Y, Ndisang JF, Tang G, Cao K, & Wang R (2004). Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol* **287**, H2316-H2323.

Cornelissen W, De Laet A, Kroese AB, Van Bogaert PP, Scheuermann DW, & Timmermans JP (2000). Electrophysiological features of morphological Dogiel type II neurons in the myenteric plexus of pig small intestine. *J Neurophysiol* **84**, 102-111.

Costa M, Brookes SJ, & Hennig GW (2000). Anatomy and physiology of the enteric nervous system. *Gut* **47 Suppl 4**, iv15-iv19.

De Man JG, De Winter BY, Herman AG, & Pelckmans PA (2007). Study on the cyclic GMP-dependency of relaxations to endogenous and exogenous nitric oxide in the mouse gastrointestinal tract. *Br J Pharmacol* **150**, 88-96.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Antonelli E, Roviezzo F, Morelli A, Cirino G, Wallace JL, & Fiorucci S (2006). Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating KATP channels. *J Pharmacol Exp Ther* **316**, 325-335.

Farre R, Auli M, Lecea B, Martinez E, & Clave P (2006). Pharmacologic characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter. *J Pharmacol Exp Ther* **316**, 1238-1248.

Farrugia G, Miller SM, Rich A, Liu X, Maines MD, Rae JL, & Szurszewski JH (1998). Distribution of heme oxygenase and effects of exogenous carbon monoxide in canine jejunum. *Am J Physiol* **274**, G350-G358.

Fiorucci S, Distrutti E, Cirino G, & Wallace JL (2006). The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. *Gastroenterology* **131**, 259-271.

Furness JB (2000). Types of neurons in the enteric nervous system. *J Auton Nerv Syst* **81**, 87-96.

Furness JB. The Enteric Nervous System. 3. 2006. Massachusetts, Blackwell Publishing.
Ref Type: Serial (Book,Monograph)

Furness JB & Costa M (1980). Types of nerves in the enteric nervous system. *Neuroscience* **5**, 1-20.

Furness JB, Jones C, Nurgali K, & Clerc N (2004). Intrinsic primary afferent neurons and nerve circuits within the intestine. *Prog Neurobiol* **72**, 143-164.

Genton L & Kudsk KA (2003). Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. *Am J Surg* **186**, 253-258.

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, & Burnstock G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**, 1313-1323.

Gibbons SJ & Farrugia G (2004). The role of carbon monoxide in the gastrointestinal tract. *J Physiol* **556**, 325-336.

Gonzalez A & Sarna SK (2001). Neural regulation of in vitro giant contractions in the rat colon. *Am J Physiol Gastrointest Liver Physiol* **281**, G275-G282.

Gonzalez AA, Farre R, & Clave P (2004). Different responsiveness of excitatory and inhibitory enteric motor neurons in the human esophagus to electrical field stimulation and to nicotine. *Am J Physiol Gastrointest Liver Physiol* **287**, G299-G306.

Grider JR (2003a). Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J Pharmacol Exp Ther* **307**, 460-467.

Grider JR (2003b). Reciprocal activity of longitudinal and circular muscle during intestinal peristaltic reflex. *Am J Physiol Gastrointest Liver Physiol* **284**, G768-G775.

Grider JR & Jin JG (1994). Distinct populations of sensory neurons mediate the peristaltic reflex elicited by muscle stretch and mucosal stimulation. *J Neurosci* **14**, 2854-2860.

Grundy D (2002). Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut* **51 Suppl 1**, i2-i5.

Grundy D (2006). Signalling the state of the digestive tract. *Auton Neurosci* **125**, 76-80.

Guyton AC & Hall JE (1996). *Tratado de fisiología médica*, 9 ed. Mc Graw-Hill interamericana, Madrid.

Hansen MB (2003a). Neurohumoral control of gastrointestinal motility. *Physiol Res* **52**, 1-30.

Hansen MB (2003b). The enteric nervous system I: organisation and classification. *Pharmacol Toxicol* **92**, 105-113.

Hansen MB (2003c). The enteric nervous system II: gastrointestinal functions. *Pharmacol Toxicol* **92**, 249-257.

Hansen MB (2003d). [The enteric nervous system]. *Ugeskr Laeger* **165**, 4097-4101.

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, & Waschek JA (1998). International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* **50**, 265-270.

Horowitz B, Ward SM, & Sanders KM (1999). Cellular and molecular basis for electrical rhythmicity in gastrointestinal muscles. *Annu Rev Physiol* **61**, 19-43.

Hosoki R, Matsuki N, & Kimura H (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* **237**, 527-531.

Husebye E (1999). The patterns of small bowel motility: physiology and implications in organic disease and functional disorders. *Neurogastroenterol Motil* **11**, 141-161.

Ishii I, Akahoshi N, Yu XN, Kobayashi Y, Namekata K, Komaki G, & Kimura H (2004). Murine cystathionine gamma-lyase: complete cDNA and genomic sequences, promoter activity, tissue distribution and developmental expression. *Biochem J* **381**, 113-123.

Jimenez M, Borderies JR, Vergara P, Wang Y, & Daniel EE (1999). Slow waves in circular muscle of porcine ileum: structural and electrophysiological studies. *Am J Physiol* **276**, G393-G406.

Jimenez M, Cayabyab FS, Vergara P, & Daniel EE (1996). Heterogeneity in electrical activity of the canine ileal circular muscle: interaction of two pacemakers. *Neurogastroenterol Motil* **8**, 339-349.

Kamoun P (2004). [H2S, a new neuromodulator]. *Med Sci (Paris)* **20**, 697-700.

Kato H, Yamamoto T, Yamamoto H, Ohi R, So N, & Iwasaki Y (1990). Immunocytochemical characterization of supporting cells in the enteric nervous system in Hirschsprung's disease. *J Pediatr Surg* **25**, 514-519.

Keef KD, Du C, Ward SM, McGregor B, & Sanders KM (1993). Enteric inhibitory neural regulation of human colonic circular muscle: role of nitric oxide. *Gastroenterology* **105**, 1009-1016.

Kellow J (2004). Small Bowel Disorders. In *Pathophysiology of the Enteric Nervous System*, eds. Spiller R & Grundy D, pp. 134-146. Blackwell Publishing, Malden (Massachusetts) USA.

Kimura H (2000). Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem Biophys Res Commun* **267**, 129-133.

Komuro T (2006). Structure and organization of interstitial cells of Cajal in the gastrointestinal tract. *J Physiol* **576**, 653-658.

Konturek SJ, Konturek JW, Pawlik T, & Brzozowski T (2004). Brain-gut axis and its role in the control of food intake. *J Physiol Pharmacol* **55**, 137-154.

Kunze WA & Furness JB (1999). The enteric nervous system and regulation of intestinal motility. *Annu Rev Physiol* **61**, 117-142.

Lecci A, Santicioli P, & Maggi CA (2002). Pharmacology of transmission to gastrointestinal muscle. *Curr Opin Pharmacol* **2**, 630-641.

Liu LW & Huizinga JD (1993). Electrical coupling of circular muscle to longitudinal muscle and interstitial cells of Cajal in canine colon. *J Physiol* **470**, 445-461.

Lu G, Qian X, Berezin I, Telford GL, Huizinga JD, & Sarna SK (1997). Inflammation modulates in vitro colonic myoelectric and contractile activity and interstitial cells of Cajal. *Am J Physiol* **273**, G1233-G1245.

Malcolm A & Camilleri M (2000). Coloanal motor coordination in association with high-amplitude colonic contractions after pharmacological stimulation. *Am J Gastroenterol* **95**, 715-719.

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, & Malagelada JR (1993). Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* **23**, 724-728.

Nagahama M, Semba R, Tsuzuki M, & Aoki E (2001). L-arginine immunoreactive enteric glial cells in the enteric nervous system of rat ileum. *Biol Signals Recept* **10**, 336-340.

Nurgali K, Stebbing MJ, & Furness JB (2004). Correlation of electrophysiological and morphological characteristics of enteric neurons in the mouse colon. *J Comp Neurol* **468**, 112-124.

O'Kelly T, Brading A, & Mortensen N (1993). Nerve mediated relaxation of the human internal anal sphincter: the role of nitric oxide. *Gut* **34**, 689-693.

Olsson C & Holmgren S (2001). The control of gut motility. *Comp Biochem Physiol A Mol Integr Physiol* **128**, 481-503.

Pluja L, Alberti E, Fernandez E, Mikkelsen HB, Thuneberg L, & Jimenez M (2001). Evidence supporting presence of two pacemakers in rat colon. *Am J Physiol Gastrointest Liver Physiol* **281**, G255-G266.

Pluja L, Fernandez E, & Jimenez M (1999). Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol* **126**, 883-892.

Pluja L, Fernandez E, & Jimenez M (2000). Electrical and mechanical effects of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide in the rat colon involve different mechanisms. *Eur J Pharmacol* **389**, 217-224.

Rae MG, Fleming N, McGregor DB, Sanders KM, & Keef KD (1998). Control of motility patterns in the human colonic circular muscle layer by pacemaker activity. *J Physiol* **510 ( Pt 1)**, 309-320.

Ralevic V, Knight G, & Burnstock G (1998). Effects of hibernation and arousal from hibernation on mesenteric arterial responses of the golden hamster. *J Pharmacol Exp Ther* **287**, 521-526.

Ramón y Cajal S (1904). Textura del sistema nervioso del hobre y los vertebrados. pp. 1168-1196. Imprenta y librería de Nicolás Moya, Madrid.

Rao SS, Sadeghi P, Beaty J, Kavlock R, & Ackerson K (2001). Ambulatory 24-h colonic manometry in healthy humans. *Am J Physiol Gastrointest Liver Physiol* **280**, G629-G639.

Rattan S, Al HR, & De Godoy MA (2004). Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation. *Am J Physiol Gastrointest Liver Physiol* **287**, G605-G611.

Robert K, Vialard F, Thiery E, Toyama K, Sinet PM, Janel N, & London J (2003). Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain. *J Histochem Cytochem* **51**, 363-371.

Rodriguez-Membrilla A, Martinez V, Jimenez M, Gonalons E, & Vergara P (1995). Is nitric oxide the final mediator regulating the migrating myoelectric complex cycle? *Am J Physiol* **268**, G207-G214.

Ruckebusch Y & Bueno L (1977). Origin of migrating myoelectric complex in sheep. Am J Physiol **233**, E483-E487.

Ruhl A (2005). Glial cells in the gut. *Neurogastroenterol Motil* **17**, 777-790.

Ruhl A, Nasser Y, & Sharkey KA (2004). Enteric glia. *Neurogastroenterol Motil* **16 Suppl 1**, 44-49.

Rumessen JJ & Thuneberg L (1991). Interstitial cells of Cajal in human small intestine. Ultrastructural identification and organization between the main smooth muscle layers. *Gastroenterology* **100**, 1417-1431.

Sanders KM (1996). A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology* **111**, 492-515.

Sanders KM, Koh SD, & Ward SM (2006). Interstitial cells of cajal as pacemakers in the gastrointestinal tract. Annu Rev Physiol **68**, 307-343.

Sarna SK (2003). Neuronal locus and cellular signaling for stimulation of ileal giant migrating and phasic contractions. *Am J Physiol Gastrointest Liver Physiol* **284**, G789-G797.

Sarna SK (2006). Molecular, functional, and pharmacological targets for the development of gut promotility drugs. *Am J Physiol Gastrointest Liver Physiol* **291**, G545-G555.

Schemann M & Neunlist M (2004). The human enteric nervous system. *Neurogastroenterol Motil* **16 Suppl 1**, 55-59.

Schicho R, Krueger D, Zeller F, Von Weyhern CW, Frieling T, Kimura H, Ishii I, De GR, Campi B, & Schemann M (2006). Hydrogen sulfide is a novel prosecretory neuromodulator in the Guinea-pig and human colon. *Gastroenterology* **131**, 1542-1552.

Schworer H, Clemens A, Katsoulis S, Kohler H, Creutzfeldt W, & Schmidt WE (1993). Pituitary adenylate cyclase-activating peptide is a potent modulator of human colonic motility. *Scand J Gastroenterol* **28**, 625-632.

Searcy DG & Lee SH (1998). Sulfur reduction by human erythrocytes. *J Exp Zool* **282**, 310-322.

Serio R, Alessandro M, Zizzo MG, Tamburello MP, & Mule F (2003). Neurotransmitters involved in the fast inhibitory junction potentials in mouse distal colon. *Eur J Pharmacol* **460**, 183-190.

Serio R, Barajas-Lopez C, Daniel EE, Berezin I, & Huizinga JD (1991). Slow-wave activity in colon: role of network of submucosal interstitial cells of Cajal. *Am J Physiol* **260**, G636-G645.

Shah V, Lyford G, Gores G, & Farrugia G (2004). Nitric oxide in gastrointestinal health and disease. *Gastroenterology* **126**, 903-913.

Smith TK, Reed JB, & Sanders KM (1987). Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. *Am J Physiol* **252**, C215-C224.

Soffer EE, Thongsawat S, & Ellerbroek S (1998). Prolonged ambulatory duodeno-jejunal manometry in humans: normal values and gender effect. *Am J Gastroenterol* **93**, 1318-1323.

Spencer NJ (2001). Control of migrating motor activity in the colon. *Curr Opin Pharmacol* **1**, 604-610.

Stark ME, Bauer AJ, Sarr MG, & Szurszewski JH (1993). Nitric oxide mediates inhibitory nerve input in human and canine jejunum. *Gastroenterology* **104**, 398-409.

Summers RW, Anuras S, & Green J (1983). Jejunal manometry patterns in health, partial intestinal obstruction, and pseudoobstruction. *Gastroenterology* **85**, 1290-1300.

Szurszewski JH, Ermilov LG, & Miller SM (2002). Prevertebral ganglia and intestinofugal afferent neurones. *Gut* **51 Suppl 1**, i6-10.

Takaki M (2003). Gut pacemaker cells: the interstitial cells of Cajal (ICC). *J Smooth Muscle Res* **39**, 137-161.

Tam FS & Hillier K (1992). The role of nitric oxide in mediating non-adrenergic non-cholinergic relaxation in longitudinal muscle of human taenia coli. *Life Sci* **51**, 1277-1284.

Tang G, Wu L, Liang W, & Wang R (2005). Direct stimulation of K(ATP) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. *Mol Pharmacol* **68**, 1757-1764.

Teague B, Asiedu S, & Moore PK (2002). The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* **137**, 139-145.

Telford GL & Sarna SK (1991). The migrating myoelectric complex of the small intestine. *Chaos* **1**, 299-302.

Timmermans JP, Adriaensen D, Cornelissen W, & Scheuermann DW (1997). Structural organization and neuropeptide distribution in the mammalian enteric nervous system, with special attention to those components involved in mucosal reflexes. *Comp Biochem Physiol A Physiol* **118**, 331-340.

Timmermans JP, Hens J, & Adriaensen D (2001). Outer submucous plexus: an intrinsic nerve network involved in both secretory and motility processes in the intestine of large mammals and humans. *Anat Rec* **262**, 71-78.

Vanden Berghe P, Bisschops R, & Tack J (2001). Imaging of neuronal activity in the gut. *Curr Opin Pharmacol* **1**, 563-567.

Vantrappen G, Janssens J, Hellemans J, & Ghoos Y (1977). The interdigestive motor complex of normal subjects and patients with bacterial overgrowth of the small intestine. *J Clin Invest* **59**, 1158-1166.

Vogalis F & Goyal RK (1997). Activation of small conductance Ca2+-dependent K+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. *Journal of Physiology-London* **502**, 497-508.

Wang GD, Hu HZ, Wang XY, Gao N, Liu SM, Fang XC, Xia Y, & Wood JD (2004). Purinergic inhibitory neuromuscular transmission mediated by the P2Y(1) receptor in guinea-pig small intestine. *Gastroenterology* **126**, A275.

Xue L, Farrugia G, Sarr MG, & Szurszewski JH (1999). ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *Am J Physiol* **276**, G1373-G1379.

Xue L, Farrugia G, & Szurszewski JH (2000). Effect of exogenous ATP on canine jejunal smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **278**, G725-G733.

Zagorodnyuk VP, Vladimirova IA, Vovk EV, & Shuba MF (1989). Studies of the inhibitory non-adrenergic neuromuscular transmission in the smooth muscle of the normal human intestine and from a case of Hirschsprung's disease. *J Auton Nerv Syst* **26**, 51-60.

Zhao W, Zhang J, Lu Y, & Wang R (2001). The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J* **20**, 6008-6016.

Zizzo MG, Mule F, & Serio R (2006). Mechanisms underlying hyperpolarization evoked by P2Y receptor activation in mouse distal colon. *Eur J Pharmacol* **544**, 174-180.

# Capítulo 1

## $P2Y_1$ Receptors Mediate Inhibitory Purinergic Neuromuscular Transmission in the Human Colon


## *Abstract*

Indirect evidence suggests that ATP is a neurotransmitter involved in inhibitory pathways in the neuromuscular junction in the gastrointestinal tract. The aim of this study was to characterize purinergic inhibitory neuromuscular transmission in the human colon. Tissue was obtained from colon resections for neoplasm. Muscle bath, microelectrode experiments and immunohistochemical techniques were performed. MRS 2179 was used as a selective inhibitor of $P2Y_1$ receptors. We found that *1)* ATP (1mM) and ADPβS (10 μM), a preferential P2Y agonist, inhibited spontaneous motility and caused smooth muscle hyperpolarization (about -12mV), *2)* MRS 2179 (10μM) and apamin (1μM) significantly reduced these effects, *3)* both the fast component of the inhibitory junction potential (IJP) and the non-nitrergic relaxation induced by electrical field stimulation were dose-dependently inhibited ($IC_{50}$ about 1μM) by MRS 2179, *4)* ADPβS reduced the IJP probably by a desensitization mechanism, *5)* apamin (1μM) reduced the fast component of the IJP (by 30%-40%) and the inhibitory effect induced by EFS and *6)* $P2Y_1$ receptors were localized in smooth muscle cells as well as in enteric neurons. These results show that ATP or a related purine is released by enteric inhibitory motor neurons causing a fast hyperpolarization and smooth muscle relaxation. The high sensitivity of MRS 2179 has revealed, for the first time in the human gastrointestinal tract, that a $P2Y_1$ receptor present in smooth muscle probably mediates this mechanism through a pathway that partially involves apamin-sensitive calcium-activated potassium channels. $P2Y_1$ receptors can be an important pharmacological target to modulate smooth muscle excitability.

## *Introduction*

The mechanisms involved in non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmission are highly important to the gastrointestinal tract. Smooth muscle relaxation is needed in several physiological functions such as sphincter relaxation, gastric accommodation, and descending phase during the peristaltic reflex. The identity of the neurotransmitter(s) implicated in the inhibitory pathway is still being debated. VIP/PACAP (Bitar *et al.*, 1980), Nitric Oxide (NO) (Bult *et al.*, 1990), ATP (Burnstock *et al.*, 1970) or Carbon Monoxide (CO) (Farrugia *et al.*, 1998) are putative NANC inhibitory mediators (Lecci *et al.*, 2002). There is a consensus that nerve mediated relaxation is complex and that probably several mediators are co-released from inhibitory motor neurons causing smooth muscle hyperpolarization and relaxation. In several species this is indicated by the inhibitory junction potential (IJP) having two phases, a fast-component (IJPf) followed by a slow component (IJPs) (Crist *et al.*, 1992; He & Goyal, 1993). In this study we investigated the hypothesis that ATP or a related purine is responsible for the NANC inhibitory transmission in the human colon.

Purinergic P2 receptors might be involved in several functions in the gastrointestinal tract, including synaptic transmission and neuromuscular interaction (Ralevic & Burnstock, 1998; Burnstock, 2004). Possible purinergic neuromuscular transmission in the small intestine of humans has been indicated in a study using the sucrose-gap technique (Zagorodnyuk *et al.*, 1989). In the jejunum and colon, the IJP has a fast component followed by a sustained component (Stark *et al.*, 1993; Keef *et al.*, 1993). The fast component is L-NNA-insensitive and therefore non-nitrergic whereas the second component is L-NNA-sensitive and might be due to the release of nitric oxide from inhibitory motorneurons (Stark *et al.*, 1993; Keef *et al.*, 1993). The fast component of the IJP is partially suramin and apamin-sensitive and it is abolished after desensitization with ADP$\beta$S and therefore considered purinergic, possibly through P2

receptors (Xue *et al.*, 1999). All these results are consistent but they are indirect evidence due to the lack of specific pharmacological tools to demonstrate a purinergic pathway through a specific receptor.

There are two families of P2-receptors: P2X receptors, which are ligand-gated ion channels and P2Y receptors which belong to the group of G-protein coupled receptors. Several subtypes of receptors ($P2X_{1-7}$ and $P2Y_{-1,2,4,6,11,12,13,14}$) in each family have been described. Purinergic receptors play a crucial role in the control of gastrointestinal motility. P2X receptors mediate fast synaptic transmission (Galligan, 2002) including transmission from interneurons to motor neurons (Bian *et al.*, 2000) and are probably located in interstitial cells of Cajal (Burnstock & Lavin, 2002) which might participate in neuromuscular interaction. Activation of P2X receptors is generally thought to mediate smooth muscle contraction, and P2Y, relaxation. The inverse effect has also been reported (Ishiguchi *et al.*, 2000). Suramin, PPADS and reactive blue have been widely used as purinoreceptor antagonists but unfortunately they do not discriminate between P2X and P2Y receptors (Glanzel *et al.*, 2003; Lambrecht, 2000; Ralevic & Burnstock, 1998). Furthermore, an interaction between the VIP/PACAP pathway and these purinergic antagonists has been demonstrated (Briejer *et al.*, 1995; Pluja *et al.*, 2000). The lack of specific antagonists has made it difficult to establish the identity of the receptor involved in NANC relaxation. In 1998 Camaioni et al showed that the compound MRS 2179, which is the N6-methyl modification of 2'-deoxyadenosine 3',5'-bisphosphate, was a potent $P2Y_1$ receptor antagonist (Camaioni *et al.*, 1998) and it is currently considered competitive and specific (Alexander *et al.*, 2005;Hu *et al.*, 2003). The suramin analogue 8,8'-(carbonyl bis(imino-3,1-phenylenecarbonylimino) bis(1,3,5-naphthalenetrisulfonic acid) (NF023) competitively antagonizes P2X receptor-mediated responses in certain vascular and visceral smooth muscles. NF023 is a P2X antagonist with preferential sensitivity to $P2X_1$ receptors (Soto *et al.*, 1999; Alexander *et al.*, 2005).

The aim of this study was to characterize NANC non-nitrergic inhibitory neurotransmission in the human colon. This study demonstrates, for the first time, a functional purinergic inhibitory neuromuscular transmission through $P2Y_1$ receptors in the human colon. Preliminary data from this study were presented at the Digestive Disease Week meeting of the American Gastroenterology Association (AGA), Chicago, Illinois 2005 (USA) and at the XX International Symposium on Neurogastroenterology and Motility, Toulouse, 2005 (France).

## *Materials and Methods*

### Tissue Preparation

Specimens of distal (n=9) and sigmoid colon (n=19) were obtained from patients (aged 38-85 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 mm x 4 mm, were cut. The experimental procedure was approved by the ethics committee of the Hospital of Mataró (Barcelona, Spain).

### Mechanical Experiments

Muscle strips were examined in a 10 ml organ bath filled with non-adrenergic non-cholinergic (NANC) Krebs solution (phentolamine, atropine and propanol 1 μM) at 37±1ºC. An isometric force transducer (Harvard VF-1) connected to an amplifier was used to record the mechanical activity. Data were digitalized (25 Hz) using Datawin1 software (Panlab-Barcelona) coupled to an ISC-16 A/D card installed in a PC computer. A tension of 4 g was applied and the tissue was allowed to equilibrate for 1 hour. After this period, strips displayed spontaneous phasic activity. Electrical Field Stimulation (EFS) was applied during 2 minutes (pulse duration 0.4 ms, frequency 2 to 20 Hz, and amplitude 50 V). To estimate the responses to drugs, the area under the curve (AUC) of spontaneous contractions from the baseline was measured before and after drug addition or before and during EFS. In order to normalize data, the value of AUC obtained before the treatment was considered 100 and the percentage of inhibition of the spontaneous motility was estimated with the AUC obtained after the treatment.

## Electrophysiological Experiments

Muscle strips were dissected parallel to the circular muscle and placed in a Sylgard-coated chamber continuously perfused with NANC Krebs solution at 37±1ºC. Strips were meticulously pinned in a cross-sectioned slab allowing microelectrode recordings from both circular and longitudinal muscles. This procedure was previously reported in the canine ileum (Jimenez *et al.*, 1996). Preparations were allowed to equilibrate for approximately 1 hour before experiments started. Circular and longitudinal muscle cells were impaled with glass microelectrodes (40-60 MΩ) filled with 3 M KCl. Membrane potential was measured using standard electrometer Duo773 (WPI Inc., FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., England), and simultaneously digitalized (100 Hz) using EGAA software coupled to an ISC-16 A/D card (RC Electronics Inc., CA, USA) installed in a computer. Electrical field stimulation (EFS) was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. Train stimulation had the following parameters: total duration, 100 ms; frequency, 30 Hz; pulse duration, 0.3 ms, and increasing amplitude strengths, (5, 10, 12, 15, 17, 20, 25, 30 and 40 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. In order to obtain stable impalements, nifedipine (1μM) was perfused to abolish mechanical activity.

## Immunohistochemistry

Tissue samples were fixed with cold 4% paraformaldehyde in 0.2 M phosphate buffer, embedded in paraffin and processed for sectioning by standard methods. Paraffin sections were mounted on glass slides and kept in a cold place until processed. Slides were deparaffinized and rehydrated. They were then washed with distilled water and PBS (pH 7.4). Endogenous peroxidase quenching was performed by incubation with 2% hydrogen peroxide in PBS (pH 7.4) for 25 minutes. Then, a standard blocking was performed with BSA 0.2% diluted in PBS with Triton X-100 0.2% and Tween 20 0.05%. The incubation with the primary antibody (Anti $P2Y_1$, from Alomone Labs Ltd, Jerusalem, Israel.) 1:50 was performed overnight at 2ºC-4ºC. After rinsing the sections with PBS they were incubated with the En Vision Kit (from Dako, Glostrup, Denmark). Color development was achieved by incubation with Sigma diaminobenzidine (DAB) adding 100 μl of hydrogen peroxide in PBS. Sections were counterstained with hematoxylin.

## Solutions and Drugs

The composition of the Krebs 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 $MgSO_4$ bubbled with a mixture of 5% $CO_2$:95% $O_2$ (pH 7.4). The following drugs were used: Nifedipine, Nω-nitro-L-arginine (L-NNA), adenosine 5'-triphosphate (ATP), adenosine 5´-O-2-thiodiphosphate (ADPβS), apamin, phentolamine (Sigma Chemicals, St. Louis, USA); tetrodotoxin (TTX), atropine sulphate, propranolol, sodium nitroprusside (NaNP) (Research Biochemicals International, Natick, USA), MRS 2179, NF023, vasoactive intestinal polypeptide (VIP) (Tocris, Bristol UK), and Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) (Peptide Institute, Osaka Japan). Stock solutions were made by dissolving drugs in distilled water except for nifedipine which was dissolved in ethanol (96%).

## Data Analysis and Statistics

Data are expressed as means±SEM. The paired Student t-test was used to compare the AUC in the absence and in the presence of drugs before and during EFS. In order to normalize data, we calculated the percentage of inhibition by the drugs considering the AUC 5 minutes before the drug addition as 100%. The differences between the amplitude of the IJPs before and after drug infusion were compared by two-way (drug and voltage) analysis of variance. 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 California USA.

## *Results*

### Characterization of the NANC, Non-nitrergic Transmission

Muscle strips from the circular layer of human colon were spontaneously active and displayed rhythmic contractions with an amplitude of 2.93±0.4 g and a frequency of 2.79±0.1/minute (n=28). Amplitude was increased by the presence of TTX (1μM) to 3.26±0.43 g (n=28), suggesting the presence of an inhibitory neural tone. Electrical field stimulation (EFS) inhibited spontaneous activity (85.58%±2.5%, n=14) with an off-response at the end of the stimulus. Incubation with the nitric oxide synthase inhibitor L-NNA (1 mM) decreased the inhibitory effect induced by EFS to 70.39%±3.6% (n=14) showing that nitric oxide is an inhibitory neurotransmitter in the human colon (Figure 1). Circular and longitudinal muscle cells had a resting membrane potential of -49±1.2 mV (n=36). No major differences were found between muscle layers. In NANC conditions, short EFS pulses caused a fast inhibitory junction potential (IJP). The amplitude of the IJP was voltage-dependent reaching transient hyperpolarizations of 30±2.5 mV at a stimulation voltage of 25-30 V. No differences between the circular (n=8) and longitudinal (n=4) muscle layer were found when the amplitude of IJPs was measured. Like earlier studies (Keef *et al.*, 1993), we observed that IJPs were L-NNA (1 mM) insensitive (n=4: ns, 3 circular, and 1 longitudinal), showing that nitric oxide does not mediate the transient fast hyperpolarization (Figure 1).

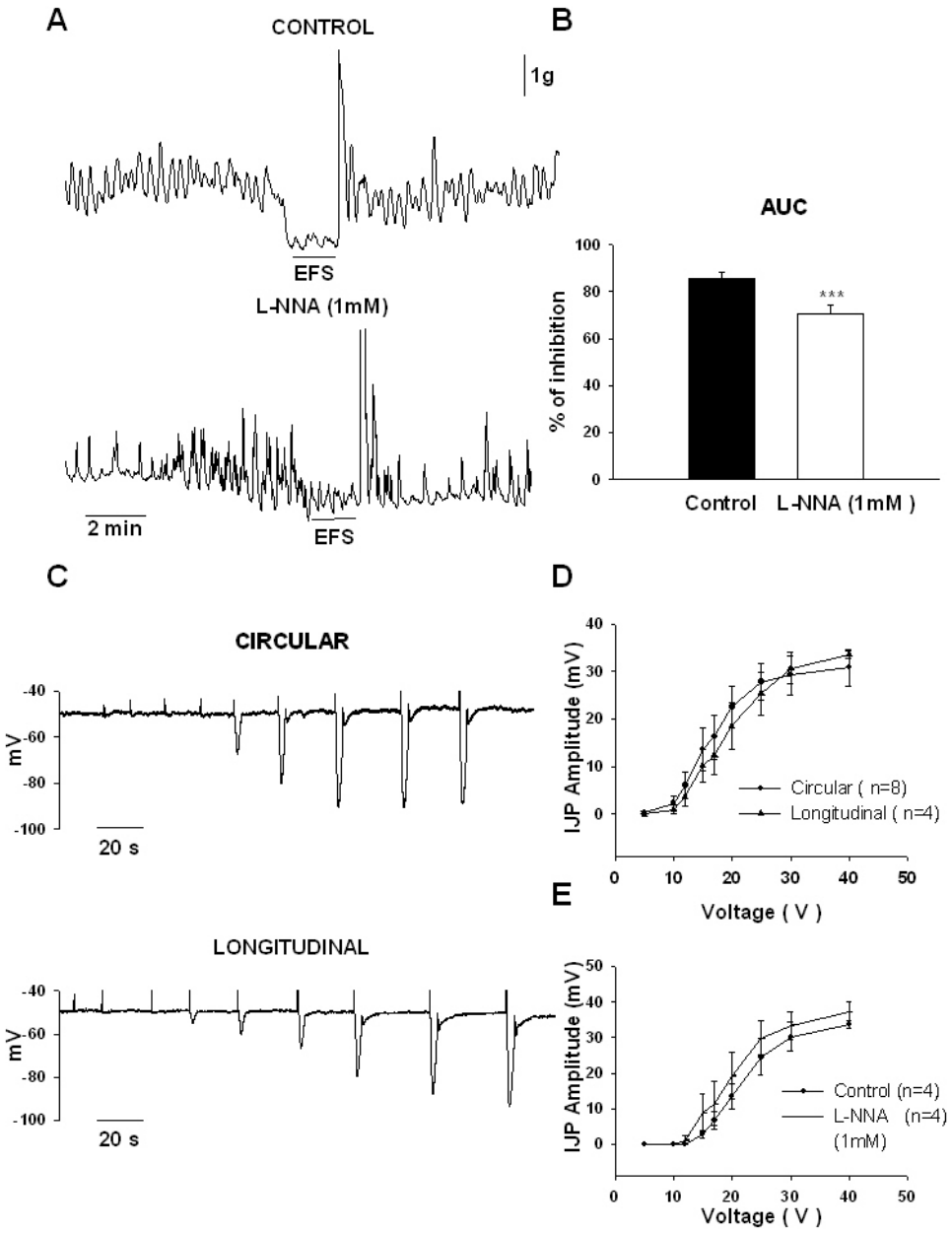



*Figure 1. (A) Mechanical recordings showing the effect of electrical field stimulation in NANC conditions (control) and in the presence of L-NNA (1 mM). (B) Histogram showing the inhibition of the spontaneous activity (AUC) in both conditions. (C) Intracellular microelectrode recordings showing IJPs induced by EFS as the stimulation voltage is increased (5, 10, 12, 15, 17, 20, 25, 30 and 40 V) from a muscular cell of the circular (top) and longitudinal (bottom) layers. (D) Comparison between the IJP amplitude from circular and longitudinal muscular cells at different stimulation voltages. (E) Effect of L-NNA (1 mM) on the IJP. Data are expressed as mean±SEM (***p<0.001).*

## Exogenous Addition of Purinergic Agonists

The purinergic pathway was studied with two agonists: ATP and ADPβS (a more stable ADP analogue). Both ATP (1mM) and ADPβS (10μM) significantly inhibited the spontaneous contractions displayed by circular muscle strips in the presence of TTX (1μM) (Figure 2). ATP (1mM) and ADPβS (10μM) both inhibited the spontaneous mechanical activity to 82%±3.9% (n=15; $p<0.001$) and 84.7%±2.2% (n=13; $p<0.001$) respectively. Transient superfusion of the tissue with ADPβS (10μM) hyperpolarized the smooth muscle to –12±0.8 mV (n=11). Hyperpolarization was recorded in the presence of TTX (1μM) (-11±2.2 mV; n=4). Just after the end of the hyperpolarization with ADPβS, the IJP was greatly reduced, progressively recovering the original values after washout (Figure 2).

## Effect of MRS 2179 on Purinergic Transmission

The inhibitory effect of both ATP and ADPβS was partially antagonized by a 10-minute preincubation with MRS 2179 (10μM). The inhibitory effect of both ATP and ADPβS was significantly reduced to 38.72%±3.6% (AUC) for ATP (n=6) and 31.56%±3.5% (AUC) for ADPβS (n=8) (Figure 3). Transient superfusion of the tissue with ADPβS (10μM) hyperpolarized the smooth muscle to –12±0.7mV. Hyperpolarization was abolished by MRS 2179 (10μM) (Figure 3).

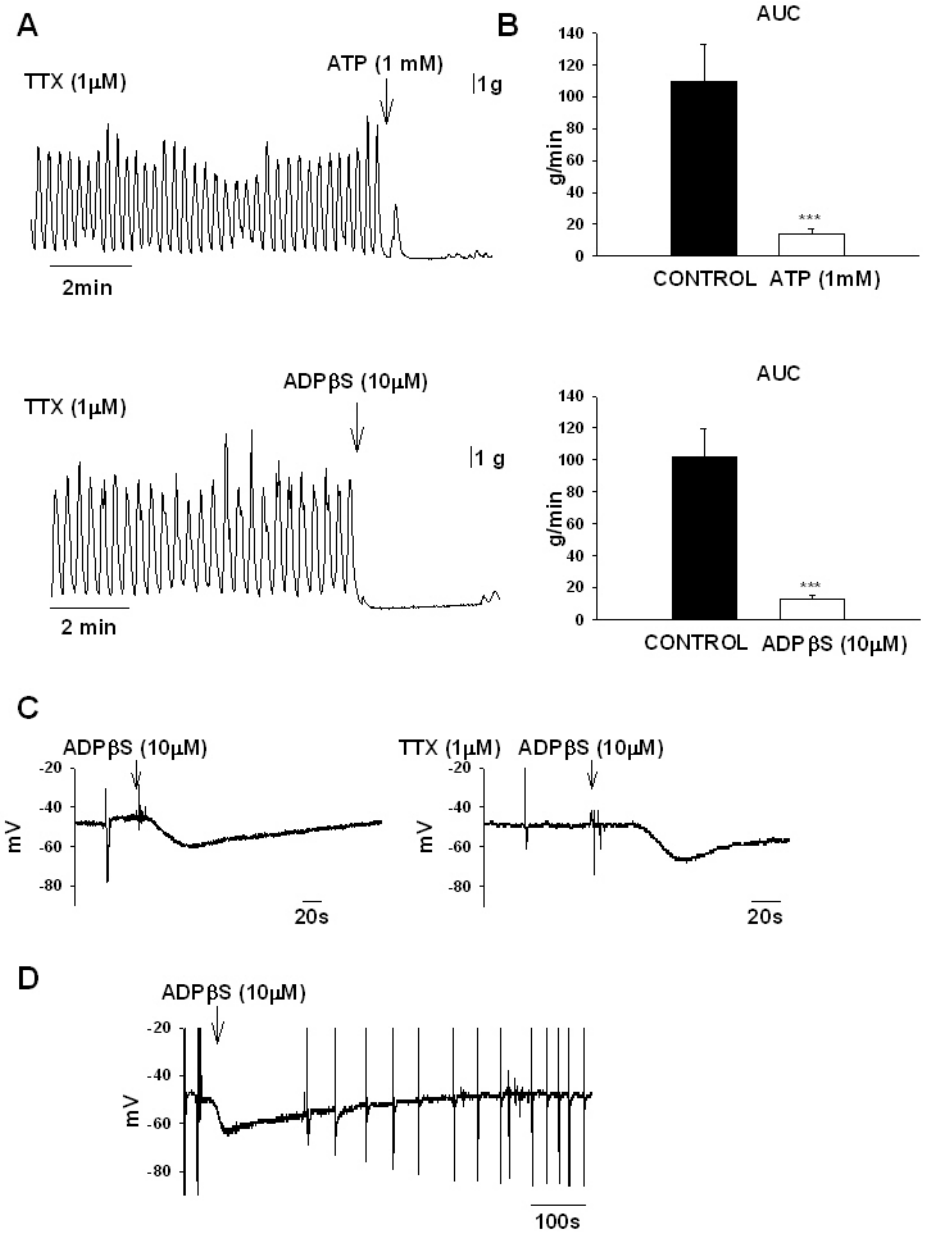



*Figure 2. (A) Mechanical recordings showing the effect of ATP (1 mM) and ADP$\beta$S (10 μM), in the presence of TTX (1 μM). (B) Histogram showing the inhibition of the spontaneous activity before and after drug addition. (C) Hyperpolarization induced by superfusion of ADP$\beta$S 10 μM in the absence and presence of the neural blocker TTX (1 μM). (D) Effect of superfusion with ADP$\beta$S 10 μM on the IJP. Data are expressed as mean±SEM (***p<0.001).*

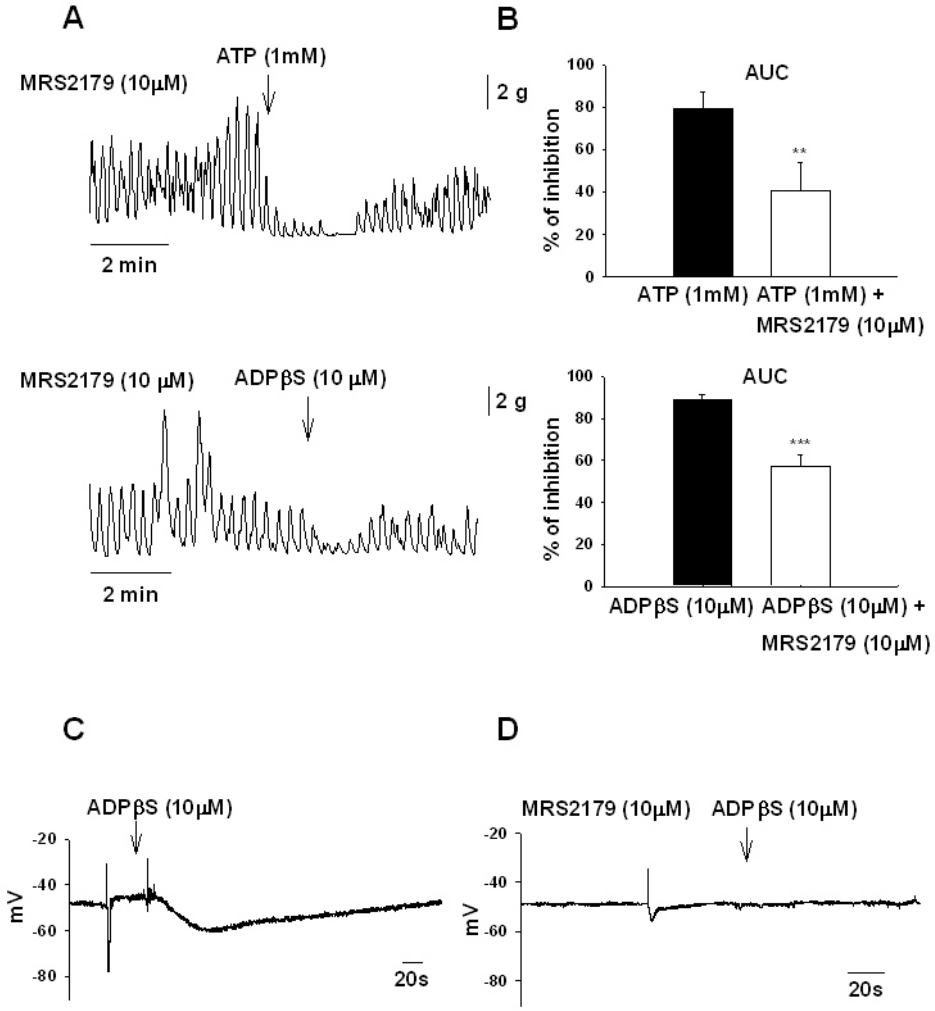



*Figure 3. (A) Effect of MRS 2179 (10$\mu$M) on the inhibition of the spontaneous motility induced by ATP (1mM) and ADP$\beta$S (10$\mu$M). (B) Histogram showing the inhibition of the spontaneous activity before and after MRS 2179 (10$\mu$M) addition. (C and D) Intracellular microelectrode recordings showing the hyperpolarization induced by ADP$\beta$S (10 $\mu$M) in the absence and presence of MRS 2179 10 $\mu$M. Data are expressed as mean±SEM (**p<0.01, ***p<0.001).*

The increase in the voltage of stimulation caused a progressive increase in the amplitude of the IJP. Using the same cell and increasing the dose of MRS 2179, we observed that IJPs were progressively reduced in both muscle layers ($p<0.0001$; circular n=5, longitudinal n=4) (Figure 4).

