Inhibitory neuromuscular transmission mediated by the P2Y1 purinergic receptor in guinea pig small intestine

Guo-Du Wang, Xi-Yu Wang, Hong-Zhen Hu, Sumei Liu, Na Gao, Xiucai Fang, Yun Xia, and Jackie D. Wood
Department of Physiology and Cell Biology, The Ohio State University College of Medicine, Columbus, Ohio

Submitted 29 September 2006; accepted in final form 8 February 2007

Wang G-D, Wang X-Y, Hu H-Z, Liu S, Gao N, Fang X, Xia Y, Wood JD. Inhibitory neuromuscular transmission mediated by the P2Y1 purinergic receptor in guinea pig small intestine. Am J Physiol Gastrointest Liver Physiol 292: G1483–G1489, 2007. First published February 22, 2007; doi:10.1152/ajpgi.00450.2006.—ATP is a putative inhibitory neurotransmitter responsible for inhibitory junction potentials (IJPs) at neuromuscular junctions (IJPs) in the intestine. This study tested the hypothesis that the purinergic P2Y1 receptor subtype mediates the IJPs. IJPs were evoked by focal electrical stimulation in the myenteric plexus and recorded with “sharp” intracellular microelectrodes in the circular muscle coat. Stimulation evoked three categories of IJPs: 1) purely purinergic IJPs, 2) partially purinergic IJPs, and 3) nonpurinergic IJPs. Purely purinergic IJPs were suppressed by the selective P2Y1 purinergic receptor antagonist MRS2179. Purely purinergic IJPs comprised 30% of the IJPs. Partially purinergic IJPs (72% of the IJPs) consisted of a component that was abolished by MRS2179 and a second unaffected component. The MRS2179-insensitive component was suppressed or abolished by inhibition of formation of nitric oxide by N^N-nitro-L-arginine methyl ester (L-NAME) in some, but not all, IJPs. An unidentified neurotransmitter, different from nitric oxide, mediated the second component in these cases. Nonpurinergic IJPs were a small third category (4%) of IJPs that were abolished by L-NAME and unaffected by MRS2179. Exogenous application of ATP evoked IJP-like hyperpolarizing responses, which were blocked by MRS2179. Application of apamin, which suppresses opening of small-conductance Ca^{2+}-activated K^+ channels in the muscle, decreased the amplitude of the purinergic IJPs and the amplitude of IJP-like responses to ATP. The results support ATP as a neurotransmitter for IJPs in the intestine and are consistent with the hypothesis that the P2Y1 purinergic receptor subtype mediates the action of ATP.

Adenosine 5'-triphosphate; neurotransmission; enteric nervous system; intestinal motility; smooth muscle

INHIBITION OF THE CIRCULAR MUSCULATURE ahead of the advancing bolus is an essential component of peristaltic propulsion in the small and large intestine. This component of the organization of propulsive motility is organized by the enteric nervous system (ENS) and reflects elevated activity in the inhibitory motor innervation of the musculature. Evidence in 1963 first implicated ATP as one of the inhibitory neurotransmitters at neuromuscular junctions in the intestine (9, 10). This role for ATP has been confirmed repeatedly in subsequent years (10, 11, 12).

Inhibitory junction potentials (IJPs) were first reported for the guinea pig taenia coli by Burnstock et al. (9) in 1963 and analyzed in more detail in 1966 (4). Excitation of ENS inhibitory motor neurons by transmural electrical field stimulation or by application of nicotinic receptor agonists is now commonly known to evoke IJPs, which are readily detected with intracellular electrophysiological recording methods, in intestinal circular muscle. In human, guinea pig, dog, and mouse preparations, the slow IJP is abolished by treatments that block the synthesis of nitric oxide and is mimicked by exogenous application of nitric oxide (3, 22, 33, 36)., which is evidence that the late, slow inhibitory postsynaptic potential (IPSP) reflects the release of nitric oxide from inhibitory motor neurons. Inhibition of nitric oxide formation does not alter the initial fast IJP in human, guinea pig, or mouse intestinal circular muscle (25, 36, 44). This suggests that the fast IJP is not mediated by release of nitric oxide.

