P2Y1 Receptors Mediate Apamin-Sensitive and -Insensitive Inhibitory Junction Potentials in Murine Colonic Circular Smooth Muscle

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ABSTRACT

Purinergic inhibitory neuromuscular transmission plays an important role in the control of intestinal motility. In most tissues this neurotransmission is apamin-sensitive, but recent studies in human colonic circular smooth muscle (CSM) suggest the presence of apamin-insensitive purinergic inhibitory junction potentials (IJPs). The current studies used conventional intracellular recordings on colonic CSM strips to characterize the purinergic IJPs in murine colonic CSM. P2Y1 receptor expression was examined by using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry. The IJP induced by nerve stimulation (NS) of one and four pulses in neuronal nitric-oxide synthase knockout mice consists of an apamin-sensitive and a dominant apamin-resistant component. These are identical to the IJPs in wild-type and CD1 mice in the presence of Nω-nitro-L-arginine methyl ester (200 μM) and were significantly inhibited by α,β-methylene ATP (50 μM), an analog of ATP. IJPs were not affected by the P2X receptor antagonist 2’,3’-o-(2,4,6-trinitrophenyl)-ATP (10 μM). Furthermore, apamin-resistant IJPs induced by single-pulse NS were abolished by pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonate (100 μM), a P2 receptor antagonist; 2’-deoxy-N6-methyl adenosine 3,5-diphosphate (MRS-2179; 10 μM), a selective P2Y1 receptor antagonist; and tetrodotoxin (1 μM). Aboral NS induced apamin-sensitive purinergic IJPs, whereas oral and circumferential NS produced apamin-sensitive and -resistant IJPs, with the latter predominating. RT-PCR and immunohistochemistry confirmed the presence of P2Y1 receptors on smooth muscle and in the myenteric plexus. These data suggest that, depending on stimulus location, activation of P2Y1 receptors produces both apamin-sensitive and apamin-resistant IJPs in murine colonic CSM.

The discovery of nonadrenergic and noncholinergic inhibitory neurotransmission to gastrointestinal (GI) smooth muscle in 1963 (Burnstock et al., 1963; Martinson and Muren, 1963) challenged the classical concept that the autonomic nervous system is composed of noradrenergic sympathetic and cholinergic parasympathetic nerves. Subsequent studies have established that this nonadrenergic and noncholinergic inhibitory neurotransmission primarily involves nitricergic and purinergic nerves (see Bennett 1997), with the latter releasing ATP or related nucleotides (Burnstock, 2006; Alexander et al., 2007).

Purinoceptors have been classified as two superfamilies, namely, P1 and P2 receptors, which are activated primarily by adenosine and ATP/ADP, respectively (Burnstock, 1978). The latter consists of two families, ligand-gated P2X receptors and G protein-coupled P2Y receptors (Alexander et al., 2007). P2Y receptors are composed of eight cloned members including P2Y1, 2, 4, 6 and 11 and P2Y11–14 (Alexander et al., 2007). Previous publications have suggested that P2Y1, 2, 4, 6, and 11 receptors are coupled to activation of phospholipase C via G11 proteins (G11-phospholipase C-IP3 pathway) and that P2Y2,2–14 receptors are preferentially linked to the inhibition of adenylate cyclase (von Kügelgen, 2006). Recent molecular and functional studies in strips of guinea pig taenia coli that were preconstricted with carbachol have shown that P2Y receptors are involved in relaxation (King and Townsend-Nicholson, 2008). A number of publications have reported that purinergic inhibitory junction potentials (IJPs) in GI smooth muscle are mediated by P2Y1 receptors, with the latter predominating. RT-PCR and immunohistochemistry confirmed the presence of P2Y1 receptors on smooth muscle and in the myenteric plexus. These data suggest that, depending on stimulus location, activation of P2Y1 receptors produces both apamin-sensitive and apamin-resistant IJPs in murine colonic CSM.
smooth muscle are caused by the opening of small conductance Ca\(^{2+}\)-activated K\(^+\) (SK) channels, as apamin, an SK channel blocker, abolishes these IJPs (see Bennett, 1997). However, recent studies have reported that a major component of purinergic IJPs is apamin-insensitive in the circular smooth muscle (CSM) of human colon (Gallego et al., 2006, 2008). Furthermore, it is known that the nature of neural responses in the gut differs depending on the orientation of the stimulating electrodes (Zhang and Paterson, 2005). Based on the aforementioned studies, we hypothesized that the nature of neural responses in the gut differs depending on the orientation of the stimulating electrodes (Zhang and Paterson, 2003). Therefore, the current study in murine colon had two aims: 1) to define subtypes of P2Y receptors that mediate the purinergic IJPs, and 2) to characterize the purinergic IJPs produced by circumferential, oral, and aboral nerve stimulation.

