P2Y \textsubscript{1} receptors mediate inhibitory purinergic neuromuscular transmission in the human colon

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P2Y\textsubscript{1} receptors mediate inhibitory purinergic neuromuscular transmission in the human colon. Am J Physiol Gastrointest Liver Physiol 291: G584–G594, 2006. First published June 1, 2006; doi:10.1152/ajpgi.00474.2005.—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 neurotransmission in the human colon. Tissue was obtained from colon resections for neoplasm. Muscle bath, microelectrode experiments, and immunohistochemical techniques were performed. 2'-deoxy-N'-methyl adenosine 3',5'-diphosphate tetraammonium salt (MRS 2179) was used as a selective inhibitor of P2Y\textsubscript{1} receptors. We found that 1) ATP (1 mM) and adenosine 5'-\beta-2-thiodiphosphate (ADP\textsubscript{β}s) (10 \mu M), a preferential P2Y\textsubscript{1} agonist, inhibited spontaneous motility and caused smooth muscle hyperpolarization (about −12 mV); 2) MRS 2179 (10 \mu M) and apamin (1 \mu M) significantly reduced these effects; 3) both the fast component of the inhibitory junction potential (IJP) and the nonintrinsic relaxation induced by electrical field stimulation were dose dependently inhibited (IC\textsubscript{50} ∼1 \mu M) by MRS 2179; 4) ADP\textsubscript{β}s reduced the IJP probably by a desensitization mechanism; 5) apamin (1 \mu M) reduced the fast component of the IJP (by 30–40\%) and the inhibitory effect induced by electrical field stimulation; and 6) P2Y\textsubscript{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 motoneurons, 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\textsubscript{1} receptor present in smooth muscle probably mediates this mechanism through a pathway that partially involves apamin-sensitive calcium-activated potassium channels. P2Y\textsubscript{1} receptors can be an important pharmacological target to modulate smooth muscle excitability.

smooth muscle relaxation; purinergic receptors; inhibitory junction potential

**THE MECHANISMS INVOLVED in nonadrenergic, noncholinergic (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/pituitary adenylate cyclase-activating peptide (PACAP) (3), nitric oxide (NO) (6), ATP (8), or carbon monoxide (16) are putative NANC inhibitory mediators (26). There is a consensus that

nerve-mediated relaxation is complex and that probably several mediators are coreleased from inhibitory motoneurons, causing smooth muscle hyperpolarization and relaxation. In several species, this is indicated by the inhibitory junction potential (IJP) having two phases, a fast component (IJP\textsubscript{f}) followed by a slow component (IJP\textsubscript{s}) (12, 20). 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 (7, 29). Possible purinergic neuromuscular transmission in the small intestine of humans has been indicated in a study using the sucrose-gap technique (33). In the jejunum and colon, the IJP has a fast component followed by a sustained component (24, 32). The fast component is \textit{N}\textsuperscript{ω}-nitro-L-arginine (l-NNA) insensitive and therefore nonnitrergic, whereas the second component is l-NNA sensitive and might be due to the release of NO from inhibitory motoneurons (24, 32). IJP\textsubscript{f} is partially suramin and apamin sensitive, and it is abolished after desensitization with adenosine 5'-\beta-2-thiodiphosphate (ADP\textsubscript{β}s) and therefore considered purinergic, possibly through P2 receptors (35). 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\textsubscript{1–7} and P2Y\textsubscript{1, 2, 4, 6, 11–14}) in each family have been described. Purinergic receptors play a crucial role in the control of gastrointestinal motility. P2X receptors mediate fast synaptic transmission (17), including transmission from interneurons to motoneurons (2), and are probably located in interstitial cells of Cajal (9), which might participate in neuromuscular interaction. Activation of P2X receptors is generally thought to mediate smooth muscle contraction, whereas P2Y is thought to modulate relaxation. The inverse effect has also been reported (22). Suramin, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), and reactive blue have been widely used as purinoreceptor antagonists, but unfortunately, they do not discriminate between P2X and P2Y receptors (19, 25, 29). Furthermore, an interaction between the VIP/PACAP pathway and these purinergic antagonists has been demonstrated (5, 27). 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. (10) showed that 2'-deoxy-N6-methyl adenosine 3',5'-diphosphate tetraammonium salt (MRS 2179) was a potent P2Y1 receptor antagonist (10), and it is currently considered competitive and specific (1, 21). The suramin analog 8,8'-[carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-naphthalene-trisulfonic acid) hexasodium salt (NF 023) competitively antagonizes P2X receptor-mediated responses in certain vascular and visceral smooth muscles. NF 023 is a P2X antagonist with preferential sensitivity to P2X1 receptors (1, 31).