In guinea pig small intestinal circular muscle, the fast IJP and the fast IJP-like action of ATP are suppressed by apamin, suramin, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS), reactive blue-2, and desensitization of postreceptor signal transduction by adenosine 5'-O-(2-thiodiphosphate) (ADPβS) (16, 22, 30, 44). Reactive blue-2, which is a P2 receptor antagonist, suppresses hyperpolarizing responses evoked by the stable ATP analog α,β-methylene ATP in strips of circular muscle from rat cecum (30). On the other hand, α,β-methylene ATP does not bind to P2Y1 receptors cloned from chick brain (41), which suggests that a second P2Y receptor might be present in the rat intestine. In guinea pig taenia coli, purine nucleotides evoke muscle relaxation with a potency order of diadenosine polyphosphate P1,P3-diadenosine triphosphate (Ap3A) = diadenosine polyphosphate P1,P4-diadenosine tetraphosphate (Ap4A) > ATP > diadenosine polyphosphate P1,P4-diadenosine tetraphosphate (Ap4A) > diadenosine polyphosphate P1,P5-diadenosine pentaphosphate (Ap5A), and these actions are suppressed by suramin with a pA2 value of ~5, which is suggestive of involvement of the P2Y1 receptor subtype (23). PPADS also acts as an antagonist at the P2Y1 receptor with potency similar to that of reactive blue-2 and suramin (28, 29). In guinea pig taenia coli, PPADS shifts the concentration-response curve for stimulus-evoked IJPs and IJP-like actions of ATP to the left, which suggests that P2Y1 receptors are expressed in this intestinal muscle (42).

Neurally evoked excitatory junction potentials (EJPs), which appear to be purinergic, have been reported for the guinea pig

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Purinergic P2Y1: Neuromuscular Transmission

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Stimulation of the P2Y1 purinergic receptor. A preliminary communication involves stimulation of phospholipase C, release of inositol trisphosphate, and elevation of free Ca
$^{2+}$ inside the smooth muscle fibers (27). On the other hand, in the mouse colon, ampin-sensitive hyperpolarization induced by activation of P2Y receptors is reported to be mediated primarily by stimulation of adenylate cyclase and release of Ca
$^{2+}$ from intracellular ryanodine-dependent stores (47). Suppression of both the fast IJP and the fast IJP-like action of ATP by apamin reflects inhibition of opening of the Ca
$^{2+}$-activated K$^+$ channels in the muscle membranes (1, 38). Suramin, PPADS, and reactive blue-2 are marginally selective antagonists at the P2 purinergic receptor subtype, and suppression of both the fast IJP and the fast IJP-like action of ATP by these drugs implicates involvement of this receptor category in the generation of the fast IPSP and the fast IPSP-like action of ATP (14, 17).

Lack of availability, until recently, of selective antagonists for P2 purinergic receptor subtypes has impeded progress toward unequivocal identification of the receptor responsible for the fast IJP and the fast IJP-like action of ATP. Recent evidence reported by Gallego et al. (21) strongly implicates P2Y$_1$ as the purinergic receptor responsible for the fast IJP and fast IJP-like action of ATP for the circular muscle coat of the human distal and sigmoid colon.

The most potent and selective P2Y$_1$ receptor antagonist available to date is MRS2179, which is the bisphosphate derivative 2'-deoxy-N$^\alpha$-methyladenosine-3',5'-bisphosphate (13, 31). It is a competitive antagonist at the turkey P2Y$_1$ receptor with a pA$_2$ value of 6.99. Moreover, it is ineffective at the human P2Y$_2$, the human P2Y$_4$, and the rat P2Y$_6$ receptors (4). MRS2179 has an apparent pK$_a$ value of 6.75 at the human P2Y$_1$ receptor and a pA$_2$ value of 6.18 for inhibition of the mimcry of purinergic slow excitatory postsynaptic potentials by ATP in neurons in the guinea pig small intestinal submucosal plexus (24).