Materials and Methods

Tissue Preparation and Conventional Intracellular Recordings. Experimental protocols were approved by the Animal Care Committee of Queen’s University, in keeping with guidelines of the Canadian Council on Animal Care. Adult mice (CD1; Charles River Canada, Montreal, QC, Canada), neuronal nitric-oxide synthase knockout nNOS\((+/−)\); B6.129S4-Nos1\(^{tm1Plh}\), and wild-type mice (B6.129SF2/J) (The Jackson Laboratory, Bar Harbor, ME) (Huang et al., 1993) of either sex were killed by cervical dislocation after isoflurane anesthesia. nNOS\((+/−)\) knockout mice were used to examine P2Y receptor expression in the colonic tunica muscularis. The abdominal cavity was then opened along the mesenteric attachment in a dissection dish. The mucosal and submucosal layers were then removed by using a dissecting microscope. Strips (7–10 × 7–10 mm) of the distal colon were pinned with the circular muscle facing upward on the bottom of a recording chamber lined with silastic elastomer (Sylgard; Dow Corning, Midland, MI) and superfused at 2 ml/min with carboxenated Krebs’ solution containing atropine (3 μM), guanethidine (3 μM), and substance P (1 μM) at 36°C to block cholinergic, adrenergic, and tachykinergic responses (Zhang et al., 2008). Nerve stimulation (NS) using either one or four square wave pulses (20 Hz) with a duration of 0.3 ms and submaximal voltage of 50 to 70 V was delivered to the muscle preparations by two pairs of silver wires that were placed at the mesenteric attachment edge (circumferential NS) and either the oral or the aboral end (oral NS or aboral NS), whereas electrical activity was recorded by using conventional intracellular electrodes in the tissue of 4 to 5 mm from each pair of the stimulating electrodes (Fig. 1). Previously defined electrical parameters (amplitude and half-amplitude duration of IJPs) were used to quantitatively analyze the electrical properties of the smooth muscle (Zhang and Paterson, 2003).

P2Y, Receptor Expression. Reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry were used to examine P2Y, receptor expression in the colonic tunica muscularis. RNA was extracted from approximately 100 mg of distal colon tunica muscularis by using an Ambion RNAqueous kit (Ambion, Austin, TX). The RNA was then precipitated by using LiCl precipitation solution (Ambion). Subsequently, cDNA was reverse-transcribed from 1 μg of total RNA using Superscript III (Invitrogen, Carlsbad, CA) and oligo(dT)\(_{12-18}\) primers (Invitrogen). P2Y1 receptor gene expression was probed by using an Eppendorf North America (New York, NY) M2704 Mastercycler gradient PCR machine with the following primers for 30 cycles: forward, CGACAGGGTTTATGCCACTT; and reverse, CGTGTCTCCATCTCTGCTGA. For immunohistochemical detection of P2Y1 receptor expression, whole mounts of tunica muscularis were dissected in ice-cold phosphate-buffered saline (PBS) before overnight fixation in 4% paraformaldehyde at 4°C. In each preparation, small strips of circular muscle were removed to reveal the underlying myenteric plexus. After 3 × 10-min washes in PBS, tissues were incubated for 1 h in 10% normal horse serum in 1% Triton-X100 in PBS. Tissues were washed three times in PBS before incubation overnight in rabbit anti-P2Y1 receptor antiserum (1:500; Alomone Labs, Jerusalem, Israel) (Van Crombruggen et al., 2007). Primary antiserum was then removed, and the preparations were washed in PBS for 3 × 10 min, followed by a 2-h incubation in donkey anti-rabbit Alexa Fluor 555 (Invitrogen). After a final 3 × 10-min wash, preparations were mounted on slides in buffered glycerol and coverslipped. Whole mounts were examined with a BX 51 epifluorescence microscope (Olympus Canada, Markham, ON, Canada), and micrographs were acquired with a charge-coupled device camera (Photometrics Coolsnap; Photon Technology International, Lawrenceville, NJ). Specificity of the P2Y1 antiserum was tested by preadsorbing the antiserum with antigenic peptide 1:1 for 90 min before addition of the mixture to colonic tissue, per the manufacturer’s instructions. Preadsorption abolished immunoreactivity in mouse colon (see Fig. 11C).