The aim of this study was to characterize NANC nonadrenergic, noncholinergic (NANC) conditions (control) and in the presence of N6-nitro-L-arginine (L-NNA) (1 mM). B: histogram showing the inhibition of the spontaneous activity (area under the curve, AUC) in both conditions. C: intracellular microelectrode recordings showing inhibitory junction potentials (IJP) 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. Values are means ± SE. ***P < 0.001.

MATERIALS AND METHODS

**Tissue preparation.** Specimens of the distal (n = 9) and sigmoid colon (n = 19) were obtained from patients (aged 38–85 yr) during colon resections for neoplasm. Colon segments from macroscopically normal regions were collected and transported to the laboratory in cold saline buffer. The tissue was placed in Krebs solution on a dissection dish, and the mucosal layer was gently removed. Circular muscle strips, 10 × 4 mm, were cut. The experimental procedure was approved by the ethics committee of the Hospital of Mataró (Barcelona, Spain).

**Mechanical experiments.** Muscle strips were examined in a 10-ml organ bath filled with NANC Krebs solution (phentolamine, atropine, and propanol, each 1 μM) at 37 ± 1°C. 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 h. After this period, strips displayed spontaneous phasic activity. Electrical field stimulation (EFS) was applied for 2 min (pulse duration, 0.4 ms; frequency, 2–20 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. To normalize data, the value of AUC obtained before the treatment was considered as 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 ± 1°C. Strips were meticulously pinned in a cross-sectioned slab, allowing micro-electrode recordings from both circular and longitudinal muscles. This procedure was previously reported in the canine ileum (23). Preparations were allowed to equilibrate for 1 h 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 the Duo773 standard electrometer (WPI, Sarasota, FL). Tracings were displayed on a 4026 oscilloscope (Racal-Dana) and simultaneously digitalized (100 Hz) using EGAA software coupled to an ISC-16 A/D card (RC Electronics, Santa Barbara, CA) 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, 100 ms; frequency, 30 Hz; pulse duration, 0.3 ms; and increasing amplitude strengths of 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. To obtain stable impalements, nifedipine (1 µM) 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 deparaffinized, rehydrated, and stained with antibodies to specific markers. The sections were then counterstained with hematoxylin.

Fig. 2. A: mechanical recordings showing the effect of ATP (1 mM) and adenosine 5’-β-2-thiodiphosphate (ADPβS) (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 ADPβS (10 µM) in the absence and presence of the neural blocker TTX (1 µM). D: effect of superfusion with ADPβS (10 µM) on the IJP. Values are means ± SE. ***P < 0.001.
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 min. Then, a standard blocking was performed with 0.2% BSA diluted in PBS with 0.2% Triton X-100 and 0.05% Tween 20. The incubation with the primary antibody (anti-P2Y1, from Alomone Labs, Jerusalem, Israel; 1:50) was performed overnight at 2–4°C. After the sections were rinsed with PBS, they were incubated with the EnVision kit (Dako, Glostrup, Denmark). Color development was achieved by incubation with diaminobenzidine (DAB; Sigma, St. Louis, MO), with the addition of 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$_2$PO$_4$, 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, L-NNA, ATP, ADP$_S$ (Sigma); TTX, atropine sulphate, propranolol, and sodium nitroprusside (NaNP) (Research Biochemicals, Natick, MA); MRS 2179, NF 023, and VIP (Tocris, Bristol UK); and 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.** Values are means ± SE. The paired Student’s t-test was used to compare the AUC in the absence and in the presence of drugs before and during EFS. To normalize data, we calculated the percentage of inhibition by the drugs, considering the AUC 5 min 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) ANOVA. A P value of <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 the NANC, nonnitrergic transmission. Muscle strips from the circular layer of the 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 contractions/min (n = 28). Amplitude was increased by the presence of TTX (1 μM) to 3.26 ± 0.43 g (n = 28), suggesting the presence of an inhibitory neural tone. EFS inhibited spontaneous activity (85.58 ± 2.5%, n = 14) with an off-response at the end of the stimulus. Incubation with the NO synthase inhibitor L-NNA (1 mM) decreased the inhibitory effect induced by EFS to 70.39 ± 3.6% (n = 14), showing that NO is an inhibitory neurotransmitter in the human colon (Fig. 1).