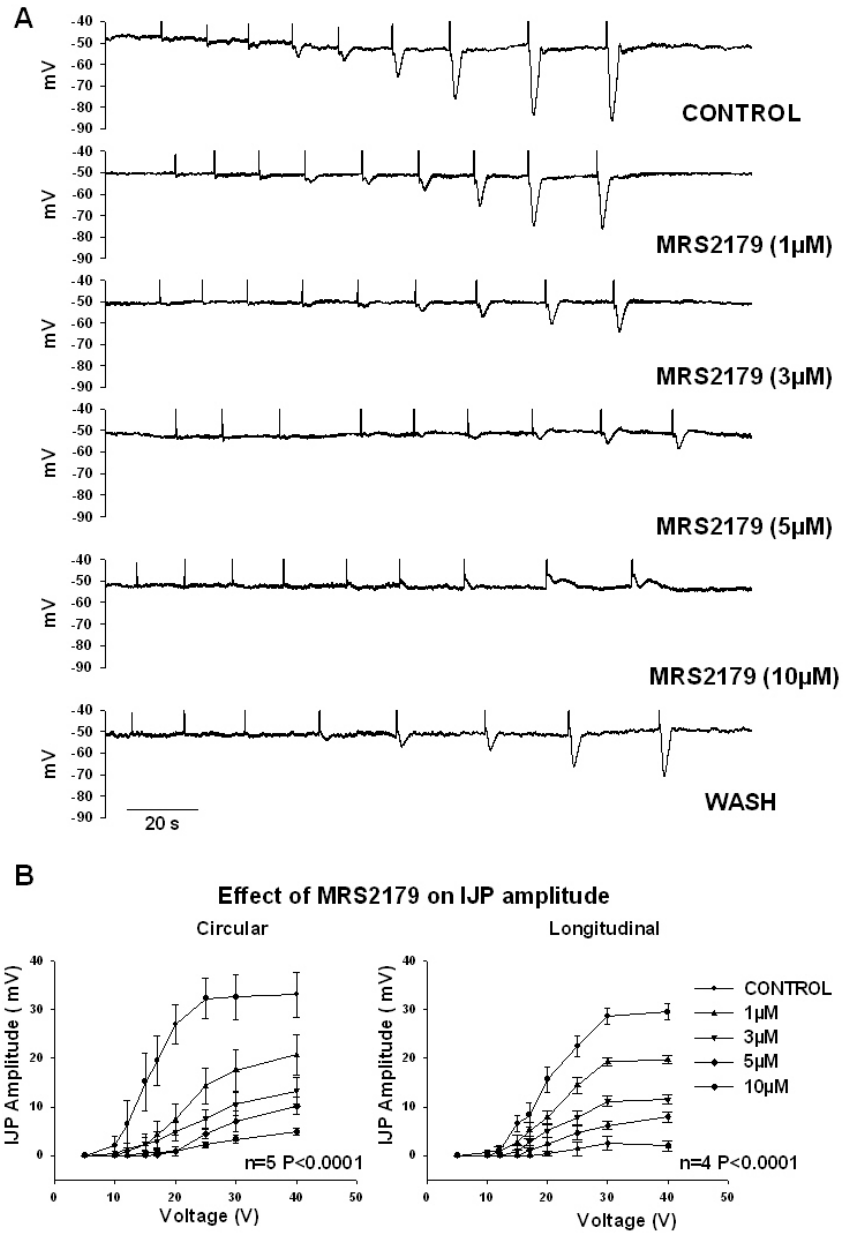



*Figure 4. (A) Intracellular microelectrode recordings showing IJPs induced by EFS as the stimulation voltage is increased (5,10,12,15,17,20,25,30 and 40 V) in control conditions (top) and in the presence of MRS 2179 ( 1, 3, 5 and 10 μM) and after washout (bottom). (B) Effect of MRS 2179 (1, 3, 5 and 10 μM) on IJP amplitude in the circular and longitudinal muscle layers. Data are expressed as mean±SEM (***p<0.001).*

In order to calculate the $IC_{50}$ of MRS 2179, a protocol using supramaximal IJPs was performed (using 30 V stimuli). The $IC_{50}$ was 1.22 μM (95% confidence interval 0.66-1.88 μM; logIC50=-5.95±0.11) in the circular muscle layer and 1.31 μM (95% confidence interval 1.05-1.69 μM, $logIC_{50}$=-5.88±0.05) in the longitudinal muscle layer (Figure 5).

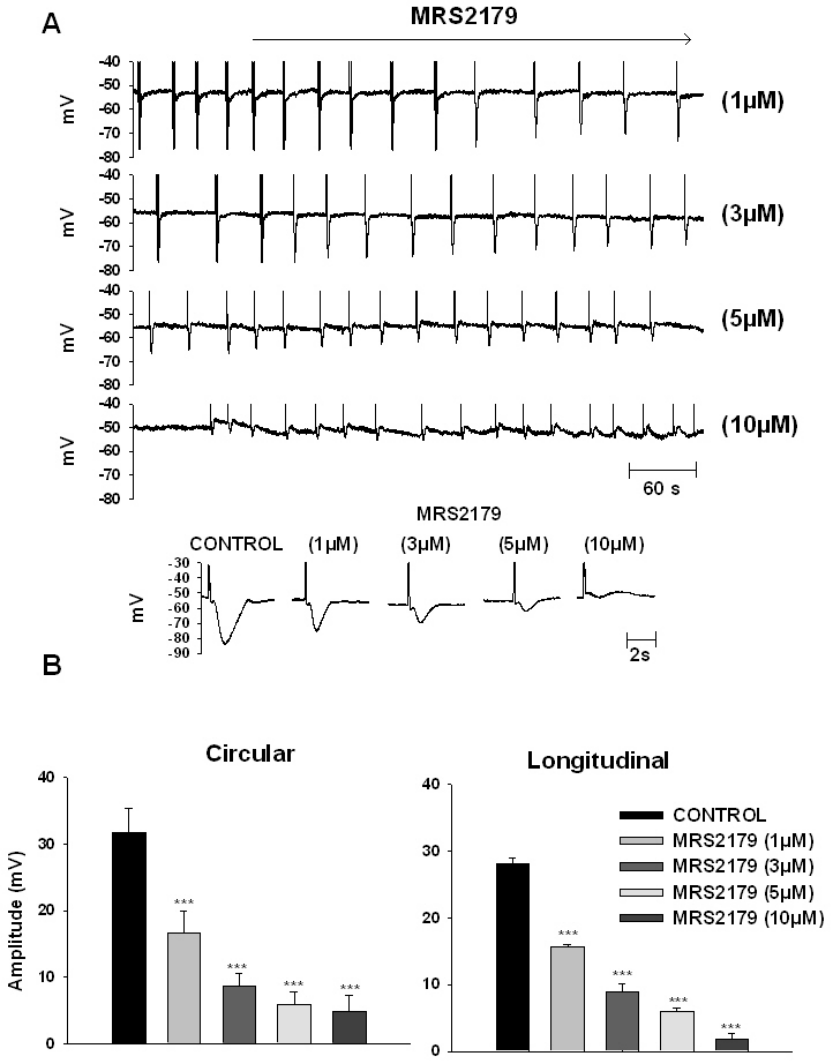



*Figure 5. (A) Intracellular microelectrode recordings showing the effect of MRS 2179 (1, 3, 5 and 10 μM) on the IJPs induced by a supramaximal EFS of 30 V of stimulation. (B) Dose dependent inhibitory effect of MRS 2179 on the IJP amplitude in the circular and longitudinal muscle layers. Data are expressed as mean±SEM (***p<0.001).*

It is important to note that in the presence of MRS 2179 (10 μM), the amplitude of the IJP was extremely low (data not different from 0 mV using a t-test analysis for both muscle layers).

The non-nitrergic relaxation induced by electrical field stimulation (2Hz) was fully antagonized by MRS 2179 in a dose-response manner (Figure 6).

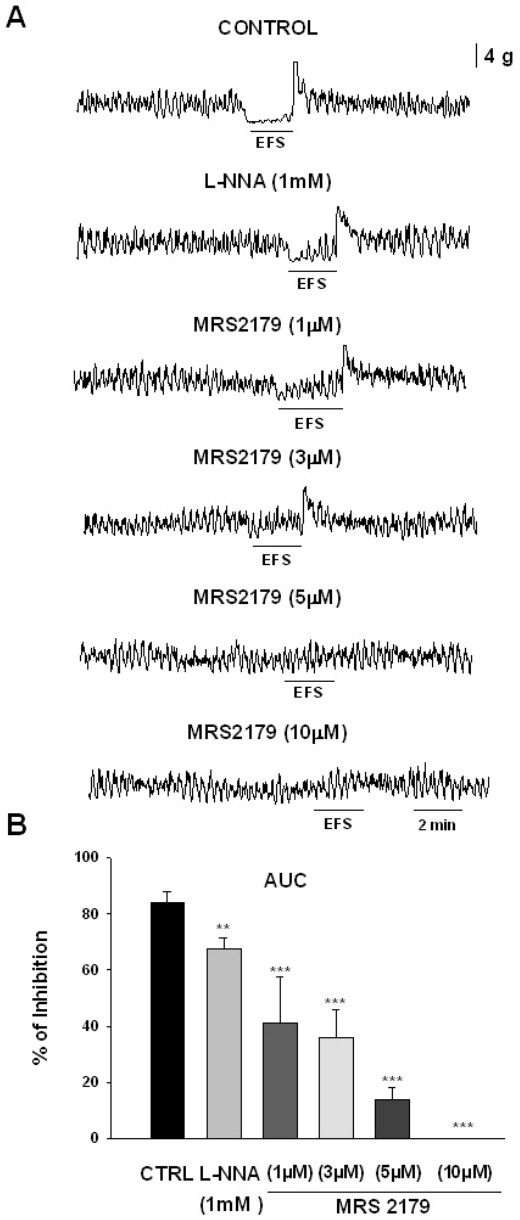



*Figure 6. (A) Effect of MRS 2179 on the inhibition of the spontaneous activity induced by EFS in the presence of L-NNA (1 mM). (B) Histogram showing the effect of MRS 2179 on the percentage of inhibition (AUC) before and during EFS. Data are expressed as mean±SEM (**p<0.01, ***p<0.001).*

The $IC_{50}$ was 0.87 μM (95% confidence interval 0.32-2.35 μM, $logIC_{50}$-6.06±0.21). In order to evaluate the effect of the stimulation frequency on the inhibitory

response, EFS was performed at 2, 5, 10 and 20 Hz. In all cases a nitrergic component (sensitive to L-NNA 1 mM) followed by a MRS 2179 (1μM) sensitive component was observed (Table 1).

| FERCUENCY | CONTROL | L-NNA (1mM) | MRS 2179 (1μM) |
|---|---|---|---|
| **2Hz** | 84±3.6 | 67±3.8 | 41±16 |
| **5Hz** | 91±4.2 | 64±3.8 | 24±6.6 |
| **10Hz** | 86±4.5 | 52±9.7 | 13±6.5 |
| **20Hz** | 85±8.4 | 41±18 | * |

* a contractile response was obtained.

*Table 1. Percentage of inhibition induced by EFS at different frequencies in control conditions in the presence of L-NNA (1mM) and MRS 2179 (1μM) (n=4; ANOVA: p<0.01)*

However, in the presence of both L-NNA (1 mM) and MRS 2179 (3-10 μM), high stimulation frequencies (5-20 Hz) caused a contractile effect possibly due to the release of non-cholinergic excitatory transmitters.

## Effect of NF023 on Purinergic Transmission

In order to evaluate a putative involvement of P2X receptors on purinergic neurotransmission, NF023 (a preferential P2X blocker) was tested. NF023 (10 μM) did not modify the non-nitrergic relaxation induced by EFS at 2 Hz (data not shown). Moreover NF023 (10 μM) did not inhibit the IJP of the circular muscle (n=4) (data not shown). A slight (about 10-20%) but significant ($p<0.05$) increase in the IJP was observed.

## Effect of MRS 2179 on Other Putative Inhibitory Neurotransmitters

In order to check the specificity of MRS 2179 in the purinergic pathway, we tested other putative NANC neurotransmitters such as VIP, PACAP and the nitric oxide

donor, NaNP. All these putative neurotransmitters caused complete cessation of the spontaneous mechanical activity in the presence of the neural blocker TTX (1μM). After incubation with MRS 2179 (10 μM), NaNP (10 μM), VIP (0.2 μM) and PACAP (0.2 μM) completely inhibited the spontaneous motility (n=5 for each drug) (Figure 7).

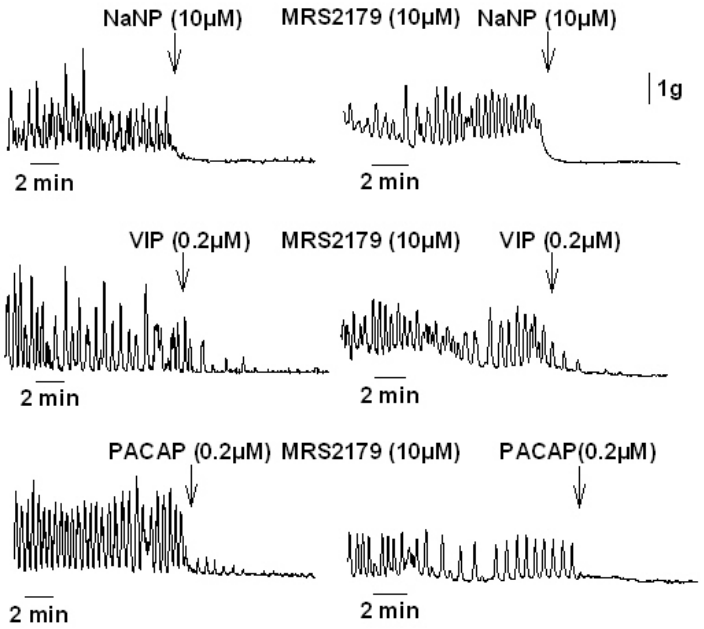



*Figure 7. Mechanical recordings showing the effect of NaNP (10 μM), VIP (0.2 μM) and PACAP (0.2 μM) before and after the incubation with MRS 2179 (10 μM) in the presence of TTX (1 μM).*

## Evaluation of the Apamin Pathway on Purinergic Neurotransmission

In order to check if small conductance calcium-activated potassium channels could be involved in the purinergic pathway, apamin (1μM) was tested. Apamin partially blocked the inhibitory effect of exogenously added ATP and ADPβS. In the presence of apamin, the inhibitory effect of ATP was reduced to 26.24%±7.7% (n=4) and ADPβS to 57.5%±12% (n=4) (both $p<0.05$).The non-nitrergic inhibition of spontaneous activity induced by EFS was also partially reduced by apamin to 35.74%±8.47% (n=4). Apamin (1 μM) reduced the amplitude of the IJP to 32.59%±4.3% (n=4) (Figure 8).

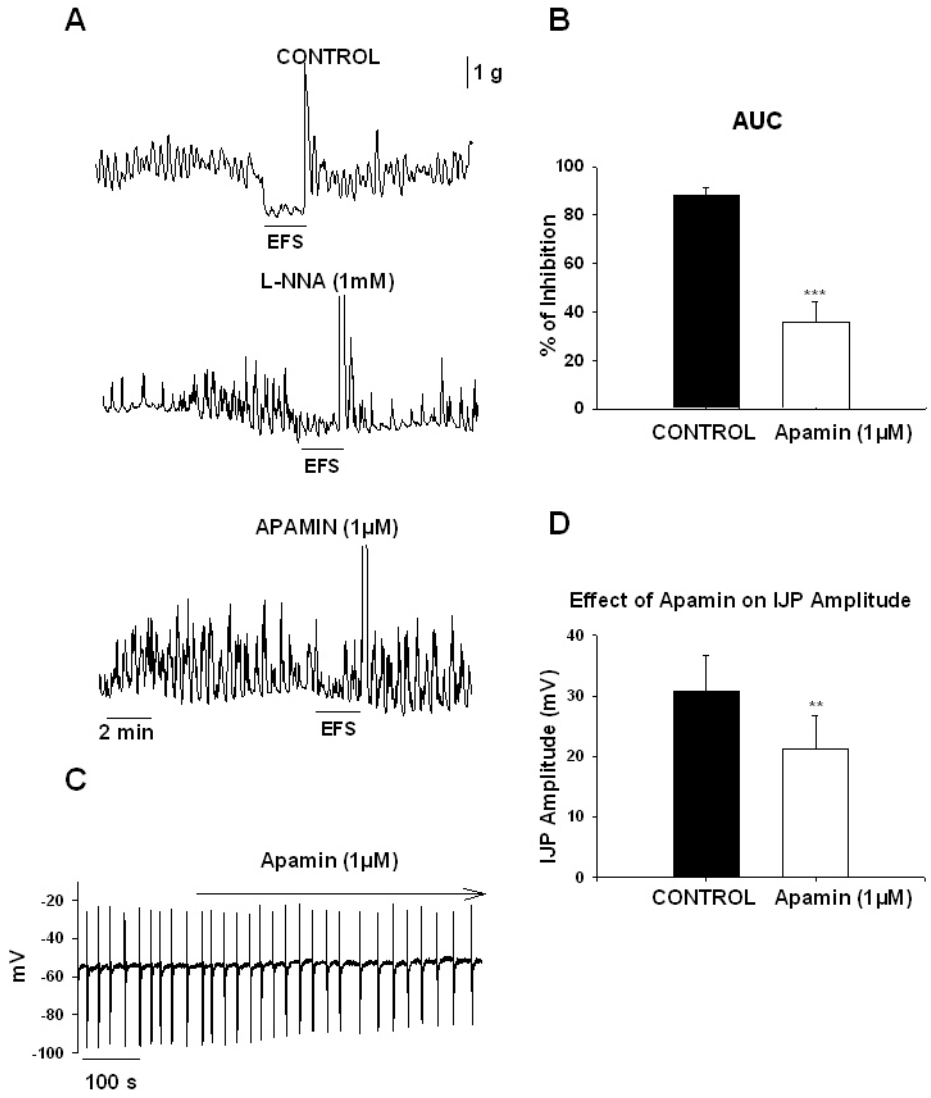



*Figure 8. (A) Effect of apamin (1$\mu$M) on the inhibition of the spontaneous activity induced by EFS. (B) Histogram showing the effect of apamin (1$\mu$M) on the percentage of inhibition (AUC) before and during EFS. (C) Effect of apamin (1$\mu$M) on the supramaximal IJP. (D) Histogram showing the effect of apamin 1 $\mu$M on the IJP amplitude. Data are expressed as mean±SEM (**p<0.01, ***p<0.001).*

## Immunohistochemistry

Positive $P2Y_1$ receptor immunoreactivity was present in the circular and longitudinal smooth muscle layers. Some neurons of the myenteric ganglia were positively marked (Figure 9). Positive immunoreactivity was detected in the tunica media of blood vessels. Minor staining was observed in the muscularis mucosae. The staining was considered specific for $P2Y_1$ since preabsorption with the antigen of the primary antibody resulted in no observed immunoreactivity. Moreover, no immunoreactivity was seen in the absence of the primary antibody.

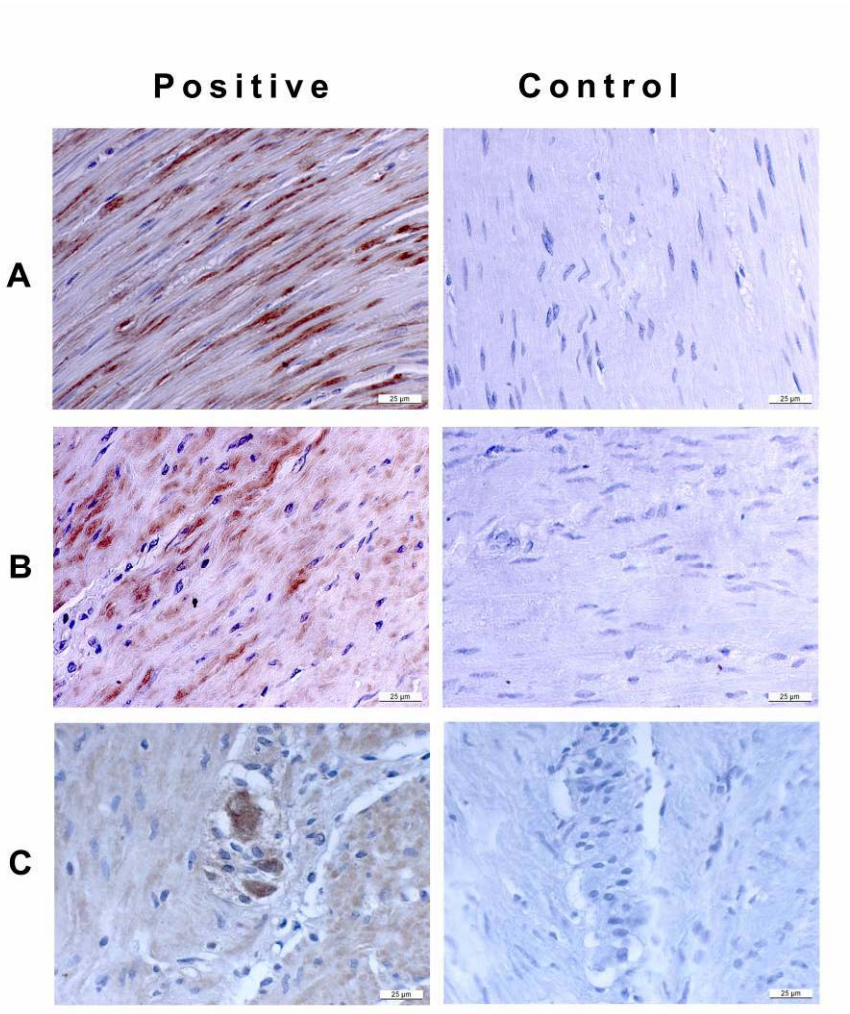



*Figure 9. Immunohistochemical localization of $P2Y_1$ receptors in (A) colonic circular smooth muscle (positive sample and control) in absence of the primary antibody, (B) colonic longitudinal smooth muscle (positive sample and control), and (C) myenteric ganglia (positive sample and control).*

## *Discussion*

In the present study we demonstrate, for the first time, a functional purinergic neurotransmission between inhibitory motor neurons and human colonic smooth muscle through $P2Y_1$ receptors. Several experimental procedures have been performed to characterize purinergic neurotransmission, including the use of non-selective P2 antagonists such as suramin and PPADS. In this study we used MRS 2179 as a selective inhibitor of $P2Y_1$ receptors (Camaioni *et al.*, 1998; Alexander *et al.*, 2005). We found that *1)* both the fast component of the IJP and the non-nitrergic relaxation induced by EFS were dose-dependently inhibited by MRS 2179, *2)* MRS 2179 inhibited the relaxation and hyperpolarization induced by ADPβS (a preferential P2Y agonist), *3)* ADPβS reduced the IJP probably by a desensitization mechanism and *4)* $P2Y_1$ receptors were localized in smooth muscle cells as well as in enteric neurons. These results show that ATP or a related purine is released by enteric inhibitory motor neurons causing a fast hyperpolarization and smooth muscle relaxation. The high sensitivity of MRS 2179 shows, for the first time in the human gastrointestinal tract, that it is probably a $P2Y_1$ receptor present in smooth muscle that mediates this mechanism through a pathway that partially involves apamin-sensitive calcium-activated potassium channels.

The spontaneous mechanical activity of human sigmoid colon consists of regular myogenic cyclic contractions. This is the most common motility pattern found in vitro in human colonic strips (Rae *et al.*, 1998). Electrical field stimulation releases inhibitory transmitters and consequently inhibits spontaneous motility (Keef *et al.*, 1993). Previous papers have demonstrated that nitric oxide is an important neurotransmitter involved in smooth muscle inhibition in the human colon (Boeckxstaens *et al.*, 1993). Consequently, inhibition of nitric oxide synthase reduces smooth muscle relaxation (Keef *et al.*, 1993) and the slow component of IJP (Xue *et al.*, 1999). However, NO is not the only NANC neurotransmitter released by inhibitory motor neurons because *1)*

an important non-nitrergic relaxation, insensitive to L-NNA, is present during EFS and *2)* the fast component of IJP (IJPf) is L-NNA insensitive (Keef *et al.*, 1993). Indirect but consistent evidence suggests that ATP might be responsible for smooth muscle inhibition in the human gastrointestinal tract. To date, ATP has been studied as an inhibitory neurotransmitter by using non-selective purinergic blockers, comparing apamin sensitivity to nitrergic and non-nitrergic components, and desensitizing receptors with unspecific agonists.

Desensitization by prolonged exposures to ADPβS, a P2Y agonist, abolishes the IJP in the human jejunal circular muscle (Xue *et al.*, 1999) and mouse colon (Serio *et al.*, 2003). In our study we found that perfusion with ADPβS caused smooth muscle hyperpolarization and temporarily inhibited the IJP when the cell recovered resting membrane potential. These results suggest that ADPβS causes a rapid desensitization of the receptor which is consistent with the putative involvement of a P2Y receptor in smooth muscle inhibition.

Apamin has been used as a pharmacological agent to discriminate between purinergic and nitrergic pathways. Previous results (and the present) obtained from colonic (Keef *et al.*, 1993) and intestinal (Xue *et al.*, 1999) samples reported that apamin reduced the IJP by about 25%. Moreover, mechanical recordings show that apamin has a major effect on non-nitrergic relaxation induced by EFS (Boeckxstaens *et al.*, 1993). These results show that the non-nitrergic mediator involves a pathway that partially activates apamin-sensitive calcium-activated potassium channels. Studies in animals using the patch-clamp technique have shown that ATP agonists open calcium-activated potassium channels (Vogalis & Goyal, 1997). It should be pointed out that apamin might also reduce the slow component of the IJP that is nitric oxide mediated in the human colon and jejunum (Keef *et al.*, 1993;Xue *et al.*, 1999). Consequently, apamin might not discriminate between the nitrergic and non-nitrergic pathways in the human gastrointestinal tract (Xue *et al.*, 1999).

Suramin is a non-selective P2 blocker that inhibits about 30% of the IJP in the human jejunum (Xue *et al.*, 1999). MRS 2179, a competitive blocker of $P2Y_1$ receptors (Camaioni *et al.*, 1998), dose-dependently inhibited both the amplitude of the IJP and the non-nitrergic inhibition of the spontaneous motility induced by EFS. Both electrophysiological and mechanical experiments showed an $IC_{50}$ close to 1 μM. These results show that $P2Y_1$ receptors mediate smooth muscle hyperpolarization and relaxation in the human colon. Recent studies performed on animals have shown similar results: *1)* MRS 2179 inhibited relaxation induced by ATP in the fundus, duodenum, ileum and colon of the mouse (Giaroni *et al.*, 2002); *2)* MRS 2179 abolished the non-nitrergic relaxation induced by electrical field stimulation in circular muscle strips from mouse jejunum (De Man *et al.*, 2003), and *3)* MRS 2179 inhibited the fast inhibitory junction potential in the guinea-pig intestine (Wang *et al.*, 2004). These studies, performed on different animals and varying sections of the gastrointestinal tract, conclude that $P2Y_1$ receptors are the main receptors involved in purinergic inhibition. Our study confirms these results in the human colon. To our knowledge, this is the first time it has been demonstrated that the non-nitrergic relaxation and the IJP are blocked by a purinergic antagonist with high specificity to $P2Y_1$ receptors.

Smooth muscle relaxation induced by endogenous release of neurotransmitters and by electrical field stimulation is completely blocked by MRS 2179. In contrast, when ADPβS is infused in the bath, MRS 2179 partially but not completely blocks the effect. This suggests that purinergic receptors, activated by the release of ATP from enteric neurons, act on $P2Y_1$ receptors, but other subclasses of P2Y receptors (see below), not located post-junctionally, might be activated by the exogenous addition of ADPβS. These receptors might use a pathway independent of the membrane potential

because hyperpolarization induced by exogenous addition of ADPβS is completely blocked by MRS 2179. Other receptors, such as subtypes of P2X receptors, might also be involved in the non-nitrergic relaxation (Ishiguchi *et al.*, 2000). In studies on the mouse jejunum and porcine lower esophageal sphincters, where the main inhibitory pathway involves the activation of the $P2Y_1$ receptor, a minor role of P2X receptors mediating inhibition has been reported (De Man *et al.*, 2003; Farre *et al.*, 2006). In the human colon, however, NF023 (10μM), a preferential P2X antagonist (Soto *et al.*, 1999), did not inhibit the non-nitrergic relaxation nor the IJP suggesting that P2X receptors are not involved in neuro-muscular inhibition.

Immunohistochemical studies were performed to determine the presence of $P2Y_1$ receptors in the human colon. Positive immunoreactivity was found in both muscle layers and in some enteric neurons. This result gives structural support to the pharmacological approach described above. A similar distribution of $P2Y_1$ receptors has been previously described in the mouse ileum (Giaroni *et al.*, 2002). Both $P2Y_1$ and $P2Y_2$ (but not $P2Y_4$) receptors are present in smooth muscle cells of the mouse ileum (Giaroni *et al.*, 2002). Further studies are needed to detect the presence of other subtypes of P2Y receptors in colonic smooth muscle cells that might participate in smooth muscle inhibition. It is interesting to note that both muscle layers are stained, indicating the presence of the receptor. This is consistent with the involvement of $P2Y_1$ receptors in mediating the fast IJP in both muscle layers with an $IC_{50}$ close to 1μM. The expression of $P2Y_1$ receptors in enteric neurons suggests that this receptor might mediate synaptic neurotransmission between enteric neurons. Moreover, $P2Y_1$ receptors mediate slow excitatory post-sinaptic potentials in the guinea pig ileum (Hu *et al.*, 2003).

It is important to note that both ATP and NO mediate smooth muscle inhibition at a wide range of stimulation frequencies (from 2-20 Hz). At 2 Hz no major excitatory component was observed in the presence of MRS 2179 (10 μM) and therefore the $IC_{50}$ was calculated at this stimulation frequency. However, a prominent excitatory non-cholinergic contraction was recorded at high stimulation frequencies (5-20 Hz). This is consistent with data reported on other species where the atropine resistant contraction was more pronounced at higher stimulation frequencies (De Schepper *et al.*, 2005). Consistent with this result, in some microelectrode recordings a small excitatory junction potential can be observed in the presence of MRS 2179 (10 μM). The origin of the non-cholinergic depolarization and contraction need further investigation but it may be due to the release of tachykinins which are important excitatory neurotransmitters in the human colon (Cao *et al.*, 2000).

ATP and nitric oxide mediate smooth muscle relaxation in the gastrointestinal tract. Nitric oxide participates in several physiological functions such as gastric accommodation, peristaltic reflex and sphincter tone. Impairment of neural nitric oxide pathways causes several diseases such as achalasia, diabetic gastroparesis and hypertrophic pyloric stenosis among others. In this paper we demonstrate that ATP, or a related purine, is a major neuromuscular inhibitory transmitter acting mainly through $P2Y_1$ receptors. Activation of $P2Y_1$ receptors causes smooth muscle hyperpolarization and relaxation. What is unknown at present is the role of these receptors in physiological functions such as those described above, or if impairment of purinergic neurotransmission occurs in these diseases. MRS 2179 might be an important pharmacological agent to investigate such effects and $P2Y_1$ receptors could be a pharmacological target to modulate smooth muscle excitability in the human gastrointestinal tract.

# *Bibliography*

Alexander SP, Mathie A, & Peters JA (2005). Guide to receptors and channels, 1st edition (2005 revision). *Br J Pharmacol* **144 Suppl 1**, S1-128.

Bian X, Bertrand PP, & Bornstein JC (2000). Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum. *J Physiol* **528**, 551-560.

Bitar KN, Said SI, Weir GC, Saffouri B, & Makhlouf GM (1980). Neural release of vasoactive intestinal peptide from the gut. *Gastroenterology* **79**, 1288-1294.

Boeckxstaens GE, Pelckmans PA, Herman AG, & Van Maercke YM (1993). Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. *Gastroenterology* **104**, 690-697.

Briejer MR, Akkermans LM, Meulemans AL, Lefebvre RA, & Schuurkes JA (1995). 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn Schmiedebergs Arch Pharmacol* **351**, 126-135.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, & Herman AG (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345**, 346-347.

Burnstock G (2004). Introduction: P2 receptors. *Curr Top Med Chem* **4**, 793-803.

Burnstock G, Campbell G, Satchell D, & Smythe A (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* **40**, 668-688.

Burnstock G & Lavin S (2002). Interstitial cells of Cajal and purinergic signalling. *Auton Neurosci* **97**, 68-72.

Camaioni E, Boyer JL, Mohanram A, Harden TK, & Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem* **41**, 183-190.

Cao W, Pricolo VE, Zhang L, Behar J, Biancani P, & Kirber MT (2000). Gq-linked NK(2) receptors mediate neurally induced contraction of human sigmoid circular smooth muscle. *Gastroenterology* **119**, 51-61.

Crist JR, He XD, & Goyal RK (1992). Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *J Physiol* **447**, 119-131.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC

neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

De Schepper HU, De Winter BY, Seerden TC, Herman AG, Pelckmans PA, & De Man JG (2005). Functional characterisation of tachykinin receptors in the circular muscle layer of the mouse ileum. *Regul Pept* **130**, 105-115.

Farre R, Auli M, Lecea B, Martinez E, & Clave P (2006). Pharmacologic characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter. *J Pharmacol Exp Ther* **316**, 1238-1248.

Farrugia G, Miller SM, Rich A, Liu X, Maines MD, Rae JL, & Szurszewski JH (1998). Distribution of heme oxygenase and effects of exogenous carbon monoxide in canine jejunum. *Am J Physiol* **274**, G350-G358.

Galligan JJ (2002). Pharmacology of synaptic transmission in the enteric nervous system. *Curr Opin Pharmacol* **2**, 623-629.

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, & Burnstock G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**, 1313-1323.

Glanzel M, Bultmann R, Starke K, & Frahm AW (2003). Constitutional isomers of Reactive Blue 2 - selective P2Y-receptor antagonists? *Eur J Med Chem* **38**, 303-312.

He XD & Goyal RK (1993). Nitric oxide involvement in the peptide VIP-associated inhibitory junction potential in the guinea-pig ileum. *J Physiol* **461**, 485-499.

Hu HZ, Gao N, Zhu MX, Liu S, Ren J, Gao C, Xia Y, & Wood JD (2003). Slow excitatory synaptic transmission mediated by P2Y1 receptors in the guinea-pig enteric nervous system. *J Physiol* **550**, 493-504.

Ishiguchi T, Takahashi T, Itoh H, & Owyang C (2000). Nitrergic and purinergic regulation of the rat pylorus. *Am J Physiol Gastrointest Liver Physiol* **279**, G740-G747.

Jimenez M, Cayabyab FS, Vergara P, & Daniel EE (1996). Heterogeneity in electrical activity of the canine ileal circular muscle: interaction of two pacemakers. *Neurogastroenterol Motil* **8**, 339-349.

Keef KD, Du C, Ward SM, McGregor B, & Sanders KM (1993). Enteric inhibitory neural regulation of human colonic circular muscle: role of nitric oxide. *Gastroenterology* **105**, 1009-1016.

Lambrecht G (2000). Agonists and antagonists acting at P2X receptors: selectivity profiles and functional implications. *Naunyn Schmiedebergs Arch Pharmacol* **362**, 340-350.

Lecci A, Santicioli P, & Maggi CA (2002). Pharmacology of transmission to gastrointestinal muscle. *Curr Opin Pharmacol* **2**, 630-641.

Pluja L, Fernandez E, & Jimenez M (2000). Electrical and mechanical effects of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide in the rat colon involve different mechanisms. *Eur J Pharmacol* **389**, 217-224.

Rae MG, Fleming N, McGregor DB, Sanders KM, & Keef KD (1998). Control of motility patterns in the human colonic circular muscle layer by pacemaker activity. *J Physiol* **510 ( Pt 1)**, 309-320.

Ralevic V & Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**, 413-492.

Serio R, Alessandro M, Zizzo MG, Tamburello MP, & Mule F (2003). Neurotransmitters involved in the fast inhibitory junction potentials in mouse distal colon. *Eur J Pharmacol* **460**, 183-190.

Soto F, Lambrecht G, Nickel P, Stuhmer W, & Busch AE (1999). Antagonistic properties of the suramin analogue NF023 at heterologously expressed P2X receptors. *Neuropharmacology* **38**, 141-149.

Stark ME, Bauer AJ, Sarr MG, & Szurszewski JH (1993). Nitric oxide mediates inhibitory nerve input in human and canine jejunum. *Gastroenterology* **104**, 398-409.

Vogalis F & Goyal RK (1997). Activation of small conductance Ca2+-dependent K+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. *Journal of Physiology-London* **502**, 497-508.

Wang GD, Hu HZ, Wang XY, Gao N, Liu SM, Fang XC, Xia Y, & Wood JD (2004). Purinergic inhibitory neuromuscular transmission mediated by the P2Y(1) receptor in guinea-pig small intestine. *Gastroenterology* **126**, A275.

Xue L, Farrugia G, Sarr MG, & Szurszewski JH (1999). ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *Am J Physiol* **276**, G1373-G1379.

Zagorodnyuk VP, Vladimirova IA, Vovk EV, & Shuba MF (1989). Studies of the inhibitory non-adrenergic neuromuscular transmission in the smooth muscle of the normal human intestine and from a case of Hirschsprung's disease. *J Auton Nerv Syst* **26**, 51-60.

# Capítulo 2

## $P2Y_1$ receptors mediate inhibitory neuromuscular transmission and enteric neuronal activation in small intestine


## *Abstract*

There is increasing evidence that ATP or a related purine plays a crucial role in smooth muscle relaxation and enteric synaptic neurotransmission. Accordingly, the aim of the present work is to investigate the role $P2Y_1$ receptors in purinergic inhibitory neurotransmission (pig ileum) and enteric neuronal activation in the small intestine (guinea-pig ileum). Using contractility measurements, microelectrode recordings and $Ca^{2+}$ imaging we found that *1)* ADPβS (10μM) caused smooth muscle relaxation and hyperpolarization that was antagonised by MRS 2179 (10μM) a $P2Y_1$ receptor antagonist and apamin (1μM). *2)* Electrical field stimulation caused a non-nitrergic IJP and relaxation that was antagonised by MRS 2179 (10μM). *3)* $P2Y_1$ receptors were immunolocalized in smooth muscle cells and enteric neurons. *4)* Superfusion of ADPβS (1μM) induced $Ca^{2+}$ transients in myenteric neurons that were inhibited by MRS 2179 (1μM), but not by TTX (1μM) *5)* EFS induced calcium transients were partially inhibited by MRS 2179 (1μM). We conclude that in the small intestine purinergic neuromuscular transmission responsible for the IJP and non-nitrergic relaxation is mediated by $P2Y_1$ receptors located in smooth muscle cells. Functional $P2Y_1$ receptors are also present in guinea-pig myenteric neurons. Therefore $P2Y_1$ receptors might be an important pharmacological target to modulate gastrointestinal functions.

