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

Víctor Gil,1 Diana Gallego,2 Laura Grasa,1–3 María Teresa Martín,1–2 and Marcel Jiménez1–2

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

Submitted 2 November 2009; accepted in final form 12 April 2010

Gil V, Gallego D, Grasa L, Martín MT, Jiménez M. Purinergic and nitricergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. Am J Physiol Gastrointest Liver Physiol 299: G158–G169, 2010. First published April 15, 2010; doi:10.1152/ajpgi.00448.2009.—Nitric oxide (NO) and ATP mediate smooth muscle relaxation in the gastrointestinal tract. However, the involvement of these neurotransmitters in spontaneous neuronal activity is unknown. The aim of the present work was to study spontaneous neuromuscular transmission in the rat midcolon. Microelectrode experiments were performed under constant stretch both in circular and longitudinal directions. Spontaneous inhibitory junction potentials (sIJP) were recorded. Tetrodotoxin (1 μM) and apamin (1 μM) depolarized smooth muscle cells and inhibited sIJP. Nω-nitro-l-arginine (l-NGA, 1 mM) depolarized smooth muscle cells but did not modify sIJP. In contrast, the P2Y1 antagonist MRS-2500 (1 μM) did not modify the resting membrane potential (RMP) but reduced sIJP. These results correlate with in vitro (muscle bath) and in vivo (strain gauges) data where l-NGA but not MRS-2500 induced a sustained increase of spontaneous motility. We concluded that, in the rat colon, inhibitory neurons regulate smooth muscle RMP and cause sIJP. In vitro, the release of inhibitory neurotransmitters is independent of nicotinic, P2X, and 5-hydroxytryptamine type 3 receptors. Neuronal NO causes a sustained smooth muscle depolarization that is responsible for a constant inhibition of spontaneous motility. In contrast, ATP acting on P2Y1 receptors is responsible for sIJP but does not mediate inhibitory neural tone. ATP and NO have complementary physiological functions in the regulation of gastrointestinal motility.

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

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

It is well known that EFS-induced IJP involves a fast followed by a slow component (6, 12, 16, 18, 24). Apamin, a calcium-sensitive K+ (sKCa) channel blocker, has been a pharmacological tool used to distinguish between the fast and the slow component of the IJP in several species, including the rat colon (24). Furthermore, the fast component is abolished by P2Y1 receptor antagonist (8, 13, 14, 24, 34), whereas the slow component is Nω-nitro-l-arginine (l-NGA) sensitive and therefore nitric oxide (NO) mediated (18, 24, 33). Accordingly, a cotransmission process between ATP or a related purine and NO has been proposed with different functions for each neurotransmitter (12). Activation of P2Y1 receptor might mediate phasic relaxation, whereas NO might mediate tonic sustained relaxation. Spontaneous and evoked IJPs might have different properties (28) although it is conceivable that the fast component of the IJP might have similar properties to spontaneous IJP because both of them are apamin sensitive (28).

Accordingly, we have studied the ongoing release of inhibitory neuromuscular transmitters in the rat colon that elicits spontaneous IJPs and modulates resting membrane potential (RMP). Briefly, we found that neural-mediated spontaneous IJPs are MRS-2500 sensitive and therefore due to P2Y1 receptor activation; in contrast, l-NGA depolarize smooth muscle cells without a major effect on spontaneous IJP. These data correlate well with mechanical data both in vitro and in vivo where NO but not ATP mediates tonic muscular inhibition. It is concluded that both neurotransmitters are released by inhibitory motor neurons but they have complementary physiological functions.
MATERIALS AND METHODS