Drugs. All drugs were purchased from Sigma-Aldrich (St. Louis, MO), but isoflurane was purchased from Baxter (Mississauga, ON, Canada). The following drugs were used: nifedipine, atropine, guanethidine, apamin, substance P, N\(^{-}\)-nitro-l-arginine methyl ester (l-NAME), α,β-methylene ATP, pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonate (PPADS), MRS-2179, 2’,3’-o-(2,4,6-trinitrophe- nyl)-ATP (TNP-ATP), and tetrodotoxin (TTX). Nifedipine was dissolved as a stock solution in alcohol, and others were dissolved in distilled and deionized water. These stocks were diluted to final concentrations with Krebs’ solution.

Statistical Analysis. Data are shown as mean ± S.E.M., and n refers to number of animals. Predrug and postdrug comparisons were made using the paired Student’s t test, and P < 0.05 was considered statistically significant.

Results

General Electrical Properties of Mouse Distal Colon CSM. Intracellular recordings of mouse distal colon CSM revealed two patterns of spontaneous electrical activity (Fig. 2) in the presence of atropine (3 μM), guanethidine (3 μM), and substance P (1 μM). Unitary membrane potentials similar to those seen in other GI smooth muscle (Edwards et al., 1999; Zhang et al., 2008) were seen in all of the tissues. In some preparations spontaneous action potentials superimposed on
Fig. 2. Patterns of spontaneous electrical activity recorded in CSM of mouse colon. A, raw recording reveals continuous membrane potential fluctuations of up to 3 mV (termed unitary potentials; Edwards et al., 1999) with superimposed spontaneous hyperpolarizations (*) and action potentials (#). The spontaneous hyperpolarizations could be abolished by application of apamin, MRS-2179, and α,β-methylene ATP (data not shown). No significant slow waves were observed in this recording. Large negative deflections represent IJPs induced by electrical stimulation. B, example of action potentials during the depolarization phase of classic slow waves. These action potentials and slow waves were blocked by nifedipine (1 μM).

Fig. 3. Nitrergic component of the IJPs in the CSM of wild-type and nNOS knockout mice. A and D, original recordings of effects of L-NAME (200 μM) on the IJPs induced by one and four pulses in the presence of atropine (3 μM), guanethidine (3 μM), substance P (1 μM), and nifedipine (1 μM). B and E, snapshots of the IJPs before and after application of L-NAME in the wild-type and nNOS knockout mice, respectively. C and F, superimposed IJPs for comparison. NS (one pulse) induced the IJP with amplitude of 35.1 mV and duration of 616 ms on average in wild-type mice (versus 32.3 mV and 608 ms in nNOS knockout), whereas four pulses produced wider IJP (35.3 mV and 1226 ms) followed by slow IJP in wild-type mice. The slow IJP was abolished by L-NAME in wild-type mice. The nitrergic slow IJP was not visible in nNOS knockout mice. However, in most nNOS knockout mice, an EJP (arrow indicated) with variable amplitude occurred during an IJP and was observed in response to NS of four pulses (20 Hz). The identity of the EJPs was not pursued further in the current studies.
unitary membrane potentials were recorded, but no slow waves were evident (Fig. 2A). In most preparations, however, action potentials were superimposed on classical slow waves (Fig. 2B). Both the action potentials and slow waves were blocked by nifedipine, in keeping with our previous studies (Zhang and Paterson, 2005). Preliminary experiments suggested that application of nifedipine (1 μM) did not significantly affect IJP amplitude or duration (data not shown). Therefore, to prevent the dislodgement of the intracellular recordings, nifedipine was routinely added to the Krebs’ solution in the remaining studies.