## *Introduction*

Purine receptors are classified as P1, activated by adenosine and P2 receptors that recognize ATP, ADP, UTP and UDP. P2 receptors are divided in two families: P2X and P2Y receptors that are ligand gated ion channels and G protein-coupled receptors respectively. Several P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ $P2Y_{11}$…), have been cloned and characterized (Ralevic & Burnstock, 1998; Burnstock, 2004). Suramin, reactive blue and PPADS are unspecific P2 blockers that do not discriminate between P2Y and P2X receptors (Glanzel *et al.*, 2003; Ralevic & Burnstock, 1998). In 1998 Camaioni and co-authors showed that the compound MRS 2179, which is the N6-methyl modification of 2'-deoxyadenosine 3',5'-Bisphosphate, was a potent $P2Y_1$ receptor antagonist (Camaioni *et al.*, 1998). This compound is considered a specific $P2Y_1$ blocker (Alexander *et al.*, 2005).

P2 receptors have many physiological functions in nervous, vascular, respiratory and immune systems among others (Ralevic & Burnstock, 1998). There is increasing evidence that ATP or a related purine plays a crucial role in the gastrointestinal tract including mechano-sensory transduction, enteric synaptic and neuromuscular transmission regulating both motility and secretion (Galligan & North, 2004; Raybould *et al.*, 2004). P2 receptors are widely distributed in guinea-pig enteric neurons and early studies showed that ATP cause depolarization in the majority of S-type neurons and hyperpolariztion in AH-type neurons (Katayama & Morita, 1989).In the guinea-pig small intestine P2X receptors are involved in fast-synaptic transmission (fEPSPs) (LePard *et al.*, 1997). Inhibitory motor neurons receive fast synaptic inputs mediated by P2X receptors (De Man *et al.*, 2003; Johnson *et al.*, 1999). P2Y receptors mediate slow synaptic transmission (sEPSPs) in the submucosal plexus and probably participate in transduction mechanisms from the mucosa (Hu *et al.*, 2003; Cooke *et al.*,

2004). Recent data suggest that $P2Y_1$ receptors mediate purinergic inhibitory neuromuscular transmission in the guinea-pig colon (Hu *et al.*, 2003) and mouse jejunum (De Man *et al.*, 2003). Interestingly, this receptor mediates the fast inhibitory junction potential (IJP) and the non-nitrergic relaxation of the human colon (Gallego *et al.*, 2006) and therefore the study of this receptor in other species and areas of the gastrointestinal tract is crucial to further establish $P2Y_1$ receptors as potential pharmacological targets in the regulation of gastrointestinal functions such as secretion and motility (Wood, 2006).

In the present work we have used MRS 2179 as a pharmacological tool to investigate the NANC relaxation in the pig ileum and the involvement of the $P2Y_1$ receptor in the activation of enteric neurons in guinea pig ileum. We have found that the fast inhibitory junction potential and the non nitrergic relaxation are inhibited by MRS 2179. Functional data suggest that $P2Y_1$ receptors are also present in enteric neurons probably participating in synaptic transmission.

## *Materials and methods*

### Tissue preparation

Pig ileum specimens were collected from a slaughterhouse. These specimens were collected and transported to the laboratory in cold Krebs solution. Once in the laboratory the tissue was placed on a dissection dish, and the mucosal and submucosal layers were gently removed. Muscle strips (1cm long and 0.4cm wide) were cut in the circular orientation.

Guinea-pigs of either sex (250–700 g) were killed by cervical dislocation and exsanguinated by severing the carotid arteries, a method approved of by the Animal Ethics Committee of the University K.U. Leuven. A portion of the proximal ileum was removed and subsequently pinned out in a Sylgard-lined Petri dish to be dissected into a longitudinal muscle-myenteric plexus preparation (LMMP). Dissection was performed under continuous superfusion of cold oxygenated Krebs solution.

### Mechanical Experiments

Muscle strips obtained from pig ileum were studied in a 10 ml organ bath filled with Krebs solution at 37±1ºC. An isometric force transducer (Harvard VF-1) connected to an amplifier was used to record the mechanical activity. Data were digitalized (25 Hz) using Datawin1 software (Panlab-Barcelona) coupled to an ISC-16 A/D card installed in a PC computer. A tension of 2 g was applied and the tissue was allowed to equilibrate for 1 hour. After this period, carbachol 10μM was added in order to obtain a spontaneous phasic activity. Electrical Field Stimulation (EFS) was applied by two parallel platinum wire electrodes placed 10 mm apart for 5 min (pulse duration 0.4 ms, frequency 1 Hz to 3 Hz, and amplitude 26 V (about 65mA current). To estimate the

responses to drugs, the area under curve (AUC) of the contractions was measured before and after agonist addition or before and during EFS.

## Electrophysiological Experiments

Muscle strips obtained from pig ileum were dissected parallel to the circular muscle and placed in a sylgard coated chamber continuously perfused with Krebs solution containing phentolamine, atropine and propranolol 1μM to block adrenergic and muscarinic receptors at 37±1ºC. Strips were meticulously pinned allowing microelectrode recordings. Preparations were allowed to equilibrate for approximately 1 hour before experiments were started. Circular muscle cells were impaled with glass microelectrodes (40-60MΩ) filled with 3M KCl. Membrane potential was measured using standard electrometer Duo773 (WPI Inc., FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., England), and simultaneously digitalized (100 Hz) using EGAA software coupled to an ISC-16 A/D card (RC Electronics Inc., CA, USA) installed in a computer. EFS was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. Train stimulation had the following parameters: total duration 100ms, frequency 30Hz, pulse duration 0.3ms and increasing amplitude voltage (5, 10, 12, 15, 17, 20 and 25V, corresponding to a current of 12, 25, 30, 35, 45, 55, and 65 mA respectively). 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. Nifedipine (1μM) was used to abolish the mechanical activity and obtain stable impalements.

## Immunohistochemistry

Pig samples were fixed with cold paraformaldehyde 4% in 0.2M phosphate buffer, embedded in paraffin and processed for sectioning by standard methods. Paraffin sections were mounted on glass slides and kept in a cold place until processed. Slides were deparaffinized and rehydrated. Afterwards, they were washed with distilled

water and PBS (pH 7.4). Endogenous peroxidase quenching was performed by incubation with 2% hydrogen peroxide in PBS (pH 7.4) for 25 min. Then, a standard blocking was performed with BSA 0.2% diluted in PBS with Triton X-100 0.2% and Tween 20 0.05%. The incubation with the primary antibody (Anti $P2Y_1$, from Alomone Labs Ltd, Jerusalem, Israel.) 1:50 was performed overnight at 2-4ºC. After rinsing the sections with PBS they were incubated with the En Vision Kit (from Dako, Glostrup, Denmark).Two negative controls were performed to ensure the specific binding to $P2Y_1$ receptors, a preabsortion control to ensure specific binding of the primary antibody and a control in the absence of the primary antibody to ensure specific binding of the secondary antibody (En Vision Kit). Color development was achieved by incubation with Sigma diaminobenzidine (DAB) adding 100µl of hydrogen peroxide in PBS. Sections were counterstained with haematoxilin.

## Calcium Imaging in enteric neurons

To study the action of $P2Y_1$ receptors involved in enteric neuronal signaling, we chose to use $Ca^{2+}$ imaging in whole-mount preparations of guinea-pig ileum a well-established technique to study ligand mediated activation in a large amount of enteric neurons (Bisschops *et al.*, 2006; Vanden Berghe *et al.*, 2001). The guinea-pig ileum is especially suitable because the ENS can be easily exposed, which is essential to allow $Ca^{2+}$ indicator molecules to diffuse into the ganglia. Pieces of LMMP were prepared by microdissection in cold Krebs solution continuously bubbled with 95% $O_2$ / 5% $CO_2$ (pH 7.4). Tissues were loaded with Fluo-4 AM ($9 \times 10^{-6}$µM, RT, 45') in Krebs solution supplemented with pluronic acid (0.02% w/v). Tissue samples (1 $cm^2$) were stretched over a metal ring, immobilized by rubber o-ring and transferred to a coverglass bottom chamber. Sequences of fluorescent images were recorded on an inverted confocal scanning microscope (Nikon TE 300 - Noran Oz®). In preliminary experiments optimal laser intensity was set to obtain sufficient image quality while minimizing phototoxicity and bleaching. To reduce contraction, tissues were immobilized and experiments were

performed in the presence of nifedipine (1μM). Residual movements were corrected for using algorithms written in Igor Pro (Wavemetrics, Lake Oswego, OR). Tissues were perfused in a constant flow of Krebs solution (1 ml/min) and an electronic valve system allowed switching between normal and drug containing solutions. In order to characterize the response to $P2Y_1$ receptor activation the following sequences were recorded: *1)* To identify neuronal cells, we exposed the tissues briefly (5 s) to high $K^+$ Krebs solution (50 mM $K^+$) causing a sudden transient $[Ca^{2+}]_i$ rise in the neurons. After 5 minutes, ADPβS (1μM) was briefly (6s) perfused to assess the proportion of responding neurons and compare the amplitudes of ADPβS and $K^+$ responses (n=3). *2)* To identify the receptor involved in the ADPβS induced response, ADPβS (1μM) was briefly infused in the absence (control) and presence (10 minutes) of MRS 2179 (1μM). A third infusion of ADPβS was performed after a 10 minutes washout of the antagonist (n=8). *3)* In order to check if the response was due to a direct activation of the receptor or was mediated by $Na^+$ mediated action potentials, ADPβS (1μM) was infused (6s) in the absence and presence of TTX (1μM) (n=4). *4)* To evaluate the effect endogenous release of purines, focal electrical stimulation (Trains of 1s, 20Hz, 0.4ms pulse duration, 30V corresponding to an approximate current of 0.2mA) was locally applied on a nerve strand using a platinum electrode. Responses were elicited twice with an interval of 10 minutes. In order to evaluate a putative rundown, the stimulus was given twice in control solution (n=3), while in a different set of experiments (n=6) the 2nd stimulus was given in the presence of the antagonist to assess its blocking effect.

## 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 bubbled with a mixture of 5% $CO_2$-95% $O_2$ (pH 7.4). The following drugs were used: Nifedipine, Nω-nitro-L-arginine (L-NNA), adenosine 5'-triphosphate (ATP), adenosine 5´-O-2-

thiodiphosphate (ADPβS), apamin, phentolamine, carbamylcholine chloride (Sigma Chemicals, St. Louis, USA); tetrodotoxin (TTX), atropine sulphate, propranolol, sodium nitroprusside (NaNP) (Research Biochemicals International, Natick, USA). MRS 2179 (Tocris, Bristol UK). Fluo-4 AM and pluronic acid were obtained from Molecular Probes (Leiden, the Netherlands). Stock solutions were made by dissolving drugs in distilled water except for nifedipine that was dissolved in ethanol 96% (0.01% final concentration).

## Data analysis and statistics

Data are expressed as mean±SEM. Paired Student's t-test was used to compare mechanical activity in the absence and in the presence of drugs. In order to normalize data, we calculated the percentage of inhibition by the drugs considering the AUC 3 minutes before the drug addition as 100%. The differences between IJP amplitude and mechanical relaxation induced by EFS, before and after drug infusion were compared by two-way ANOVA for repeated measures (drug and voltage) followed by a post hoc test (Bonferroni) using Graph Pad (Prism) software. All image analysis was performed using custom written routines in Igor Pro (Wavemetrics, Lakes Oswego, OR, USA). Regions of interest (ROIs) 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. Individual traces were analyzed and peaks were included if they reached above a set threshold. This threshold was determined by the baseline plus 5 times the standard deviation (as a measure for noise) of the each trace at rest. Paired student's t-test or ANOVA test was used before and after drug addition. A $P < 0.05$ was considered statistically significant.

## *Results*

### Exogenous addition of ADPβS

In order to evaluate the role of $P2Y_1$ receptors, ADPβS, a stable ADP analogue, was used as agonist in the pig ileum. Regular rhythmic contractions were induced by carbachol (10μM) and experiments were performed in the presence of the neural blocker TTX (1μM). These contractions were abolished by the addition of ADPβS 10μM (90.69±1.9 %, n=6; p<0.001) (Figure 1A). The inhibitory effect of ADPβS was partially antagonised by a 10-minute preincubation with MRS 2179 10μM (55.76±2.2%, n=4; p<0.005) (Figure 1B). Apamin (1μM) reversed the inhibitory effect to a similar extent (52.8±10.9%; n=5; p<0.05).

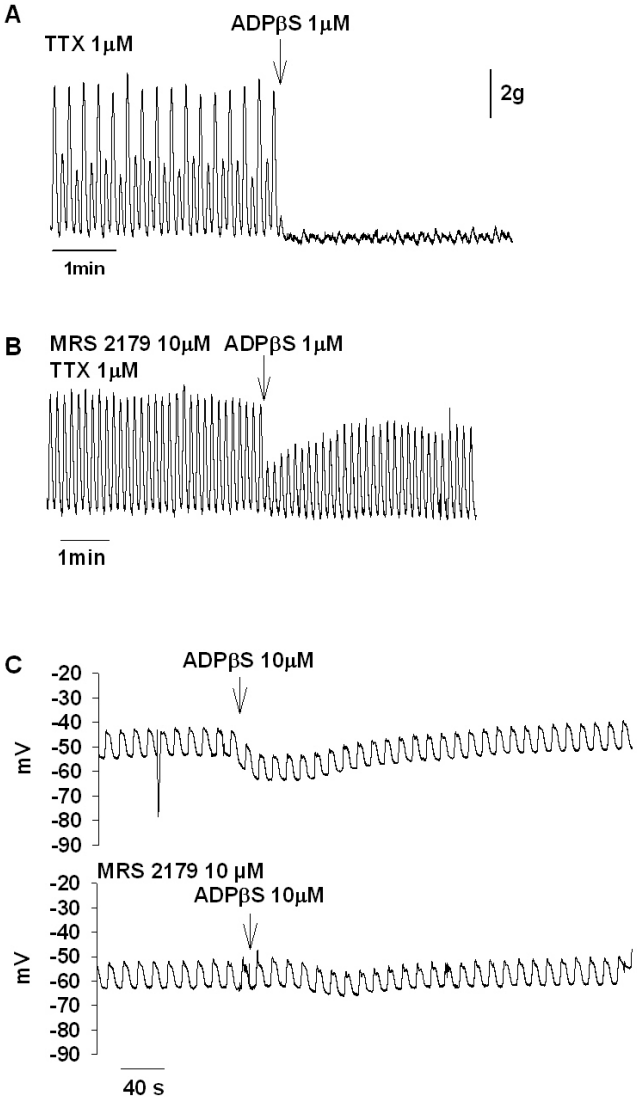



*Figure 1. (A) Mechanical recordings showing the effect of ADPβS (1μM), in the presence of TTX (1μM) (B) Effect of MRS 2179 (10μM) on the inhibition of the spontaneous motility induced by ADPβS (1μM) (C) Intracellular microelectrode recordings showing the hyperpolarization induced by ADPβS (10μM) in the absence and presence of MRS 2179 (10μM) in the pig ileum. Notice the presence of slow wave activity of about 6 cpm and 10 mV amplitude.*

Microelectrode recordings showed slow wave activity at a frequency of about 10 cpm and resting membrane potential of -60 mV. ADPβS (10μM) caused a14.5±0.9 mV (n=6) smooth muscle hyperpolarization (Figure 1C). The hyperpolarization was not influenced by L-NNA or TTX, but was dose-dependently antagonised by MRS 2179 (5 and 10μM) (Figure 1C). Apamin (1μM) abolished the hyperpolarization induced by ADPβS (n=3) (Table 1).

| Protocol | N | Hyperpolarization (mV) | Bonferroni |
|---|---|---|---|
| ADPβS (10μM) | 6 | -14.5 ± 1.0 | - |
| L-NNA + ADPβS (10μM) | 11 | -14.2 ± 1.4 | ns |
| MRS 2179 (5μM)+ ADPβS (10μM) | 3 | -3.9 ± 2.2 | P < 0.01 |
| MRS 2179 (10μM)+ ADPβS (10μM) | 6 | -1.7 ± 1.0 | P < 0.001 |
| TTX (1μM)+ ADPβS (10μM) | 4 | -9.5 ± 3.3 | ns |
| Apamin (1μM)+ ADPβS (10μM) | 3 | 0.0 ± 0.0 | P < 0.001 |

*Table 1. Hyperpolarisations (mV) induced by ADPβS (10μM) in the absence and presence of several drugs.*

*Data are expressed as mean± sem and compared with ANOVA (p<0.001). A post-hoc Bonferroni test was performed comparing the hyperpolarisation induced by ADPβS with the other protocols (p values are listed in the last column).*

## Effect of MRS 2179 on the purinergic neuromuscular transmission

Electrical field stimulation (EFS) caused voltage dependent inhibitory junction potentials (IJP) in the pig ileal circular muscle. With increasing doses of MRS 2179 (1, 3, 5 and 10μM), the IJPs were progressively reduced (n=4, p<0.0001) (Figure 2).

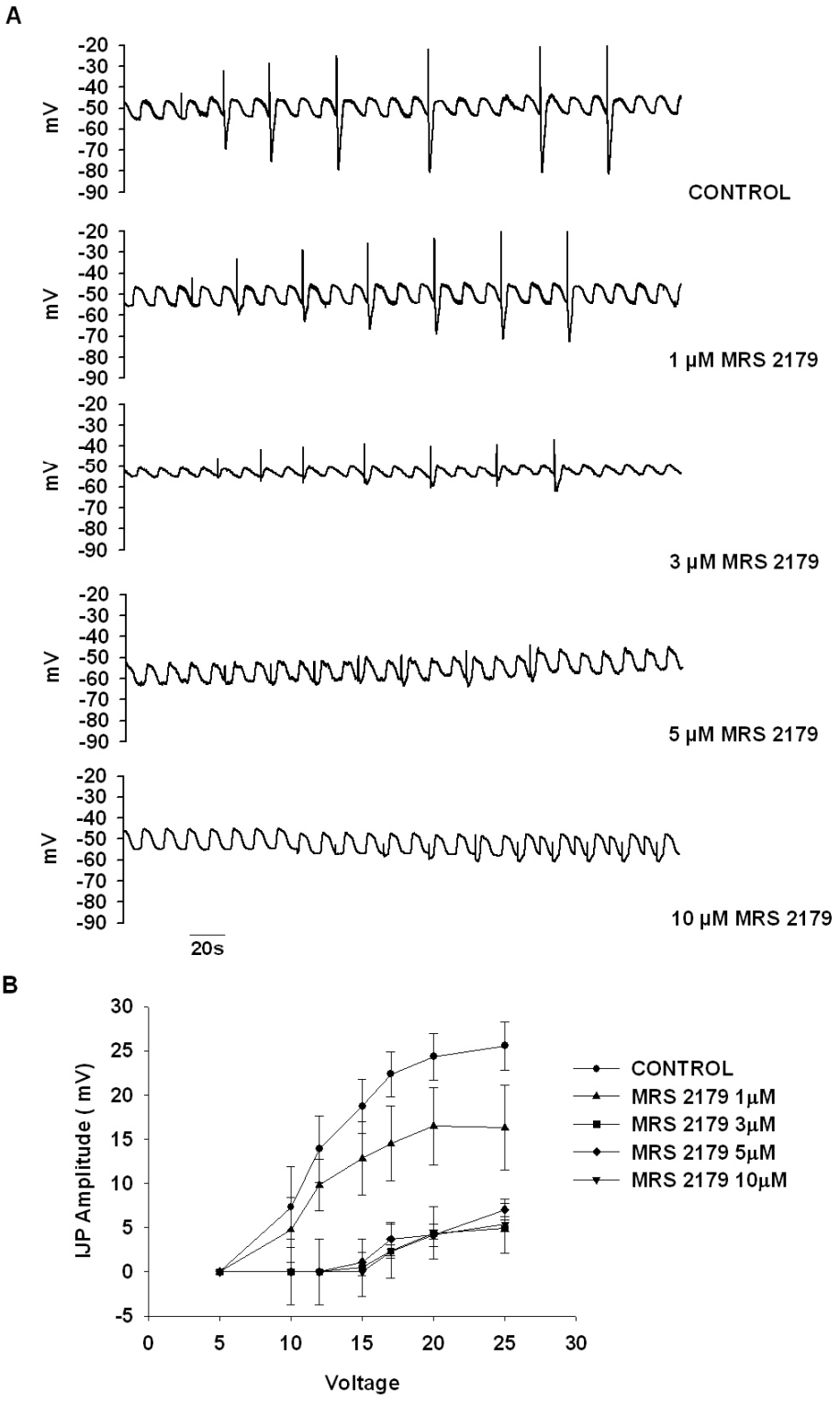



*Figure 2 .(A) Intracellular microelectrode recordings showing IJPs elicited by EFS as the stimulation voltage is increased (5,10,12,15,17,20 and 25V) in control conditions (top) and in the presence of MRS 2179 ( 1, 3, 5 and 10$\mu$M) (bottom) (B) Effect of MRS 2179 ( 1, 3, 5 and 10$\mu$M) on IJP amplitude in the pig ileal circular muscle .*

To calculate the $IC_{50}$ of MRS 2179 we performed a protocol using supramaximal IJPs (usually 20V stimuli). The $IC_{50}$ was 0.7μM (log $IC_{50}$: -6.15±0.23; n=4) (Figure 3).

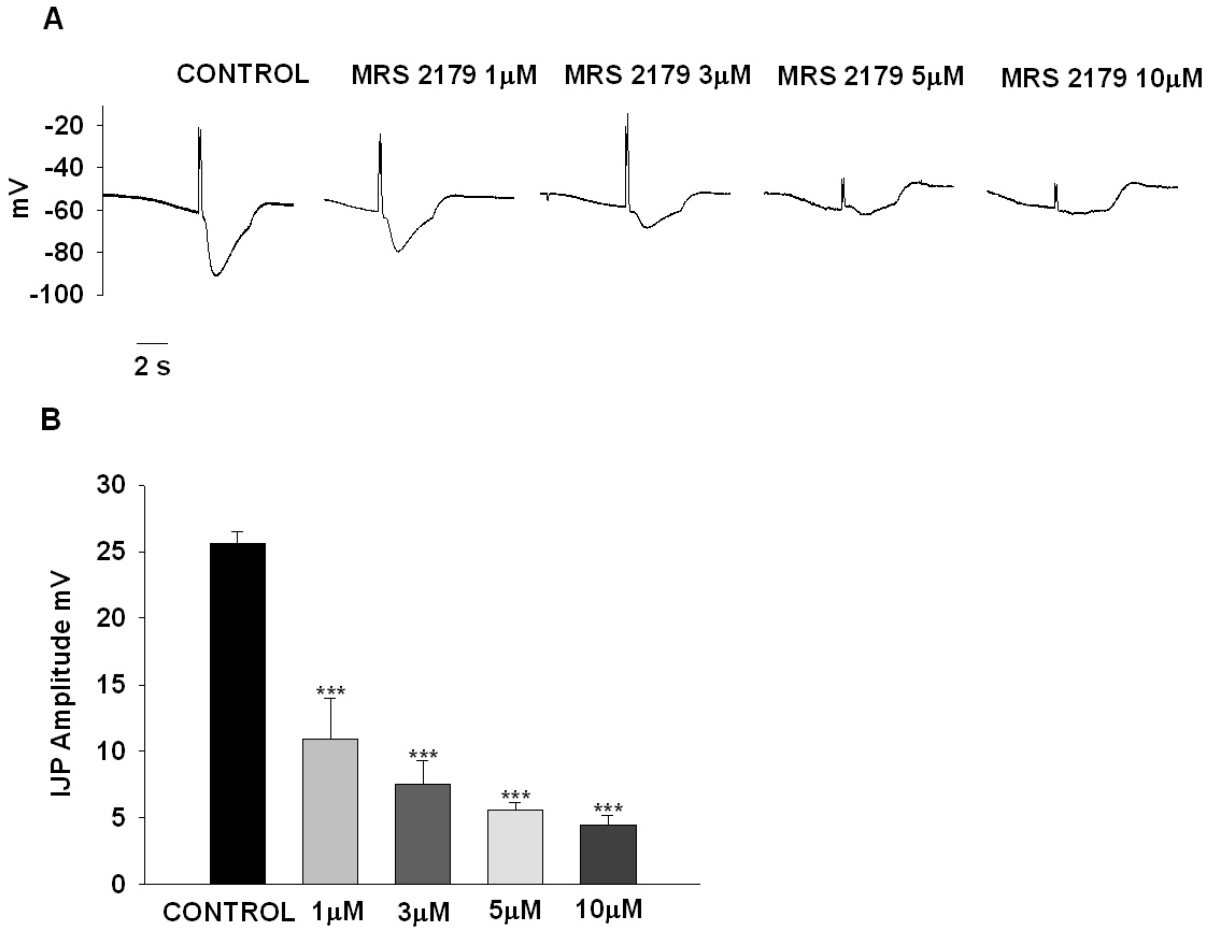



*Figure 3. (A) Intracellular microelectrode recordings showing the effect of increased doses of MRS 2179 on the supramaximal IJP. (B) Effect of MRS 2179 on IJP amplitude in the pig ileal circular muscle. Data are expressed as mean±SEM (***p<0.001).*

Inhibition of spontaneous motility was induced by EFS (1, 2 and 3 Hz) in control conditions and in the presence of L-NNA. The non-nitrergic inhibition of spontaneous activity induced by EFS was reduced by MRS 2179 10 μM to 17.94±9.08 % at 1Hz; 31.03±11.13 at 2Hz and 36.63±22.46% at 3Hz (n=4 each, p=0.0001) (Figure 4).

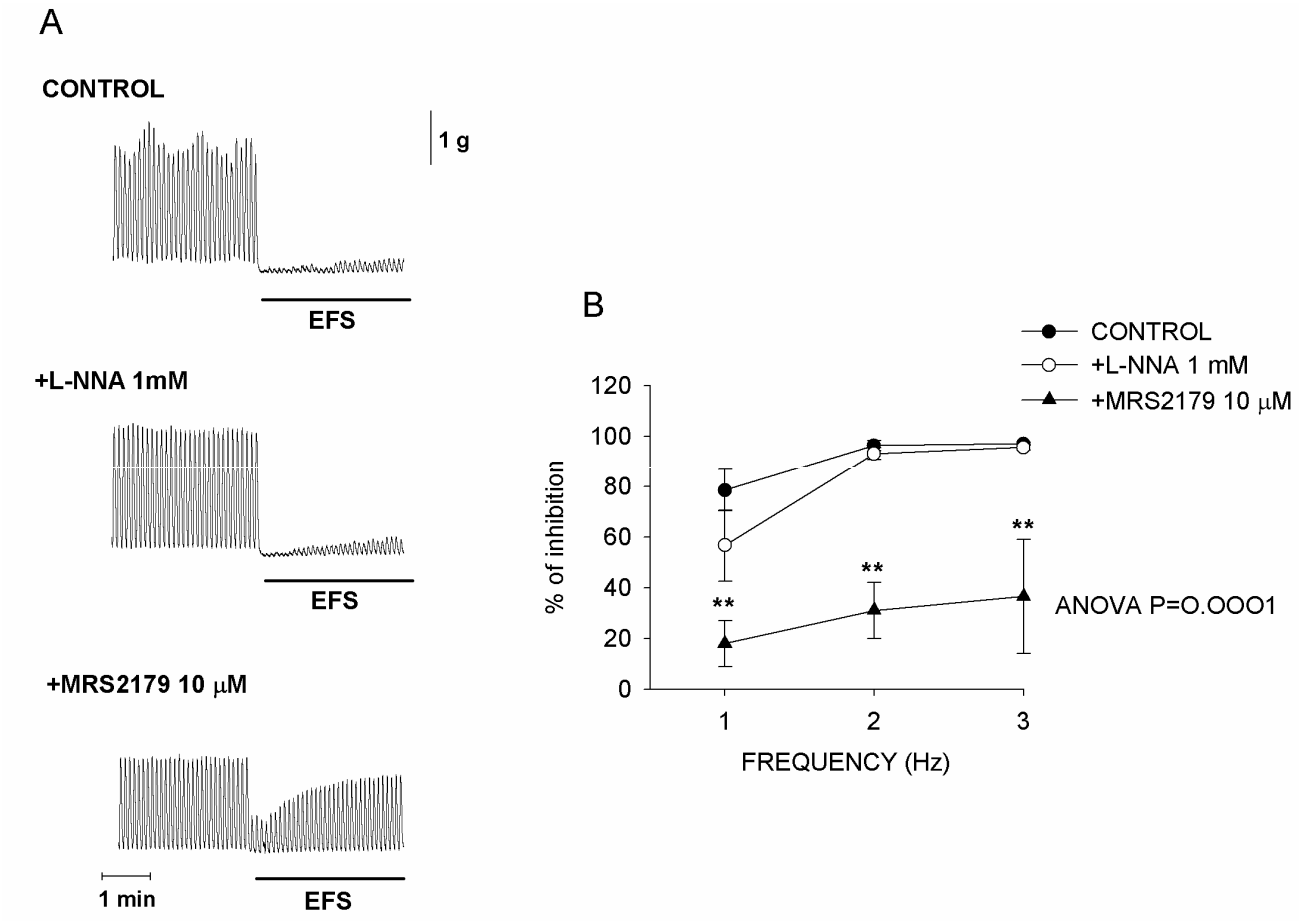



*Figure 4. (A) Mechanical recordings showing the inhibitory effect of EFS (frequency of stimulation of 2 Hz) showing the effect of L-NNA 1 mM and MRS 2179 (10μM) on the EFS response.(B) The effect of L-NNA and MRS 2179 on the percentage of inhibition (AUC) elicited by EFS at different frequencies (1, 2 and 3Hz) in the pig ileum. Data are expressed as mean±SEM (**p<0.01).*

## Evaluation of the apamin pathway on the purinergic neuromuscular transmission

Apamin 1μM was used to test whether small conductance calcium activated potassium channels were involved in the purinergic pathway in the pig ileal circular muscle. The non-nitrergic inhibition of mechanical activity induced by EFS was reduced

by apamin (1μM) to 15.70±7.85 % at 1Hz; 6.78±6.78 % at 2Hz and 3.56±3.56 % at 3Hz (n=4, p<0.0001) (Figure 5). In the presence of apamin (1μM) the IJP was abolished (n=4, p<0.01).

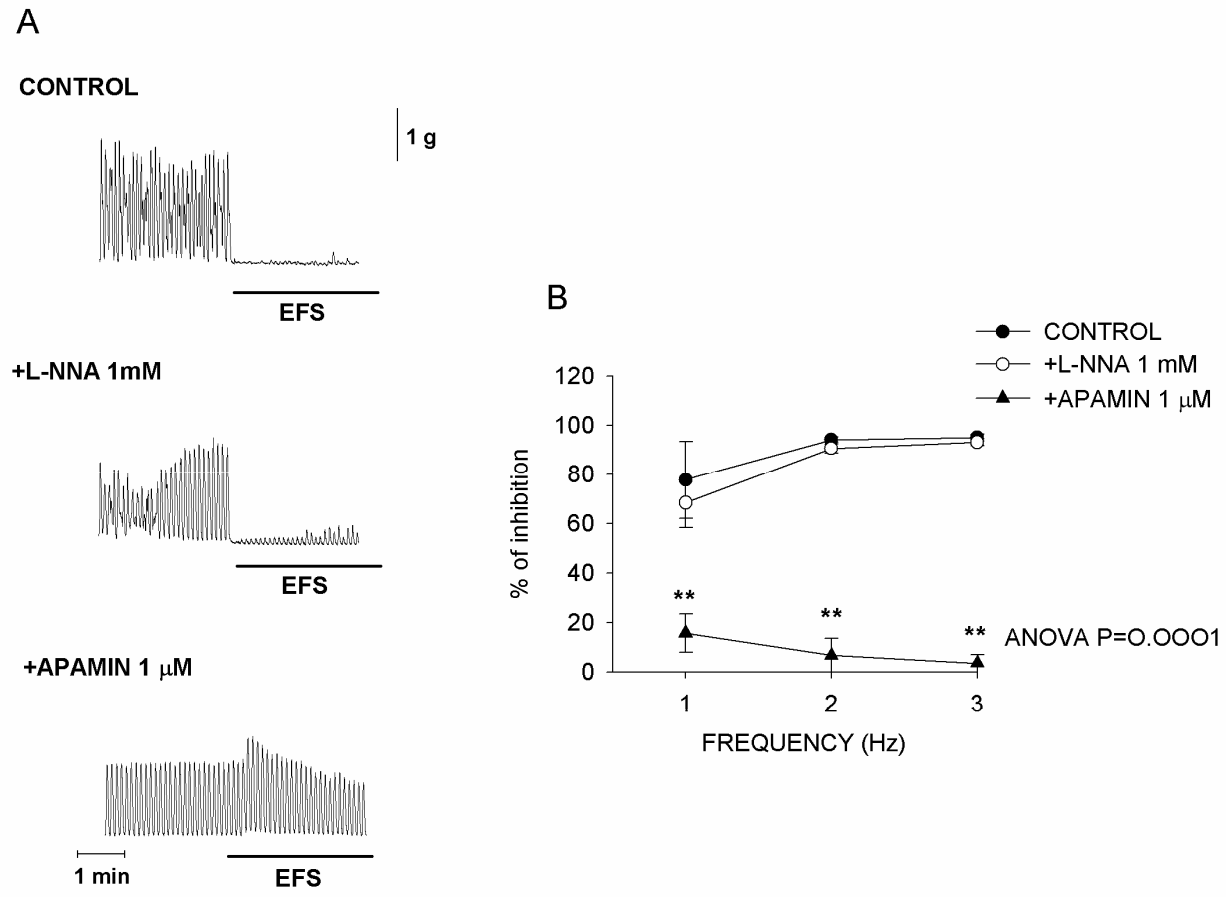



*Figure 5 .(A) Mechanical recordings showing the inhibitory effect of EFS (frequency of stimulation of 2 Hz) showing the effect of L-NNA 1 mM and apamin 1μM on the EFS response.(B) The effect of L-NNA and apamin on the percentage of inhibition (AUC) elicited by EFS at different frequencies ( 1, 2 and 3Hz) in the pig ileum. Data are expressed as mean±SEM (**p<0.01).*

## Effect of MRS 2179 on the NaNP-induced inhibition

To test whether MRS 2179 was acting on purinergic receptos and not downstream within the pathway, its effects on the inhibition induced by the nitric oxide donor NaNP were examined. After incubation of MRS 2179 (10μM), NaNP (10μM) caused a complete cessation of the spontaneous motility (n=4).

## Effect of $P2Y_1$ receptors in neuronal activation

In the guinea-pig LMMP preparation, $K^+$ (50 mM) induces a transient rise in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) neurons with an average in relative amplitude of 1.73 ± 0.02 (256 neurons from 21 ganglia, n = 3 guinea-pigs). ADPβS (1μM) induced a $[Ca^{2+}]_i$ rise in 43 ± 6 % of the neurons and only very few cells that were not identified by $K^+$ (10 cells in all of the recordings) responded to ADPβS. The relative amplitude of the ADPβS (1μM) responses -based on a series of different sets of recordings (see methods) was 1.55 ± 0.02 (382 neurons; n=11). The responses were not affected by the neural blocker TTX (1μM) (49 cells; n=4).

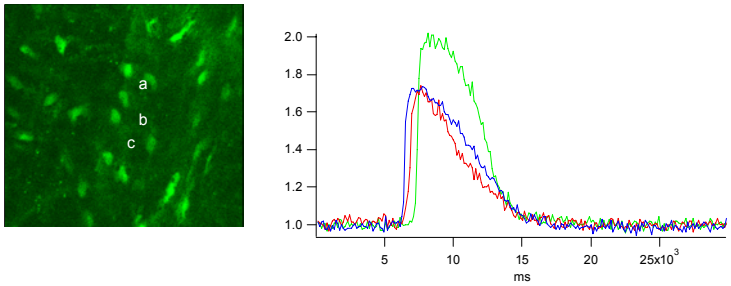
The putative antagonism of MRS 2179 (1μM) on the calcium transients induced by ADPβS was tested on 71 cells from 5 animals that were responders to ADPβS (Table 2). In the presence of MRS 2179 (1μM) 44 neurons did not respond to ADPβS. The remaining responding 27 neurons showed a decrease in the relative amplitude of the response and an increase in the time to peak and duration (Table 2).

| | ADPβS (1μM) | MRS 2179(1μM) +ADPβS (1μM) | Wash (10 min) ADPβS (1μM) |
|---|---|---|---|
| Responding cells | 71 | 27 | 65 |
| Amplitude (ratio) | 1.69±0.03 | 1.27±0.04 | 1.51±0.03 |
| Duration (s) | 9.04±0.20 | 13.18±0.64 | 9.42±0.31 |
| Time to peak (s) | 3.67±0.26 | 8.33±0.78 | 4.99±0.31 |

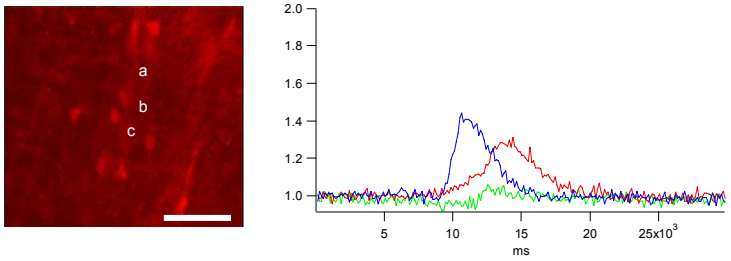
*Table 2. Calcium transients induced by ADPβS in control, in the presence of MRS 2179 and after washout. Data are expressed as mean± sem and compared with ANOVA ($p<0.001$) and post-hoc Bonferroni tests (** $p<0.001$).*

The localization of non-responding and partially responding cells was visualised by overlaying maximum intensity pictures that represent the maximal $[Ca^{2+}]_i$ responses in the presence and absence of the antagonist (Figure 6).

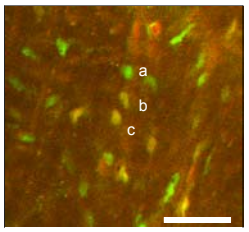
A- ADPβS




B- MRS 2179 (1μM) + ADPβS




C- Picture obtained matching A (green) and B




C- Washout (10 min.)

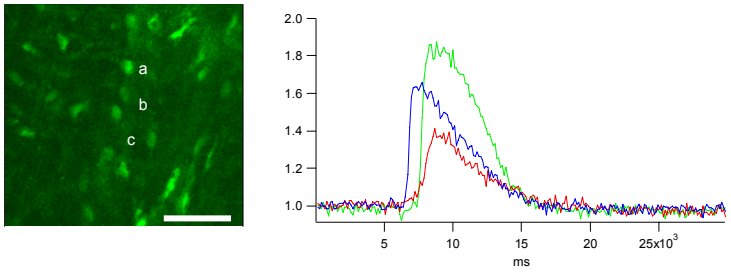



*Figure 6. The increase in fluorescence from the movie was calculated (tracing in right panels: a green; b blue and c red) and plotted in a single picture (left panels). A, B and D represent the increase in florescence after stimulation with ADPβS (1μM) in control (A), after incubation with MRS 2179 (1μM) (B) and after washout (D). Picture on C was obtained matching picture A (green) and B (red). In this picture, green cells (i.e. a) were neurons responding to ADPβS and non-responders in the presence of the antagonists and orange cells (i.e. b and c) were neurons with a decrease in the increase in fluorescence in guinea-pig LMMP preparations. The bar indicates 50 μm.*

The response to ADPβS partially was recovered after 10 minutes washout of the antagonist (Figure 6 and Table 2). Electrical Field Stimulation of a nerve strand caused a calcium transient in neurons of the nearby ganglion. 58 responding neurons (relative amplitude: 1.46±0.04) from 6 different animals were analysed. In the presence of MRS 2179 (1μM), the number of responding cells decreased to 38 and a drop in the relative amplitude (1.22±0.02; $p<0.001$) was observed. No time dependent effects or rundown could be observed (n=3).

## Immunohistochemistry

Positive $P2Y_1$ receptor immunoreactivity was present in the pig ileal circular and longitudinal smooth muscle layers. Some neurons of pig myenteric and submucosal ganglia were positively marked (Figure 7). A minor staining was observed in the muscularis mucosae. The staining was considered specific for $P2Y_1$ since preabsorption of the primary antibody abolished the immunoreactivity. Moreover, no immunoreactivity was seen in the absence of the primary antibody.