Circular and longitudinal muscle cells had a resting membrane potential of -49 ± 2.5 mV (n = 36). No major differences were found between muscle layers. In NANC conditions, short EFS pulses caused a 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 layers were found when the amplitudes of IJPs were measured. Like earlier studies (24), we observed that IJPs were L-NNA (1 mM) insensitive (n = 4; not significant, 3 circular and 1 longitudinal), showing that NO does not mediate the transient fast hyperpolarization (Fig. 1).
Exogenous addition of purinergic agonists. The purinergic pathway was studied with two agonists, ATP and ADPβS (a more stable ADP analog). Both ATP (1 mM) and ADPβS (10 μM) significantly inhibited the spontaneous contractions displayed by circular muscle strips in the presence of TTX (1 μM) (Fig. 2). ATP (1 mM) and ADPβS (10 μM) 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 (10 μM) hyperpolarized the smooth muscle to −12 ± 0.8 mV (n = 11). Hyperpolarization was recorded in the presence of TTX (1 μM) (−11 ± 2.2 mV; n = 4). Just after the end of the hyperpolarization with ADPβS, the IJP was greatly reduced and progressively recovered to the original values after washout (Fig. 2).

Effect of MRS 2179 on purinergic transmission. The inhibitory effect of both ATP and ADPβS was partially antagonized by a 10-min preincubation with MRS 2179 (10 μM). 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) (Fig. 3). Transient superfusion of the tissue with ADPβS (10 μM) hyperpolarized the smooth muscle to −12 ± 0.7 mV. Hyperpolarization was abolished by MRS 2179 (10 μM) (Fig. 3).

The increase in the voltage of stimulation caused a progressive increase in the amplitude of the IJP. Using the same

Fig. 4. A: intracellular microelectrode recordings showing IJP 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. Values are means ± SE. ***P < 0.001.
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, $n = 4$) (Fig. 4). To calculate the IC$_{50}$ of MRS 2179, a protocol using supramaximal IJPs was performed (using 30-V stimuli). The IC$_{50}$ was $1.22 \mu M$ (95% confidence interval of $0.66–1.88 \mu M$; logIC$_{50} = -5.95 \pm 0.11$) in the circular muscle layer and $1.31 \mu M$ (95% confidence interval of $1.05–1.69 \mu M$, logIC$_{50} = -5.88 \pm 0.05$) in the longitudinal muscle layer (Fig. 5). It is important to note that in the presence of MRS 2179 (10 $\mu M$), the amplitude of the IJP was extremely low (data not different from 0 mV using a t-test analysis for both muscle layers).

The nonnitrergic relaxation induced by EFS (2 Hz) was fully antagonized by MRS 2179 in a dose-response manner (Fig. 6). The IC$_{50}$ was $0.87 \mu M$ (95% confidence interval of $0.32–2.35 \mu M$, logIC$_{50} = -6.06 \pm 0.21$). 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 1 mM l-NNA) followed by a MRS 2179 (1 $\mu M$)-sensitive component was observed (Table 1).

However, in the presence of both l-NNA (1 mM) and MRS 2179 (3–10 $\mu M$), high stimulation frequencies (5–20 Hz) caused a contractile effect, possibly due to the release of noncholinergic excitatory transmitters.

**Effect of NF 023 on purinergic transmission.** To evaluate a putative involvement of P2X receptors on purinergic neurotransmission, NF 023 (a preferential P2X blocker) was tested. NF 023 (10 $\mu M$) did not modify the nonnitrergic relaxation induced by EFS at 2 Hz (data not shown). Moreover, NF 023 (10 $\mu M$) did not inhibit the IJP of the circular muscle ($n = 4$) (data not shown). A slight (~10–20%) but significant ($P < 0.05$) increase in the IJP was observed.