Animals

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

Tissue Samples

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

Intracellular Microelectrode Recording

The tissue was pinned with the circular muscle layer facing upward in a Sylgard-coated chamber, and manual stretch (2.1 ± 0.1-fold increase in the length of the circular axis and 1.8 ± 0.1-fold increase in the length of the longitudinal axis, n = 38) was applied. Strips were continuously perfused with carbogenated physiological saline solution at 37 ± 1°C and were allowed to equilibrate for 1 h. Circular smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl (30–60 MΩ of resistance). Membrane potential was measured by using the standard electrometer Duo773 (WPI, Sarasota, FL). Tracings were displayed on an oscilloscope 4026 (Racal-Dana, Windsor, England) and simultaneously digitalized (100 Hz) with a PowerLab 4/30 system and Chart 5 software for Windows (both from ADInstruments, Castle Hill, NSW, Australia). To maintain stable impalements, experiments were performed in the presence of nifedipine (1 μM). Spontaneous IJP s (sIJP) were measured by two different methods: (1) frequency distribution (0.5-mV bins) of the values of the membrane potential (30–60 s) expressed as bin probability from 0 to 1. In this case, when sIJP are recorded, the frequency distribution has a tail toward the most negative values (Fig. 1), and, when sIJP are inhibited, the tail is not present; and (2) according to this difference in the frequency distribution, the standard deviation (SD; expressed in mV) indicative of the presence of sIJP was measured using the following expression: SD of the recording inside the cell − SD of the recording outside the cell (both of them for a period of at least 30–60 s). Mean ± SD has been previously described as a valid method to measure sIJP because the amplitude of sIJP is correlated (r² = 0.83) to the SD of the recording (25). Because of the presence of ongoing sIJP, the RMP cannot be estimated measuring the mean of the recording. Accordingly, the RMP (expressed in mV) was calculated as the most probable bin of the frequency distribution (0.1-mV bins; 30-60-s recordings) (Fig. 1).

Muscle Bath Studies

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

In Vivo Motility Studies

Animal preparation. After a fasting period of 6 h, animals were anesthetized by inhalation of isofluorane to allow cannulation of the right jugular vein as previously described (31). Level III of anesthesia was maintained with intravenous thiopental sodium as required, and rats were tracheotomized to facilitate spontaneous breathing. Body temperature was maintained at 37°C by placing the animal on a heating pad. A laparatomy was performed to suture a strain-gauge (3 × 5 mm; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) to the wall of the colon (2 cm from de cecum) to record circular muscle activity. The strain gauge was connected toVF-1 Harvard Apparatus, Holliston, MA) connected to a computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. A tension of 1 g was applied, and tissues were allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. The area under the curve (AUC) of contractions from the baseline was measured to estimate the mechanical activity, and the result was expressed in grams per minute (g/min).

In Vivo Motility Studies

Animal preparation. After a fasting period of 6 h, animals were anesthetized by inhalation of isofluorane to allow cannulation of the right jugular vein as previously described (31). Level III of anesthesia was maintained with intravenous thiopental sodium as required, and rats were tracheotomized to facilitate spontaneous breathing. Body temperature was maintained at 37°C by placing the animal on a heating pad. A laparatomy was performed to suture a strain-gauge (3 × 5 mm; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) to the wall of the colon (2 cm from de cecum) to record circular muscle activity. The strain gauge was connected toVF-1 Harvard Apparatus, Holliston, MA) connected to a computer through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain) coupled to an A/D converter installed in the computer. A tension of 1 g was applied, and tissues were allowed to equilibrate for 1 h. After this period, strips displayed spontaneous phasic activity. The area under the curve (AUC) of contractions from the baseline was measured to estimate the mechanical activity, and the result was expressed in grams per minute (g/min).

In Vivo Motility Studies

Animal preparation. After a fasting period of 6 h, animals were anesthetized by inhalation of isofluorane to allow cannulation of the right jugular vein as previously described (31). Level III of anesthesia was maintained with intravenous thiopental sodium as required, and rats were tracheotomized to facilitate spontaneous breathing. Body temperature was maintained at 37°C by placing the animal on a heating pad. A laparatomy was performed to suture a strain-gauge (3 × 5 mm; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) to the wall of the colon (2 cm from de cecum) to record circular muscle activity. The strain gauge was connected to
a high-gain amplifier (MT8P; Lectromed, Herts, UK), and signals were sent to a recording unit connected to a computer (PowerLab/800; ADInstruments).