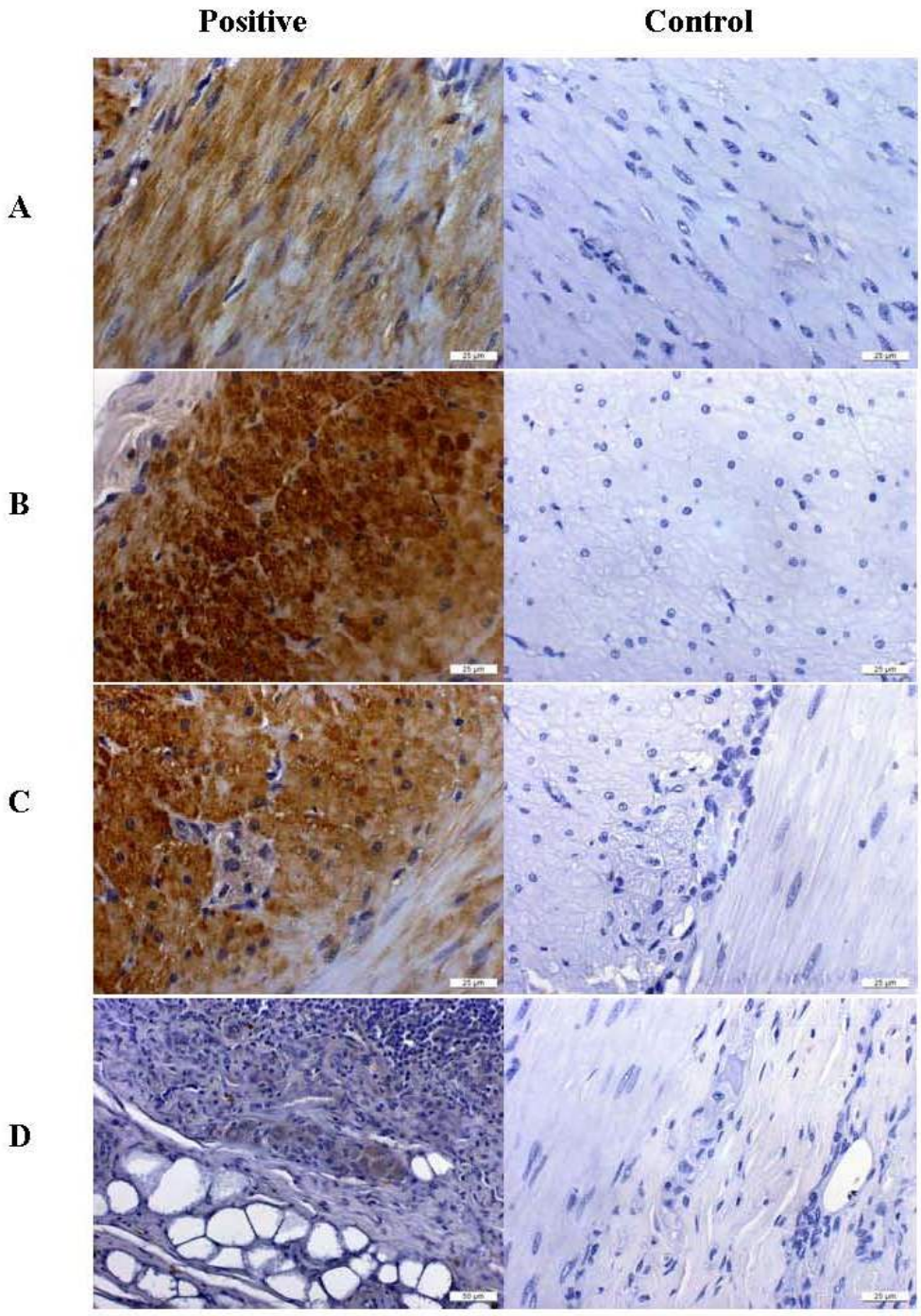



***Figure 7**. Immunohistochemical localization of $P2Y_1$ receptors in (A) pig ileal circular smooth muscle (positive sample and control) in absence of the primary antibody, (B) pig ileal longitudinal smooth muscle (positive sample and control), and (C) pig myenteric ganglia and (D) submucous ganglia (positive sample and control).*

## *Discussion*

The identity of the NANC inhibitory transmitters in the porcine gastrointestinal tract is still debated despite the importance of this animal as a potential source for intestinal xenotransplantation to humans (Farre *et al.*, 2006; Fernandez *et al.*, 1998; Jager & Van der Schaar, 1988; Matsuda *et al.*, 2004). Inhibitory neurotransmission can be mediated by PACAP (Matsuda *et al.*, 2004), nitric oxide and/or ATP (Farre *et al.*, 2006; Fernandez *et al.*, 1998). Probably a combination of several neurotransmitters in a complex mechanism of co-transmission is present in the gastrointestinal tract as suggested for other species (see for review Lecci *et al.*, 2002). However, the inhibitory junction potential induced by electrical field stimulation in the pig small intestine is L-NNA insensitive (Fernandez *et al.*, 1998) and shows a fast hyperpolarisation followed by an induced slow wave as it has been previously showed in other species (Cayabyab *et al.*, 1996). N-type calcium channels sensitive to ω-conotoxin (GVIA) are pre-junctionally involved in the release of the neurotransmitter responsible for the IJP (Borderies *et al.*, 1997; Cayabyab *et al.*, 1996; Matsuda *et al.*, 2004). The IJP in the pig ileum is sensitive to apamin (Fernandez *et al.*, 1998; Jager & Van der Schaar, 1988; Matsuda *et al.*, 2004) suggesting that small conductance calcium activated potassium channels are involved in the hyperpolarization as it has been shown in murine colonic isolated smooth muscle cells (Koh *et al.*, 1997). According to the original hypothesis of Burnstock (Burnstock *et al.*, 1970), in a previous paper we have suggested that ATP was mediating the IJP in the porcine ileum (Fernandez *et al.*, 1998) similar to the fast-component of the IJP described in several species including humans (Crist *et al.*, 1992; Xue *et al.*, 1999; Pluja *et al.*, 1999). However, due to the lack of specific antagonists it has been difficult to characterize the receptor involved in neuromuscular junction. MRS 2179 is considered a competitive selective $P2Y_1$ receptor antagonist (Alexander *et al.*, 2005; Gao *et al.*, 2006). MRS 2179 partially antagonised *1)* the effect of exogenous applied ATP in the murine gastrointestinal tract (Giaroni *et al.*, 2002) *2)* non nitrergic

relaxation induced by electrical field stimulation in the mouse jejunum and porcine lower oesophageal sphincter (De Man *et al.*, 2003; Farre *et al.*, 2006) and *3)* the fast component of the inhibitory junction potential in the guinea-pig ileum (Wang *et al.*, 2004; Wang *et al.*, 2007). We have recently shown that $P2Y_1$ receptors mediate the fast-component of the IJP and the non-nitrergic relaxation in the human colon (Gallego *et al.*, 2006). In the present paper we confirm our previous hypothesis showing that ATP was responsible for the fast component of the IJP and $P2Y_1$ receptors mediate the hyperpolarisation and relaxation in the pig small intestine. Our conclusions data are based on the following results: *1)* Purinergic agonists caused hyperpolarization and inhibition of motility in the presence of the neural blocker TTX, *2)* This inhibition and hyperpolarization are antagonised by MRS 2179, *3)* MRS 2179 antagonised the IJP and the non-nitrergic relaxation. *4)* $P2Y_1$ receptors are present in smooth muscle cells as well as in enteric neurons. Taken together, these results provide reasonable functional and pharmacological evidence to conclude that $P2Y_1$ receptors mediate purinergic neurotransmission in the porcine small intestine. In a previous paper it has been shown that PACAP mediated hyperpolarization was mediated by activation of apamin sensitive calcium activated potassium channels and ATP did not cause significant smooth muscle hyperpolarizations (Matsuda *et al.*, 2004). We do not have an explanation for these discrepancies although it is possible a co-transmission between ATP and a second neurotransmitter (NO, PACAP etc.) and the residual response observed in the mechanical recordings in the presence of MRS 2179 was attributable to a non purinergic neurotransmitter. Data about the nature of the receptor that mediates the fast component of the IJP on the human small intestine are still unavailable but it is plausible that also here ATP mediates the fast IJP (Xue *et al.*, 1999). To confirm the involvement of $P2Y_1$, the sensitivity of the small intestine human IJP to MRS 2179 has to be evaluated.

The presence of the receptor in a subpopulation of enteric neurons has been confirmed by inmunohistochemistry in several species including mouse ileum (Giaroni

*et al.*, 2002), pig small intestine (present work) and human colon (Gallego *et al.*, 2006). In order to visualize $P2Y_1$ activation and provide functional data about neuronal activity and receptor presence in myenteric neurons we have studied the effect of MRS 2179 on calcium transient induced by ADPβS and electrical stimulation in neurons of the guinea-pig ileum. This is a suitable method to study neuronal activity that involves a rise in intracellular calcium in neurons (Bisschops *et al.*, 2006; Vanden Berghe *et al.*, 2001). The transduction mechanism after activation of $P2Y_1$ receptors involves activation of phospholipase C, release of inositol 1,4,5-triphosphate and elevation of cytosolic free calcium (Gao *et al.*, 2006; Hu *et al.*, 2003). In the present work we have measured calcium transients in intact ganglia of guinea-pig ileum mediated by $P2Y_1$ receptor and activation of the signalling cascade described above. Our results confirm previous data obtained from a heterologous expression of cloned guinea-pig $P2Y_1$ receptors in HEK293 cells, where receptor activation caused an increase in intracellular calcium sensitive to MRS 2179 (Gao *et al.*, 2006). It is interesting to notice that neural blockade did not modify calcium transients indicating that the majority of the effect arises from direct activation of the $P2Y_1$ receptor in myenteric neurons. The functional role of $P2Y_1$ receptor expressing neurons in the myenteric plexus is unknown. In our experiments from myenteric plexus we have found that transients elicited by stimulation of nerve strands were decreased by MRS 2179 confirming the presence of synaptic transmission through $P2Y_1$ receptors in the myenteric plexus. In the mouse ileum the colocalization of $P2Y_1$ receptors and NOS in neurons of myenteric ganglia as well as in smooth muscle cells, suggests that activation of $P2Y_1$ receptors located in neurons stimulate NO/ATP release causing relaxation due to activation of cGMP (NO) and $P2Y_1$ receptors on smooth muscle (Giaroni *et al.*, 2002). Altogether these data suggest that $P2Y_1$ receptors participate in synaptic transmission between myenteric neurons and participate in inhibitory neuromuscular transmission. In contrast, in the guinea-pig submucosal plexus, the role of $P2Y_1$ receptors seems better characterised, here $P2Y_1$ receptors mediate slow EPSPs (Hu *et al.*, 2003;Monro *et al.*, 2004) and neurons

receiving purinergic excitatory inputs participate in mucosal secretion (Cooke *et al.*, 2004; Fang *et al.*, 2006).

We conclude that in the pig small intestine, purinergic neuromuscular transmission responsible for the IJP and non-nitrergic relaxation is mediated by $P2Y_1$ receptors located in smooth muscle cells. Functional $P2Y_1$ receptors are present in myenteric neurons of the guinea-pig ileum participating in synaptic transmission. $P2Y_1$ receptors might be important pharmacological targets to modulate several gastrointestinal functions such as transduction, motility and secretion. Although the presence of $P2Y_1$ receptors have recently been confirmed in the human colon (Gallego *et al.*, 2006), their role in the human small intestine needs further investigation.

# *Bibliography*

Alexander SP, Mathie A, & Peters JA (2005). Guide to receptors and channels, 1st edition (2005 revision). *Br J Pharmacol* **144 Suppl 1**, S1-128.

Bisschops R, Vanden Berghe P, Sarnelli G, Janssens J, & Tack J (2006). CRF-induced calcium signaling in guinea pig small intestine myenteric neurons involves CRF-1 receptors and activation of voltage-sensitive calcium channels. *Am J Physiol Gastrointest Liver Physiol* **290**, G1252-G1260.

Borderies JR, Gonalons E, Angel F, Vergara P, & Jimenez M (1997). Effect of different calcium channel blockers on inhibitory junction potentials and slow waves in porcine ileum. *Life Sci* **60**, 883-892.

Burnstock G (2004). Introduction: P2 receptors. *Curr Top Med Chem* **4**, 793-803.

Burnstock G, Campbell G, Satchell D, & Smythe A (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* **40**, 668-688.

Camaioni E, Boyer JL, Mohanram A, Harden TK, & Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem* **41**, 183-190.

Cayabyab FS, deBruin H, Jimenez M, & Daniel EE (1996). Ca2+ role in myogenic and neurogenic activities of canine ileum circular muscle. *Am J Physiol* **271**, G1053-G1066.

Cooke HJ, Xue J, Yu JG, Wunderlich J, Wang YZ, Guzman J, Javed N, & Christofi FL (2004). Mechanical stimulation releases nucleotides that activate P2Y1 receptors to trigger neural reflex chloride secretion in guinea pig distal colon. *J Comp Neurol* **469**, 1-15.

Crist JR, He XD, & Goyal RK (1992). Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *J Physiol* **447**, 119-131.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

Fang X, Hu HZ, Gao N, Liu S, Wang GD, Wang XY, Xia Y, & Wood JD (2006). Neurogenic secretion mediated by the purinergic P2Y1 receptor in guinea-pig small intestine. *Eur J Pharmacol* **536**, 113-122.

Farre R, Auli M, Lecea B, Martinez E, & Clave P (2006). Pharmacologic characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter. *J Pharmacol Exp Ther* **316**, 1238-1248.

Fernandez E, Guo X, Vergara P, & Jimenez M (1998). Evidence supporting a role for ATP as non-adrenergic non-cholinergic inhibitory transmitter in the porcine ileum. *Life Sci* **62**, 1303-1315.

Gallego D, Hernandez P, Clave P, & Jimenez M (2006). P2Y1 receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. *Am J Physiol Gastrointest Liver Physiol*.

Galligan JJ & North RA (2004). Pharmacology and function of nicotinic acetylcholine and P2X receptors in the enteric nervous system. *Neurogastroenterol Motil* **16 Suppl 1**, 64-70.

Gao N, Hu HZ, Zhu MX, Fang X, Liu S, Gao C, & Wood JD (2006). The P2Y purinergic receptor expressed by enteric neurones in guinea-pig intestine. *Neurogastroenterol Motil* **18**, 316-323.

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, & Burnstock G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**, 1313-1323.

Glanzel M, Bultmann R, Starke K, & Frahm AW (2003). Constitutional isomers of Reactive Blue 2 - selective P2Y-receptor antagonists? *Eur J Med Chem* **38**, 303-312.

Hu HZ, Gao N, Zhu MX, Liu S, Ren J, Gao C, Xia Y, & Wood JD (2003). Slow excitatory synaptic transmission mediated by P2Y1 receptors in the guinea-pig enteric nervous system. *J Physiol* **550**, 493-504.

Jager LP & Van der Schaar MW (1988). Characterization of inhibitory innervation in porcine colonic circular muscle. *Can J Physiol Pharmacol* **66**, 1386-1397.

Johnson PJ, Shum OR, Thornton PD, & Bornstein JC (1999). Evidence that inhibitory motor neurons of the guinea-pig small intestine exhibit fast excitatory synaptic potentials mediated via P2X receptors. *Neurosci Lett* **266**, 169-172.

Katayama Y & Morita K (1989). Adenosine 5'-triphosphate modulates membrane potassium conductance in guinea-pig myenteric neurones. *J Physiol* **408**, 373-390.

Koh SD, Dick GM, & Sanders KM (1997). Small-conductance Ca(2+)-dependent K+ channels activated by ATP in murine colonic smooth muscle. *Am J Physiol* **273**, C2010-C2021.

Lecci A, Santicioli P, & Maggi CA (2002). Pharmacology of transmission to gastrointestinal muscle. *Curr Opin Pharmacol* **2**, 630-641.

LePard KJ, Messori E, & Galligan JJ (1997). Purinergic fast excitatory postsynaptic potentials in myenteric neurons of guinea pig: distribution and pharmacology. *Gastroenterology* **113**, 1522-1534.

Matsuda NM, Miller SM, Sha L, Farrugia G, & Szurszewski JH (2004). Mediators of non-adrenergic non-cholinergic inhibitory neurotransmission in porcine jejunum. *Neurogastroenterol Motil* **16**, 605-612.

Monro RL, Bertrand PP, & Bornstein JC (2004). ATP participates in three excitatory postsynaptic potentials in the submucous plexus of the guinea pig ileum. *J Physiol* **556**, 571-584.

Pluja L, Fernandez E, & Jimenez M (1999). Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol* **126**, 883-892.

Ralevic V & Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**, 413-492.

Raybould HE, Cooke HJ, & Christofi FL (2004). Sensory mechanisms: transmitters, modulators and reflexes. *Neurogastroenterol Motil* **16 Suppl 1**, 60-63.

Vanden Berghe P, Missiaen L, Bellon E, Vanderwinden JM, Janssens J, & Tack J (2001). Free cytosolic Ca2+ recordings from myenteric neurones in multilayer intestinal preparations. *Neurogastroenterol Motil* **13**, 493-502.

Wang GD, Hu HZ, Wang XY, Gao N, Liu SM, Fang XC, Xia Y, & Wood JD (2004). Purinergic inhibitory neuromuscular transmission mediated by the P2Y(1) receptor in guinea-pig small intestine. *Gastroenterology* **126**, A275.

Wang GD, Wang XY, Hu HZ, Liu S, Gao N, Fang X, Xia Y, & Wood JD (2007). Inhibitory Neuromuscular Transmission Mediated by the P2Y1 Purinergic Receptor in Guinea-Pig Small Intestine. *Am J Physiol Gastrointest Liver Physiol*.

Wood JD (2006). The enteric purinergic P2Y1 receptor. *Curr Opin Pharmacol* **6**, 564-570.

Xue L, Farrugia G, Sarr MG, & Szurszewski JH (1999). ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *Am J Physiol* **276**, G1373-G1379.

# Capítulo 3

## Purinergic and Nitrergic junction potential in the human colon


### *Abstract*

The aim of the present work is to investigate a putative junction transmission (NO and ATP) in the human colon and to characterize the electrophysiological and mechanical responses that might explain different functions from both neurotransmitters. Muscle bath and microelectrode techniques were performed on human colonic circular muscle strips obtained from surgical specimens. Results: *1)* The nitric oxide donor NaNP (10 μM) but not the P2Y receptor agonist ADPβS (10μM) is able to cause a sustained relaxation. *2)* Electrical field stimulation (EFS) at 1Hz causes fast Inhibitory Junction Potententials (IJPs) and a relaxation sensitive to MRS 2179 (10 μM). *3)* EFS at higher frequencies (5Hz) shows a biphasic IJP with fast hyperpolarization sensitive to MRS 2179 (10μM) followed by sustained hyperpolarization sensitive to L-NNA (1mM); this electrophysiological response is well correlated to the mechanical inhibition also obtained at 2 and 5 Hz when both drugs are needed to fully block the EFS-relaxation. *4)* Two consecutive single pulses induce MRS 2179-sensitive fast IJPs that show a rundown. *5)* The rundown mechanism is not dependent on the degree of hyperpolarization and it is present after incubation with L-NNA (1mM), Hexamethonium (100 μM), MRS 2179 (1μM) or NF023 (10μM). We conclude *1)* Single pulses elicit ATP release from enteric motor neurons that cause a fast IJP and a transient relaxation that is difficult to maintain over time; *2)* Nitric oxide is released at higher frequencies causing a sustained hyperpolarization and relaxation. These differences might be responsible for complementary mechanisms being phasic (ATP) and tonic (NO) relaxations.

## *Introduction*

The mechanisms involved in non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmission are responsible for several physiological functions such as gastric accommodation, sphincter relaxation, and descending phase during the peristaltic reflex. The final step of these mechanisms is the release of inhibitory neurotransmitter(s) at the neuromuscular junction responsible for smooth muscle relaxation. The identity of the neurotransmitter(s) implicated in the inhibitory pathway is still being debated, but ATP and Nitric Oxide are recognized nowadays as two of the major inhibitory mediators in the gastrointestinal (GI) tract. It is possible that other neurotransmitters such as CO or VIP/PACAP might be released from inhibitory motor-neurons contributing to smooth muscle relaxation. However, most of the data available in the literature concerns animal samples and little data is published translating these results to the human gastrointestinal tract.

Several experiments show that nitric oxide is a potent inhibitory neurotransmitter in the GI tract (Bult *et al.*, 1990): *1)* Nitrergic neurons expressing neural nitric oxide synthase (nNOS) are present in the gut (Matini *et al.*, 1995;Timmermans *et al.*, 1994), *2)* Inhibitors of NOS, such as L-NNA or L-NAME, inhibit neural mediated relaxation (Boeckxstaens *et al.*, 1993), inhibit the slow component of the inhibitory junction potential (IJPs) and increase muscular contractions (Keef *et al.*, 1993) *3)* Nitric oxide (NO) donors cause smooth muscle hyperpolarization and relaxation (Boeckxstaens *et al.*, 1993; Keef *et al.*, 1993) *4)* NO causes activation of Guanylate cyclase increasing intracellular cGMP levels (De Man *et al.*, 2007). All these results demonstrate that nitrergic neurons have the apparatus of NO synthesis and NO released from inhibitory motor neurons cause smooth muscle relaxation. However, several data are inconsistent with the hypothesis that nitric oxide is the sole NANC inhibitory neurotransmitter in the human GI tract: *1)* The fast component of the inhibitory junction potential (IJPf) is L-NNA insensitive (Gallego *et al.*,

2006; Keef *et al.*, 1993) *2)* In the presence of NOS inhibitors, an important NANC-non nitrergic relaxation is present (Boeckxstaens *et al.*, 1993; Tam & Hillier, 1992; Undi *et al.*, 2006). These results are consistent with the presence of at least a complementary inhibitory neurotransmitter.

In 1970, ATP was proposed as an inhibitory neurotransmitter in the gastrointestinal tract (Burnstock *et al.*, 1970). However, due to the lack of specific antagonist, the identity of the receptor involved in the relaxation has been difficult to establish. Suramin and PPADS are nonselective pururinoceptor antagonists that have been used to study the non-nitrergic relaxation in the human ileum and colon (Benko *et al.*, 2007; Undi *et al.*, 2006). However, MRS 2179, which is the N6-methyl modification of 2'-deoxyadenosine 3',5'-bisphosphate, is a potent $P2Y_1$ receptor antagonist (Camaioni *et al.*, 1998) and it is currently considered competitive and specific (Alexander *et al.*, 2005;Hu *et al.*, 2003). $P2Y_1$ receptors have several physiological functions in the gastrointestinal tract (Wood, 2006). $P2Y_1$ receptors are present both in enteric neurons (Gao *et al.*, 2006) and smooth muscle cells (Gallego *et al.*, 2006) and might participate in smooth muscle relaxation (De Man *et al.*, 2003), synaptic transmission (Hu *et al.*, 2003) and neurogenic secretion (Fang *et al.*, 2006).We have recently shown that $P2Y_1$ receptors are localized in smooth muscle cells and are responsible for the fast component of the IJP and the NANC non-nitrergic relaxation (Gallego *et al.*, 2006). In addition, we have found that both L-NNA and MRS 2179 are required to fully block mechanical relaxation induced by stimulation of inhibitory motorneurons by EFS or through nicotinic receptors in the human colon (Auli *et al.*, 2005). All these data are consistent with a putative role of purinergic inhibitory neurons causing the release of ATP, or a related purine, acting through $P2Y_1$ receptors and causing smooth muscle hyperpolarization and relaxation (Gallego *et al.*, 2006). At present, the relative contribution of purinergic and nitrergic neurotransmission mediating smooth muscle hyperpolarization and relaxation is unknown. The aim of the present paper is to investigate the electrophysiological basis of inhibitory transmission

in the human colon and to examine whether both inhibitory neurotransmitters are redundant or complementary.

## *Materials and methods*

### Tissue Preparation

Specimens of distal and sigmoid colon (N=31) were obtained from patients aged 47 to 80 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 in a dissection dish, and the mucosal layer was gently removed. Circular muscle strips, 10 mm x 4 mm, were cut. The patients provided informed consent and the experimental procedure was approved by the ethics committee of the Hospital of Mataró (Barcelona, Spain).

### Mechanical Experiments

Muscle strips were examined in a 10 ml organ bath filled with Krebs solution at 37±1ºC containing phentolamine, atropine and propranolol (each 1μM) to block adrenergic and muscarinic receptors. An isometric force transducer (Harvard VF-1) connected to an amplifier was used to record the mechanical activity. Data were digitalized (25 Hz) using Datawin1 software (Panlab-Barcelona) coupled to an ISC-16 A/D card installed in a PC computer. A tension of 4 g was applied and the tissue was allowed to equilibrate for 1 hour. After this period, strips displayed spontaneous phasic activity. Electrical field stimulation (EFS) was applied for 2 minutes (pulse duration 0.4 ms, frequency 1, 2 and 5 Hz, and amplitude 50 V).

### Data analyses and statistics

To estimate the responses to drugs, the area under the curve (AUC) of spontaneous contractions from the baseline was measured before and after drug addition (each 2 min) or before and during EFS (each 30s). In order to normalize data, the value of AUC obtained before the treatment was considered to be 100 and the percentage of inhibition of the spontaneous motility was estimated with the AUC

obtained after the treatment. One-way ANOVA followed by a Bonferroni post-hoc test was performed to compare the response measured in each interval of time and control. N values represent the number of strips from different patients.

## Electrophysiological Experiments

Muscle strips were dissected parallel to the circular muscle and placed in a Sylgard-coated chamber continuously perfused with NANC Krebs solution at 37±1ºC. Strips were meticulously pinned in a cross-sectioned slab allowing microelectrode recordings from both circular and longitudinal muscles. This procedure was previously reported with canine ileum (Jimenez *et al.*, 1996). Preparations were allowed to equilibrate for approximately 1 hour before experiments started. Circular and longitudinal muscle cells were impaled with glass microelectrodes (40-60 MΩ) filled with 3 M KCl. Membrane potential was measured using standard electrometer Duo773 (WPI Inc., FL, USA). Tracings were displayed on an oscilloscope 4026 (Racal-Dana Ltd., England), and simultaneously digitalized (100 Hz) using EGAA software coupled to an ISC-16 A/D card (RC Electronics Inc., CA, USA) installed in a computer. Electrical field stimulation (EFS) was applied using two silver chloride plates placed perpendicular to the longitudinal axis of the preparation and 1.5 cm apart. To obtain stable impalements, nifedipine (1μM) was perfused to abolish mechanical activity.

## Stimulation protocols and data analysis

Trains (supramaximal voltage 50V, 0.3ms) of 5 seconds were performed at 1Hz (5 pulses: P1 to P5) and 5Hz (25 pulses). The amplitude of the 5 IJPs was calculated when 1Hz (5 pulses) were applied. At 5Hz, the response consisted of a fast component followed by a slow one (see results). In this case the fast component was estimated with the maximum amplitude and the slow component was estimated at 2.5 and 3.75s after the beginning of the stimulus. A two way ANOVA test was performed to compare

data before and after drug addition. N values represent the number of strips from different patients

Two pulses (pulse duration: 0.3 ms) were performed at the same time from 1 to 20s. Both pulses had the same voltage and depending on the protocol the voltage was 20V, 30V or 50V. The response consisted of a fast IJP1 elicited by the first pulse followed by a second IJP2 elicited by the second pulse. The ratio Y=IJP2/IJP1 was plotted vs X=time interval between pulses. Data were fitted with an curve with an initial plateau at 0 (IF:X<X0 Y=0) followed by an exponential curve (IF: X>X0 Y=100*(1 - exp(-k*(X-X0)))). X0, K and $R^2$ were calculated using a non linear regression with GraphPad Prism software version 4.00 (GraphPad Software, San Diego California USA). N values represent the number of strips from different patients and n values the number of pair data analyzed.

## Solutions and Drugs

The composition of the Krebs 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 $MgSO_4$ bubbled with a mixture of 5% $CO_2$:95% $O_2$ (pH 7.4). The following drugs were used: Nifedipine, Nω-nitro-L-arginine (L-NNA), adenosine 5'-triphosphate (ATP), adenosine 5´-O-2-thiodiphosphate (ADPβS), apamin, phentolamine; tetrodotoxin (TTX), atropine sulphate, propranolol, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), adenosine, hexamethonium chloride (Sigma Chemicals, St. Louis, USA) sodium nitroprusside (NaNP) (Research Biochemicals International, Natick, USA), MRS 2179, NF023 (Tocris, Bristol UK). Stock solutions were made by dissolving drugs in distilled water except for nifedipine which was dissolved in ethanol (96%) (0.01% final concentration), and DPCPX which was dissolved in DMSO (0.01% final concentration).

# *Results*

## Mechanical responses in the human colon

Circular muscle strips showed spontaneous mechanical activity at a frequency of 2.79 ± 0.1 contractions/min and amplitude 2.93 ± 0.4 g (N=28). In the presence of the neural blocker TTX, the nitric oxide donor NaNP 10 μM abolished the spontaneous motility. The inhibitory effect was prominent for more than 10 minutes (n=5) (Figure 1). In contrast, ATP (1mM, n=4) and ADPβS (10 μM, n=6) (a preferential and stable P2Y agonist) transiently inhibited the motility but spontaneous contractions were recorded during the incubation of purinergic agonist starting about 5 to 6 minutes after drug addition (Figure 1).

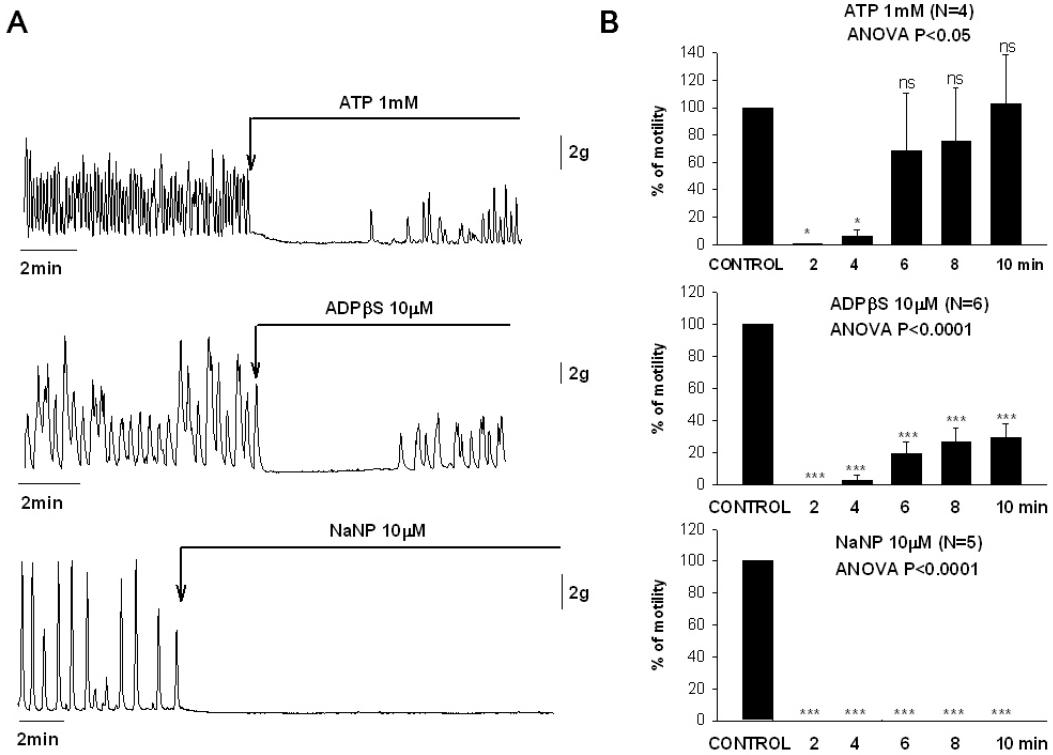



*Figure 1. (A) Mechanical recordings from human colonic circular muscle strips showing the inhibition of the spontaneous motility caused by ATP 1mM, ADPβS 10μM and NaNP 10μM (B) Histograms showing the time course of the inhibition during drug incubation. Data are expressed as mean±sem. *P < 0.05; ***P < 0.001.*

Electrical field stimulation (EFS) (1, 2, 5 Hz) for 2 minutes caused almost complete cessation of spontaneous motility (Figures 2, 3 and 4).

In all cases, during the period of EFS, spontaneous motility was <20% of the basal values. In order to study the putative nitrergic and purinergic co-transmission the following protocols were performed for each frequency of stimulation: *1)* Study of the nonnitrergic inhibitory component: EFS was applied in the presence of L-NNA (1mM); *2)* Study of the nonpurinergic inhibitory component: EFS was applied in the presence of MRS 2179 (10μM) and *3)* Study of the response during EFS during simultaneous blockade of both nitrergic and purinergic components by both L-NNA and MRS 2179 (Figures 2, 3 and 4).

At 1Hz (Figure 2), the EFS-inhibition was still observed in the presence of L-NNA (1mM) (ANOVA $p<0.001$ vs. control) but MRS 2179 (10μM) partially antagonized EFS-relaxation (ANOVA: ns vs. basal values). Simultaneous addition of both MRS 2179 and L-NNA fully blocked the inhibition of spontaneous motility induced by EFS (ANOVA: ns. vs. control). These results suggest that at this frequency of stimulation the response was mainly MRS 2179 (10μM)-sensitive and was therefore considered mainly purinergic with a minor nitrergic component.

At 2 Hz and 5 Hz (Figure 3 and 4) a strong inhibition was observed in the presence of MRS 2179 (10μM) and in the presence of L-NNA (1mM). In the presence of both MRS 2179 and L-NNA the inhibition of spontaneous motility during EFS was not observed (n.s). In some recordings a non-cholinergic contractile response was observed. These results suggest that at this range of frequencies the response was partially MRS 2179 and L-NNA sensitive and therefore was considered both purinergic and nitrergic.

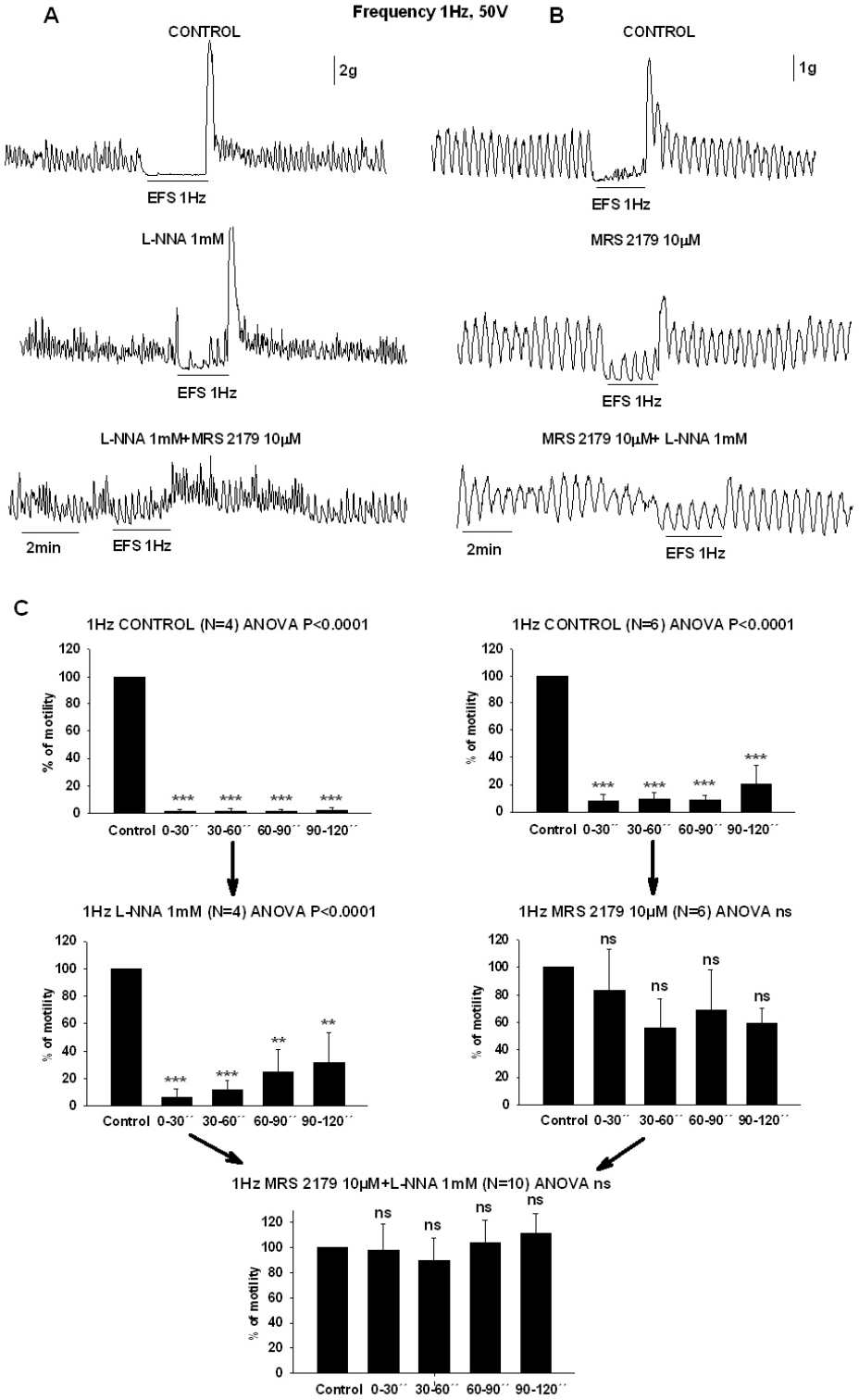



*Figure 2. Mechanical recordings from human colonic circular muscle strips showing the inhibition of the spontaneous motility caused by EFS (1Hz, 50V) in control conditions (A and B, top) , after L-NNA (1mM) incubation (A middle), MRS 2179 (10 µM) incubation (B middle) and incubation with both L-NNA and MRS 2179 (A and B bottom). (C) Histograms showing the percentage of inhibition measured at 30s interval during the stimulation period and in each of the above experimental conditions. Data are expressed as mean±sem. **P < 0.01; ***P < 0.001.*

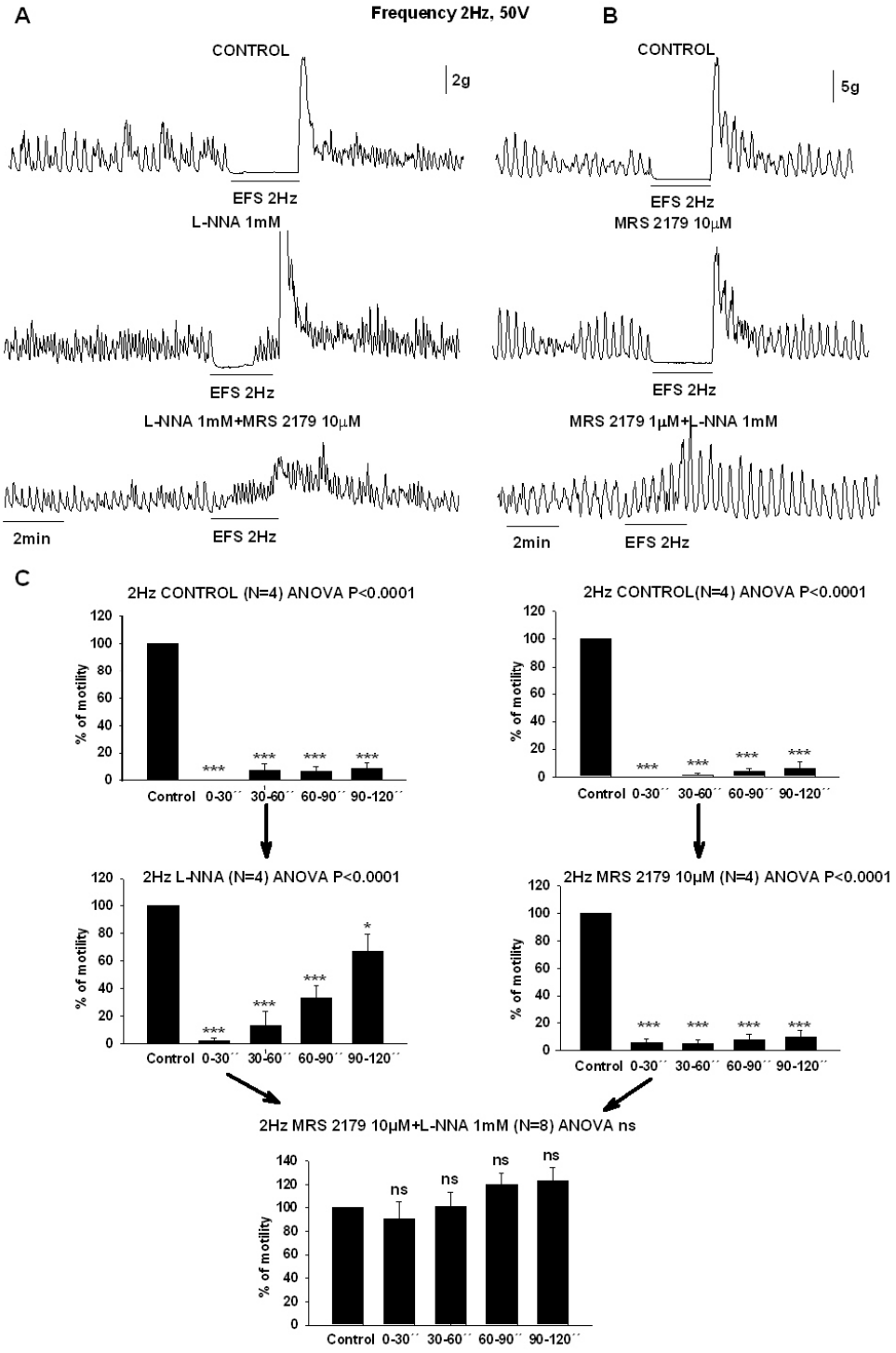



*Figure 3. Mechanical recordings from human colonic circular muscle strips showing the inhibition of the spontaneous motility caused by EFS (2Hz, 50V) in control conditions (A and B, top), after L-NNA (1mM) incubation (A middle), MRS 2179 (10μM) incubation (B middle) and incubation with both L-NNA and MRS 2179 (A and B bottom). (C) Histograms showing the percentage of inhibition measured at 30s interval during the stimulation period and in each of the above experimental conditions. Data are expressed as mean±sem. *P < 0.05; ***P < 0.001.*

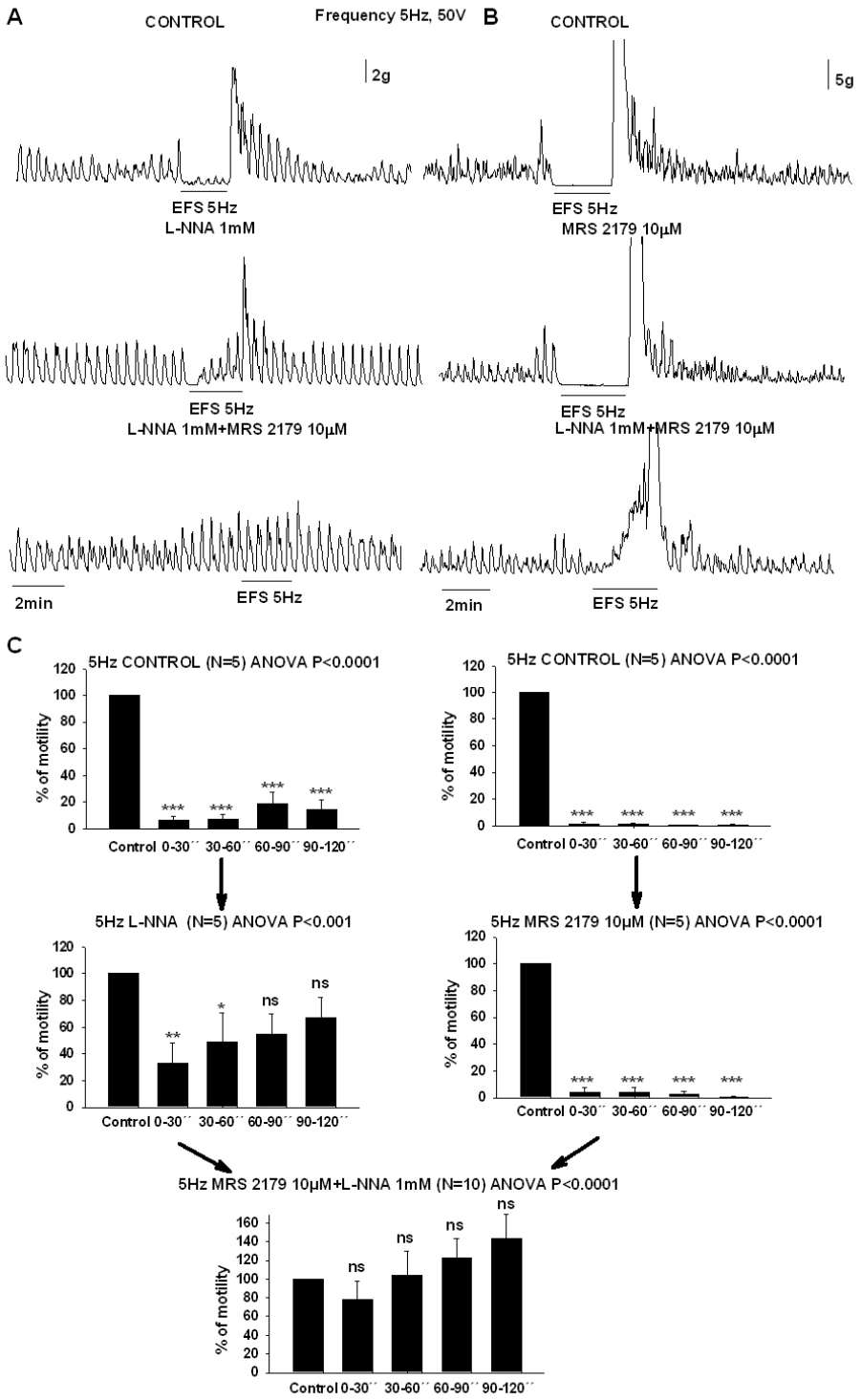



*Figure 4. Mechanical recordings from human colonic circular muscle strips showing the inhibition of the spontaneous motility caused by EFS (5Hz, 50V) in control conditions (A and B, top), after L-NNA (1mM) incubation (A middle), MRS 2179 (10µM) incubation (B middle) and combination of both L-NNA and MRS 2179 (A and B bottom). (C) Histograms showing the percentage of inhibition measured at 30s interval during the stimulation period and in each of the above experimental conditions. Data are expressed as mean±sem. *P < 0.05; **P < 0.01; ***P < 0.001.*

It is important to notice that at 2 Hz and 5 Hz when the purinegic component was blocked with MRS 2179 (10μM) the remaining nitrergic component could abolish the mechanical activity during all the EFS. In contrast, when the nitrergic component was inhibited, the time course of the inhibitory response was different, being a greater inhibition at the beginning of the EFS and partially recovering with time (Figures 3 and 4).