**Effect of MRS 2179 on other putative inhibitory neurotransmitters.** To check the specificity of MRS 2179 in the purinergic pathway, we tested other putative NANC neurotransmitters such as VIP, PACAP, and the NO donor NaNP. All these putative neurotransmitters caused complete cessation of the spontaneous mechanical activity in the presence of the neural blocker TTX (1 $\mu M$). After the incubation with MRS 2179 (10

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**Fig. 5.** A: intracellular microelectrode recordings showing the effect of MRS 2179 (1, 3, 5, and 10 $\mu M$) on IJP 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. Values are means ± SE. ***$p < 0.001$. 

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NaNP (10 μM), VIP (0.2 μM), and PACAP (0.2 μM) completely inhibited the spontaneous motility (n = 5 for each drug) (Fig. 7).

**Evaluation of the apamin pathway on purinergic neurotransmission.** To check whether small-conductance calcium-activated potassium channels could be involved in the purinergic pathway, apamin (1 μM) 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 nonnitrergic 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) (Fig. 8).

**Immunohistochemistry.** Positive P2Y1 receptor immunoreactivity was present in the circular and longitudinal smooth muscle layers. Some neurons of the myenteric ganglia were positively marked (Fig. 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 P2Y1 because 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.

**DISCUSSION**

In the present study, we demonstrated, for the first time, a functional purinergic neurotransmission between inhibitory motoneurons and human colonic smooth muscle through P2Y1 receptors. Several experimental procedures have been performed to characterize purinergic neurotransmission, including the use of nonselective P2 antagonists such as suramin and PPADS. In this study, we used MRS 2179 as a selective

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**Table 1. Percentage of inhibition induced by electrical field stimulation**

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Control</th>
<th>L-NNA (1 mM)</th>
<th>MRS 2179 (1 μM)</th>
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<tr>
<td>2 Hz</td>
<td>84±3.6</td>
<td>67±3.8</td>
<td>41±16</td>
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<td>5 Hz</td>
<td>91±4.2</td>
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<tr>
<td>10 Hz</td>
<td>86±4.5</td>
<td>52±9.7</td>
<td>13±6.5</td>
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<tr>
<td>20 Hz</td>
<td>85±8.4</td>
<td>41±18</td>
<td>*</td>
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Values are means ± SE; n = 4. Percentages of inhibition induced by electrical field stimulation at different frequencies in control conditions and in the presence of L-nitro-L-arginine (L-NNA) (1 mM) and 2′-deoxy-L-N6-methyl adenosine 3′,5′-diphosphate tetraammonium salt (MRS 2179) (1 μM) are shown. *A contractile response was obtained. P < 0.01 by ANOVA.
inhibitor of P2Y1 receptors (1, 10). We found that I) both IJPf and the nonnitrergic relaxation induced by EFS were dose dependently inhibited by MRS 2179, 2) MRS 2179 inhibited the relaxation and hyperpolarization induced by ADP\textsuperscript{S (a preferential P2Y agonist)}, 3) ADP\textsuperscript{S} reduced the IJP probably by a desensitization mechanism, and 4) P2Y\textsubscript{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 motoneurons, 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\textsubscript{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 the human sigmoid colon consists of regular myogenic cyclic contractions. This is the most common motility pattern found in vitro in human colonic strips (28). EFS releases inhibitory transmitters and consequently inhibits spontaneous motility (24). Previous studies have demonstrated that NO is an important neurotransmitter involved in smooth muscle inhibition in the human colon (4). Consequently, inhibition of NO synthase reduces smooth muscle relaxation (24) and IJPs (35). However, NO is not the only NANC neurotransmitter released by inhibitory motoneurons because 1) an important nonnitrergic relaxation, insensitive to l-NNA, is present during EFS and 2) IJPf is l-NNA insensitive (24). 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 nonselective purinergic blockers, comparing apamin sensitivity with nitrergic and nonnitrergic components, and comparing desensitizing receptors with unspecific agonists.

Desensitization by prolonged exposures to ADP\textsuperscript{S}, a P2Y agonist, abolishes the IJP in the human jejunal circular muscle (35) and mouse colon (30). In our study, we found that perfusion with ADP\textsuperscript{S} caused smooth muscle hyperpolarization and temporarily inhibited the IJP when the cell recovered resting membrane potential. These results suggest that ADP\textsuperscript{S} 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 (24) and intestinal (35) samples reported that apamin reduced the IJP by about 25%. Moreover, mechanical recordings show that apamin has a major effect on nonnitrergic relaxation induced by EFS (4). These results show that the nonnitrergic 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 (33). It should be pointed out that apamin might also reduce the slow component of the IJP that is NO mediated in the human colon and jejunum (24, 35). Consequently, apamin might not discriminate between the nitrergic and nonnitrergic pathways in the human gastrointestinal tract (35).