In the presence of nifedipine (1 μM), spontaneous hyperpolarizations with variable amplitudes up to 15 mV were recorded in 31 of 35 CD1 mice, all the nNOS knockout mice, and all the wild-type mice (Figs. 2A, 3, A and D, and see Figs. 5A and 8A). α,β-Methylene ATP (50 μM), a stable analog of ATP; MRS-2179 (10 μM), a selective P2Y1 receptor antagonist; and apamin, an SK channel blocker, abolished the spontaneous hyperpolarizations, suggesting that these result from the opening of SK channels via activation of P2Y1 receptors by the spontaneous release of ATP or related nucleotides at neuromuscular junctions. The findings are consistent with previous studies in the distal colon of mice and guinea pigs (Spencer et al., 1998b; Dickson et al., 2007).

**Inhibitory Innervation to the CSM Cells.** It was previously shown that nitrergic and purinergic neurotransmission accounts mainly for the neural inhibition in the murine upper digestive tract (Burns et al., 1996; Zhang et al., 2008). Therefore, the nitrergic and purinergic innervation to the colonic CSM was first examined in wild-type, nNOS knockout, and CD1 mice (Figs. 3 and 4 and see Figs. 9 and 10; Tables 1 and 2). NS (1 pulse) induced an IJP in wild-type, nNOS knockout (Fig. 3, Ba and Ea), and CD1 mice. NS of four pulses produced a rapid initial IJP followed by a slow IJP in the wild-type mice (Fig. 3Bb), but the slow IJP was not observed in the nNOS knockout mice (Fig. 3Eb) or control mice in the presence of the nitric-oxide synthase inhibitor L-NAME (200 μM), suggesting its nitrergic identity. In most cases, application of L-NAME unmasked an excitatory junction potential (EJP) (arrows in Fig. 3E) of variable amplitude that occurred during an IJP evoked by NS of four pulses (20 Hz). The pharmacological identity of the noncholinergic, nontachykininergic EJPs was not pursued further in the current studies. IJPs were identical in wild-type, nNOS, and CD1 mice in the presence of L-NAME (Fig. 3, B and D). Apamin-sensitive and -insensitive IJPs were identical in wild-type and nNOS knockout mice. A and D, raw recordings showed effects of apamin (300 nM) on the RMP and IJPs in the presence of L-NAME in wild-type and nNOS knockout mice. B and E, IJPs from A and D in an expanded time scale. C and F, showed superimposed IJPs before and after application of apamin.
The pharmacological properties of apamin-resistant IJPs in CSM of murine colon are investigated. Apamin-resistant IJPs are defined as 1.7 mV after 1 min and 300 nM apamin, respectively; n = 6). MRS-2179 (10 μM) hyperpolarized the resting membrane potential (RMP) (Fig. 5A) and inhibited IJPs (Fig. 5B). Surprisingly, NS evoked marked EJPs that followed the residual IJPs (Fig. 5B). The RMP hyperpolarization and EJPs were prevented by pretreatment with apamin (Fig. 5C). The latter effect was not prevented by pretreatment with the P2X<sub>1</sub>, P2X<sub>2</sub>, and P2X<sub>3</sub> receptor antagonist TNP-ATP (Virginio et al., 1998) (10 μM) or TTX (1 μM) (Fig. 6). α,β-Methylene ATP also inhibited the apamin-resistant IJPs (Table 2; Fig. 5D). PPADS (100 μM) significantly depolarized the RMP (Fig. 7A) in the tissues that were pretreated with apamin (300 nM). PPADS decreased the apamin-resistant IJPs by 2.8 mV on average 5 min after bath administration (Fig. 5Bb) but abolished apamin-resistant IJPs at 20 min (Fig. 7Bd). The effects of PPADS were apparently not reversible because the inhibition of IJPs did not recover 40 min after washing out. Furthermore, TNP-ATP (10 μM) had no effect on the IJP (Table 2). The combined effects of α,β-methylene ATP and PPADS on the IJPs suggest that both the apamin-sensitive and -resistant IJPs are purinergic but not P2X receptor-mediated.