## Junction potential in the human colon

Electrical field stimulation at 1 Hz and 50V for 5 seconds generates 5 consecutive pulses (P1 to P5). The response consisted of a first fast IJP of about 15 to 20mV recorded after the first pulse (P1), followed by a very small response after the second pulse (P2) and fast IJPs of about 5 to 10 mV after the other three pulses (P3, P4 and P5) (Figure 5). The response was almost abolished by MRS 2179 10 μM (N=5) and a minor difference was observed in the presence of L-NNA 1mM (N=5) (Figure 5). The response obtained when the tissue was incubated with both MRS 2179 10 μM and L-NNA 1mM (n=10) was similar to the response obtained with MRS 2179 10 μM alone. Accordingly, the response at 1 Hz was considered mainly purinergic through $P2Y_1$ receptors.

Electrical field stimulation at 5 Hz for 5 seconds generates 25 consecutive pulses. At this frequency the response consisted of an IJP with a fast component followed by a sustained one (Figure 6). The amplitude of the fast component was about 20 to 25 mV whereas the slow component measured at 2.5 s and 3.75 s after the beginning of the stimulus was about 10 to 15mV. L-NNA (1mM) did not modify the fast component but significantly reduced the slow component measured at 2.5 s and 3.75 s after the beginning of the stimulus. In contrast, addition of MRS 2179 (10μM) decreased the fast component and no major effect on the slow component was observed (Figure 6).

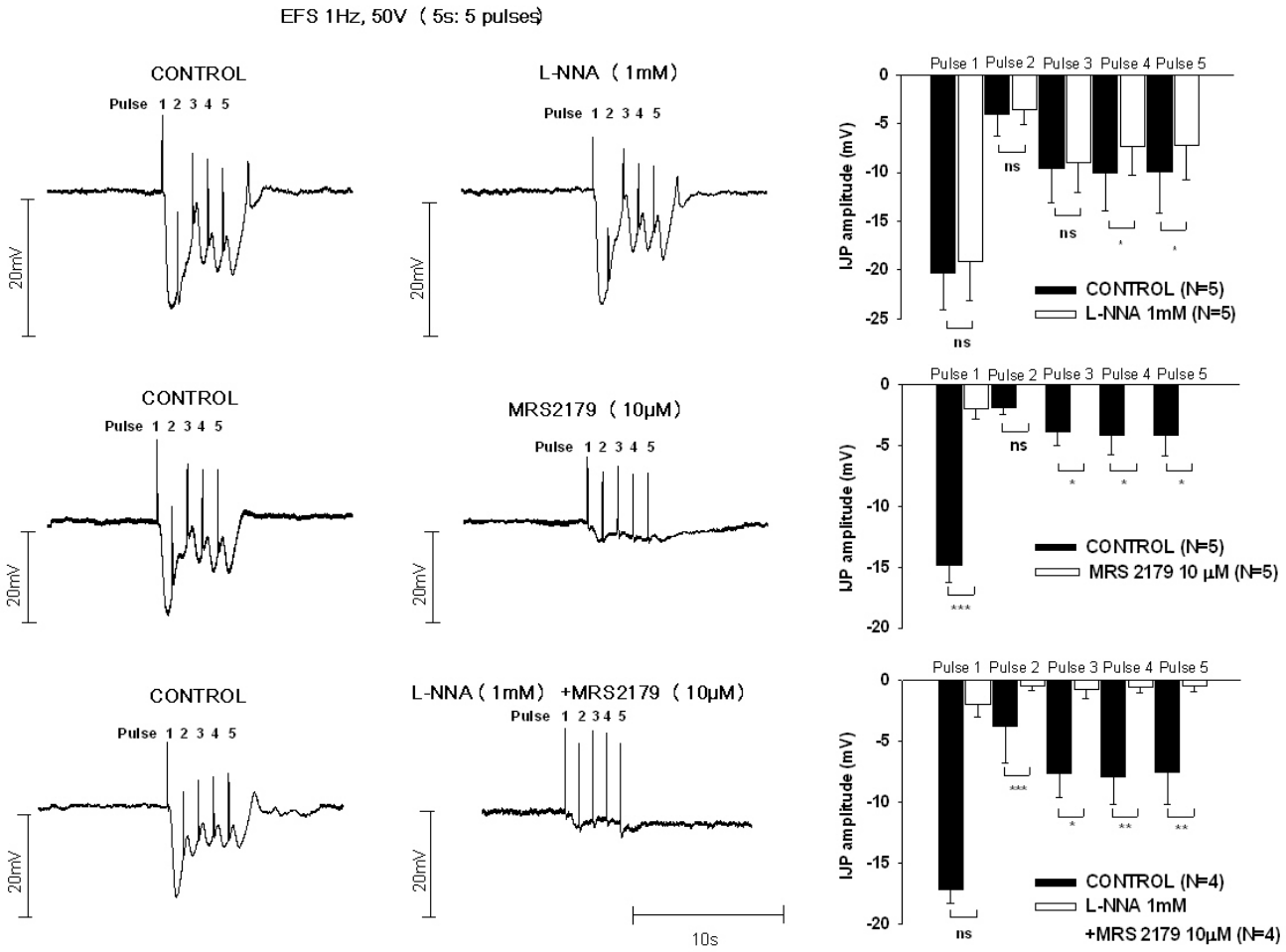



*Figure 5. (A) Representative recordings of IJPs from human colonic circular muscle cells obtained after 1Hz and 5s stimulation (5 pulses: P1 to P5) in control conditions, after L-NNA (1mM), MRS 2179 (10$\mu$M) or incubation of both inhibitors. (B) Histogram showing the response from each pulse (P1 to P5) in each experimental condition. *P < 0.05; **P < 0.01; ***P < 0.001.*

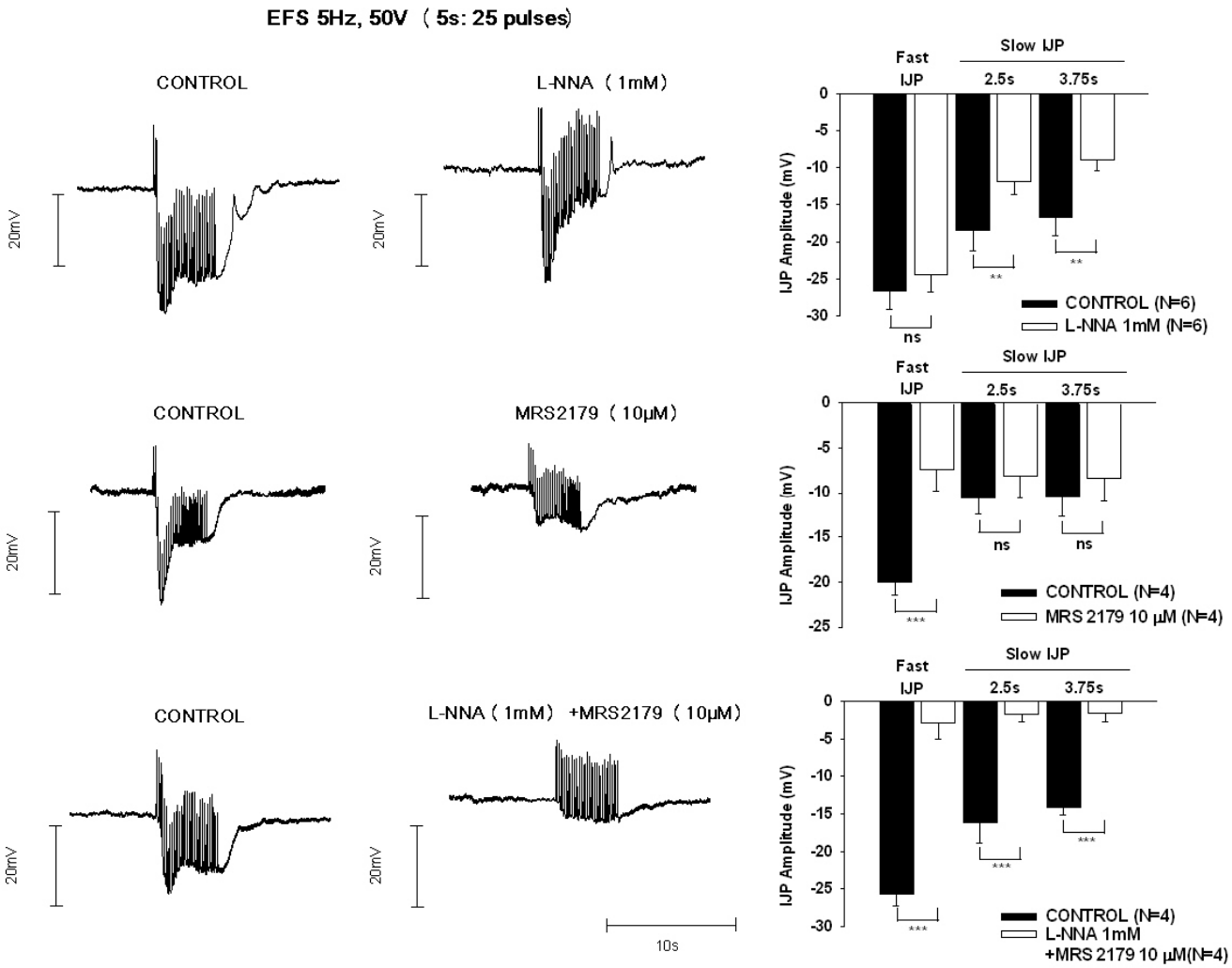



*Figure 6. (A) Representative recordings of IJPs from human colonic circular muscle cells obtained after 5Hz and 5s stimulation (25 pulses) in control conditions, after L-NNA (1mM), MRS 2179 (10μM) or incubation of both inhibitors. (B) Histogram showing the response from the fast component and sustained component (measured at 2.5 and 3.75s after the beginning of the stimuli) in each experimental condition. **P < 0.01; ***P < 0.001.*

Addition of both L-NNA (1mM) and MRS 2179 (10 μM) completely inhibited both the slow and fast component. In order to illustrate the co-transmission mechanism a subtraction between the response in control conditions and the response in the

presence of both L-NNA and MRS 2179 was performed (Figure 6). In this case, the subtraction of the response obtained in control minus the response obtained in the presence of L-NNA consisted mainly of a slow component, corresponding to the response attributable to NO release. In contrast, the subtraction of the response obtained in control-MRS 2179 consisted of a fast component corresponding to the response attributable to a purine acting on $P2Y_1$ receptors. Notice that when the subtraction of the response in control and the response obtained after incubation with L-NNA and MRS 2179 both the fast and slow components were present (Figure 7).

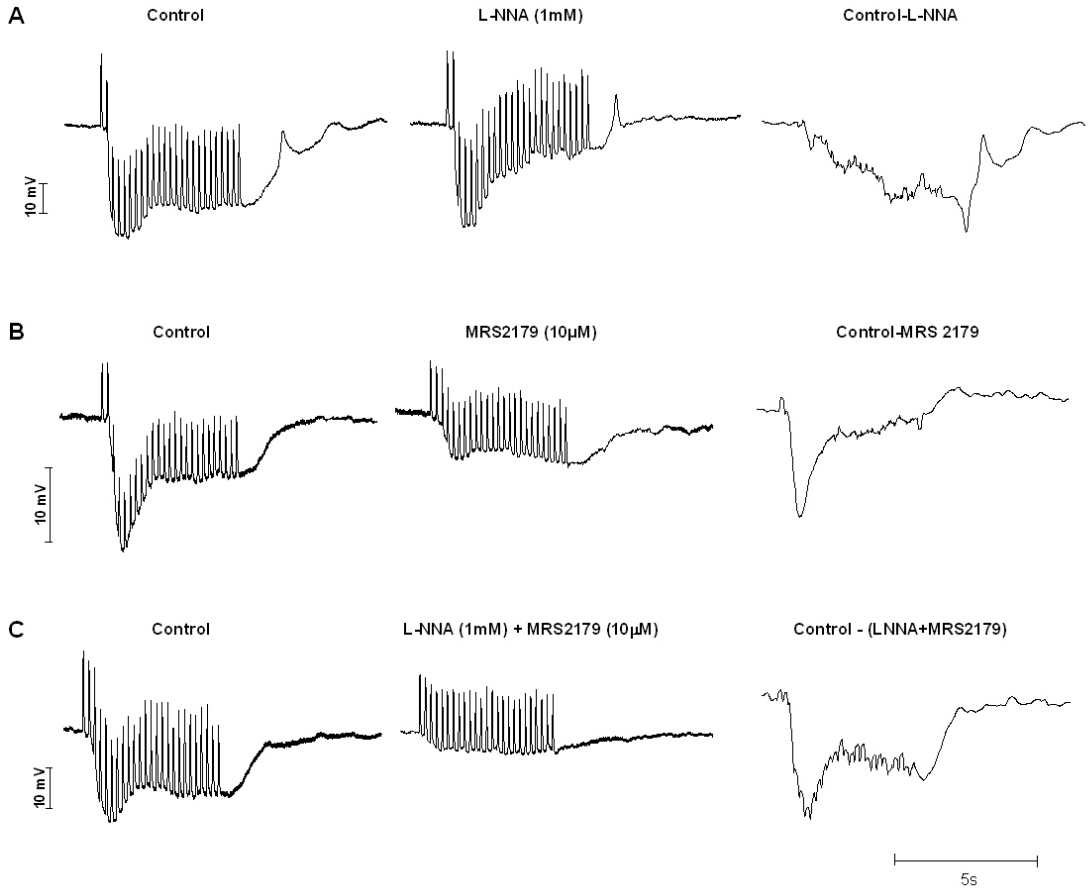



*Figure 7. To illustrate the co-transmission mechanism, a substraction (right) from the electrophysiological response obtained in control (left) and the response obtained after incubation with L-NNA (1mM), MRS 2179 (10µM) or both drugs (middle) was performed. Notice that the L-NNA sensitive response, probably attributable to NO release is mainly the sustained IJP causing a sustained hiperpolarization and the MRS 2179 sensitive response probably attributable to activation of $P2Y_1$ receptors is mainly the fast component of the IJP.*

## Characterization of the rundown of the fast component of the IJP in the human colon

The fast component of the IJP that is attributable to activation of $P2Y_1$ receptors shows a rundown i.e. a decrease in the response observed when two stimuli are close together (Figure 8).

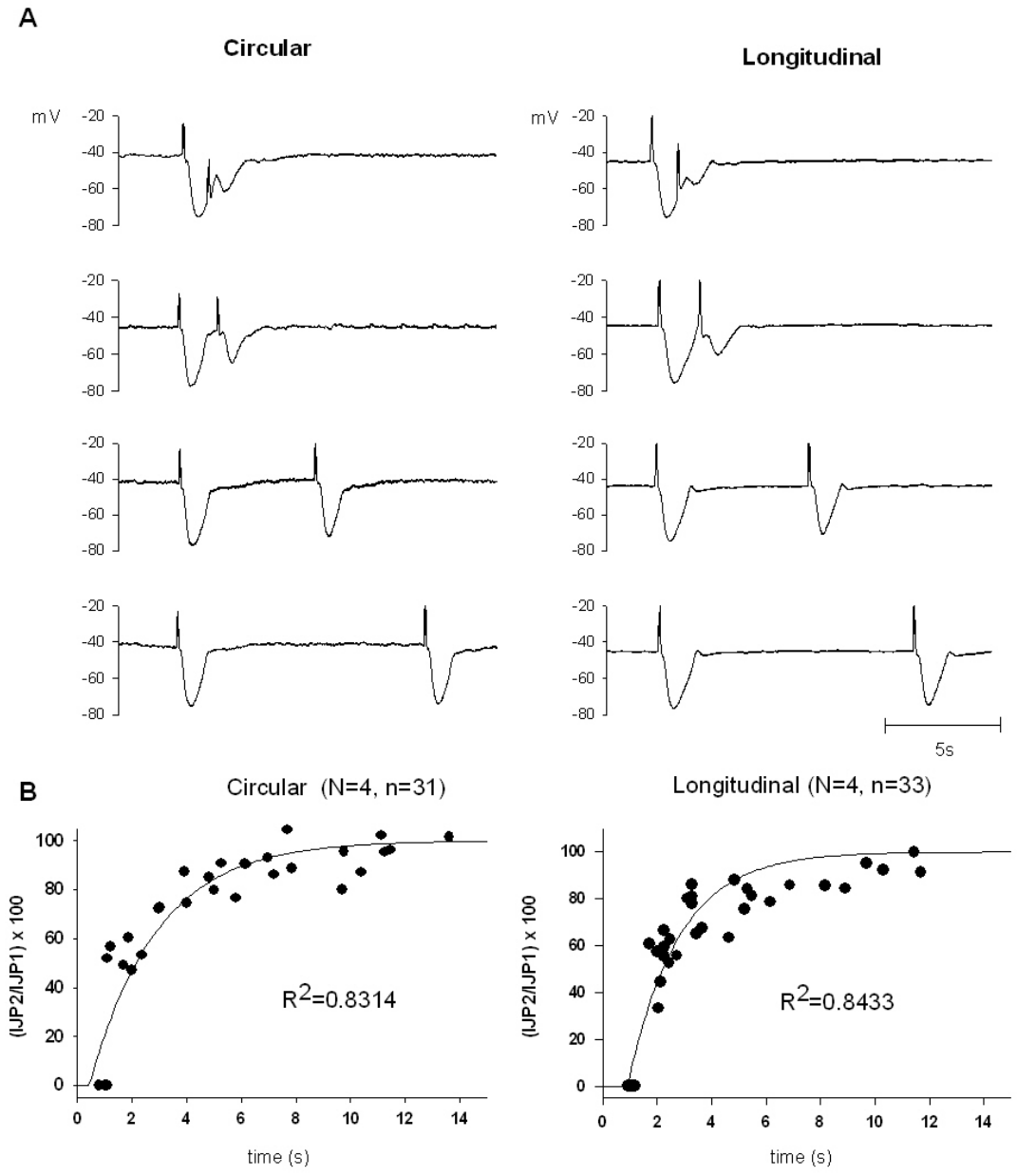



*Figure 8. Rundown response obtained after two consecutive stimuli obtained from the circular and longitudinal layer (A). Percentage of the first response (Y axis) obtained at different time intervals (X axis) (B). Experimental data (dots) were fitted with an exponential curve (see methods) and $R^2$ was estimated.*

The mathematical model proposed in the present work shows high correlation values (Figures 8, 9 and 10).

About 2.5 s after the first pulse, the amplitude of the response obtained after the second pulse (IJPf2) was about 50% of the IJP elicited by the first pulse. 80% to 90% of recovery was achieved with intervals from 5s to 8s after the first pulse. This rundown mechanism is present both in the circular and longitudinal muscle layers (Figure 8). In order to characterize the IJP rundown, the rundown mechanism might be attributable to the percentage of receptor occupation. Accordingly, we have performed the same analysis in the presence of graded responses i.e supramaximal IJPs (about 30mV hyperpolarization), intermediate IJPs (about 20mV hyperpolarization) and small IJPs (about 10 mV hyperpolarization) (Figure 9).

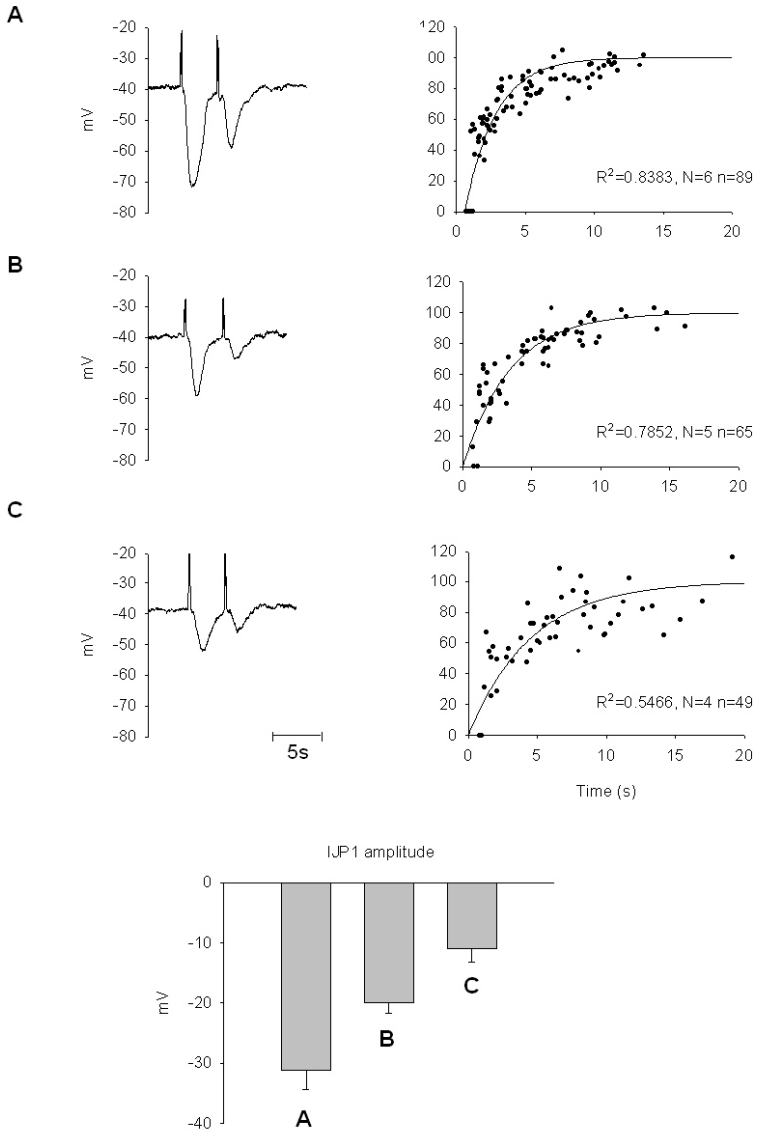



*Figure 9. Effect of the amplitude of the first response (IJP1) on the rundown mechanism. Rundown recorded with 30mV IJP1 amplitude (A), 20mV IJP1 amplitude (B) and 10mV IJP1 amplitude (C). Experimental data (dots) were fitted with an exponential curve (see methods) and $R^2$ was estimated (Left).*

In each case, the voltage of stimulation was selected to obtain an appropriate response. All these responses are MRS 2179 sensitive (Gallego *et al.*, 2006). The rundown mechanism was equally present independently of the amplitude of the first hyperpolarization (Figure 9).

The rundown mechanism was present after incubation of the tissue with L-NNA (1mM); MRS 2179 (1μM), NF023 (10 μM) or Hexamethonium (100μM) (Figure 10). Neither adenosine (0.5nM-1μM) nor DPCPX (10 μM) had any effect on the IJP amplitude and rundown, data not shown. It is important to notice that MRS 2179 (1μM) reduced the amplitude of the IJPf by about 50% (Gallego *et al.*, 2006) without modifying the rundown mechanism.

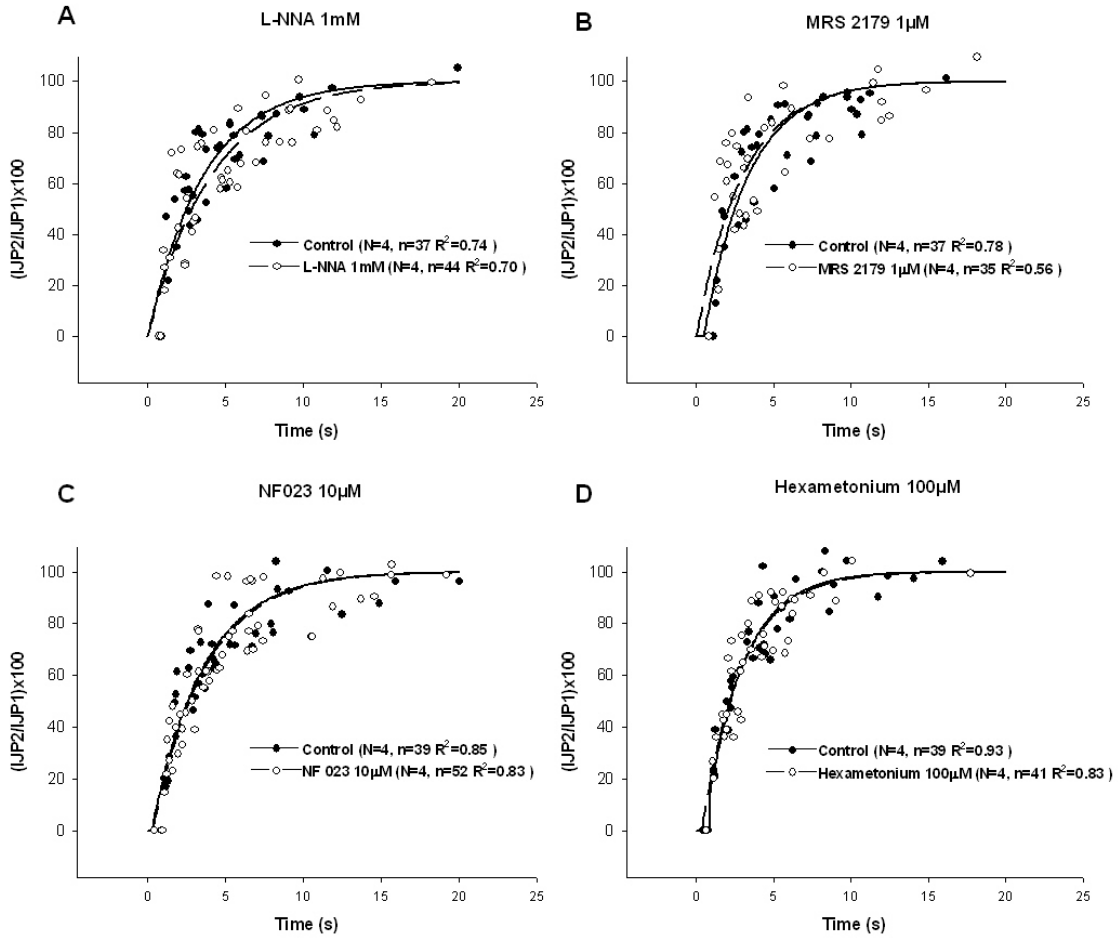



*Figure 10. Rundown recorded after incubation with L-NNA (1mM) (A), MRS 2179 (1μM) (B), NF023 (10μM) (C) or Hexametonium (100μM) (D). Black filled dots are experimental data obtained in control and white filled dots experimental data after drug incubation.*

## *Discussion*

In the present paper we demonstrate that an inhibitory neurotransmission in the human colon has two main components that might be attributable to ATP and nitric oxide. The release of these neurotransmitters depends on the frequency of stimulation causing different types of junction potentials. Fast junction potentials are elicited by single or low frequency stimuli and are mainly MRS 2179 sensitive, whereas higher frequencies are needed to elicit a fast followed by a sustained component that has an MRS 2179 (fast) and L-NNA (sustained) sensitive junction potential. These two components are complementary in causing smooth muscle inhibition. In the present work we propose a new pharmacological approach to characterize the junction potential in the human gastrointestinal tract using different kinds of electrical pulses. Our electrophysiological findings can explain the inhibitory neurotransmission in the human colon.

### Evidence suggesting co-transmission

In the human colon single pulses or short trains induce fast inhibitory junction potentials (fIJP) that are insensitive to L-NNA (Gallego *et al.*, 2006; Keef *et al.*, 1993) and consequently fIJP are not mediated by nitric oxide. In contrast, in other species short pulses cause a biphasic IJP with a fast component followed by a sustained one (Crist *et al.*, 1992; He & Goyal, 1993; Pluja *et al.*, 1999). Usually, the fast component is L-NNA insensitive and the sustained component is L-NNA sensitive, showing a co-transmission between nitric oxide and a second neurotransmitter. In a recent and relevant paper from Dr. Wood's laboratory, the authors showed that the majority of IJPs in the guinea-pig small intestine consist of a fast IJP, sensitive to MRS 2179, followed by a sustained IJP mainly sensitive to L-NNA (Wang *et al.*, 2007), showing that purinergic components through $P2Y_1$ receptors and NO mediate inhibitory neurotransmission in that species. It is important to notice that in the guinea-pig small

intestine, single pulses of electrical field stimulation did not elicit a biphasic IJP in all the recordings (26% of them were pure purinergic, consiting of a fast IJP) and a small percentage of junction potential have an L-NNA insensitive sustained component. In the human colon this co-transmission is much more difficult to record because long pulses are needed to demonstrate the presence of these two components. In the present work we confirm previous data from Dr. Sanders and Dr Keef's laboratory showing that single pulses elicit fIJPs that are L-NNA insensitive, whereas long pulses elicit a fast component followed by a sustained one (Keef *et al.*, 1993). Apamin has been used as a pharmacological tool to discriminate between the fast and the slow component of the IJP (He & Goyal, 1993; Pluja *et al.*, 1999; Wang *et al.*, 2007). However, it seems that in the human gastrointestinal tract apamin does not discriminate between the fast and the slow component of the IJP because both components are partially sensitive to the drug (Keef *et al.*, 1993; Xue *et al.*, 1999). In a previous paper we demonstrated that the fast component of the IJP was sensitive to MRS 2179 and therefore it might be attributable to a purinergic mediator acting at $P2Y_1$ receptors (Gallego *et al.*, 2006). In the present work we have studied the co-transmission between NO and ATP using this pharmacological approach.

## Characterization of the purinergic component: the IJP-rundown

Single or short train pulses elicit fast IJP that show a rundown when a second pulse (test pulse) is applied at different time intervals after the first conditioning pulse. This mechanism has been previously denominated as IJP-rundown in animal studies (King, 1994; Matsuyama *et al.*, 2003). In our study, the amplitude of the IJP elicited by the test pulse was very small at short time intervals and recovered when time intervals were increased. The time to recover 50% of the first IJP was around 2.5 s both in circular and longitudinal muscle layers which is quite similar to those previously described in animal studies (King, 1994; Matsuyama *et al.*, 2003). Our data show that

this mechanism is largely independent of the amplitude of the conditioning IJP. The rundown mechanism can be attributable to a pre-junctional mechanism i.e. pre-juntional receptors causing the inhibition of the release of the inhibitory mediator; or alternatively to a post-juntional mechanism including receptor or intracellular pathways. Regarding the pre-junctional hypothesis, a previous study on hamster proximal colon demonstrated that nitric oxide might be responsible for the IJP-rundown (Matsuyama *et al.*, 2003). In this species, short pulses elicited a fast component followed by a sustained nitrergic one and the IJP-rundown was inhibited with L-NNA incubation. In the colon of the hamster nitric oxide probably caused the inhibition of the release of the neurotransmitter responsible for the first component of the IJP (Matsuyama *et al.*, 2003). Unfortunately, we could not demonstrate this mechanism in the human colon because single pulses elicit fast IJPs that are L-NNA insensitive and incubation with L-NNA did not inhibit the rundown mechanism. Moreover when pulses of 1 Hz were applied (see below) the response was not modified in the presence of L-NNA. These results do not support the hypothesis that nitric oxide causes pre-junctional inhibition of fast IJP in the human colon. Another putative mechanism of pre-junctional inhibition might be through adenosine receptors. Adenosine might cause a pre-junctional inhibition of neurotransmitter release as has been previously demonstrated for excitatory neurotransmitters in animal models (Lee *et al.*, 2001). Moreover, adenosine inhibits IJP in the guinea-pig ileum but pre-junctional P1 purinoceptors are not responsible for the IJP-rundown (King, 1994). In the human colon we did not find an inhibitory effect of adenosine on the IJP and inhibitors of adenosine receptors such as DCPX did not inhibit the IJP-rundown. Other antagonists such as low concentration of MRS 2179 (0.1 μM) that inhibits the IJP by about 50% or NF023, a putative P2X antagonist, do not inhibit the IJP-rundown, suggesting that these receptors are not involved in the pre-junctional inhibition of the inhibitory transmitter. Hexamethonium did not affect the IJP- rundown. Although we do not have a final explanation of the mechanism in the human colon, a post-junctional mechanism might be responsible for

the IJP- rundown. Incubation of P2Y agonist causes transient hyperpolarization and when the membrane potential is recovered a partial inhibition of the IJP ocurrs (Gallego *et al.*, 2006). Moreover, stable agonists have been widely used as “desensitizers” of purinergic receptors.

## Characterization of the purinergic component: pulses at 1 Hz

Pulses elicited at 1 Hz for 5s (5 pulses) elicited 5 fast IJPs of variable amplitude. The first IJP was of high amplitude compared to the others probably due to the rundown mechanism. It is important to notice that the second IJP was usually absent or was very small. This result fits with the rundown mechanism described in the present paper. However, the other 3 IJPs had similar amplitude although smaller than the first one. A similar result has been reported in the guinea-pig ileum where trains of 1 Hz caused IJPs of smaller amplitude compared to the first response (King, 1994). In this case, the recovery of rundown may coincide with the release of new inhibitory neurotransmitters with the next pulse and a compromise between both mechanisms exists. The mechanical response of this protocol of electrical field stimulation consists of a sustained inhibition of spontaneous motility, probably based on the successive fast IJPs. Both the electrophysiological and the mechanical responses are mainly inhibited by MRS 2179, showing the involvement of ATP or a related purine acting at $P2Y_1$ receptors.

## Characterization of the purinergic and nitrergic component: pulses at 5 Hz

Electrical field stimulation at 5 Hz for 5 s (25 pulses) clearly demonstrates the co-transmission mechanism. The electrophysiological response shows a fast component followed by a sustained one. It is important to notice that the fast component is mainly MRS 2179 sensitive whereas the second component is mainly L-

NNA sensitive showing a co-transmission between ATP, or a related purine acting on $P2Y_1$ receptors, and nitric oxide. This pharmacological approach has been recently used in the guinea-pig ileum where the majority of IJPs with a fast and sustained component were MRS 2179 and L-NNA sensitive respectively (Wang *et al.*, 2007). The mechanical response of this protocol of EFS consisted of a complete inhibition of spontaneous motility. Incubation of the tissue with both MRS 2179 and L-NNA blocked the inhibitory effect induced by EFS.

It is important to notice that electrical and mechanical responses are correlated when a single inhibitor is infused. In the presence of MRS 2179 the fast component is inhibited and the sustained component is present. This sustained hyperpolarization is probably responsible for the sustained relaxation observed in the presence of the $P2Y_1$ antagonist. This is an interesting result because it demonstrates that nitrergic neurotransmission can cause a sustained hyperpolarization causing a sustained relaxation in the absence of purinergic inhibitory neurotransmission. This result fits with the effect of NO donors on the spontaneous motility because NO causes sustained hyperpolarization (Gallego *et al.*, 2006) and causes long lasting inhibition of spontaneous motility. In contrast, when nitrergic neurotransmission is inhibited, the sustained component is reduced and the mechanical activity is only transiently inhibited but this inhibition can not be maintained over time. This response appears both at 2 and 5 Hz of stimulation. It is possible that continuous release of ATP or a related purine causes a rundown mechanism that is unable to cause hyperpolarization and relaxation of smooth muscle cells. According to this result incubation of the tissue with ADP$\beta$S or ATP causes transient relaxation that partially recovers with time.