**Evaluation of motor parameters.** The effects of both MRS-2500 (0.2 μmol/kg) and t-NNA (10 μmol/kg) were evaluated in a separate set of animals. After an equilibration period of 30 min, the amplitude and frequency of contractions were determined before and after intravenous administration of drugs. Amplitude of a single contraction was determined by measuring the AUC delimited by the tracing, and the result was expressed in square millimeters (mm²). To test the effects of MRS-2500 and t-NNA, the mean amplitude of the five consecutive contractions appearing just before the drug administration was compared with the mean amplitude of the five consecutive contractions appearing after the drug administration. To determine the effects of both MRS-2500 and t-NNA in the frequency, contractions recorded during the 10 min before and after drug administration were counted, and the results were expressed as number of contractions per hour.

**Solutions and Drugs**

The composition of the physiological saline solution was (in mM) 10.10 glucose, 115.48 NaCl, 21.90 NaHCO₃, 4.61 KCl, 1.14 NaH₂PO₄, 1.50 CaCl₂, and 1.16 MgSO₄ (pH 7.3–7.4). In all of the experiments, physiological saline solution contained atropine, phentolamine, and pranopanol (1 μM each one) and was continuously bubbled with carbogen (95% O₂ and 5% CO₂). The following drugs were used: apamin, hexamethonium, nifedipine, t-NNA, and phentolamine (Sigma Chemicals, St. Louis, MO); atropine sulfate (Merck, Darmstadt, Germany); and (1R,2S,4S,5S)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonoxy) bicyclo[3.1.0]hexane-1-methanol dihydrogen iodo-6-(methylamino)-9H-purin-9-yl]-2-[imino-3,1-phenylenecarbonylimino]bis-1,3,5-naphthalene-trisulfonic acid, hexasodium salt (NF-023), ondansetron hydrochloride, propanolol (Tocris, Bristol, UK). Stock solutions were dissolved in physiological saline solution by sonication.

**Data Analysis and Statistics**

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

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

### RESULTS

**Characterization of sIJP and RMP**

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

**Neural Regulation of sIJP and RMP**

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

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

The NO synthase inhibitor t-NNA (1 mM) depolarized 5.3 ± 0.7 mV smooth muscle cells but did not modify sIJP (Table 1 and Fig. 3A). In contrast, the selective P2Y₁ antagonist MRS-2500 (1 μM) did not modify the RMP membrane potential but inhibited sIJP (Table 1 and Fig. 3A). Notice that, in the presence of t-NNA, the value of maximum probability is shifted to the right, indicative of the depolarization without a...
Hexamethonium (200 μM) did not depolarize smooth muscle cells and did not modify sIJP (Table 1 and Fig. 4). These data demonstrate a minor role of nicotinic, P2X, and 5-HT<sub>3</sub> receptors in the regulation of the RMP and sIJP.

### Neural Regulation of Spontaneous Motility In Vitro

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

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

To evaluate the putative interaction between P2Y<sub>1</sub> receptors and NO, the effect of l-NNA was tested in the presence of MRS-2500. The depolarization induced by l-NNA (1 mM) was not affected by pretreating the tissue with MRS-2500 (1 μM) (8.1 ± 1.4 mV depolarization, MRS-2500: 39.8 ± 4.7 vs. l-NNA + MRS-2500: 31.7 ± 3.5 mV, P < 0.01, n = 6; Fig. 6A). Notice that MRS-2500 abolished spontaneous IJP, and shift to the right in the frequency distribution of the membrane potential (indicative of depolarization) is observed after l-NNA (Fig. 6B). Moreover, l-NNA (1 mM) induced an important increase in the spontaneous motility when it was added to the muscle bath in the presence of MRS-2500 (1 μM) [MRS-2500: 11.4 ± 2.3 vs. l-NNA + MRS-2500: 40.2 ± 9.3 g/min (AUC), P < 0.05, n = 7; Fig. 6C].