### Purinoceptor Subtypes that Mediate Apamin-Sensitive and -Resistant IJPs

It has been reported that MRS-2179 (10 μM) did not change the RMP (Fig. 8, A and B) but abolished the IJPs produced by single pulses (Fig. 8, C and D) either in the absence or presence of apamin (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Effect of L-NAME on IJP in CSM of murine colon</th>
<th>MP</th>
<th>Amplitude</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>Wild-type n = 3</td>
<td>One-Pulse NS</td>
<td>Control</td>
<td>−43.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Four-Pulse NS</td>
<td>Control</td>
<td>−46.6 ± 6.8</td>
</tr>
<tr>
<td>nNOS knockout n = 4</td>
<td>One-Pulse NS</td>
<td>Control</td>
<td>−42.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Four-Pulse NS</td>
<td>Control</td>
<td>−45.5 ± 1.1</td>
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* Represents the significance before and after an application of pharmacological agent.

### Table 2

<table>
<thead>
<tr>
<th>Pharmacological properties of apamin-resistant IJPs in CSM of murine colon</th>
<th>MP</th>
<th>Amplitude</th>
<th>Duration</th>
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<tbody>
<tr>
<td>Apamin&lt;sup&gt;1&lt;/sup&gt; (n = 20)</td>
<td>Control</td>
<td>−40.2 ± 1.6</td>
<td>30.0 ± 1.7</td>
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<tr>
<td>α,β-methylene ATP&lt;sup&gt;2&lt;/sup&gt; (n = 6)</td>
<td>Control</td>
<td>−42.0 ± 1.1</td>
<td>36.5 ± 1.2</td>
</tr>
<tr>
<td>α,β-methylene ATP&lt;sup&gt;3&lt;/sup&gt; (n = 6)</td>
<td>Control</td>
<td>−54.3 ± 1.8</td>
<td>18.8 ± 0.8</td>
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<tr>
<td>PPADS&lt;sup&gt;4&lt;/sup&gt; (n = 7)</td>
<td>Control</td>
<td>−42.5 ± 0.8</td>
<td>22.5 ± 1.5</td>
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<tr>
<td>TTX&lt;sup&gt;5&lt;/sup&gt; (n = 5)</td>
<td>Control</td>
<td>−43.9 ± 3.0</td>
<td>13.5 ± 2.1</td>
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<tr>
<td>MRS-2179&lt;sup&gt;6&lt;/sup&gt; (n = 4)</td>
<td>Control</td>
<td>−44.1 ± 1.6</td>
<td>16.0 ± 0.7</td>
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<tr>
<td>MRS-2179&lt;sup&gt;7&lt;/sup&gt; (n = 5)</td>
<td>Control</td>
<td>−37.1 ± 1.5</td>
<td>33.3 ± 1.6</td>
</tr>
<tr>
<td>TNP-ATP&lt;sup&gt;8&lt;/sup&gt; (n = 4)</td>
<td>Control</td>
<td>−39.3 ± 2.2</td>
<td>17.4 ± 3.5</td>
</tr>
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</table>

All experiments were performed in the presence of atropine, guanethidine, substance P, and nifedipine. Additional agents were applied as footnoted below.

1 Represents the significance before and after an application of pharmacological agent.
2 In the presence of L-NAME in wild-type (n = 3) and knockout (n = 4) and CD1 (n = 11) mice.
3 In the presence of L-NAME and apamin in wild-type (n = 3) and knockout (n = 3) mice.
4 In the presence of L-NAME and apamin in wild-type (n = 4) and knockout (n = 3) mice.
5 In the presence of L-NAME and apamin in wild-type (n = 3) and knockout (n = 2) mice.
6 In the presence of L-NAME and apamin in CD1 mice.
7 In the presence of L-NAME and apamin in CD1 mice.
8 In the presence of L-NAME in CD1 mice.
also prevented the membrane hyperpolarization induced by αβ-methylene ATP (Fig. 6). The abolition of the purinergic IJPs by MRS-2179 strongly implies that P2Y1 receptors mediate both the apamin-sensitive and -resistant purinergic IJPs.