The results of the present paper show that single-pulse stimulation (or probably also short trains) is able to stimulate purinergic neurons. In contrast, nitrergic neurons need trains of stimuli to release nitric oxide. At present it is not known whether both

inhibitory neurotransmitters are released from the same neuron or different subsets of inhibitory neurons are alternatively present in the GI tract. However, a co-localization of nitric oxide synthase (NOS) and quinacrine fluorescence (indicative of vesicular adenosine 5'-triphosphate, ATP), has been reported giving structural support to a co-transmission process (Belai & Burnstock, 2000).

Taken together, our results demonstrate that the two inhibitory neurotransmitters have different functions inhibiting colonic motility. Nitric Oxide: *1)* is responsible for the sustained hyperpolarization; *2)* can cause sustained relaxation; *3)* can be tonically released from inhibitory motor neurons. In contrast, the purinergic mediator acting on $P2Y_1$ receptors: *1)* is responsible for the fast hyperpolarization (which usually has bigger amplitude than the sustained component) *2)* might cause a transient relaxation that is difficult to maintain over time due to the rundown mechanism *3)* is probably not tonically released from enteric neurons.

It is conceivable that both neurotransmitters are involved in inhibiting the motility of other areas of the gastrointestinal tract such as the small intestine (Undi *et al.*, 2006) or LES (Farre *et al.*, 2006). When a tonic relaxation should occur such as in gastric accommodation, probably nitric oxide can accomplish the function without a purinergic input. In contrast when a transient and sudden relaxation is needed probably ATP through $P2Y_1$ receptors can accomplish this function. More studies are needed in other areas of the human GI tract to demonstrate this hypothesis and it might be important in the future to try to investigate the putative role of the co-transmission impairment in human neuropathologies that affect the motor pathway.

# *Bibliography*

Alexander SP, Mathie A, & Peters JA (2005). Guide to receptors and channels, 1st edition (2005 revision). *Br J Pharmacol* **144 Suppl 1**, S1-128.

Auli M, Farre R, Hernandez P, Gallego D, Martinez E, Marti-Rague J, Sunol X, Jimenez M, & Clave P (2005). Nitrergic and purinergic co-neurotransmission in human sigmoid colon. *Neurogastroenterol Motil* **17**, 27.

Belai A & Burnstock G (2000). Pattern of distribution and co-localization of NOS and ATP in the myenteric plexus of human fetal stomach and intestine. *Neuroreport* **11**, 5-8.

Benko R, Undi S, Wolf M, Vereczkei A, Illenyi L, Kassai M, Cseke L, Kelemen D, Horvath OP, Antal A, Magyar K, & Bartho L (2007). P(2) purinoceptor antagonists inhibit the non-adrenergic, non-cholinergic relaxation of the human colon in vitro. *Neuroscience* **147**, 146-152.

Boeckxstaens GE, Pelckmans PA, Herman AG, & Van Maercke YM (1993). Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. *Gastroenterology* **104**, 690-697.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, & Herman AG (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345**, 346-347.

Burnstock G, Campbell G, Satchell D, & Smythe A (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* **40**, 668-688.

Camaioni E, Boyer JL, Mohanram A, Harden TK, & Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem* **41**, 183-190.

Crist JR, He XD, & Goyal RK (1992). Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *J Physiol* **447**, 119-131.

De Man JG, De Winter BY, Herman AG, & Pelckmans PA (2007). Study on the cyclic GMP-dependency of relaxations to endogenous and exogenous nitric oxide in the mouse gastrointestinal tract. *Br J Pharmacol* **150**, 88-96.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

Fang X, Hu HZ, Gao N, Liu S, Wang GD, Wang XY, Xia Y, & Wood JD (2006). Neurogenic secretion mediated by the purinergic P2Y1 receptor in guinea-pig small intestine. *Eur J Pharmacol* **536**, 113-122.

Farre R, Auli M, Lecea B, Martinez E, & Clave P (2006). Pharmacologic characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter. *J Pharmacol Exp Ther* **316**, 1238-1248.

Gallego D, Hernandez P, Clave P, & Jimenez M (2006). P2Y1 receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. *Am J Physiol Gastrointest Liver Physiol* **291**, G584-G594.

Gao N, Hu HZ, Zhu MX, Fang X, Liu S, Gao C, & Wood JD (2006). The P2Y purinergic receptor expressed by enteric neurones in guinea-pig intestine. *Neurogastroenterol Motil* **18**, 316-323.

He XD & Goyal RK (1993). Nitric oxide involvement in the peptide VIP-associated inhibitory junction potential in the guinea-pig ileum. *J Physiol* **461**, 485-499.

Hu HZ, Gao N, Zhu MX, Liu S, Ren J, Gao C, Xia Y, & Wood JD (2003). Slow excitatory synaptic transmission mediated by P2Y1 receptors in the guinea-pig enteric nervous system. *J Physiol* **550**, 493-504.

Jimenez M, Cayabyab FS, Vergara P, & Daniel EE (1996). Heterogeneity in electrical activity of the canine ileal circular muscle: interaction of two pacemakers. *Neurogastroenterol Motil* **8**, 339-349.

Keef KD, Du C, Ward SM, McGregor B, & Sanders KM (1993). Enteric inhibitory neural regulation of human colonic circular muscle: role of nitric oxide. *Gastroenterology* **105**, 1009-1016.

King BF (1994). Prejunctional autoinhibition of purinergic transmission in circular muscle of guinea-pig ileum; a mechanism distinct from P1-purinoceptor activation. *J Auton Nerv Syst* **48**, 55-63.

Lee JJ, Talubmook C, & Parsons ME (2001). Activation of presynaptic A1-receptors by endogenous adenosine inhibits acetylcholine release in the guinea-pig ileum. *J Auton Pharmacol* **21**, 29-38.

Matini P, Faussone-Pellegrini MS, Cortesini C, & Mayer B (1995). Vasoactive intestinal polypeptide and nitric oxide synthase distribution in the enteric plexuses of the human colon: an histochemical study and quantitative analysis. *Histochem Cell Biol* **103**, 415-423.

Matsuyama H, El-Mahmoudy A, Shimizu Y, & Takewaki T (2003). Nitrergic prejunctional inhibition of purinergic neuromuscular transmission in the hamster proximal colon. *J Neurophysiol* **89**, 2346-2353.

Pluja L, Fernandez E, & Jimenez M (1999). Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol* **126**, 883-892.

Tam FS & Hillier K (1992). The role of nitric oxide in mediating non-adrenergic non-cholinergic relaxation in longitudinal muscle of human taenia coli. *Life Sci* **51**, 1277-1284.

Timmermans JP, Barbiers M, Scheuermann DW, Bogers JJ, Adriaensen D, Fekete E, Mayer B, van Marck EA, & De Groodt-Lasseel MH (1994). Nitric oxide synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. *Cell Tissue Res* **275**, 235-245.

Undi S, Benko R, Wolf M, Illenyi L, Horvath OP, Antal A, Csontos Z, Vereczkei A, & Bartho L (2006). Purinergic nerves mediate the non-nitrergic relaxation of the human ileum in response to electrical field stimulation. *Brain Res Bull* **71**, 242-244.

Wang GD, Wang XY, Hu HZ, Liu S, Gao N, Fang X, Xia Y, & Wood JD (2007). Inhibitory neuromuscular transmission mediated by the P2Y1 purinergic receptor in guinea pig small intestine. *Am J Physiol Gastrointest Liver Physiol* **292**, G1483-G1489.

Wood JD (2006). The enteric purinergic P2Y1 receptor. *Curr Opin Pharmacol* **6**, 564-570.

Xue L, Farrugia G, Sarr MG, & Szurszewski JH (1999). ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *Am J Physiol* **276**, G1373-G1379.

# Capítulo 4

## The gaseous mediator, hydrogen sulphide, inhibits *In Vitro* motor paterns in the human, rat and mouse colon and jejunum


### *Abstract*

Hydrogen sulphide ($H_2S$) has been recently proposed as a transmitter in the brain and peripheral tissues. Its role in the gastrointestinal tract is still unknown despite some data suggest an involvement of this gasotransmitter mediating smooth muscle relaxation. The aim of this study is to investigate the effect of this gas in intestinal segments from mouse jejunum and colon and muscular strips from the human and rat colon.

In isolated segments of both the mouse colon and jejunum, bath applied NaHS (a $H_2S$ donor) caused a concentration-dependent inhibition of spontaneous motor complexes (MC's) ($IC_{50}$ 121μM in the colon and 150 μM in the jejunum). This inhibitory effect of NaHS on MC's was *1)* unaffected by TTX, capsaicin, PPADS, and L-NNA suggesting therefore a direct effect on the smooth muscle; *2)* significantly reduced by apamin 3μM.

NaHS concentration dependently inhibited the spontaneous motility in strips from human colon ($IC_{50}$ 261μM) and rat colon ($IC_{50}$ 31μM).The inhibitory effect of NaHS on colonic strips was: *1)* unaffected by the neural blocker tetrodotoxin (TTX 1μM) with $IC_{50}$ 183μM for the human colon and of 26μM for the rat colon, and *2)* significantly reduced by Glybenclamide (10μM), Apamin (3μM) and TEA (10mM) with $IC_{50}$ values of 2464μM, 1307μM, 2421μM for human strips, and 80μM, 167μM, and 674μM for rat strips, respectively.

We conclude that $H_2S$ causes a strong inhibitory action on in vitro intestinal and colonic motor patterns in rodents and humans. This effect appears to be critically

dependent on K channels particularly apamin sensitive sKCa channels and glybenclamide sensitive K (ATP) channels.

## *Introduction*

The foul smelling gas, hydrogen sulphide ($H_2S$) has been proposed as a third gaseous neuromodulator, after nitric oxide (NO) and carbon monoxide (CO). $H_2S$ can be produced via the reduction of the amino acid cysteine, through two endogenous enzyme systems. The first enzyme is cystathionine β- synthase (CBS) which is present in brain and peripheral tissues, including the gut (*Fiorucci at al., 2006*) The second enzyme is cystathionine γ lyase (CSE) , found only in peripheral tissues (Moore *et al.*, 2003;Bhatia *et al.*, 2005). Both CBS and CSE have been found in enteric neurons. (*Schicho et al., 2006*) Also a third enzyme, a non-pyridoxal-phosphate-dependent one, the Mercaptopyruvate sulfurtransferase has been proposed as a hydrogen sulfide-generating enzyme (Kamoun, 2004). Hydrogen sulfide could be generated through non-enzymatic reactions since it has been shown that washed human erythrocytes incubated with glucose and elemental sulfur produce hydrogen sulfide at a constant rate. As sulfur and glucose are both available in circulating blood this could be a possible pathway of production in vivo (Searcy & Lee, 1998). In addition, in the gastrointestinal tract, large quantities of $H_2S$ are produced by endogenous sulphate reducing bacteria, and concentrations can reach 3 mM in the colon (Suarez *et al.*, 1998). Emerging evidence indicates that $H_2S$ can have important effects on excitable tissues, such as nerves and smooth muscle (Abe & Kimura, 1996; Kimura, 2002; Kimura *et al.*, 2005; Qu *et al.*, 2007).

In the urinary bladder, Pataccini et al (Patacchini *et al.*, 2004) have demonstrated an excitatory action of $H_2S$ on bladder contraction, which was mediated by capsaicin sensitive nerves. In contrast in isolated ileal muscle strips, $H_2S$ caused relaxation (Hosoki *et al.*, 1997).In vascular smooth muscle, $H_2S$ has been shown to facilitate the release of NO, acting in synergy with endogenously released NO to cause relaxation (Bhatia, 2005). Recent work by Shichio et al has demonstrated a pro secretory effect of $H_2S$ in the human colon. This effect is mediated by capsaicin sensitive nerves,

suggesting it activates extrinsic afferents to mediate secretion (Schicho *et al.*, 2006). These data show that $H_2S$ can cause either contraction or relaxation and the actions of $H_2S$ might be nerve mediated or alternatively a putative effect on smooth muscle could be postulated.

The effects of $H_2S$ on an integrated motor pattern such as peristalsis have not yet been examined, nor have the effects on human gastrointestinal smooth muscle been identified In the present experiments we examined the effects of $H_2S$ on spontaneous distension induced motor patterns in the mouse jejunum and colon, and subsequently characterized the effects and the mechanism of $H_2S$ induced relaxation in the rat and human colon.

## *Materials and Methods*

### Mouse tissue preparation

Experiments were performed on isolated segments of jejunum and colon from C57Bl6 mice of either sex. To examine the role of TRPV1 receptors, TRPV1 -/- animals were used (Davis *et al.,* 2000). Animals were killed by overdose of sodium pentobarbital (300 mg/kg; Sagatal, Rhone Poulenc Fr.) followed by exsanguination. The abdomen was opened and the mid jejunum and distal colon were removed, flushed of their contents and placed in oxygenated Kreb's solution.

### Rat tissue preparation

Male Sprague-Dawley rats (Charles River, Lyon, France) 8-10 weeks old and weighing 300-350g, were used. They were kept under conventional conditions in an environmentally controlled room (20-21ºC, 60% humidity, 12:12h light-dark cycle) in groups of three animals and had unlimited access to water and food. Before the in vitro studies, rats were kept individually and fasted for 16-18h with ad libitum access to water. Animals were decapitated and bled. All the experimental protocols were approved by the ethical committee of the Universitat Autònoma de Barcelona (Spain).

The entire colon was carefully removed and placed on a dissection dish containing carbogenated krebs solution. The mesenteric fat was removed and the colon was opened along the mesenteric border. A small segment of mid colon was pinned to a Sylgard-base with the mucosa facing upwards and the mucosal and submucosal layers were gently removed in order to study circular muscle strips.

### Human Tissue Preparation

Specimens of distal and sigmoid colon were obtained from patients (aged 47-78 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 mm x 4 mm, were cut. The patients provided informed consent and experimental procedure was approved by the ethics committee of the Hospital of Mataró (Barcelona, Spain).

## Mechanical Experiments

**A) Isolated mouse colon and jejunum.** In the experiments with isolated mouse intestine, a 3cm segment of gut was then placed in an organ bath (10 ml) perfused with warm (36°C) oxygenated Kreb's and was cannulated at both ends, with a pressure transducer at the aboral end. The lumen was distended with saline to a pressure of 2-3 cm$H_2O$ and spontaneous motor complexes recorded. Signals were amplified using a Neurolog NL108 (Digitimer UK) pressure amplifier, digitized at 100 Hz using a CED 1401 interface (Cambridge Electronic Designs, Cambridge UK) and displayed on a PC running the Spike2 sofware package.

Drugs were added directly to the bath solution, except for a few cases when luminal application of $H_2S$ was examined. Agonists were perfused for 10 min, followed by a washout period of 30 min prior to further agonist application. Antagonists were added to the bath solution 30 min prior to agonist application. The roles of endogenous ATP and NO release were examined using PPADS (30$\mu$M) and L-NAME (100$\mu$M) respectively. The potential role of TRPV1 receptors was assessed using TRPV1 -/- transgenic mice. To allow examination of direct effects on smooth muscle contraction, TTX (1$\mu$M) was used, which blocks all neurally mediated motor complexes in this preparation.

**B) Colonic strips.** Human and rat tissue muscle strips were examined in a 10 ml organ bath filled with Krebs solution at 37$\pm$1ºC. An isometric force transducer (Harvard VF-1) connected to an amplifier was used to record the mechanical activity.

Data were digitalized (25 Hz) using Datawin1 software (Panlab-Barcelona) coupled to an ISC-16 A/D card installed in a PC computer. A tension of 4g was applied to the human tissue and of 1g to the rat tissue and they were allowed to equilibrate for 1 hour. After this period, strips displayed spontaneous phasic activity. To estimate the responses to drugs, the area under the curve (AUC) of spontaneous contractions from the baseline was measured before and after drug addition. In order to normalize data, the value of AUC obtained before the treatment was considered 100 and the percentage of inhibition of the spontaneous motility was estimated with the AUC obtained after the treatment.

## Solutions and Drugs

The composition of the Krebs 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 $MgSO_4$ bubbled with a mixture of 5% $CO_2$:95% $O_2$ (pH 7.4). The following drugs were used: Sodium hydrogen sulfide (NaHS), Apamin, Glybenclamide, Tetraethylammonium chloride (TEA), Capsaicin, ATP, L-NAME, bethanechol chloride (Sigma Chemicals, St. Louis, USA) tetrodotoxin (TTX) (Latoxan, Valence, France). PPADS (Tocris UK) Stock solutions were made by dissolving drugs in distilled water except for Glybenclamide which was dissolved in DMSO and capsaicin which was dissolved in a solution of 80% DMSO / 20% Tween 80.

## Data Analysis and Statistics

In the human and rat muscle strip experiments cumulative concentration-response curves of $H_2S$, using NaHS as a donor, were calculated in order to estimate the $IC_{50}$. To normalize data, we calculated the percentage inhibition by the drugs considering the AUC before the addition of the $H_2S$ donor as 100%. The differences between groups were compared by two-way analysis of variance (two way ANOVA). 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 California US.

In the mouse isolated intestine experiments, motor complexes were analysed with respect to peak amplitude (relative to basal pressure). The mean amplitude of the 5 contractions before drug application was considered as baseline and inhibitory effects were expressed as % change from baseline.

## *Results*

In both the mouse colon and jejunum, bath applied NaHS caused an inhibition of spontaneous motor complexes. This effect was dose dependent, with $IC_{50}$ values of 121μM in the colon and 150 μM in the jejunum, and maximal (100%) inhibition occurring between 300μM and 1000μM. NaHS applied luminally also caused an inhibition of motility, but the effect was less consistent, and required higher concentrations compared to bath application. For all further experiments, NaHS was directly applied to the bath. As the effects of NaHS were similar in the jejunum and colon, data are pooled here. (Figure 1)

### Role of TRPV1 receptors

As previous work on urinary bladder muscle and intestinal secretion have suggested a role for capsaicin sensitive nerves the effects of $H_2S$, we hypothesized that TRPV1 channels may be involved in the inhibitory effects of NaHS on spontaneous motor complexes. In the mouse jejunum and colon capsaicin (100nM) causes an inhibition of motor complexes (see figure 2). In contrast this effect is absent in intestine from TRPV1-/- animals. However, in both TRPV1 +/+ and -/- intestine, NaHS caused a similar concentration- dependent inhibition, with nearly identical concentration response relationships (n=6 of each)

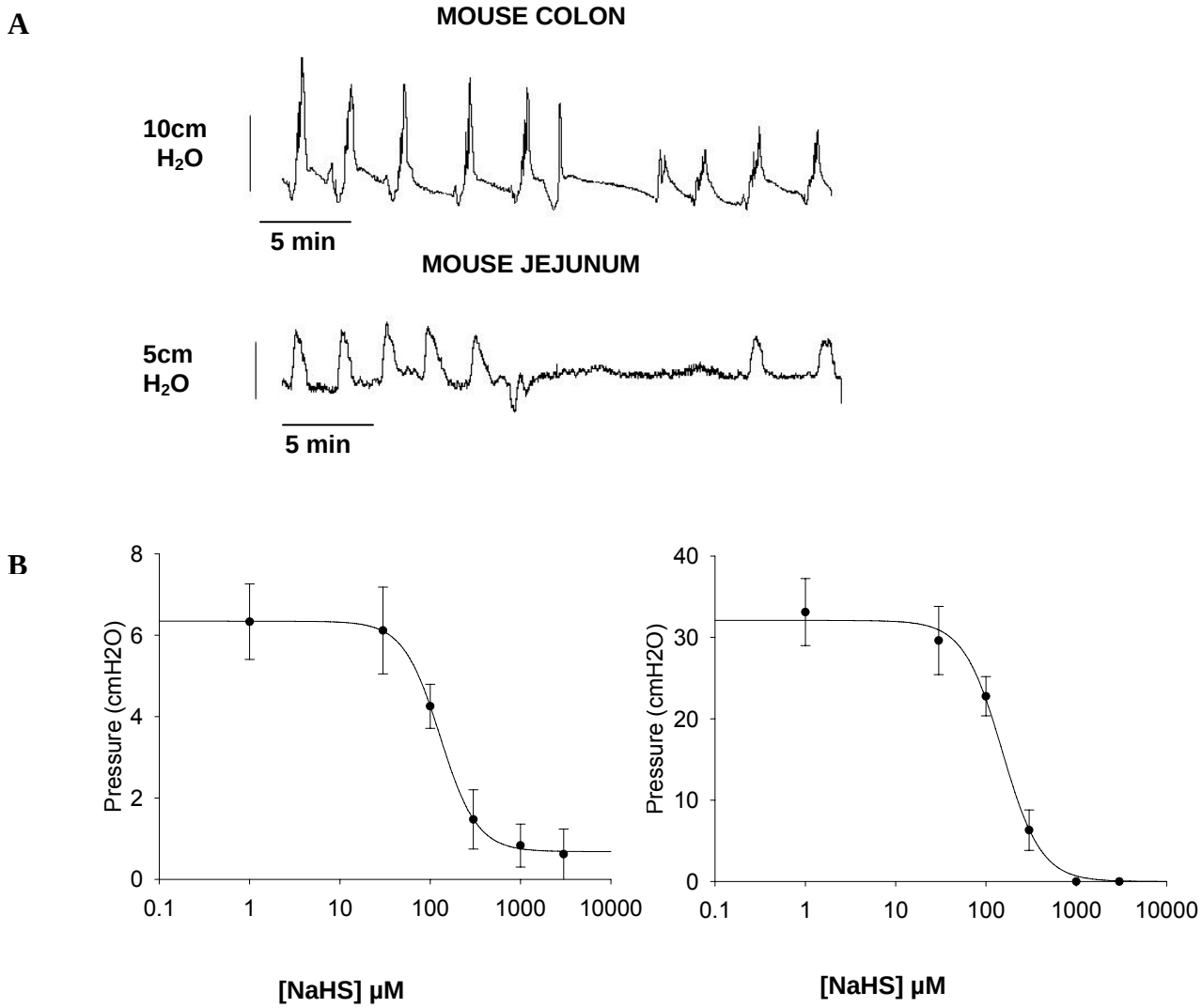



*Figure 1. A) Recordings of spontaneous motor complexes in the isolated mouse jejunum and colon. NaHS (300µM) causes a significant inhibition of the amplitude of these motor complexes both the colon (top trace) and jejunum (bottom trace) B) Concentration response relationship for NaHS in mouse jejunum and colon. NaHS caused a concentration dependent inhibition with similar IC50 values in colon and jejunum.*

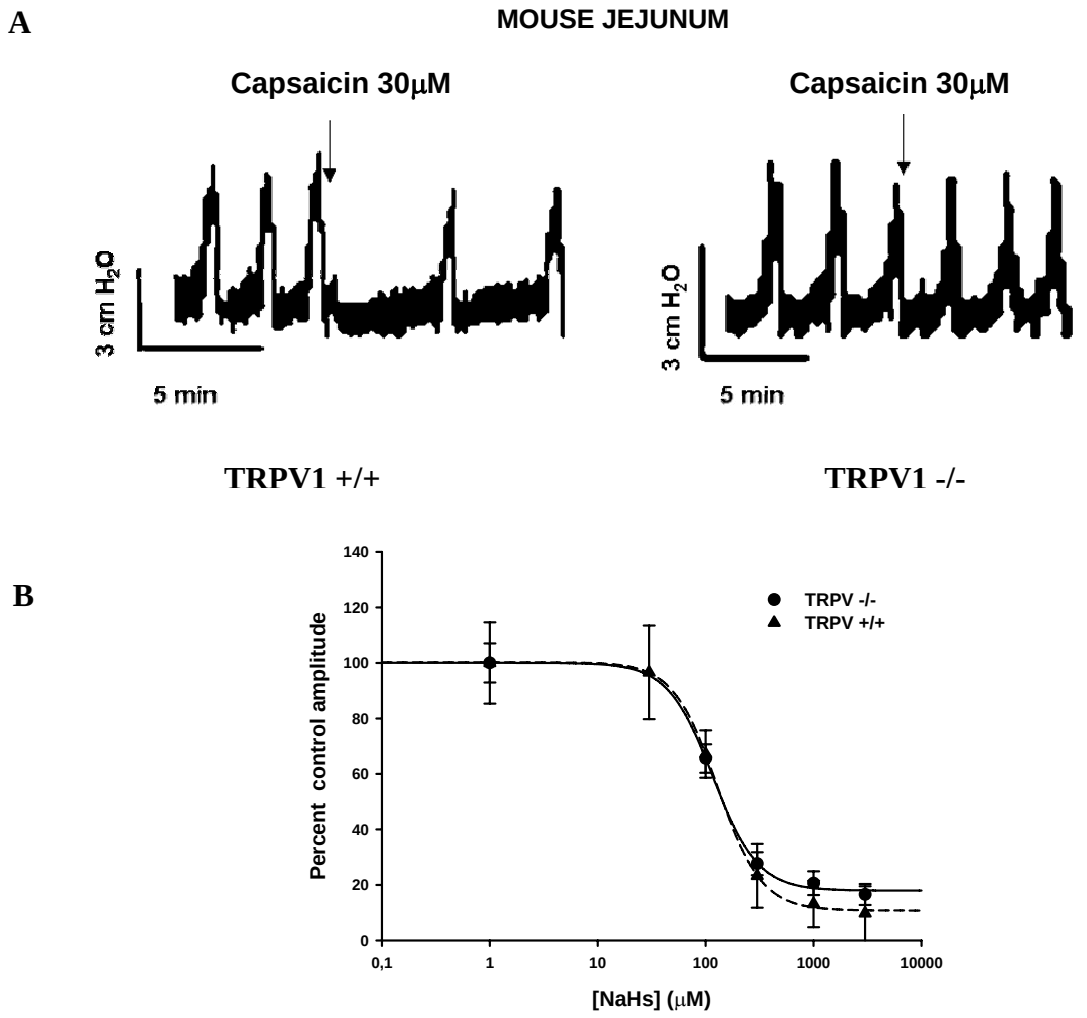



*Figure 2. A) Recordings of spontaneous motor complexes in the isolated mouse jejunum showing the effect of capsaicin 100nM in TRPV+/+ and -/- mice.B) Concentration response curves to NaHS in TRPV+/+ and -/- mice. Concentration response relationships to NaHS do not differ significantly between the two groups.*

## Role of Purinergic receptors

As ATP is an important inhibitory neurotransmitter in the intestine, and since our own preliminary experiments have indicated a role for P2 receptors in $H_2S$ mediated excitation of visceral afferent nerves in the mouse (unpublished observations), we

sought to examine the role of endogenously released ATP in the $H_2S$ mediated relaxation of jejunal and colonic motility. As seen in figure 3, ATP (300μM) causes inhibition of MCs, an effect that can be prevented by 30μM PPADS. In contrast the effect of NaHS (300μM) was not significantly inhibited by PPADS (80.1±8.8%vs76.33±9.3% inhibition p>0.05, n=5) (Figure 3).

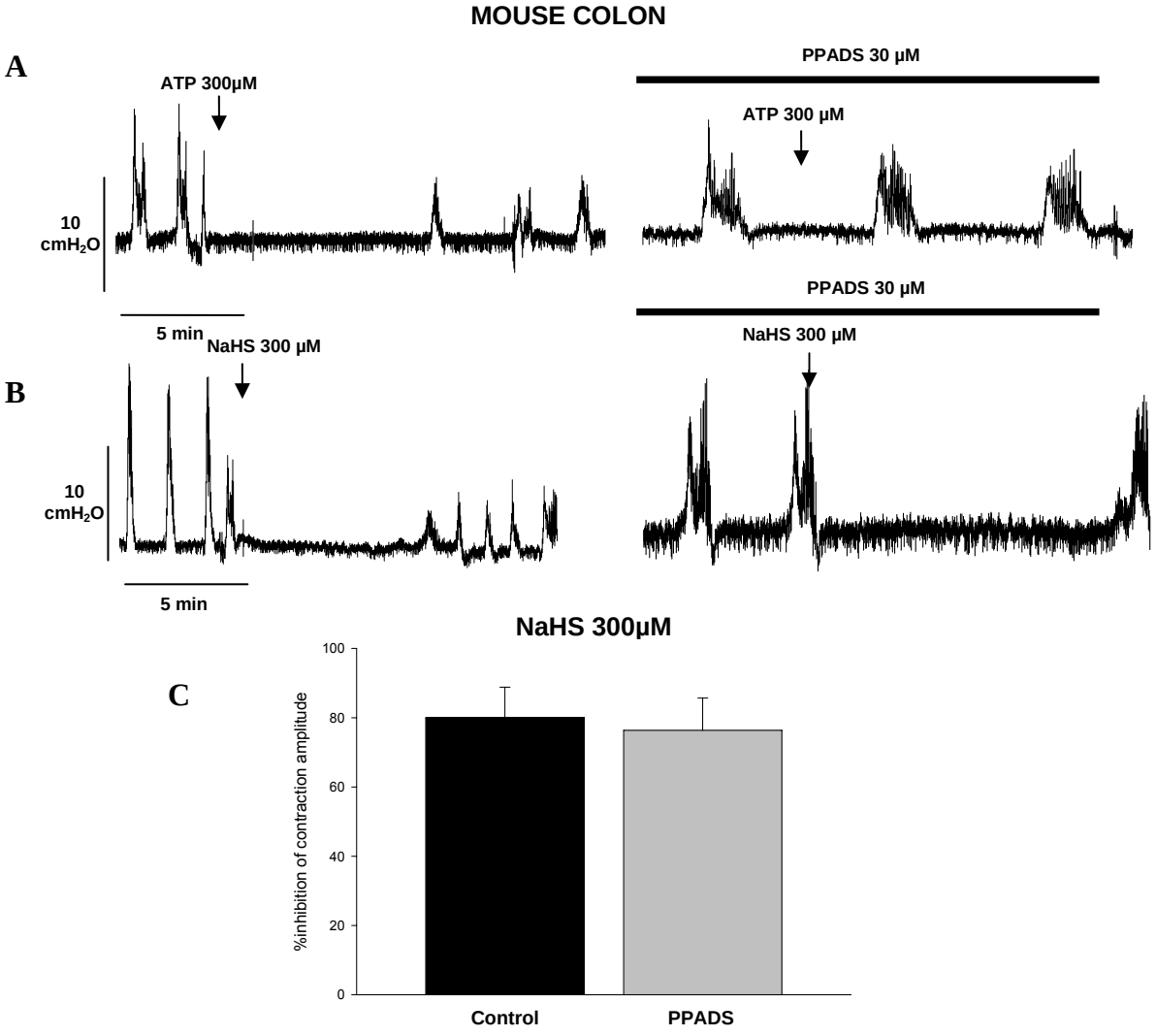



*Figure 3. Mechanical recordings showing the inhibitory effect of ATP (300μM) on the MCs and the reversion of this inhibitory effect by PPADS (30μM) in the mouse colon (A) and mechanical recordings showing the inhibitory effect of NaHS (300μM) on the MCs notice that this inhibitory effect was unaffected by PPADS (30μM) (B). C) Summary of effect of PPADS on MC amplitude (n=6 p>0.05)*

## Role of NO release

As NO is an important inhibitory neurotransmitter in the gut, and because of the evidence, at least in some vascular smooth muscles, that NO may mediate some of the effects of $H_2S$, we examined the role of NO release using the NO synthase blocker, L-NAME. In the mouse jejunum, L-NAME (100μM) increased MC amplitude and frequency, while in the colon, MC amplitude was inhibited. However in neither region was the inhibitory effect of NaHS (300μM) attenuated, causing similar inhibition of MC amplitude in both regions (74.3±8.9% control vs 80.3±10.1% L-NAME, N=6).

## NaHS Directly inhibits smooth muscle contraction

To examine the effects of NaHS on smooth muscle contraction, independent of neural influences, we blocked intrinsic neurotransmission using TTX (1μM). This abolished spontaneous motor complexes and increased tone. Under these conditions, NaHS resulted in a reduction in basal tone (3.5±0.4 vs1.2±0.22 cm$H_2O$, $p<0.05$ n=5). To study the effect of NaHS on a pharmacologically induced contraction, we examined the effect of perfusing 300μM NaHS on the contraction induced by 30μM bethanechol under control conditions, in the presence of TTX, bethanechol induced a contraction that partially relaxed during a 5 minute perfusion. The amplitude of this contraction was significantly attenuated in the presence of 300μM NaHS. (25.8±6.6 vs 10.2±3.5, $p<0.05$ n=6). Contraction amplitude was restored after washout of NaHS. (Figure 4)

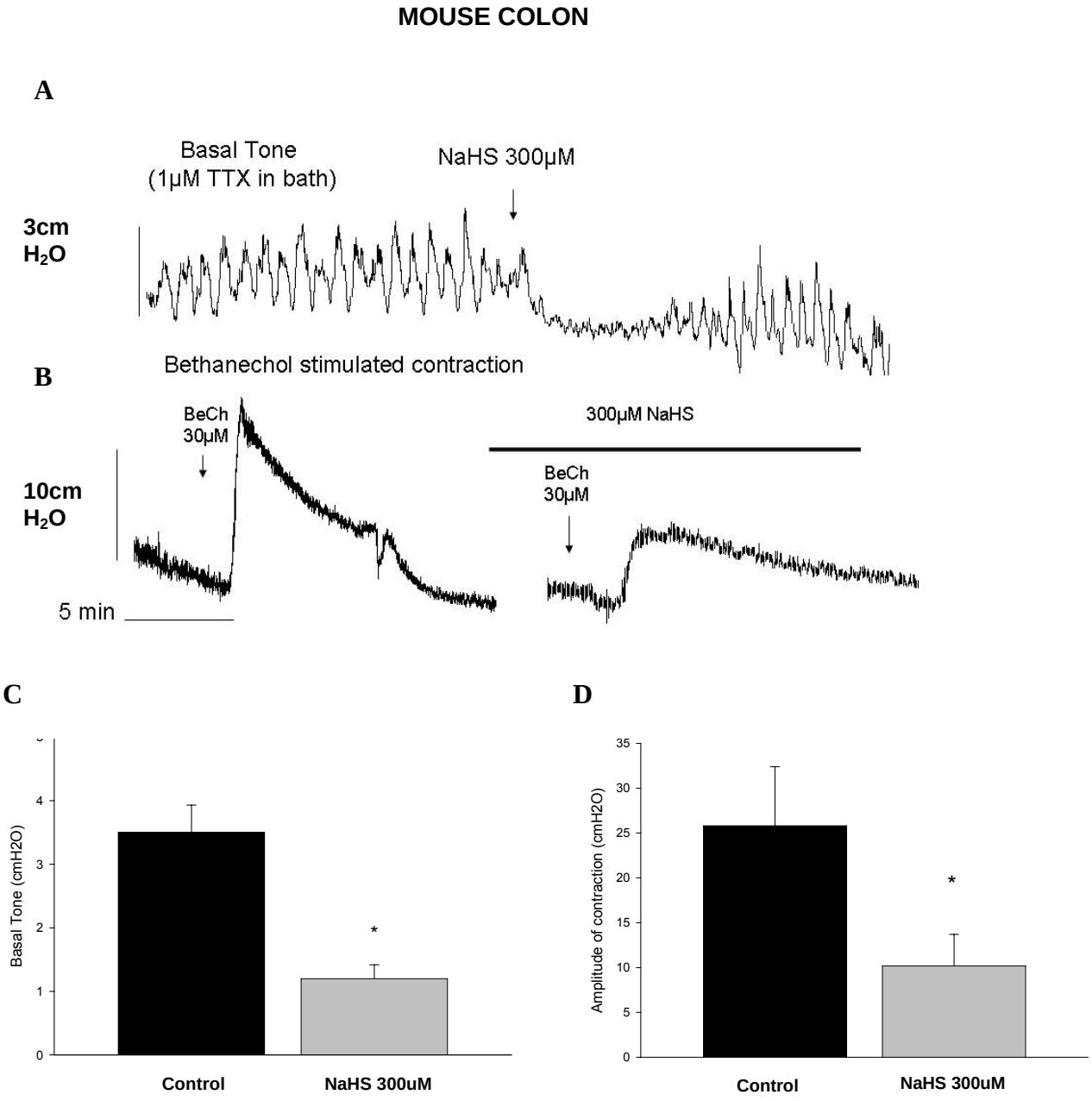



*Figure 4. NaHs directly inhibits smooth muscle contraction in the mouse colon. In the presence of TTX, motor complexes are abolished and only oscillating slow wave-like activity is seen. NaHS causes a fall in basal (myogenic) tone (A). When a contraction is stimulated by 30 nM bethanechol, the amplitude is significantly reduced in the presence of NaHS (B). Summary of the effect of NaHS on basal tone (C) and bethanechol contraction amplitude.*

## Effects of NaHS in human and rat colon muscle strips

NaHS, the $H_2S$ donor, dose dependently inhibited spontaneous motility. In the human colon $IC_{50}$ was 261μM (95% confidence interval log $IC_{50}$= -3.58± 0.05; n=5) and in the rat colon $IC_{50}$ was 31μM (95% confidence interval log $IC_{50}$= -4.5±0.04 n=6). No major differences were found when dose response curves were performed in the presence of the neural blocker tetrodotoxin (TTX 1μM). In the Human: $IC_{50}$ was 183μM (95% confidence interval log $IC_{50}$= -3.73±0.08; n=5, ns) and in the rat colon $IC_{50}$ 26μM (95% confidence interval log $IC_{50}$= -4.57±0.04; n=6, ns). (Figure 5)

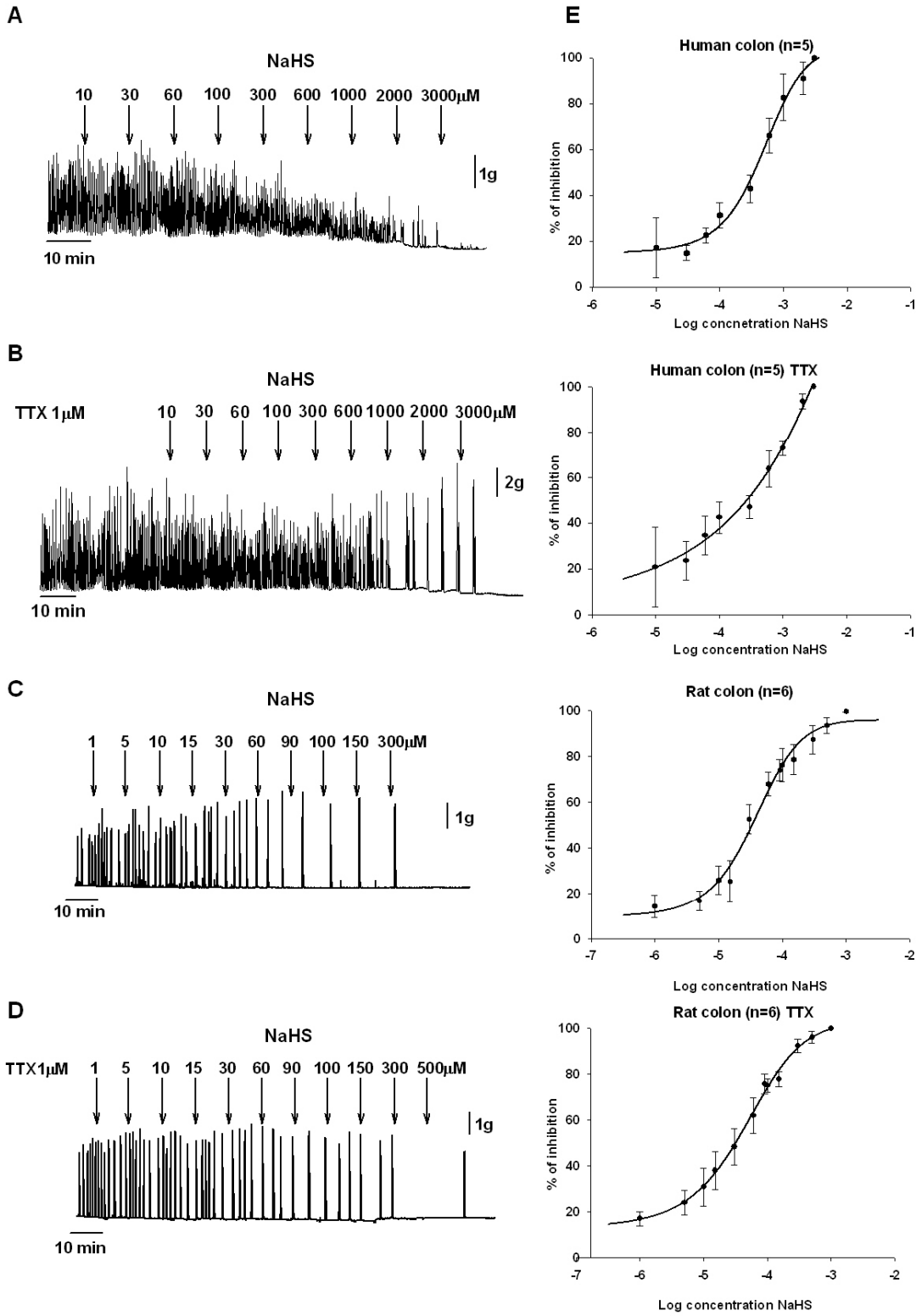



*Figure 5.(A) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (10-3000$\mu$M) in control conditions and (B) after the incubation with TTX 1$\mu$M in the human colon. (C) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (1-1000 $\mu$M) in control conditions and (D) after the incubation with TTX 1$\mu$M in the rat colon.(E) Dose response curves.*

## Role of potassium channels

Available evidence suggests that some of the effects of $H_2S$ may be mediated via activation of one or more potassium channel subtypes. We therefore designed a number of experiments to examine the role of K channels in the actions of $H_2S$ in the GI tract. In the presence of TTX, TEA (10mM), a K channel blocker, significantly reduced the inhibitory effect induced by $H_2S$. The NaHS $IC_{50}$ was reduced to 2421μM (95% confidence interval log $IC_{50}$= 2.6±0.06; n=5, P<0.0001) for the human colon and 674μM (95% confidence interval log $IC_{50}$= -3.1±0.04; n=6, P<0.0001) for the rat colon. (Figure 6)

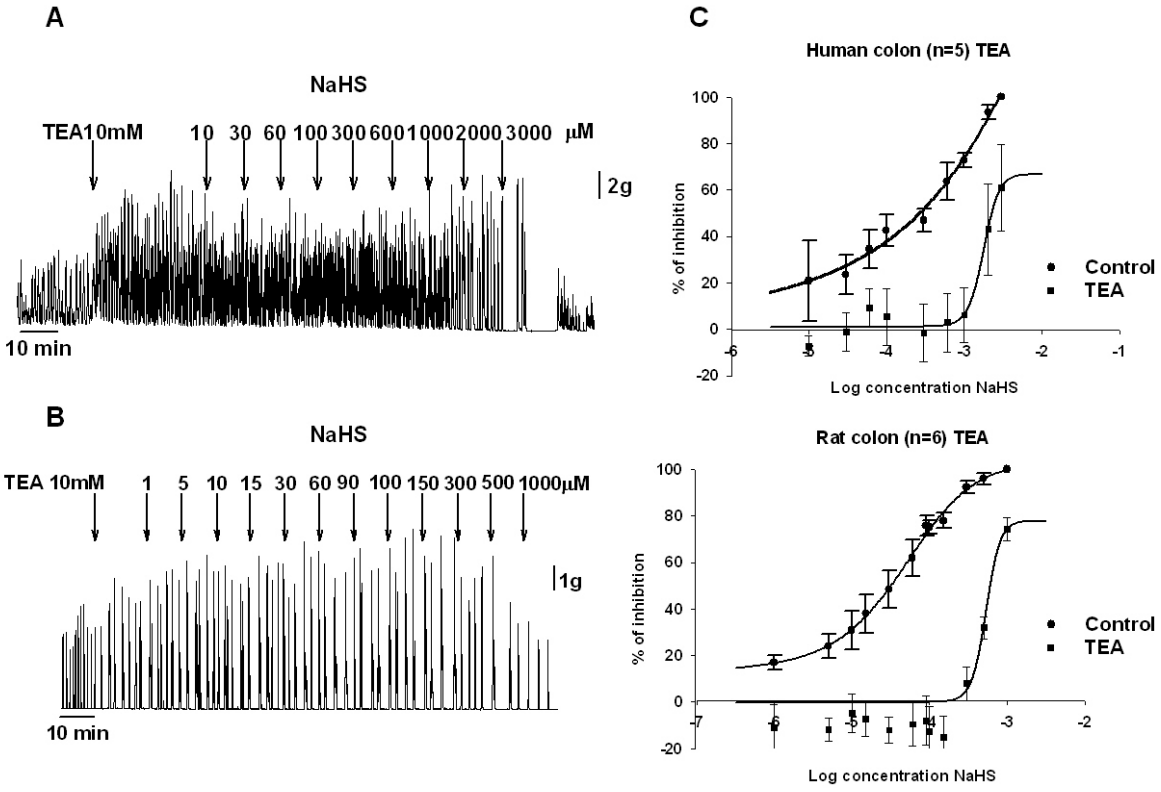



*Figure 6. (A) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (10-3000μM) after the incubation with TEA 1mM in the human colon (B) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (1-1000 μM) after the incubation with TEA 1mM in the rat colon. (C) Dose response curves.*

In the presence of TTX (1μM) (Figure 7), the ATP sensitive channel blocker glybenclamide (10μM) significantly reduced the inhibitory effect of $H_2S$. Glybenclamide increased the NaHS $IC_{50}$ to 2464μM (95% confidence interval log $IC_{50}$= -2.6±0.1; n=5, P<0.0001) for the human colon and to 80μM (95% confidence interval log $IC_{50}$= -4.0±0.04; n=6 P<0.0001) for the rat colon.