The general aim of our study was to test the hypothesis that the fast IJP and fast IJP-like action of exogenously applied ATP in guinea pig small intestinal circular muscle reflect stimulation of the P2Y$_1$ purinergic receptor. A preliminary report has been published in abstract form (40).

MATERIALS AND METHODS

Adult male Hartley-strain guinea pigs (300–350 g) were stunned by a sharp blow to the head and exsanguinated from the cervical vessels according to protocols approved by the Ohio State University Laboratory Animal Care and Use Committee and United States Department of Agriculture Veterinary Inspectors. Whole mount preparations were obtained from the midjejunum and ileum. The segments were microdissected for electrophysiological recording as described previously (20, 45).

Conventional intracellular electrophysiological recording methods with “sharp” glass microelectrodes were used to record stimulus-evoked IJPs in the circular muscle coat of mucosa-free preparations that had strips of longitudinal muscle removed to expose the myenteric plexus. The preparations were pinned to Sylgard resin at the bottom of a 2.0-ml recording chamber that was perfused at a rate of 10–15 ml/min with Krebs solution warmed to 37°C and gassed with 95% O$_2$-5% CO$_2$ to buffer at pH 7.3–7.4. The composition of the Krebs solution was (in mM) 120 NaCl, 6 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 1.35 Na$_2$HPO$_4$, 14.4 NaHCO$_3$, and 1.15 glucose. Nifedipine and scopolamine (1 µM) were added to the Krebs solution to suppress muscle movements during electrophysiological recording. The microelectrodes were filled with 2 M KCl or 4 M potassium acetate and had resistances of 80–120 MΩ. The preamplifier (M-767; World Precision Instruments, Sarasota, FL) was equipped with a bridge circuit for intraneuronal injection of electrical current. Constant-current rectangular pulses were driven by a Grass S9D stimulator (Grass Instrument Division, Astro-Med, Warwick, RI). Electrometer output was amplified and observed on oscilloscopes (Tektronics 3012; Tektronics, Beaverton, OR) and Astro-Med thermal recorders and saved on digital recording tape. Neuromuscle junction potentials were evoked by focal electrical stimulation of interganglionic fiber tracts in the myenteric plexus with bipolar insulated tungsten electrodes placed perpendicularly to the longitudinal axis of the preparation and connected through stimulus-isolation units (Grass SINS) to Grass S48 stimulators (Astro-Med). Stimulus parameters were single pulses with durations of 2 ms and amplitudes of 2 mA.

Pharmacological agents were applied by addition to the bathing solution. Agents used were ATP, N$^\alpha$-nitro-L-arginine methyl ester hydrochloride (l-NNAME), scopolamine, nifedipine, apamin, vasoactive intestinal peptide (VIP), tetrodotoxin (TTX), and VIP$_{28}$, each of which was obtained from Sigma (St. Louis, MO). MRS2179 (as tetraammonium salt), [2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP), and suramin were obtained from Tocris Bioscience (Ellisville, MO). PPADS was purchased from RBI (Natick, MA).

Data analysis. Data are presented as means ± SE with n referring to the number of muscle cells. Continuous curves for concentration-response relationships were constructed with the following least-

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\text{AJP-Gastrointest Liver Physiol} \cdot \text{VOL 292} \cdot \text{JUNE 2007} \cdot \text{www.ajpgi.org}
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Fig. 1. MRS2179 and apamin suppressed partially purinergic inhibitory junction potentials (IJPs). A: MRS2179 suppressed the fast component of the IJP in a concentration-dependent manner in the guinea pig ileum. The IJPs were evoked by focal application of a single electrical stimulus pulse to a ganglion in the myenteric plexus. B: concentration-response relation for suppression of fast IJPs by MRS2179 in 12 preparations. The IC$_{50}$ for suppression of the IJPs by MRS2179 was 0.2 ± 0.3 µM. C: concentration-response relation for suppression of fast IJPs by apamin in 17 preparations. The IC$_{50}$ for suppression of the IJPs by apamin was 0.03 ± 0.01 µM.