**Polarity of Purinergic Innervation to the Colonic CSM.** To test the hypothesis that polarization of purinergic innervation to the colonic CSM accounts for the diversity of the purinergic IJPs (i.e., apamin-sensitive and -resistant purinergic IJPs), we recorded purinergic IJPs produced by circumferential and oral NS, and circumferential and aboral NS in the same CSM cells (Fig. 1). Figure 9 is an example of electrical responses of the CSM cells to circumferential and oral NS. There was no difference in apamin-sensitive and -resistant IJPs induced by the circumferential NS (single-pulse) (Fig. 9, A, D, and G) versus oral NS (Fig. 9, B, E, and H). The comparison of apamin-resistant IJPs evoked by circumferential and aboral NS using single pulses is summarized in Table 3. Figure 10 shows snapshots of IJPs recorded before and after application of apamin (300 nM) and highlights the difference in the IJPs evoked by circumferential versus aboral NS. These data indicate there is predominance of apamin-resistant IJPs in circumferential and descending inhibition.

**P2Y1 Receptor mRNA and Protein Expression.** RT-PCR with primers designed to amplify P2Y1 receptor cDNA detected P2Y1 receptor in the distal colon tunica muscularis of each of three CD1 mice examined (Fig. 11A). Protein expression was also examined by using immunohistochemistry with anti-P2Y1 receptor antiserum. Immunoreactivity was evident within the CSM and a subset of myenteric neurons (Fig. 11B). Similar patterns of immunoreactivity were observed in each of four distal colon preparations examined. Preadsorption of the antiserum with the antigen against which it was raised abolished all of the immunoreactivity (Fig. 11C).

**Discussion**

In the current study, we have shown both apamin-sensitive and -resistant purinergic IJPs in murine distal colonic CSM. Furthermore, the effects of P2 receptor agonists and antag-
onists suggest that both apamin-sensitive and -resistant purinergic IJPs are mediated by P2Y₁ receptors. This physiological evidence is further strengthened by the detection of the mRNA encoding P2Y₁ receptors in the colonic CSM layer and immunohistochemical evidence of the receptor on the membrane of colonic CSM cells. Interestingly, the observation that the apamin-resistant IJP is preferentially evoked by circumferential and oral NS, compared with aboral NS, Fig. 7. Abolition of the apamin-resistant IJPs by PPADS (100 μM), a P2 receptor antagonist. A, the RMP depolarization produced by PPADS. B, a–d, snapshots of the IJPs depicted in A. Superimposed IJPs are shown in B. e, PPADS eradicated the apamin-resistant IJPs induced by single-pulse electrical stimulation. The inhibitory effect of PPADS was maximal at 20 min when the RMP had largely recovered from initial depolarization, making it unlikely that the effect on the IJP was caused by membrane depolarization.

Fig. 8. Inhibitory effects of MRS-2179 (10 μM), a competitive and selective P2Y₁ purinoceptor antagonist, on the purinergic IJPs. A and B, raw recordings showed no effects of MRS-2179 on the RMP in the absence and presence of apamin. C and D, shown are IJPs in the expanded time scale depicted in A and B. MRS-2179 blocked the IJPs in the absence and presence of apamin, implying that P2Y₁ purinoceptor mediates both the apamin-sensitive and apamin-resistant IJPs.
Purinergic fast IJPs result from opening of SK channels, murine lower esophageal sphincter (Zhang et al., 2008). The both guinea pig small intestine (He and Goyal, 1993) and nitrergic nerves, respectively, have been clearly shown in guinea pig taenia coli (King and Townsend-Nicholson, 2008) have suggested that the inhibitory effect of α,β-methylene ATP is caused by activation of P2X receptors on myenteric neurons, which in turn stimulate the release of ATP. In the current study, however, α,β-methylene ATP induced TTX-insensitive circular muscle hyperpolarization that was prevented by pretreatment with MRS-2179. In addition, the P2X receptor antagonist TNP-ATP did not affect the purinergic IJP or 4) MRS-2179 eliminated apamin-sensitive and -resistant purinergic IJPs; and 4) P2Y1 receptors were shown in the colon CSM layer by both RT-PCR and immunohistochemistry.