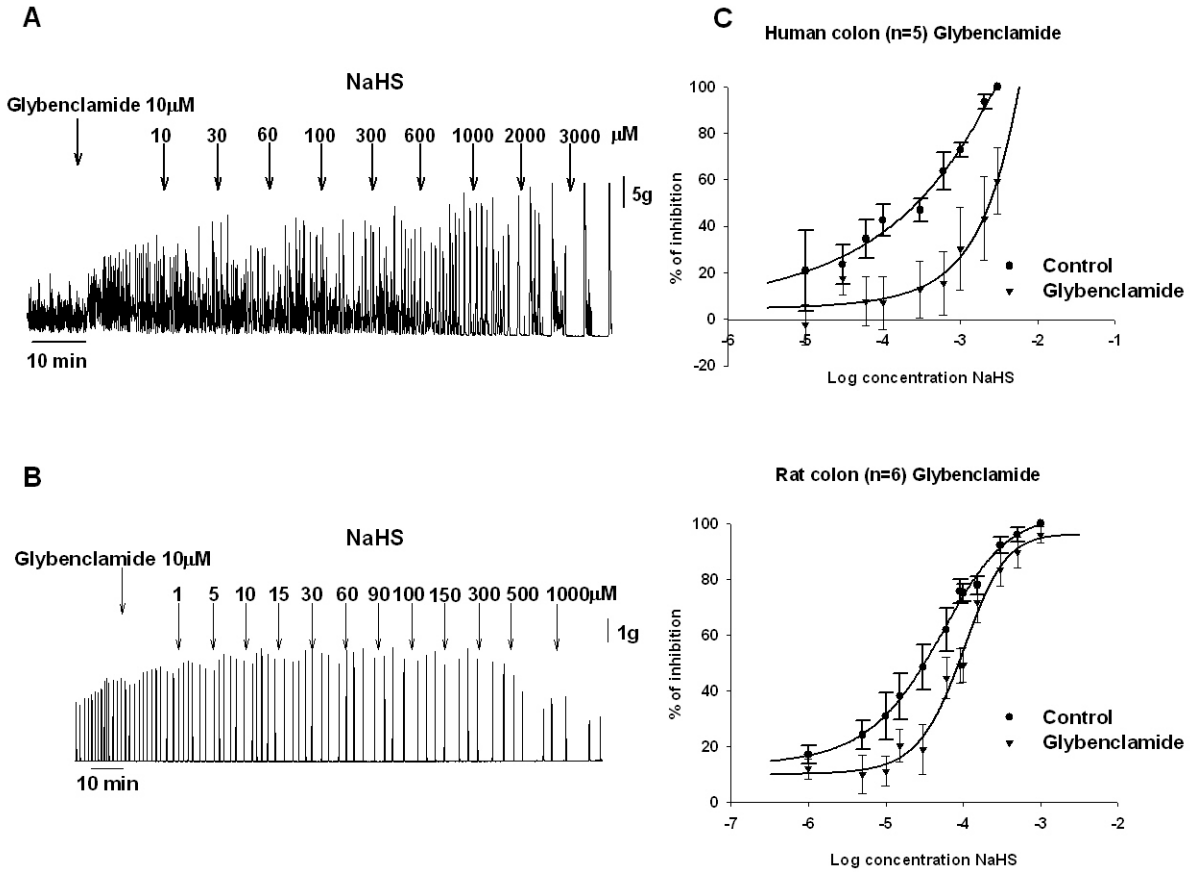



*Figure 7. (A) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (10-3000μM) after the incubation with Glybenclamide 10μM in the human colon (B) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (1-1000 μM) after the incubation with Glybenclamide 10μM in the rat colon. (C) Dose response curves.*

In the presence of TTX, the small conductance calcium activated potassium channel blocker apamin (3μM) significantly reduced the inhibitory effect of $H_2S$. Apamin increased the NaHS $IC_{50}$ to 1307μM (95% confidence interval log $IC_{50}$= -2.88±0.07; n=5, P<0.0001) in the human colon and to 167μM (95% confidence interval log $IC_{50}$= -3.77±0.06, n=6, P<0.0001) in the rat colon (Figure 8).

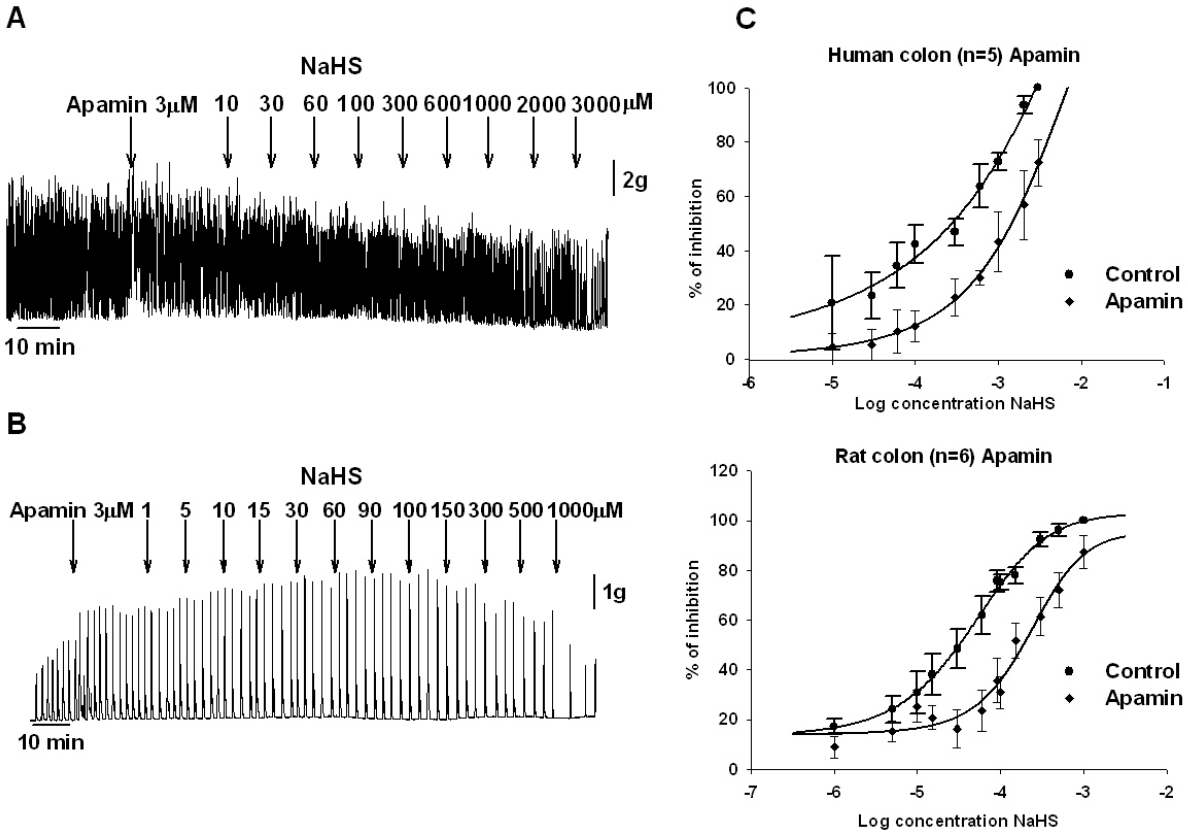



*Figure 8. (A) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (10- 3000 μM) after the incubation with Apamin 3μM in the human colon (B) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (1- 1000 μM) after the incubation with Apamin 3μ M in the rat colon. (C) Dose response curves.*

Based on these results we examined the effect of apamin on the NaHS induced inhibition of motor complexes in the mouse colon. In the mouse colon preparation, the inhibitory effect of NaHS on spontaneous motor complexes was significantly attenuated by apamin (3μM). In some cases (see Figure 9) the inhibitory effect was converted to excitation in the presence of apamin, suggesting a crucial role for SK channels in the effects of NaHS on colonic motility.

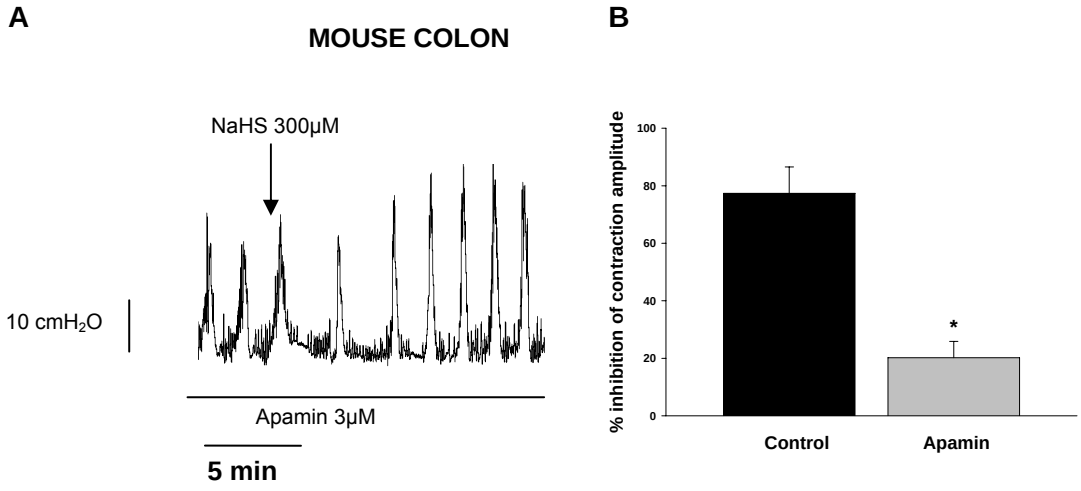



*Figure 9. Mechanical recordings showing reversal of the inhibitory effect of NaHS (300μM) in the MCs by Apamin (3μM) in the mouse colon (**left**) and percentage of inhibition induced by NaHS in the absence and presence of apamin (**right**).*

In the presence of TTX, glybenclamide (10μM) and apamin (3μM) had a cumulative effect reducing the inhibition caused by NaHS, the $IC_{50}$ was 3294 μM (95% confidence interval log $IC_{50}$= -2.51±0.08; n=5, P<0.0001) for the human colon and 263 μM (95% confidence interval log $IC_{50}$= -3.57±0.05; n=6, P<0.0001) for the rat colon. These results were statistically different from those obtained with apamin and glybenclamide alone. (Figure 10)

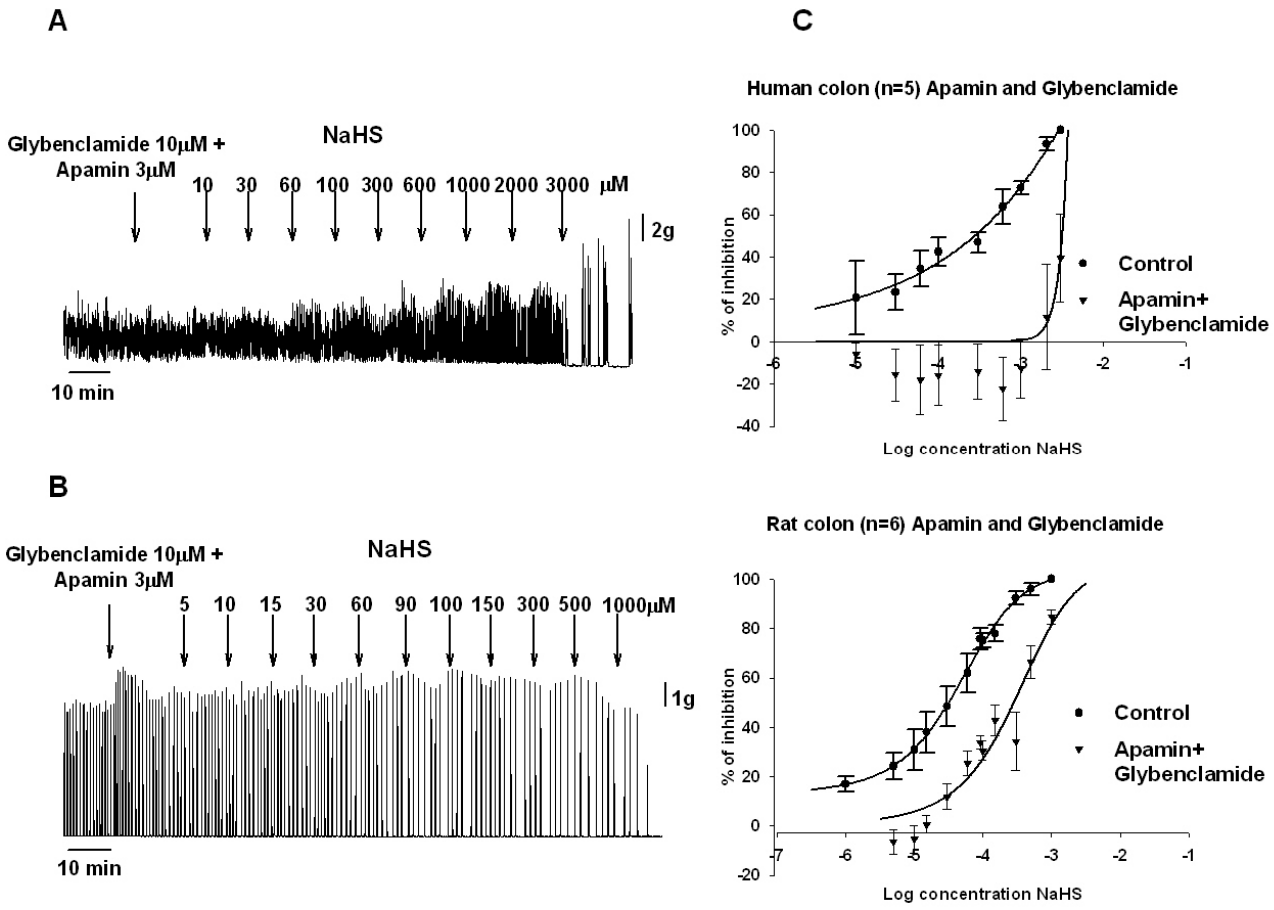



*Figure 10. (A) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (10-3000$\mu$M) after the incubation with Glybenclamide 10$\mu$M and Apamin 3$\mu$M in the human colon (B) Mechanical recordings showing the inhibitory effect of cumulative doses of NaHS a $H_2S$ donor (1-1000 $\mu$M) after the incubation with Glybenclamide 10$\mu$M and Apamin 3$\mu$M in the rat colon. (C) Dose response curves.*

## *Discussion*

The toxic gas, hydrogen sulphide, has recently been described in a number of tissues as a novel gaseous mediator. $H_2S$ has been shown to have effects on a variety of smooth muscle preparations, including vascular, bladder and intestine. Because of the large amounts of $H_2S$ present in the intestine and colon, we sought to examine the effects of the $H_2S$ donor, NaHS on motor patterns in isolated jejunum and colon. Our experiments clearly demonstrate that NaHS, at physiologically relevant concentrations, inhibits spontaneous motor complexes in the isolated jejunum and colon. These effects are not mediated by TRPV1 receptors on capsaicin sensitive nerves (as suggested in bladder, and GI secretory effects) nor are they dependent on endogenous inhibitory reflexes utilizing NO or ATP. We have however shown that NaHS inhibits motility by directly inhibiting smooth muscle contraction, largely through an action on multiple potassium channels.

Work by Pattachini et al has demonstrated that in isolated urinary bladder muscle that NaHS caused contraction that was abolished by capsaicin pre-treatment (Patacchini *et al.*, 2004). Furthermore, the effects of NaHS were similar to that of capsaicin, and could be blocked by neurokinin antagonists. These observations, taken together were interpreted to suggest that NaHS stimulated endogenous capsaicin sensitive nerves, resulting in tachykinin release, and subsequent smooth muscle contraction. Recent work by Schichio et al has also demonstrated a pro secretory effect of $H_2S$ in the rodent and human colon, as well as stimulation of enteric nerves. Capsaicin sensitive nerves were also implicated in these effects (Schicho *et al.*, 2006). This raised the possibility that NaHS might act via the capsaicin receptor, TRPV1. Indeed in our experiments, capsaicin inhibited motor complexes, similar to the effect of NaHS. However, utilizing TRPV1 deficient mice, we found that the effects of NaHS were almost identical to the wildtype controls. Experiments performed by Pataccini et al showed that the effects of NaHS in bladder could not be blocked by the TRPV1

antagonists capsazepine and SB366791, however the effect of NaHS was antagonized with ruthenium red. The authors suggested that this raised the possibility of an action on TRPV1 receptors at a novel site or other TRP channels. In a separate study, TRPV1 antagonists attenuated NaHS induced neurogenic inflammation in the lung (Patacchini *et al.*, 2005; Trevisani *et al.*, 2005). However our experiments with transgenic mice effectively rule out, at least in the mouse intestine, a role for the TRPV1 receptor in mediating NaHS inhibition of motility, our results here point to other mechanisms of action.

Previous work in vascular smooth muscle, has suggested that nitric oxide may mediate, or synergize with the effects of $H_2S$ (Zhao *et al.*, 2001). Given that NO is one of the most important inhibitory neurotransmitters in the gut, we examined its role in the inhibition of peristalsis induced by NaHS. L-NAME, at a concentration that effectively blocks endogenous NO synthase, altered peristaltic contractions in the jejunum and colon in different ways. In the jejunum, contraction amplitude and frequency were increased, as was basal tone. In contrast in the colon, contraction amplitude was decreased. The reasons for these regional differences are not clear. (*Powell et al., 2003*) have made a similar observation in the mouse colon, using another NOS inhibitor. It may be that NO plays a predominantly inhibitory role in the jejunum, while in the colon, NO may activate some excitatory neural circuits itself, or mediate a post inhibitory “rebound” contraction. Another possibility is that NO excites the longitudinal muscle, as has been demonstrated in the esophagus (Zhang *et al.*, 2000). Nonetheless, despite alterations in their basal motility patterns, L-NAME failed to prevent the inhibition of peristalsis by NaHS, suggesting that endogenous NO does not play a role in NaHS induced inhibition.

ATP is increasingly recognised as an important inhibitory neurotransmitter in the gut. Acting through P2Y receptors it mediates a fast inhibitory junction potential (Xue *et al.*, 1999; Serio *et al.*, 2003; Gallego *et al.*, 2006). Furthermore, ATP release can be stimulated by a variety of noxious stimuli in the viscera (eg distention, hypoxia etc.)

(Burnstock & Wood, 1996). Our own results show that exogenous ATP can inhibit MCs in the jejunum and colon. Additional preliminary data suggests that $H_2S$ can excite intestinal afferent fibres, in part via ATP release and an action on PPADS sensitive receptors (Rong *et al.*, 2005). We therefore examined the effects of PPADS on NaHS inhibition of MCs. At concentrations that prevent the inhibition of MCs by exogenous ATP, there is no effect on the inhibition of MCs by NaHS, suggesting that ATP does not mediate the inhibitory effects.

There is recent literature suggesting that $H_2S$ may directly inhibit contraction in a variety of smooth muscle such as bladder, vascular, and in ileal smooth muscle strips (Hosoki *et al.*, 1997). To examine this possibility in our preparation, we blocked neurally mediated MCs with TTX, thus permitting examination of the direct effects on smooth muscle. Under these conditions, $H_2S$ inhibited both basal tone, and inhibited the contraction evoked by the muscarinic agonist bethanechol. Subsequently, utilizing muscle strips from the rat and human colon, we used a number of pharmacological approaches to examine the ionic mechanism of action of $H_2S$ on these tissues. Blockade of potassium channels with the non selective potassium channel blocker TEA resulted in significant attenuation of the inhibitory effect of NaHS. This led us to utilize more selective blockers of specific potassium channel families. The selective sKCa channel blocker apamin resulted in significant inhibition of the relaxant effects of NaHS. In some cases the inhibitory effect was converted by apamin to an excitatory one. In addition glybenclamide, a K(ATP) channel antagonist, also significantly diminished the inhibitory effect of NaHS. These results are in agreement with that in vascular smooth muscle and in the rabbit ileum. It appears that from our experiments here that the relaxant effect of $H_2S$ in human and rodent GI smooth muscle is direct via the activation of smooth muscle K channels. Interestingly in the isolated mouse colon prepration, in some cases, in the presence of apamin, an excitatory effect of NaHS was unmasked. The mechanism of these remains to be seen, however the action of NaHS may depend critically on the relative balance between inhibitory and excitatory actions. The finding

of an important role for calcium activated potassium channels suggests that increase in intracellular calcium may be important, this may explain the finding of excitation in the presence of apamin. The source of this intracellular calcium rise remains to be determined. Further experiments using intracellular microelectrode and patch clamp techniques are needed to fully characterize the ionic mechanisms underlying $H_2S$ induced smooth muscle relaxation.

The finding that $H_2S$ is a potent inhibitor of motility is perhaps surprising, given the large quantities present in the GI tract. Our experiments did however demonstrate that luminal application of NaHS far less effectively and consistently inhibited motility compared to bath application. This suggests that, even in the jejunum, the mucosa serves as a particularly effective barrier to the diffusion of $H_2S$ across to the muscle layers. Indeed, the colonic mucosa is endowed an efficient $H_2S$ detoxifying mechanism, oxidizing more than 300μmol of $H_2S$ to thiol compounds daily in the rat colon (Suarez *et al.*, 1998). However the possibility remains that when this barrier is broken down, such as in severe colitis, that a greater amount of $H_2S$ can access the muscle layers and inhibit motility, possibly becoming clinically relevant in conditions such as toxic megacolon which can complicate severe colitis. It is of particular interest that $H_2S$ has been implicated in the pathogenesis of acute pancreatitis, and lipopolysaccharide-induced systemic inflammation (Bhatia *et al.*, 2005; Li *et al.*, 2005; Hu *et al.*, 2007) two conditions known to be associated with increased gut permeability and bacterial translocation. Recent work has also demonstrated an antinociceptive effect of NaHS in the colon (Distrutti *et al.*, 2006), how much of this is due to muscle relaxation is not clear.

In summary, our studies have demonstrated a novel inhibitory action of $H_2S$ on rodent and now for the first time, human colonic motility. This effect was direct, and did not depend on TRPV1, P2 receptors or an NOS dependent mechanism. The inhibitory action of $H_2S$ appears to be critically dependent on K channels, particularly apamin sensitive sKCa channels and glybenclamide sensitive K(ATP) channels. These

observations may have physiological and pathophysiological relevance in conditions where $H_2S$ production is increased or where detoxification mechanisms are impaired.

# *Bibliography*

Abe K & Kimura H (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* **16**, 1066-1071.

Bhatia M (2005). Hydrogen sulfide as a vasodilator. *Iubmb Life* **57**, 603-606.

Bhatia M, Wong FL, Fu D, Lau HY, Moochhala SM, & Moore PK (2005). Role Of Hydrogen Sulfide In Acute Pancreatitis And Associated Lung Injury. *Faseb J* **19**, 623-625.

Burnstock G & Wood JN (1996). Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Curr Opin Neurobiol* **6**, 526-532.

Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Antonelli E, Roviezzo F, Morelli A, Cirino G, Wallace Jl, & Fiorucci S (2006). Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating Katp channels. *J Pharmacol Exp Ther* **316**, 325-335.

Gallego D, Hernandez P, Clave P, & Jimenez M (2006). P2Y1 receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. *Am J Physiol Gastrointest Liver Physiol* **291**, G584-G594.

Hosoki R, Matsuki N, & Kimura H (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* **237**, 527-531.

Hu LF, Wong PT, Moore PK, & Bian JS (2007). Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia. *J Neurochem* **100**, 1121-1128.

Kamoun P (2004). [H2S, a new neuromodulator]. *Med Sci (Paris)* **20**, 697-700.

Kimura H (2002). Hydrogen sulfide as a neuromodulator. *Mol Neurobiol* **26**, 13-19.

Kimura H, Nagai Y, Umemura K, & Kimura Y (2005). Physiological roles of hydrogen sulfide: synaptic modulation, neuroprotection, and smooth muscle relaxation. *Antioxid Redox Signal* **7**, 795-803.

Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M, & Moore PK (2005). hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *Faseb J* **19**, 1196-1198.

Moore PK, Bhatia M, & Moochhala S (2003). Hydrogen sulfide: from the smell of the past to the mediator of the future? *Trends Pharmacol Sci* **24**, 609-611.

Patacchini R, Santicioli P, Giuliani S, & Maggi CA (2004). Hydrogen Sulfide (H2s) Stimulates Capsaicin-Sensitive Primary Afferent Neurons In The Rat Urinary Bladder. *Br J Pharmacol* **142**, 31-34.

Patacchini R, Santicioli P, Giuliani S, & Maggi CA (2005). Pharmacological investigation of hydrogen sulfide (H2S) contractile activity in rat detrusor muscle. *Eur J Pharmacol* **509**, 171-177.

Qu K, Lee SW, Bian JS, Low CM, & Wong PT (2007). Hydrogen sulfide: neurochemistry and neurobiology. *Neurochem Int*.

Rong WF, Beyak M, Bulmer D, Jiang W, Winchester WJ, & Grundy D (2005). Hydrogen sulphide activates small intestinal afferent fibres through a purinergic mechanism. *Gastroenterology* **128**, A225.

Schicho R, Krueger D, Zeller F, Von Weyhern CW, Frieling T, Kimura H, Ishii I, De GR, Campi B, & Schemann M (2006). Hydrogen sulfide is a novel prosecretory neuromodulator in the guinea-pig and human colon. *Gastroenterology* **131**, 1542-1552.

Searcy DG & Lee SH (1998). Sulfur reduction by human erythrocytes. *J Exp Zool* **282**, 310-322.

Serio R, Alessandro M, Zizzo MG, Tamburello MP, & Mule F (2003). Neurotransmitters involved in the fast inhibitory junction potentials in mouse distal colon. *Eur J Pharmacol* **460**, 183-190.

Suarez F, Furne J, Springfield J, & Levitt M (1998). Production and elimination of sulfur-containing gases in the rat colon. *Am J Physiol* **274**, G727-G733.

Trevisani M, Patacchini R, Nicoletti P, Gatti R, Gazzieri D, Lissi N, Zagli G, Creminon C, Geppetti P, & Harrison S (2005). Hydrogen sulfide causes vanilloid receptor 1-mediated neurogenic inflammation in the airways. *Br J Pharmacol* **145**, 1123-1131.

Xue L, Farrugia G, Sarr MG, & Szurszewski JH (1999). ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *Am J Physiol* **276**, G1373-G1379.

Zhang Y, Miller DV & Paterson WG (2000). Opposing roles of K(+) and Cl(-) channels in maintenance of opossum lower esophageal sphincter tone. *Am J Physiol Gastrointest Liver Physiol* **279**, G1226-G1234.

Zhao W, Zhang J, Lu Y, & Wang R (2001). The vasorelaxant effect Of H(2)S as a novel Endogenous gaseous K(ATP) channel opener. *Embo J* **20**, 6008-6016.

# Discusión general

La discusión particular de cada uno de los uno de los apartados ha sido presentada en en su capítulo correspondiente. En este apartadado se abordan los grandes conceptos en base a una discusión general que pretende ser integradora entre las diversas discusiones.

La neurotransmisión no-adrenérgica no-colinérgica (NANC) inhibitoria es fundamental para asegurar diversas funciones del aparato digestivo, entre las que se encuentran: la acomodación gástrica (Andrews *et al.*, 2007), la relajación de esfínteres como el esfínter esofágico inferior (LES) (Gonzalez *et al.*, 2004;Farre *et al.*, 2006) o el anal interno (O'Kelly *et al.*, 1993) y la relajación descendente del reflejo peristáltico (Grider & Jin, 1994; Bornstein *et al.*, 2004; Grider, 2003a;Grider, 2003b;Kunze & Furness, 1999;Olsson & Holmgren, 2001), así como la relajación de las diversas áreas del tracto gastrointestinal (Allescher *et al.*, 1992; Bitar & Makhlouf, 1982; Bult *et al.*, 1990; Christinck *et al.*, 1991; De Man *et al.*, 2003; Burnstock *et al.*, 1970; Farrugia *et al.*, 2003; Stark *et al.*, 1993). La identidad de estos neurotransmisores NANC es todavía un tema en discusión, aunque se piensa que están bastante conservados en las diferentes especies de mamíferos y en las diferentes áreas del tracto gastrointestinal (Hansen, 2003; Schemann & Neunlist, 2004). Se han propuesto diversos neurotransmisores como inhibitorios, entre ellos el ATP, el óxido nítrico (NO), el VIP, el PACAP, el CO e incluso el $H_2S$ (Lecci *et al.*, 2002; Teague *et al.*, 2002; Hosoki *et al.*, 1997).

Este estudio se ha centrado en la interacción de estos neurotransmisores NANC inhibitorios, principalmente el ATP y el NO, en la unión neuromuscular y en el efecto que los neurotransmisores causan a nivel de potencial de membrana (hiperpolarizando las células musculares lisas) y a nivel motor (relajando el músculo

liso e inhibiendo la motilidad). Además de interaccionar a nivel muscular mediando las relajaciones en las diversas áreas del tubo digestivo, los neurotransmisores NANC, poseen otras funciones como la prosecretora a nivel de plexo submucoso, como es el caso del ATP en el cobayo (Hu *et al.*, 2003), o bien el caso del sulfhídrico en el colon humano y de cobayo (Schicho *et al.*, 2006). Para los péptidos como el VIP o el PACAP se han propuesto funciones inmunitarias (Genton & Kudsk, 2003). Estos neurotransmisores podrían interferir también en el mecanismo marcapasos, ya que se ha descrito que las ICCs poseen receptores purinérgicos y que el ATP podría modular las ondas lentas (Burnstock & Lavin, 2002).

Para considerar que una determinada sustancia participa en un determinado proceso de neurotransmisión deben cumplirse diversos criterios:

En primer lugar, el neurotransmisor debe sintetizarse y almacenarse en los terminales pre-sinápticos.

En segundo lugar, cuando esta sustancia se administra de forma exógena (como fármaco), debe imitar exactamente la acción del transmisor liberado de forma endógena.

En tercer lugar, debe ser liberada en cantidad suficiente como para ejercer una acción definida sobre receptores post-sinápticos, específicos, en la neurona post-sináptica u órgano efector.

En cuarto y último lugar, debe existir un mecanismo específico para eliminarla del lugar de acción (hendidura sináptica, placa motora…), es decir, enzimas para su degradación o inactivación, o bien mecanismos de recaptación (Kandel *et al.*, 2001; Burnstock, 2007).

En base a estos criterios se pueden discutir los resultados obtenidos en esta tesis doctoral.

## *Síntesis y almacenamiento de ATP y NO*

En el caso del ATP, está ampliamente aceptado que la síntesis de este neurotransmisor se produce a nivel mitocondrial, y que éste es almacenado en vesículas (Burnstock, 2007).

El ATP se almacena en vesículas pre-sinápticas de dos tipos: vesículas claras que se suelen encontrar en la zona activa de la terminal pre-sináptica, que almacenan moléculas pequeñas como acetilcolina o aminas biógenas, y vesículas de núcleo denso que se pueden localizar a lo largo de todo el citoplasma de la neurona pre-sináptica, que almacenan moléculas de gan peso molecular como péptidos. Estas últimas podrían estar implicadas en neuromodulación (Kandel *et al.*, 2001).

En este estudio no hemos demostrado la síntesis o presencia de ATP en las neuronas pre-sinápticas. Usualmente, el marcador empleado para demostrar la presencia a nivel pre-sináptico de ATP, es una proteína fluorescente, la quinacrina, que se une a proteínas vesiculares ligadas a ATP (Belai & Burnstock, 1994; Belai & Burnstock, 2000; Burnstock, 2004; Burnstock, 2007; Burnstock, 2006).

El óxido nítrico es sintetizado a partir del aminoácido L-arginina, que pasa a citrulina en presencia de NADPH, tetrahidrobiopterina y oxígeno. Esta reacción es catalizada por la sintasa de óxido nítrico o NOS (Moncada & Bolanos, 2006). Existen marcadores bastante específicos para detectar la presencia de NO a nivel pre-sináptico, se suelen utilizar marcadores para la NOS neural, su enzima de síntesis, y marcadores para la NADPH diaforasa, ya que el NADPH es cofactor de la NOS. Este último marcador está actualmente en entredicho ya que, en algunos trabajos de investigación, se ha visto que no se puede correlacionar el marcaje de NOS con la

NAPDH diaforasa (Belai & Burnstock, 2000), o bien que marca de forma inespecífica tipos celulares que no sintetizan NO como células conectivas (Keranen *et al.*, 1995). Numerosos estudios se han realizado marcando óxido nítrico, y han demostrado una correlación entre la pérdida de neuronas nitrérgicas y las diversas patologías en las que está implicado, como es el caso de la acalasia (Mearin *et al.*, 1995;Mearin *et al.*, 1993). Respecto a este neurotransmisor, se ha sugerido que se sintetiza en función de la demanda y que por tanto no estaría almacenado en vesículas, aunque también se ha podido detectar NOS unida a membranas vesiculares. Otra teoría es que, el óxido nítrico, se almacenaría en moléculas más estables que contuvieran este gas, como los S-nitrosotioles, aunque este tema está todavía en discusión (Olgart *et al.*, 2000).

Es un objetivo de futuras investigaciones desarrollar mejores marcadores purinérgicos y estandarizar su detección mediante técnicas inmunohistoquímicas. Esto permitiría valorar la pérdida de inervación purinérgica en diversas patologías en que se ha propuesto una pérdida o alteración de la neurotransmisión purinérgica, como es la enfermedad de Hirschprung (Zagorodnyuk *et al.*, 1989).

## *Mecanismos de liberación de ATP y NO*

Para que se produzca la liberación a nivel pre-sináptico de estas vesículas es necesario que haya un incremento del calcio intracelular en la zona del botón sináptico (zona activa). Se ha demostrado en el íleon de cerdo que los canales de calcio implicados en la liberación del ATP son los canales de calcio tipo N, que son bloqueados con ω-conotoxina GVIA (Borderies *et al.*, 1997). Estos datos obtenidos en el íleon de cerdo han sido corroborados, en el colon humano, mediante la técnica de microelectrodos. Hemos podido observar que la ω-conotoxina GVIA bloquea el componente rápido, purinérgico, del IJP (IJPf) (observaciones no publicadas). Respecto al NO, los mecanismos de liberación, están todavía en discusión, puesto que no se sabe si se encuentra o no en vesículas pre-sinápticas, si su síntesis se da según

la demanda o bien si se encuentra almacenado en otras estructuras como los S-nitrosotioles. Hay evidencias que apoyan a cada una de las tres teorías (Olgart *et al.*, 2000); sin embargo, sí se sabe que su liberación es dependiente de calcio. Se han publicado estudios llevados a cabo en el colon de cobayo que muestran que la ω-conotoxina GVIA abole el componente rápido del IJP, el mediado por ATP, pero no el componente lento, el mediado por NO, por lo tanto, según estas observaciones, la liberación de NO sería independiente de los canales de calcio tipo N (Bridgewater *et al.*, 1995). Nuestros estudios, sin embargo, no respaldan estas observaciones obtenidas en el colon de cobayo, ya que en el colon humano la ω-conotoxina GVIA, bloquea además del componente rápido del IJP, el componente lento (IJPs) o nitrérgico (observaciones no publicadas).

## *Administración exógena del neurotransmisor*

En este estudio hemos demostrado que, la administración exógena de ADPβS (agonista P2Y), produce, tanto en el colon humano como en el íleon de cerdo, una hiperpolarización del potencial de membrana en las células musculares lisas de ambas preparaciones de aproximadamente unos diez mili voltios y, tanto el ATP como el ADP βS, producen una relajación a nivel mecánico (de la motilidad espontánea o inducida por carbacol). Diversos estudios llevados a cabo en el tracto gastrointestinal de varias especies respaldan estos resultados.