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

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

Role of NO and P2Y1 Receptors on In Vivo Spontaneous Motility

Spontaneous contractions were measured in vivo with strain gauge transducers. Spontaneous motor activity was characterized by isolated phasic contractions with a regular frequency of 25.5 ± 6.5 contractions/h (n = 8). L-NNA (10 \( \mu \text{mol/kg} \)) dramatically increased the frequency of spontaneous contractions (frequency of contractions, control: 22.5 ± 8.6 vs. L-NNA: 60.0 ± 8.8 h\(^{-1} \), \( P < 0.05 \), n = 4) and increased the basal tone (0.5 ± 0.1 g, \( P < 0.05 \), n = 4) but did not modify the amplitude of spontaneous contractions. The P2Y1 antagonist MRS-2500 (0.2 \( \mu \text{mol/kg} \)) did not modify either the basal tone or the frequency of spontaneous contractions measured in vivo with the strain gauge transducer. In contrast, a single contraction with high amplitude (n = 4, ANOVA, \( P < 0.05 \)) was measured in all of the recordings, but the following contractions had the same amplitude and frequency as those recorded in the basal state (Fig. 9).

DISCUSSION

In the present paper, we demonstrate that spontaneous neuronal activity causes the release of ATP or a related purine and NO with complementary physiological functions: 1) P2Y1 receptors are responsible for the spontaneous IJP with a minor effect on the regulation of the membrane potential; 2) tonically released NO regulates the membrane potential; and 3) NO inhibition but not P2Y1 blockade increases spontaneous motility both in vitro and in vivo. Furthermore, in vitro, neuronal activity.
activation is partially independent of synaptic inputs involving nicotinic, P2X, and 5-HT3 receptors.

Quantification of Spontaneous IJP and RMP

The amplitude of sIJP was quite variable, ranging from \(-10\) to \(-20\) mV; therefore, the amplitude of individual sIJP was difficult to measure. To illustrate and quantify spontaneous IJP, we used two methodologies previously reported (25): 1) the presence of a tail in the frequency distribution of the membrane potential; and 2) the SD of the membrane potential. When spontaneous IJP were recorded, the frequency distribution of the values of the tracing had a tail toward the most negative values, and, accordingly, an increase in the SD of the points is obtained. The presence of this tail is illustrative of the sIJP, and it disappears when sIJP are inhibited (see Figs. 1 and 2). SD was measured to quantify sIJP and used for statistical purposes.

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

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

Values are means ± SE; n, no. of samples. AUC, area under the curve. The statistical significance of differences was assessed by using paired Student’s t-test.

*Drug effect on spontaneous mechanical activity was expressed as net effect (AUC after drug administration - AUC in control) and percentage [(AUC after drug administration - AUC in control)/AUC in control]·100].

Fig. 4. **Top:** intracellular microelectrode tracings showing the effect of hexamethonium (200 μM) (left) and hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) (right) on RMP and sIJP. Expanded recordings represent sIJP in control conditions (1) and after hexamethonium (200 μM) (left) and hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) (right) administration (2). **Bottom:** frequency distribution (0.5-mV bins) of the membrane potential (60-s recording) from a circular muscle cell in control conditions (solid line) and after hexamethonium (200 μM) administration (short broken line) (left) and in control conditions (solid line) and after hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) administration (short broken line) (right).
It has been previously shown that SD correlates well with the amplitude of the sIJP, and therefore, when spontaneous sIJP are inhibited, SD decreases (25). Another difficulty has been to calculate the RMP during ongoing release of inhibitory neurotransmitters. A possible approach was to measure the mean of the values of the recordings. However, this would have been inappropriate because, when sIJP were recorded, the value of RMP would have been lower than those found in the absence of sIJP. To avoid this difficulty, we calculated the value with maximum probability of the frequency distribution (0.1 mV), and we assume that this was the RMP of the cell. The difference in the estimation of the RMP and the way to stretch the tissue might contribute to the difference between the value of RMP found in the present study and our previous published data (14, 24).