It is well known that PPADS is an antagonist of P2 purinoceptors at prejunctional and postjunctional sites (Lambrecht et al., 1992; Ziganshin et al., 1993). PPADS produced a significant RMP depolarization in the presence of apamin (Fig. 7A; Table 2). The time course of effects of PPADS on the RMP and the purinergic IJPs excluded the possibility that abolition of the purinergic IJPs resulted from the RMP depolarization. PPADS significantly depolarized the RMP by 12.4 mV over control and inhibited the amplitude of the IJPs by only 2.8 mV over control in 5 min, whereas it produced the RMP depolarization by only 6.4 mV over control and abolished apamin-resistant IJPs at 20 min (Fig. 7B, b and d). A mechanism for the RMP depolarization induced by PPADS remains unknown.

α,β-Methylene ATP is a stable analog of ATP that activates P2 receptors (Burnstock and Kennedy, 1985; Pinna et al., 2005). Bath application of α,β-methylene ATP induced RMP hyperpolarization, which was prevented by pretreatment with apamin, implying that it resulted from opening of SK channels. Interestingly, after application of α,β-methylene ATP, nerve-mediated responses were composed of an initial IJP followed by a prominent EJP (Fig. 5Bb). The latter was also blocked by apamin. This EJP was not further characterized in the current study. α,β-Methylene ATP is an agonist of P2X receptors, which are reported to be primarily present on GI myenteric plexus neurons (Van Crombruggen et al., 2007; King and Townsend-Nicholson, 2008). Furthermore, this agent has been reported to not bind to P2Y1 receptors cloned from chick brain (Webb et al., 1993). Studies in guinea pig taenia coli (King and Townsend-Nicholson, 2008) have suggested that the inhibitory effect of α,β-methylene ATP is caused by activation of P2X receptors on myenteric neurons, which in turn stimulate the release of ATP. In the current study, however, α,β-methylene ATP induced TTX-insensitive circular muscle hyperpolarization that was prevented by pretreatment with MRS-2179. In addition, the P2X receptor antagonist TNP-ATP did not affect the purinergic IJP or

**TABLE 3**

<table>
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<tr>
<th>Condition</th>
<th>MP</th>
<th>Amplitude</th>
<th>Duration</th>
</tr>
</thead>
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<tr>
<td>Circumferential vs. oral NS</td>
<td>Circumferential</td>
<td>−39.4 ± 0.8</td>
<td>12.2 ± 2.1</td>
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<td>Circumferential vs. aboral NS</td>
<td>Oral</td>
<td>−38.3 ± 0.4</td>
<td>12.3 ± 1.5</td>
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<tr>
<td>Circumferential vs. aboral NS</td>
<td>Circumferential</td>
<td>−41.6 ± 1.9</td>
<td>18.3 ± 3.0</td>
</tr>
<tr>
<td>Circumferential vs. aboral NS</td>
<td>Aboral</td>
<td>−42.4 ± 1.6</td>
<td>7.5 ± 1.6*</td>
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</table>

* Statistically significant between circumferential and aboral NS.
antagonize the membrane hyperpolarization induced by α,β-methylene ATP (Fig. 6). This is similar to a recent study in human sigmoid CSM (Auli et al., 2008) in which the α,β-methylene ATP-induced inhibition of spontaneous phasic contractions was prevented by pretreatment with apamin or MRS-2179 but unaffected by TTX or P2X receptor blockade. This suggests that α,β-methylene ATP may indeed activate P2Y1 receptors.