Suramin is a nonselective P2 blocker that inhibits about 30% of the IJP in the human jejunum (35). MRS 2179, a competitive blocker of P2Y1 receptors (10), dose dependently inhibited both the amplitude of the IJP and the nonnitrergic inhibition of the spontaneous motility induced by EFS. Both electrophysiological and mechanical experiments showed an IC50 close to 1 μM. These results show that P2Y1 receptors mediate smooth muscle hyperpolarization and relaxation in the human colon. Recent studies performed on animals have shown the following similar results: 1) MRS 2179 inhibited relaxation induced by ATP in the fundus, duodenum, ileum, and colon of the mouse (18); 2) MRS 2179 abolished the nonnitrergic relaxation induced by EFS in circular muscle strips from mouse jejunum (13); and 3) MRS 2179 inhibited the fast inhibitory junction potential in the guinea pig intestine (34). These studies, performed on different animals and varying sections of the gastrointestinal tract, conclude that P2Y1 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 nonnitrergic relaxation and the IJP are blocked by a purinergic antagonist with high specificity to P2Y1 receptors.

Smooth muscle relaxation induced by endogenous release of neurotransmitters and by EFS is completely blocked by MRS 2179. In contrast, when ADP 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 P2Y1 receptors, but other subclasses of P2Y receptors (see below), not located postjunctionally, might be activated by the exogenous addition of ADP. These receptors might use a pathway independent of the membrane potential, because hyperpolarization induced by exogenous addition of ADP is completely blocked by MRS 2179. Other receptors, such as subtypes of P2X receptors, might also be involved in the nonnitrergic relaxation (22). In studies on the mouse jejunum and porcine lower esophageal sphincters, where the main inhibitory pathway involves the activation of the P2Y1 receptor, a minor role of P2X receptors mediating inhibition has been reported (13, 15). In the human colon, however, NF 023 (10 μM), a preferential P2X antagonist (31), did not inhibit the nonnitrergic relaxation nor the IJP.

![Immunohistochemical localization of P2Y1 receptors in colonic circular smooth muscle (positive sample and control) in absence of the primary antibody (A), colonic longitudinal smooth muscle (positive sample and control) (B), and myenteric ganglia (positive sample and control) (C).](http://ajpgi.physiology.org)
suggesting that P2X receptors are not involved in neuromuscular inhibition.

Immunohistochemical studies were performed to determine the presence of P2Y1 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 P2Y1 receptors has been previously described in the mouse ileum (18). Both P2Y1 and P2Y2 (but not P2Y3) receptors are present in smooth muscle cells of the mouse ileum (18). 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 P2Y1 receptors in mediating IJP in both muscle layers with an IC50 close to 1 μM. The expression of P2Y1 receptors in enteric tissues suggests that this receptor might mediate synaptic neurotransmission between enteric neurons. Moreover, P2Y1 receptors mediate slow excitatory postsynaptic potentials in the guinea pig ileum (21).

It is important to note that both ATP and NO mediate smooth muscle inhibition at a wide range of stimulation frequencies (from 2 to 20 Hz). At 2 Hz, no major excitatory component was observed in the presence of MRS 2179 (10 μM), and, therefore, the IC50 was calculated at this stimulation frequency. However, a prominent excitatory noncholinergic 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 (14). 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 noncholinergic depolarization and contraction needs further investigation, but it may be due to the release of tachykinins, which are important excitatory neurotransmitters in the human colon (11).

ATP and NO mediate smooth muscle relaxation in the gastrointestinal tract. NO participates in several physiological functions such as gastric accommodation, peristaltic reflex, and sphincter tone. Impairment of neural NO 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 neuro-muscular inhibitory transmitter acting mainly through P2Y1 receptors. Activation of P2Y1 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 whether impairment of purinergic neurotransmission occurs in these diseases. MRS 2179 might be an important pharmacological agent to investigate such effects, and P2Y1 receptors could be a pharmacological target to modulate smooth muscle excitability in the human gastrointestinal tract.

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