**Spontaneous Junction Potentials**

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

**Origin of Spontaneous IJP**

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

---

**Fig. 5. Mechanical recordings showing the effect of TTX (1 μM) (A), L-NNA (1 mM) (B), MRS-2500 (1 μM) (C), hexamethonium (200 μM) (D), and hexamethonium (200 μM) + NF-023 (10 μM) + ondansetron (1 μM) (E) on spontaneous motility in vitro.**
ous IJP. In the mouse colon, hexamethonium abolishes the neurogenic motor complex (5, 22). However, hexamethonium did not modify the RMP (5) or it caused a marked depolarization (22). The discrepancies between these two studies are unknown, but they might be the result of different stretch applied during the pinning to the preparation (22). It is important to notice that hexamethonium did not cause a major effect on spontaneous IJP (22). However, in another study, 70% of spontaneous IJP were still recorded in the presence of hexamethonium, and no major change in RMP was observed in mucosa-free preparations of the mouse colon (25). These data suggest that the major source of inputs to inhibitory motor neurons is nonnicotinic. ATP acting on P2X receptors and 5-hydroxytryptamine acting on 5-HT3 receptors mediate fast synaptic transmission in myenteric neurons of the guinea pig ileum (20, 35). However, combination of hexamethonium with P2X and 5-HT3 antagonists (NF-023 and ondansetron) did not modify the result obtained with hexamethonium alone. All of these data might suggest that, in the rat colon, sIJP are neurally mediated but mainly independent of synaptic inputs. Mechanosensitivity in both excitatory and inhibitory motor neurons have been recently described in the guinea pig ileum. However, in these cases, motor neurons rapidly adapted to the stimuli (23). Neural-mediated relaxation, caused by circular stretch, independent of synaptic inputs has also been reported recently in the mouse lower esophageal sphincter (17). Alternatively, other neurotransmitters not investigated in the present study might be responsible for the neural inhibitory tone. It is important to note that TTX caused: 1) an inhibition of spontaneous IJPs and 2) a marked depolarization of the membrane potential. Because a cotransmission process (ATP and NO) is present at the neuromuscular junction (14, 24), we analyzed which neurotransmitter is involved in each response and if the effect might be correlated with the mechanical activity.

Role of P2Y1 Receptors and NO on Spontaneous IJP and RMP

In the present study, we used MRS-2500 as a P2Y1 antagonist and we found that MRS-2500 reduced sIJP without a major change in RMP. To our knowledge, this is the first report to analyze the effect of selective P2Y1 antagon-
nists on spontaneous IJPs. Previous data from our laboratory showed that P2Y1 antagonists such as MRS-2179, MRS-2279, and MRS-2500 are useful pharmacological tools to inhibit the fast component of the IJP induced by EFS in the rat colon (14). MRS-2500 was the most potent antagonist (IC50 = 16.5 nM); therefore, we used MRS-2500 to antagonize sIJP (present work: IC50 = 3.1 nM). This result demonstrates that P2Y1 receptors are responsible for spontaneous IJP probably acting at the postjunctional level. It is important to notice that MRS-2500 did not modify the RMP. This result demonstrates that 1) prejunctional P2Y1 receptors are not involved in NO release (see below) and 2) ATP or a related purine is not tonically released from inhibitory motor neurons, causing sustained smooth muscle hyperpolarization. It is conceivable that a quanta release of ATP causes transient sIJP.

In contrast to the effect of MRS-2500, NO inhibition with L-NNA caused a depolarization of the RMP without a major effect on sIJP. These data demonstrate that 1) NO is tonically released from enteric motor neurons causing a sustained hyperpolarization of smooth muscle cells and 2) spontaneous IJPs are not NO mediated. Our results are in agreement with those reported in the mouse colon where sIJP were TTX and apamin sensitive and insensitive to NO synthase inhibitors. It was suggested that NO does not mediate sIJP and that probably another mediator involving sKCa channels might participate in this mechanism (28). In the present paper, we demonstrate that ATP or a related purine acting on P2Y1 receptor is probably the transmitter involved in this response. It is important to note that, in the presence of MRS-2500, L-NNA was still able to cause smooth muscle depolarization, suggesting that prejunctional P2Y1 receptors that might mediate sEPSP (15) are not involved in NO release.