There is now extensive literature supporting the specificity of MRS-2179 as a P2Y1 receptor antagonist (Boyer et al., 2002; King, 2002; von Kügelgen, 2006). In smooth muscle of guinea pig and murine small intestines, it blocks the apamin-sensitive IJPs with an IC_{50} of 0.2 μM, supporting the involvement of P2Y_{1} receptors in the apamin-sensitive IJPs (Wang et al., 2004, 2007). Furthermore, MRS-2179 also blocks the hyperpolarization induced by bath application of ATP in the small intestine of guinea pigs but not in mice. In the circular and longitudinal smooth muscle of human colon, a tissue where the apamin-resistant purinergic IJPs were predominant, the IC_{50} of MRS-2179 for the suppression of purinergic IJPs was 1.22 and 1.31 μM, respectively (Gallego et al., 2006, 2008). The concentration of MRS-2179 used in the current study was 10 μM. Abolition of both apamin-sensitive and -resistant IJPs by MRS-2179 (Fig. 8, Cc and Dc) suggests that purinergic neurotransmission in the murine colon is mediated by P2Y_{1} receptors, in keeping with that in the human colon. It also implies that P2Y_{1} receptors mediate divergent purinergic signaling via apamin-sensitive and -insensitive mechanisms.

The existence of apamin-sensitive and -insensitive purinergic IJPs depends on the species and tissue studied. Apamin-sensitive purinergic IJPs were recorded in the small intestine of guinea pigs (Crist et al., 1992) and lower esophageal sphincter (Zhang et al., 2008) of mice, whereas apamin-sensitive and -resistant IJPs, with the latter predominating, were shown in the human colon (Gallego et al., 2006) and mouse internal anal sphincter (McDonnell et al., 2008) and colon (Spencer et al., 1998b). Orientation of the tissues was not specified in the aforementioned reports. Zhang and Paterson (2005) and Dickson et al. (2008) have described polarized intrinsic innervation to the intestinal smooth muscle. Our finding that a major component of the apamin-resistant IJP was produced predominantly by circumferential and oral but not by aboral NS (Table 3) suggests that purinergic nerve fibers that project circumferentially and orally produce apamin-sensitive and -resistant IJPs, whereas those that project aborally induce apamin-sensitive IJPs. However, we noted that the amplitude of apamin-resistant purinergic IJPs approaches that of the IJPs induced by either oral or circumferential NS when the oral intracellular recording site is moved closer to the aboral NS location (data not shown). Thus, a possible alternative explanation is that the purinergic nerve fibers preferentially activated by oral and circumferential NS project further than those projecting aborally.

In summary, the current studies suggest that both apamin-sensitive and -insensitive purinergic IJPs are present in murine small intestines, it blocks the apamin-sensitive IJPs with an IC_{50} of 0.2 μM, supporting the involvement of P2Y_{1} receptors in the apamin-sensitive IJPs (Wang et al., 2004, 2007). Furthermore, MRS-2179 also blocks the hyperpolarization induced by bath application of ATP in the small intestine of guinea pigs but not in mice. In the circular and longitudinal smooth muscle of human colon, a tissue where the apamin-resistant purinergic IJPs were predominant, the IC_{50} of MRS-2179 for the suppression of purinergic IJPs was 1.22 and 1.31 μM, respectively (Gallego et al., 2006, 2008). The concentration of MRS-2179 used in the current study was 10 μM. Abolition of both apamin-sensitive and -resistant IJPs by MRS-2179 (Fig. 8, Cc and Dc) suggests that purinergic neurotransmission in the murine colon is mediated by P2Y_{1} receptors, in keeping with that in the human colon. It also implies that P2Y_{1} receptors mediate divergent purinergic signaling via apamin-sensitive and -insensitive mechanisms.

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In summary, the current studies suggest that both apamin-sensitive and -insensitive purinergic IJPs are present in murine small intestines.
colonic CSM. Furthermore, it appears that both components of this purinergic IJP are mediated by P2Y receptors. Purinergic nerve fibers that project circumferentially and orally produce both apamin-sensitive and -resistant IJPs, whereas those that project aborally predominantly induce apamin-sensitive IJPs. Further investigations are required to characterize the different signaling pathways and ionic mechanisms involved in the apamin-sensitive and -insensitive purinergic IJPs.

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References


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