Uno de los primeros estudios en que se evaluó la adición exógena de purinas, fue llevado a cabo por Burnstock y colaboradores en 1970. En este estudio se testó el efecto de diversas purinas y pirimidinas sobre preparaciones de tenia de cobayo, y se pudo observar como, con diferente potencia, éstas relajaban el músculo liso (Burnstock *et al.* 1970). Posteriormente en estudios más recientes se han llevado a cabo las siguientes observaciones:

En el yeyuno de ratón, tanto la adición exógena de ATP como de ADPβS producen una relajación mecánica (De Man *et al.*, 2003). En esta misma especie, se ha estudidado la potencia de relajación de diferentes agonistas purinérgicos por adición exógena y se ha establecido que, la purina de mayor efecto relajante es el 2-MeSADP (fármaco que presenta igual potencia de agonismo que el ADPβS) (Giaroni *et al.*, 2002). En el esfínter esofágico inferior (LES) porcino la adición de ATP produjo una relajación del tono de forma dosis-dependiente (Farre *et al.*, 2006). En el colon proximal de cobayo la adición exógena de ATP produjo también una relajación dosis-dependiente (Briejer *et al.*, 1995). Mediante la técnica de sucrose gap se ha mostrado que, en el músculo circular liso del intestino humano, el ATP produce tanto relajación como hiperpolarización de este tejido (Zagorodnyuk *et al.*, 1989). En el colon de ratón, mediante la técnica de microelectrodos, se ha podido demostrar que la adición exógena de ADPβS hiperpolariza las células musculares de la capa circular (Zizzo *et al.*, 2006). Utilizando la misma técnica en el músculo liso circular del yeyuno humano, se ha demostrado que, el ATP produce una hiperpolarización fásica que se asemeja al componente rápido del IJP (Xue *et al.*, 1999), mientras que la adición de ATP en el músculo liso circular del yeyuno canino, produjo una liberación a nivel local de óxido nítrico y una hiperpolarización similar al componente lento del IJP (IJPs) (Xue *et al.*, 2000). En el colon medio de rata el ATP administrado de forma exógena hiperpolariza las células musculares lisas e inhibe la motilidad espontánea (Pluja *et al.*, 1999). Estos resultados demuestran que, el ATP cumple el criterio para ser considerado como neurotransmisor inhibitorio en el tracto gastrointestinal. (En la Tabla I se sumarizan estos resultados)

| Especie | Zona del tracto gastrointestinal | Referencia |
|---|---|---|
| Cobayo | tenia | Burnstock *et al.* 1970 |
| Ratón | yeyuno | De Man *et al.* 2003 |
| Ratón | diversas áreas | Giaroni *et al.* 2002 |
| Cerdo | LES | Farré *et al.* 2006 |
| Cobayo | colon proximal | Briejer *et al.* 1995 |
| Humano | intestino | Zaragorodyuk *et al.*1989 |
| Ratón | colon | Zizzo *et al.* 2002 |
| Humano | yeyuno | Xue *et al.* 1999 |
| Perro | yeyuno | Xue *et al.* 2000 |
| Rata | colon medio | Plujà *et al.* 1999 |

*Tabla I. Tabla sumario del efecto de diversos agonistas purinérgicos sobre diferentes áreas del tracto gastrointestinal. (Nota: se han incluido los datos tanto eléctricos como mecánicos para simplificar la tabla).*

## *Receptor post-sináptico: bloqueo endógeno*

Para evaluar los receptores implicados, y el efecto a nivel post-sináptico, hasta la fecha se habían utilizado antagonistas no específicos, como el PPADs, la suramina o el Reactive Blue. Estudios muy recientes llevados a cabo en el intestino y el colon humano utilizan estos antagonistas (PPADs y suramina) para demostrar que hay receptores purinérgicos post-sinápticos que median la relajación de ambos tejidos (Benko *et al.*, 2007; Undi *et al.*, 2006). Estos estudios son sólo un ejemplo, puesto que los antagonistas previamente citados, han sido utilizados en numerosos estudios, aunque se ha demostrado que, no sólo no son específicos sobre una clase de receptor, sino que, además, en algunos casos no son totalmente específicos para la vía purinérgica. Por ejemplo, la suramina revierte la relajación causada por ATP pero también revierte la causada por VIP en colon proximal de cobayo (Briejer *et al.*, 1995), e inhibe las hiperpolarizaciones causadas por VIP y PACAP, además de las causadas por ATP en el colon de rata (Pluja *et al.*, 2000).

Gracias al desarrollo del fármaco MRS 2179 (Boyer *et al.*, 1996; Camaioni *et al.*, 1998) que actualmente se considera un antagonista específico y competitivo de los receptores $P2Y_1$ (Alexander *et al.*, 2005), se ha podido llevar a cabo este trabajo de investigación, en el que se demuestra que este fármaco, en el colon humano inhibe el IJPf y la relajación no nitrérgica inducida por estimulación eléctrica, con una $IC_{50}$ aproximada de uno micro molar. En el íleon de cerdo inhibe también el IJPf con una $IC_{50}$ similar a la del colon humano e inhibe además la relajación no nitrérgica inducida por estimulación eléctrica.

En otros estudios se han observado resultados similares a los nuestros, por ejemplo, en el yeyuno de ratón. Utilizando el mismo antagonista (el MRS 2179) se ha demostrado que la relajación no nitrérgica inducida por campo eléctrico es purinérgica mediada por receptores $P2Y_1$ (De Man *et al.*, 2003). En esta misma especie se han inmunolocalizado estos receptores en diversas áreas del tracto gastrointestinal, y se ha puesto de manifiesto que éstos son, principalmente, los que median la relajación purinérgica (Giaroni *et al.*, 2002). Estudios llevados a cabo con MRS 2179 en el LES porcino han demostrado que, son estos, los receptores purinérgicos que median la relajación en el tono (Farre *et al.*, 2006). Además, en un estudio muy similar al nuestro, se ha descrito que en el colon de cobayo, el MRS 2179 inhibe el componente rápido del IJP o IJPf (Wang *et al.*, 2004; Wang *et al.*, 2007).

En este trabajo también hemos detectado la presencia de este receptor, el $P2Y_1$, a nivel neuronal, ya que a nivel de plexo submucoso de cobayo se ha descrito que este receptor posee acciones pro-secretoras. El papel de los receptores $P2Y_1$ a nivel neuronal es todavía desconocido, aunque probablemente participen en la neurotransmisión entre neuronas, puesto que se ha descrito que participan en la generación de sEPSP (Hu *et al.*, 2003).

## *Receptor post-sináptico: bloqueo exógeno*

En este estudio se ha demostrado que el MRS 2179 revierte la hiperpolarización causada por el ADPβS en el colon humano y en el íleon de cerdo. Además revierte parcialmente la inhibición de la motilidad causada por este agonista en ambos casos.

Hay muy pocos trabajos en los que se haya estudiado el bloqueo de la adición exógena: en el colon de cobayo el MRS 2179 bloquea la hiperpolarización fásica producida por ATP (Wang *et al.*, 2007), en el tracto gastrointestinal de ratón, este antagonista, revirtió la relajación mecánica causada por ATP (Giaroni *et al.*, 2002). Recientemente se ha demostrado en el colon de ratón, que la hiperpolarización producida por βNAD (agonista propuesto como el neurotransmisor que activa los receptores $P2Y_1$) es revertida por el MRS 2179, y sin embargo, este antagonista no revierte la hiperpolarización generada por el ATP en esta preparación (Mutafova-Yambolieva *et al.*, 2007).

A pesar de todos estos datos, que respaldan que la adición exógena de ATP, o purinas relacionadas, imita el efecto de la liberación exógena, este criterio puede llevar a equívoco, puesto que, puede haber receptores fuera de la unión neuromuscular o del terminal sináptico. En este caso, estos agonistas activarían todos los receptores (con mayor, o menor potencia de agonismo), enmascarando o cambiando la respuesta que tendría si se liberara de forma endógena (localizada en la unión neuromuscular).

## *Vía intracelular*

La vía intracelular que media la relajación está bastante establecida en algunos casos como el del óxido nítrico. Hay numerosos estudios publicados que respaldan la teoría de que este neurotransmisor gaseoso perfunde a través de la membrana plasmática y activa el enzima guanilato ciclasa (De Man *et al.*, 2003), aunque también se ha descrito que, de forma minoritaria, provoca la activación de canales iónicos

(Bolotina *et al.*, 1994) y la nitrosilación de receptores (Shah *et al.*, 2004). Es también el caso del monóxido de carbono, ya que está muy establecido que este gas actúa principalmente a través de la misma vía que el óxido nítrico (Farrugia *et al.*, 1998; Gibbons & Farrugia, 2004; Chakder *et al.*, 2000).

Las primeras aproximaciones para esclarecer la vía intracelular del ATP, fueron llevadas a cabo con apamina, ya que parece ser que la hiperpolarización que produce en las células musculares lisas, podría estar, al menos en parte, mediada por la activación de canales de potasio sensibles a calcio de baja conductancia (sKCa) (Zagorodnyuk *et al.*, 1989; Xue *et al.*, 1999; Xue *et al.*, 2000; Pluja *et al.*, 2000; Boeckxstaens *et al.*, 1993; Keef *et al.*, 1993). En estudios previos se ha demostrado que la apamina bloquea completamente el IJPf en el íleon de cerdo (Fernandez *et al.*, 1998). En el colon humano, sin embargo la reducción del IJPf con apamina es sólo de un treintitres por ciento, por lo que se deduce que debe haber otros mediadores finales de la vía en el colon humano, cosa que no sucedería en el íleon de cerdo, donde los mediadores finales de la estimulación de los receptores purinérgicos $P2Y_1$ serían los canales sKCa sensibles a apamina, aunque diversas evidencias muestran que este fármaco no es totalmente específico para esta vía. Se ha demostrado que, la apamina, inhibe las hiperpolarizaciones causadas por PACAP además de las causadas por ATP en el colon de rata (Pluja *et al.*, 2000), y que bloquea parte del componente lento del IJP mediado por óxido nítrico en el intestino humano (Xue *et al.*, 1999).

Actualmente, hay una discrepancia a la hora de esclarecer la vía intracelular. Se ha descrito que la mayoría de receptores P2Y, incluyendo el $P2Y_1$, están unidos a una proteína G, que al ser activada, hidroliza un lípido de la membrana plasmática dando lugar a la formación de diacilglicerol (DAG) e inositoltrifosfato ($IP_3$), y desencadenando entre otras cosas, la abertura de canales de calcio del retículo sarcoplasmático (que activaría la abertura de canales de potasio activados por calcio sensibles a apamina, entre otros) y la activación de una proteinquinasa la PKC

(Ralevic & Burnstock, 1998). Sin embargo, en el tracto gastrointestinal hay dos hipótesis, la descrita anteriormente, que ha sido corroborada en el intestino y colon de cobayo (Hu *et al.*, 2003) y otra teoría alternativa en el colon de ratón, en que se propone que estos receptores purinérgicos activan una protein kinasa A PKA y activan la formación de AMP cíclico (Zizzo *et al.*, 2006). En este trabajo de investigación no se ha llegado a comprobar ninguna de estas dos hipótesis y en un futuro sería importante poder llevar a cabo este estudio, ya que permitiría conocer mejor la fisiología del tracto gastrointestinal humano con las posibles implicaciones clínicas que esto conllevaría para el futuro desarrollo de fármacos.

Respecto a la degradación del ATP en la unión neuromuscular, se ha demostrado que hay numerosas ectonucleasas (ectoATPasas) presentes en la mayoría de tejidos (Burnstock, 2007). En este estudio, no se ha tratado la degradación post-sináptica del ATP. Sin embargo, en la mayoría de los casos, hemos utilizado ADPβS (un análogo no hidrolizable del ADP), por lo tanto, la recuperación del efecto contráctil y del potencial de membrana se daría por el lavado, en el caso del la técnica de microelectrodos (ya que el ADPβS se aplica por superfusión) y por una desensibilización del receptor, en el caso de baño de órganos. En diversos estudios se ha utilizado la técnica de desensibilización con agonistas con diferente potencia de agonismo para intentar esclarecer los receptores implicados en la neurotransmisión purinérgica (Xue *et al.*, 1999; Xue *et al.*, 2000).

## *Co-transmisión*

En los años 50, se estableció el principio de Dale. Este principio postulaba que una neurona liberaba un solo neurotransmisor, aunque probablemente esta teoría fue interpretada erróneamente por Eccles contemporáneo de Dale. Seguramente, lo que pretendía Dale, era proponer que se puede liberar un mismo neurotransmisor químico desde todos los terminales sinápticos de una neurona. Aunque Dale era consciente de

que lo más probable era, que más de un neurotransmisor podía ser liberado por una misma neurona (Eccles, 1957; Strata & Harvey, 1999). A pesar de que se demostró en diversos trabajos de investigación que las neuronas poseían los genes para producir más de un neurotransmisor, no fue hasta finales de los 70, que Burnstock propuso el concepto de co-transmisión (Burnstock, 1976). Actualmente, hay numerosas evidencias de que el ATP es un co-transmisor en muchas de las neuronas del sistema nervioso central y periférico. Un ejemplo es la co-transmisión entre noradrenalina y ATP en los nervios simpáticos que inervan el sistema nervioso entérico (Burnstock, 2004). No podemos afirmar, con los datos experimentales obtenidos en este trabajo, que el ATP y el óxido nítrico co-localicen en el plexo mientérico de las zonas que hemos estudiado, pero esta co-localización se ha demostrado previamente en el tracto gastrointestinal de rata y en el plexo mientérico humano en estadios fetales (Belai & Burnstock, 2000; Belai & Burnstock, 1994).

En este trabajo, sin embargo, sí se confirma, en el colon humano y en el íleo de cerdo, la hipótesis cada vez más extendida de que se da una co-transmisión funcional entre el óxido nítrico y el ATP, o una purina relacionada, ya que el componente rápido del IJP es inhibido por el antagonista purinérgico (MRS 2179) y el componente lento es inhibido por el inhibidor de la NO sintasa (L-NNA). Además, estos antagonistas revierten la relajación causada por estimulación eléctrica a diferentes frecuencias en el colon humano. Este hecho de que se da una co-transmisión funcional entre óxido nítrico y ATP se ha sugerido en diversas áreas del tracto gastrointestinal y en diversas especies como el hombre, el cobayo, el hámster, la rata o el cerdo (Keef *et al.*, 1993;Boeckxstaens *et al.*, 1993; Xue *et al.*, 1999; Benko *et al.*, 2007; Undi *et al.*, 2006; Rozsai *et al.*, 2001; Pluja *et al.*, 1999; Van & Lefebvre, 2004; Ishiguchi *et al.*, 2000). En estos estudios se han utilizado inhibidores no específicos del componente purinérgico como la suramina o el PPADS o bien apamina que inhibe la parte final de la vía purinérgica, de forma no del todo específica. Por tanto, este es el primer trabajo en

que se demuestra de forma específica una co-transmisión entre el NO y el ATP en el tracto gastrointestinal humano.

Hay estudios, sin embargo, que no respaldan esta hipótesis. Estos estudios proponen una neurotransmisión principalmente nitrérgica (O'Kelly *et al.*, 1993), una co-transmisión entre el NO y el VIP/PACAP (Matsuda *et al.*, 2004) o una neurotransmisión principalmente mediada por el CO (Chakder *et al.*, 2000; Rattan *et al.*, 2004). A pesar de estas discrepancias, lo más probable sea que en la mayoría de mamíferos se de una co-transmisión entre óxido nítrico y ATP y, que según el área del tracto gastrointestinal y la especie, haya una pequeña implicación de otros neurotransmisores como el CO (Farre *et al.*, 2006), de los péptidos (VIP y PACAP) (Grider, 2003a;Pluja *et al.*, 2000) e incluso de otros neurotransmisores que todavía no están ampliamente aceptados como inhibitorios en el tubo digestivo, como podría ser el sulfhídrico (Teague *et al.*, 2002;Hosoki *et al.*, 1997).Estos neurotransmisores minoritarios podrían actuar como neuromoduladores, como por ejemplo es el caso del VIP, que se ha descrito que puede provocar la producción y la liberación a nivel pre-sináptico de óxido nítrico (Said & Rattan, 2004).

En este trabajo, se demuestra que ambos neurotransmisores poseen funciones complementarias en el colon humano. El óxido nítrico estaría implicado en producir una relajación sostenida, ya que hiperpolariza de forma duradera y permite una inhibición constante de la motilidad. En cambio el ATP, o una purina relacionada, produciría una relajación fásica ya que produce una hiperpolarización rápida pero de corta duración. Esta hiperpolarización o IJPf presenta el fenómeno de “rundown” (periodo en que el IJPf se ve reducido o abolido justo después de otro IJP). Este “rundown” se da, en el caso del colon humano, por una desensibilización post-sináptica del receptor $P2Y_1$. Si extrapolamos los resultados obtenidos en el colon humano a otras áreas del tracto gastrointestinal, las diferentes propiedades del ATP y el NO, sugieren, que el NO estaría implicado en mecanismos lentos pero duraderos

como por ejemplo la acomodación gástrica (Andrews *et al.*, 2007). Mientras que el ATP, participaría en mecanismos rápidos, pero necesariamente de corta duración y por tanto podría mediar funciones como la fase descendente del reflejo peristáltico.

## *El sulfhídrico como neurotransmisor inhibitorio*

En este trabajo, también se ha demostrado, por primera vez, que el sulfhídrico podría ser considerado como neurotransmisor o neuromodulador inhibitorio en el colon humano y murino. La adición exógena de un donador de sulfhídrico, produjo una relajación dosis-dependiente tanto en el colon humano como en el colon medio de rata. Este resultado es similar al obtenido previamente en músculo liso vascular y gastrointestinal de diversas especies, donde este gas produjo una inhibición de la contracción en el músculo liso (Hosoki *et al.*, 1997; Teague *et al.*, 2002). Además, hemos demostrado que, la vía intracelular tiene como efectores finales los canales de potasio activados por calcio de baja conductancia sensibles a apamina, y los canales de potasio sensibles a ATP bloqueados con glibenclamida. Previamente, en el músculo liso vascular, se propuso que el efecto de este gas estaba mediado por canales de potasio sensibles a ATP (Bhatia, 2005; Tang *et al.*, 2005). En condiciones fisiológicas hay numerosas fuentes de producción de este gas, se ha descrito, que las neuronas del sistema nervioso entérico lo producen (Schicho *et al.*, 2006) en el colon humano y de cobayo. Se han detectado también los enzimas de síntesis de $H_2S$ en el músculo liso de estas especies (Linden *et al.*, 2006), incluso se puede producir en sangre a través de sulfuro elemental y glucosa (Searcy & Lee, 1998). Sin embargo, a nivel de colon, la mayor parte de la producción de sulfhídrico se da por parte de la flora bacteriana, en condiciones normales, la mucosa detoxifica por oxidación grandes cantidades de $H_2S$ (Suarez *et al.*, 1998), pero en los casos en los que haya una disrupción de la función barrera y una translocación bacteriana el sulfhídrico podría agravar la situación inhibiendo la motilidad.

Es importante continuar en la investigación del papel del sulfhídrico en la regulación de la motilidad intestinal, puesto que este gas puede suponer un neurotransmisor/neuromodulador clave para poder entender importantes mecanismos fisiopatológicos

## *Aplicaciones y desarrollo de futuros fármacos*

Este trabajo abre nuevas posibilidades de estudio, ya que en la mayoría de áreas del tracto gastrointestinal se conoce la función del óxido nítrico y la implicación de éste en diversas patologías (Mearin *et al.*, 1995; Mearin *et al.*, 1993;Rodriguez-Membrilla *et al.*, 1995;Tomita *et al.*, 2002;Fenton *et al.*, 2006). Sin embargo, la función del ATP o de la purina que media la neurotransmisión purinérgica, así como su papel en las diversas patologías gastrointestinales, se desconoce.

Se han realizado estudios *In Vivo* e *In Vitro* que han demostrado que el MRS 2179 inhibe la agregación plaquetaria inducida por ADP (Baurand *et al.*, 2000), y por tanto, se ha propuesto que éste podría ser un buen fármaco para ser utilizado como antitrombótico. En este estudio se ha puesto de manifiesto que el MRS 2179 bloquea la relajación no nitrérgica en el colon humano y, por tanto, que los receptores $P2Y_1$ son responsables, en parte, de la relajación del músculo liso intestinal. Este descubrimiento podría ser utilizado también para desarrollar fármacos que estimulen el receptor como el MRS 2365, que es un agonista $P2Y_1$ (Bourdon *et al.*, 2006). Estos agonistas $P2Y_1$ podrían ser utilizados, por ejemplo, como espasmolíticos combinándolos con donadores de óxido nítrico. Esta aproximación podría complementar a los bloqueadores de canales de calcio de tipo L, que actualmente se están utilizando como espasmolíticos (Strege *et al.*, 2004; Martin *et al.*, 2004).

También, en el caso del estreñimiento por tránsito lento, en el que se ha demostrado que la expresión de la NO sintasa se ve incrementada (Tomita *et al.*, 2002), podría haber una sobre expresión de receptores $P2Y_1$ o un cambio en su

estructura que evitara su desensibilización. En este caso la administración de antagonistas no sólo nitrérgicos, sino también purinérgicos específicos como el MRS 2179, serían una posible terapia.

En diversas patologías, como la fisura del esfínter anal interno, se está aplicando ya la terapia de administración exógena de donadores de óxido nítrico, como el trinitrato de glicerina de forma tópica (puesto que el NO disminuye el tono y la presión aliviando el dolor) (Shah *et al.*, 2004). Esta terapia posee numerosos efectos secundarios ya que el NO es absorbido y pasa al torrente sanguíneo provocando vasodilatación y migrañas. Pese a que se ha descrito que la neurotransmisión en el esfínter anal interno es principalmente nitrérgica (O'Kelly *et al.*, 1993), es posible, que con otro tipo de estimulación eléctrica, se pusiera de manifiesto la presencia de un componente purinérgico; como se demuestra en este estudio a nivel de colon distal y sigma, y en ese caso, una terapia con agonistas $P2Y_1$ sería una buena aproximación terapéutica, siempre y cuando, estos agonistas no fueran absorbidos a nivel sistémico, ya que el ADP es un potente agregador plaquetario y podría producir problemas a nivel de coagulación.

El descubrimiento del sulfhídrico como posible neurotransmisor o neuromodulador inhibitorio también tiene implicaciones clínicas. Hay estudios llevados a cabo en un modelo de inflamación intestinal en rata donde se ha visto que el sobrecreciendo la translocación bacteriana producen disfunciones motoras (Porras *et al.*, 2004; Porras *et al.*, 2006b). El sulfhídrico podría tener un papel importante en las alteraciones motoras en casos de inflamación intestinal, siempre que se pierda la función barrera. En estos casos se ha propuesto un papel fundamental de la iNOS y la nNOS (Sintasas de Óxido Nítrico) (Porras *et al.*, 2006a) y se ha descrito un tratamiento con fármacos interaccionando con la vía nitrérgica (Porras *et al.*, 2007). En vista de los resultados obtenidos en el colon humano, de rata y de ratón, además de la posible implicación del $H_2S$ en estos procesos, se podría interaccionar sobre esta vía. Puesto

que en este estudio se muestra, que la relajación producida por este gas se ve inhibida por bloqueadores de canales de potasio (canales de potasio de baja conductancia sensibles a apamina y canales de potasio sensibles a ATP), estos bloqueadores de potasio podrían ser una futura herramienta farmacológica en estos procesos.

En conclusión en este trabajo de investigación se ha avanzado en el conocimiento de la fisiología de diversas regiones del tracto gastrointestinal humano y de otras especies y abre nuevas posibilidades para la investigación de los mecanismos que quedan todavía por esclarecer, además de aportar nuevos conocimientos, que en un futuro permitirán avanzar la investigación clínica, para que permita encontrar un tratamiento para las patologías que afectan al tracto gastrointestinal.

# *Bibliografía*

Alexander SP, Mathie A, & Peters JA (2005). Guide to receptors and channels, 1st edition (2005 revision). *Br J Pharmacol* **144 Suppl 1**, S1-128.

Allescher HD, Tougas G, Vergara P, Lu S, & Daniel EE (1992). Nitric oxide as a putative nonadrenergic noncholinergic inhibitory transmitter in the canine pylorus in vivo. *Am J Physiol* **262**, G695-G702.

Andrews CN, Bharucha AE, Camilleri M, Low PA, Seide B, Burton D, Baxter K, & Zinsmeister AR (2007). Nitrergic contribution to gastric relaxation induced by glucagon-like peptide-1 (GLP-1) in healthy adults. *Am J Physiol Gastrointest Liver Physiol* **292**, G1359-G1365.

Baurand A, Eckly A, Bari N, Leon C, Hechler B, Cazenave JP, & Gachet C (2000). Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. *Thromb Haemost* **84**, 484-491.

Belai A & Burnstock G (1994). Evidence for coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the rat ileum, colon and anococcygeus muscle. *Cell Tissue Res* **278**, 197-200.

Belai A & Burnstock G (2000). Pattern of distribution and co-localization of NOS and ATP in the myenteric plexus of human fetal stomach and intestine. *Neuroreport* **11**, 5-8.

Benko R, Undi S, Wolf M, Vereczkei A, Illenyi L, Kassai M, Cseke L, Kelemen D, Horvath OP, Antal A, Magyar K, & Bartho L (2007). P2 purinoceptor antagonists inhibit the non-adrenergic, non-cholinergic relaxation of the human colon in vitro. *Neuroscience* **147**, 146-152.

Bhatia M (2005). Hydrogen sulfide as a vasodilator. *IUBMB Life* **57**, 603-606.

Bitar KN & Makhlouf GM (1982). Relaxation of isolated gastric smooth muscle cells by vasoactive intestinal peptide. *Science* **216**, 531-533.

Boeckxstaens GE, Pelckmans PA, Herman AG, & Van Maercke YM (1993). Involvement of nitric oxide in the inhibitory innervation of the human isolated colon. *Gastroenterology* **104**, 690-697.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, & Cohen RA (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**, 850-853.

Borderies JR, Gonalons E, Angel F, Vergara P, & Jimenez M (1997). Effect of different calcium channel blockers on inhibitory junction potentials and slow waves in porcine ileum. *Life Sci* **60**, 883-892.

Bornstein JC, Costa M, & Grider JR (2004). Enteric motor and interneuronal circuits controlling motility. *Neurogastroenterol Motil* **16 Suppl 1**, 34-38.

Bourdon DM, Mahanty SK, Jacobson KA, Boyer JL, & Harden TK (2006). (N)-methanocarba-2MeSADP (MRS2365) is a subtype-specific agonist that induces rapid desensitization of the P2Y1 receptor of human platelets. *J Thromb Haemost* **4**, 861-868.

Boyer JL, Romero-Avila T, Schachter JB, & Harden TK (1996). Identification of competitive antagonists of the P2Y1 receptor. *Mol Pharmacol* **50**, 1323-1329.

Bridgewater M, Cunnane TC, & Brading AF (1995). Characteristic features of inhibitory junction potentials evoked by single stimuli in the guinea-pig isolated taenia caeci. *J Physiol* **485 ( Pt 1)**, 145-155.

Briejer MR, Akkermans LM, Meulemans AL, Lefebvre RA, & Schuurkes JA (1995). 5-HT-induced neurogenic relaxations of the guinea-pig proximal colon: investigation into the role of ATP and VIP in addition to nitric oxide. *Naunyn Schmiedebergs Arch Pharmacol* **351**, 126-135.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, & Herman AG (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345**, 346-347.

Burnstock G (1976). Do some nerve cells release more than one transmitter? *Neuroscience* **1**, 239-248.

Burnstock G (2004). Cotransmission. *Curr Opin Pharmacol* **4**, 47-52.

Burnstock G (2006). Historical review: ATP as a neurotransmitter. *Trends Pharmacol Sci* **27**, 166-176.

Burnstock G (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87**, 659-797.

Burnstock G, Campbell G, Satchell D, & Smythe A (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* **40**, 668-688.

Burnstock G & Lavin S (2002). Interstitial cells of Cajal and purinergic signalling. *Auton Neurosci* **97**, 68-72.

Camaioni E, Boyer JL, Mohanram A, Harden TK, & Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem* **41**, 183-190.

Chakder S, Cao GY, Lynn RB, & Rattan S (2000). Heme oxygenase activity in the internal anal sphincter: effects of nonadrenergic, noncholinergic nerve stimulation. *Gastroenterology* **118**, 477-486.

Christinck F, Jury J, Cayabyab F, & Daniel EE (1991). Nitric oxide may be the final mediator of nonadrenergic, noncholinergic inhibitory junction potentials in the gut. *Can J Physiol Pharmacol* **69**, 1448-1458.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

Eccles JC (1957). The clinical significance of research work on the chemical transmitter substances of the nervous system. *Med J Aust* **44**, 745-753.

Farre R, Auli M, Lecea B, Martinez E, & Clave P (2006). Pharmacologic characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter. *J Pharmacol Exp Ther* **316**, 1238-1248.

Farrugia G, Lei S, Lin X, Miller SM, Nath KA, Ferris CD, Levitt M, & Szurszewski JH (2003). A major role for carbon monoxide as an endogenous hyperpolarizing factor in the gastrointestinal tract. *Proc Natl Acad Sci U S A* **100**, 8567-8570.

Farrugia G, Miller SM, Rich A, Liu X, Maines MD, Rae JL, & Szurszewski JH (1998). Distribution of heme oxygenase and effects of exogenous carbon monoxide in canine jejunum. *Am J Physiol* **274**, G350-G358.

Fenton C, Wellington K, & Easthope SE (2006). 0.4% nitroglycerin ointment : in the treatment of chronic anal fissure pain. *Drugs* **66**, 343-349.

Fernandez E, Guo X, Vergara P, & Jimenez M (1998). Evidence supporting a role for ATP as non-adrenergic non-cholinergic inhibitory transmitter in the porcine ileum. *Life Sci* **62**, 1303-1315.

Genton L & Kudsk KA (2003). Interactions between the enteric nervous system and the immune system: role of neuropeptides and nutrition. *Am J Surg* **186**, 253-258.

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, & Burnstock G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**, 1313-1323.

Gibbons SJ & Farrugia G (2004). The role of carbon monoxide in the gastrointestinal tract. *J Physiol* **556**, 325-336.

Gonzalez AA, Farre R, & Clave P (2004). Different responsiveness of excitatory and inhibitory enteric motor neurons in the human esophagus to electrical field stimulation and to nicotine. *Am J Physiol Gastrointest Liver Physiol* **287**, G299-G306.

Grider JR (2003a). Neurotransmitters mediating the intestinal peristaltic reflex in the mouse. *J Pharmacol Exp Ther* **307**, 460-467.

Grider JR (2003b). Reciprocal activity of longitudinal and circular muscle during intestinal peristaltic reflex. *Am J Physiol Gastrointest Liver Physiol* **284**, G768-G775.

Grider JR & Jin JG (1994). Distinct populations of sensory neurons mediate the peristaltic reflex elicited by muscle stretch and mucosal stimulation. *J Neurosci* **14**, 2854-2860.

Hansen MB (2003). [The enteric nervous system]. *Ugeskr Laeger* **165**, 4097-4101.

Hosoki R, Matsuki N, & Kimura H (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* **237**, 527-531.

Hu HZ, Gao N, Zhu MX, Liu S, Ren J, Gao C, Xia Y, & Wood JD (2003). Slow excitatory synaptic transmission mediated by P2Y1 receptors in the guinea-pig enteric nervous system. *J Physiol* **550**, 493-504.

Ishiguchi T, Takahashi T, Itoh H, & Owyang C (2000). Nitrergic and purinergic regulation of the rat pylorus. *Am J Physiol Gastrointest Liver Physiol* **279**, G740-G747.

Kandel ER, Schwartz HS, & Jessel TM (2001). *Principios de neurociencia*, 4 ed. McGraw-Hill, Madrid.

Keef KD, Du C, Ward SM, McGregor B, & Sanders KM (1993). Enteric inhibitory neural regulation of human colonic circular muscle: role of nitric oxide. *Gastroenterology* **105**, 1009-1016.

Keranen U, Vanhatalo S, Kiviluoto T, Kivilaakso E, & Soinila S (1995). Co-localization of NADPH diaphorase reactivity and vasoactive intestinal polypeptide in human colon. *J Auton Nerv Syst* **54**, 177-183.

Kunze WA & Furness JB (1999). The enteric nervous system and regulation of intestinal motility. *Annu Rev Physiol* **61**, 117-142.

Lecci A, Santicioli P, & Maggi CA (2002). Pharmacology of transmission to gastrointestinal muscle. *Curr Opin Pharmacol* **2**, 630-641.

Linden DR, Sha L, Mazzone A, Stoltz G, Bernard C, Furne JK, Levitt M, Farrugia G, & Szurszewski JH (2006).Hydrogen sulfide is synthesized in the muscle layers of the colon and in prevertebral ganglia and alters the electrical properties of neurons. Neurogastroenterol.Motil. **18**, 702.

Martin MT, Hove-Madsen L, & Jimenez M (2004). Otilonium bromide inhibits muscle contractions via L-type calcium channels in the rat colon. *Neurogastroenterol Motil* **16**, 175-183.

Matsuda NM, Miller SM, Sha L, Farrugia G, & Szurszewski JH (2004). Mediators of non-adrenergic non-cholinergic inhibitory neurotransmission in porcine jejunum. *Neurogastroenterol Motil* **16**, 605-612.

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, & Malagelada JR (1993). Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* **23**, 724-728.

Mearin F, Papo M, & Malagelada JR (1995). Impaired gastric relaxation in patients with achalasia. *Gut* **36**, 363-368.

Moncada S & Bolanos JP (2006). Nitric oxide, cell bioenergetics and neurodegeneration. *J Neurochem* **97**, 1676-1689.

Mutafova-Yambolieva VN, Hwang SJ, Hao X, Chen H, Zhu MX, Wood JD, Ward SM, & Sanders KM (2007). Beta-nicotinamide adenine dinucleotide is an inhibitory neurotransmitter in visceral smooth muscle. *Proc Natl Acad Sci U S A* **104**, 16359-16364.

O'Kelly T, Brading A, & Mortensen N (1993). Nerve mediated relaxation of the human internal anal sphincter: the role of nitric oxide. *Gut* **34**, 689-693.

Olgart C, Gustafsson LE, & Wiklund NP (2000). Evidence for nonvesicular nitric oxide release evoked by nerve activation. *Eur J Neurosci* **12**, 1303-1309.

Olsson C & Holmgren S (2001). The control of gut motility. *Comp Biochem Physiol A Mol Integr Physiol* **128**, 481-503.

Pluja L, Fernandez E, & Jimenez M (1999). Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol* **126**, 883-892.

Pluja L, Fernandez E, & Jimenez M (2000). Electrical and mechanical effects of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide in the rat colon involve different mechanisms. *Eur J Pharmacol* **389**, 217-224.

Porras M, Martin MT, Soler M, & Vergara P (2004). Intestinal motor disorders associated with cyclical bacterial overgrowth in a rat model of enteritis. *Am J Physiol Gastrointest Liver Physiol* **287**, G58-G64.

Porras M, Martin MT, Teran E, Mourelle M, & Vergara P (2007). The NO-donor LA-419 prevents intestinal dismotility, bacterial translocation and inflammation in a rat model of enteritis. *J Pharmacol Exp Ther*.

Porras M, Martin MT, Torres R, & Vergara P (2006a). Cyclical upregulated iNOS and long-term downregulated nNOS are the bases for relapse and quiescent phases in a rat model of IBD. *Am J Physiol Gastrointest Liver Physiol* **290**, G423-G430.

Porras M, Martin MT, Yang PC, Jury J, Perdue MH, & Vergara P (2006b). Correlation between cyclical epithelial barrier dysfunction and bacterial translocation in the relapses of intestinal inflammation. *Inflamm Bowel Dis* **12**, 843-852.

Ralevic V & Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**, 413-492.

Rattan S, Al HR, & De Godoy MA (2004). Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation. *Am J Physiol Gastrointest Liver Physiol* **287**, G605-G611.

Rodriguez-Membrilla A, Martinez V, Jimenez M, Gonalons E, & Vergara P (1995). Is nitric oxide the final mediator regulating the migrating myoelectric complex cycle? *Am J Physiol* **268**, G207-G214.

Rozsai B, Lazar Z, Benko R, & Bartho L (2001). Inhibition of the NANC relaxation of the guinea-pig proximal colon longitudinal muscle by the purinoceptor antagonist PPADS, inhibition of nitric oxide synthase, but not by a PACAP/VIP antagonist. *Pharmacol Res* **43**, 83-87.

Said SI & Rattan S (2004). The multiple mediators of neurogenic smooth muscle relaxation. *Trends Endocrinol Metab* **15**, 189-191.

Schemann M & Neunlist M (2004). The human enteric nervous system. *Neurogastroenterol Motil* **16 Suppl 1**, 55-59.

Schicho R, Krueger D, Zeller F, Von Weyhern CW, Frieling T, Kimura H, Ishii I, De GR, Campi B, & Schemann M (2006). Hydrogen sulfide is a novel prosecretory neuromodulator in the Guinea-pig and human colon. *Gastroenterology* **131**, 1542-1552.

Searcy DG & Lee SH (1998). Sulfur reduction by human erythrocytes. *J Exp Zool* **282**, 310-322.

Shah V, Lyford G, Gores G, & Farrugia G (2004). Nitric oxide in gastrointestinal health and disease. *Gastroenterology* **126**, 903-913.

Stark ME, Bauer AJ, Sarr MG, & Szurszewski JH (1993). Nitric oxide mediates inhibitory nerve input in human and canine jejunum. *Gastroenterology* **104**, 398-409.

Strata P & Harvey R (1999). Dale's principle. *Brain Res Bull* **50**, 349-350.

Strege PR, Evangelista S, Lyford GL, Sarr MG, & Farrugia G (2004). Otilonium bromide inhibits calcium entry through L-type calcium channels in human intestinal smooth muscle. *Neurogastroenterol Motil* **16**, 167-173.

Suarez F, Furne J, Springfield J, & Levitt M (1998). Production and elimination of sulfur-containing gases in the rat colon. *Am J Physiol* **274**, G727-G733.

Tang G, Wu L, Liang W, & Wang R (2005). Direct stimulation of K(ATP) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. *Mol Pharmacol* **68**, 1757-1764.

Teague B, Asiedu S, & Moore PK (2002). The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* **137**, 139-145.

Tomita R, Fujisaki S, Ikeda T, & Fukuzawa M (2002). Role of nitric oxide in the colon of patients with slow-transit constipation. *Dis Colon Rectum* **45**, 593-600.

Undi S, Benko R, Wolf M, Illenyi L, Horvath OP, Antal A, Csontos Z, Vereczkei A, & Bartho L (2006). Purinergic nerves mediate the non-nitrergic relaxation of the human ileum in response to electrical field stimulation. *Brain Res Bull* **71**, 242-244.

Van CK & Lefebvre RA (2004). Nitrergic-purinergic interactions in rat distal colon motility. *Neurogastroenterol Motil* **16**, 81-98.

Wang GD, Hu HZ, Wang XY, Gao N, Liu SM, Fang XC, Xia Y, & Wood JD (2004). Purinergic inhibitory neuromuscular transmission mediated by the P2Y(1) receptor in guinea-pig small intestine. *Gastroenterology* **126**, A275.

Wang GD, Wang XY, Hu HZ, Liu S, Gao N, Fang X, Xia Y, & Wood JD (2007). Inhibitory neuromuscular transmission mediated by the P2Y1 purinergic receptor in guinea pig small intestine. *Am J Physiol Gastrointest Liver Physiol* **292**, G1483-G1489.

Xue L, Farrugia G, Sarr MG, & Szurszewski JH (1999). ATP is a mediator of the fast inhibitory junction potential in human jejunal circular smooth muscle. *Am J Physiol* **276**, G1373-G1379.

Xue L, Farrugia G, & Szurszewski JH (2000). Effect of exogenous ATP on canine jejunal smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **278**, G725-G733.

Zagorodnyuk VP, Vladimirova IA, Vovk EV, & Shuba MF (1989). Studies of the inhibitory non-adrenergic neuromuscular transmission in the smooth muscle of the normal human intestine and from a case of Hirschsprung's disease. *J Auton Nerv Syst* **26**, 51-60.

Zizzo MG, Mule F, & Serio R (2006). Mechanisms underlying hyperpolarization evoked by P2Y receptor activation in mouse distal colon. *Eur J Pharmacol* **544**, 174-180.

# Conclusiones

*1)* El receptor purinérgico responsable de la relajación intestinal (colon humano e intestino delgado de cerdo) es el $P2Y_1$. Este receptor media el componente rápido del potencial de unión neuromuscular y de la relajación mecánica correspondiente a este fenómeno eléctrico.

*2)* La relajación a nivel del colon humano responde, probablemente, a un mecanismo de co-transmisión entre ATP (a través de receptores $P2Y_1$) y óxido nítrico. El ATP es responsable de la relajación fásica, mientras que el NO sería responsable de la relajación tónica.

*3)* El sulfhídrico causa relajación de la musculatura lisa intestinal a través de canales de potasio de baja conductancia sensibles a apamina y canales de potasio sensibles a ATP. En estos momentos se desconoce el papel que juega el sulfhídrico endógeno en la relajación intestinal.

*4)* El conocimiento de los receptores y los mecanismos implicados en la relajación intestinal puede contribuir en un futuro a diseñar estrategias terapéuticas en alteraciones motoras ocasionadas por patologías en el tracto gastrointestinal humano.