Role of P2Y1 Receptors and NO on Mechanical Activity

To correlate electrophysiological data with mechanical activity, we performed muscle bath studies and in vivo studies where colonic contractions were recorded with strain gauges. A direct correlation cannot be completely performed because 1) electrophysiological studies are always performed under a certain degree of stretch and therefore a strict parallelism between electrophysiological and mechanical recordings cannot be established; 2) electrophysiological but not mechanical recordings were performed in the presence of nifedipine; 3) the neural circuitry that might be activated in vivo in intact tissue is probably different from the circuitry present in a small strip both in mechanical and electrophysiological experiments; and 4) stretch in muscle bath studies can activate neuronal activity but also L-type calcium channels that are also stretch dependent (7, 21). Taking into account all these limitations, some data are coincident and other show slight differences: 1) both hexamethonium and the combination of hexamethonium, NF-
023, and ondansetron caused a slight increase in spontane-
ous motility without a major effect on RMP and sIJP. It is
possible that a slight effect on the RMP (~1 mV) that does
not reach statistical significance might explain this result.

However, it is important to note that both the depolariza-
tion and increase of spontaneous motility is not comparable to
the result obtained with TTX, suggesting that the vast
majority of the neural inhibitory tone in vitro is independent
of nicotinic, 5-HT3, and P2X receptors; 2) both in vitro and in vivo NO inhibition cause an important increase in
spontaneous motility. The underlying mechanism responsi-
ble for the increase in spontaneous motility is probably
caused by the depolarization observed in the electrophysi-
ological recordings. Mechanical recordings obtained both in
vitro and in vivo had an increase in the frequency of
spontaneous contractions. However, it is important to note
that tone (measured at the baseline level) is increased in
vitro but not in vivo (data not shown), and the amplitude
of the phasic contraction is increased in vitro but not in vivo.

Accordingly, the increase in AUC caused by l-NNA varies
according to different experimental conditions that might
contribute to different ongoing release of neurotransmitters.
In contrast, P2Y1 inhibition with MRS-2500 did not cause a
sustained and constant increase in motility (in fact, in vitro,
a decrease of ~20% of the spontaneous motility is ob-
served), suggesting that inhibition of P2Y1 receptors and
therefore inhibition of sIJP are not translated to an increase
in spontaneous motility.

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

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

Fig. 9. Mechanical recordings showing the effect of l-NNA (10 μmol/kg)
(A) and MRS-2500 (0.2 μmol/kg) (B) on spontaneous motility in vivo.
ancy between the effect of MRS-2500 and apamin might be attributable to the different properties of both drugs. Ongoing calcium entry during cyclic contractions might activate sKCa. A complex distribution of sKCa channels in different structures in the gastrointestinal tract such as enteric neurons, interstitial cells of Cajal, and fibroblast-like cells might contribute to these discrepancies (9, 10, 32). Alternatively, a partial interaction between NO and sKCa channels has also been reported (26). At the moment, we cannot explain the inhibitory effect of MRS-2500 on the spontaneous motility found in vitro, and further studies are needed to investigate this effect. In vivo, a single high-amplitude contraction was recorded after MRS-2500 infusion. Probably, prejunctional and/or postjunctional P2Y1 receptors might participate in this effect. The subsequent contractions did not differ from those recorded in control and therefore suggest that P2Y1 receptors are not involved in the inhibitory tone present in vivo.

In the present paper, we propose that P2Y1 antagonists in combination with NO synthase inhibitors are valuable pharmacological tools to investigate the cotransmission process causing smooth muscle relaxation in the gastrointestinal tract. Both neurotransmitters might have complementary physiological functions that need further investigations, and its relative role causing smooth muscle relaxation might vary depending on the activation of a neural pattern and subsequent smooth muscle relaxation. Further studies will be required to study the effect of these antagonists in well-defined motor patterns such as, for example, the peristaltic reflex.

ACKNOWLEDGMENTS

We thank Antonio Acosta, Claudia Arenas, and Emma Martinez for technical assistance.

GRANTS

Victor Gil is supported by the Ministerio de Ciencia e Innovación (AP2007-01583). This work has been funded by the following grant: BFU2009-11118 (Ministerio de Ciencia e Innovación, España).

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