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squares fitting routine using SigmaPlot software (SPSS, Chicago, IL):
\[ V = V_{\text{max}}\left[1 + \left(\frac{C}{EC_{50}}\right)^{n_H}\right], \]
where \( V \) is the observed membrane potential response, \( V_{\text{max}} \) is the maximal response, \( C \) is the corresponding drug concentration, \( EC_{50} \) is the concentration that induces the half-maximal response, and \( n_H \) is the apparent Hill coefficient. Student’s \( t \)-test was used to determine significance with \( P < 0.05 \) considered to be significant.

RESULTS

Focal electrical stimulation applied to ganglia or interganglionic fiber tracts in the myenteric plexus evoked IJPs of variable amplitude and duration as well as EJPs in the circular muscle (Figs. 1–4). EJPs were not always apparent. When apparent, they were seen as transient membrane depolarization beyond the resting potential, which occurred at the end of the IJPs (Fig. 2, 3). We identified three kinds of IJPs, which were referred to as purely purinergic, partially purinergic, and non-purinergic. Purely purinergic IJPs were identified as those abolished by the selective P2Y1 purinergic receptor antagonist MRS2179 (Fig. 2). Partially purinergic IJPs were composed of a MRS2179-sensitive component and a non-MRS2179-sensitive component. The non-MRS2179-sensitive component was sometimes, but not always, suppressed by inhibition of nitric oxide formation. Table 1 provides data for the proportion of IJPs in the study that were purely purinergic, partially purinergic, or nonpurinergic in the jejunum and ileum. All kinds of stimulus-evoked junction potentials were suppressed or abolished by TTX.

**Fig. 2.** Pharmacological analysis of purely purinergic IJPs. A: purely purinergic IJPs were abolished by 2 μM L-NAME and neural blockade with TTX. The vasoactive intestinal peptide receptor antagonist VIP6-28 (0.5 μM; VIP), 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP; 10 μM), and N'-(nitro-L-arginine methyl ester (L-NAME; 200 μM) did not suppress the IJPs. B: quantitative data for actions of pharmacological agents on purely purinergic (fast) IJPs. Numbers of preparations are given in parentheses. Concentrations were as follows: MRS2179, 2 μM; apamin, 0.5 μM; TNP-ATP, 10 μM; TTX, 1 μM; VIP6-28, 0.5 μM; L-NAME, 200 μM; and suramin, 2 μM.

**Fig. 3.** Pharmacological analysis of nonpurinergic IJPs. A: nonpurinergic IJPs were abolished by 200 μM L-NAME and neural blockade with TTX. Neither 10 μM MRS2179 nor 1 μM apamin suppressed the IJPs. B: quantitative data for actions of pharmacological agents on nonpurinergic (slow) IJPs. Numbers of preparations are given in parentheses. Concentrations were as follows: MRS2179, 2 μM; apamin, 1 μM; TNP-ATP, 10 μM; TTX, 1 μM; VIP6-28, 0.5 μM; and L-NAME, 200 μM.
were suppressed, but not abolished, by MRS2179 (Figs. 1, 4, and 5). Of 72 partially purinergic IJPs, 33% showed a hyperpolarizing component in the presence of MRS2179 that was suppressed after addition of L-NAME to the bathing medium (Fig. 4). This component activated more slowly and had smaller amplitude relative to the MRS2179-sensitive component. Neither component occurred when a combination of sufficient concentrations of MRS2179 and L-NAME was present in the bathing solution (Fig. 4). These IJPs fit into a two-component subcategory of partially purinergic IJPs, composed of a purinergic (fast) and nitricergic (slow) component. Putative blockade of small-conductance Ca\(^{2+}\)/H1\(^{+}\)-activated K\(^{+}\) channels by apamin, blockade of P2X receptors by TNP-ATP, or VIP receptor blockade by VIP6-28 did not suppress the small L-NAME-sensitive component of the IJP (Fig. 4).

The remainder of the partially purinergic IJPs also included the second small hyperpolarizing component; however, this component was resistant to suppression by a combination of MRS2179 and L-NAME. Putative blockade of small-conductance Ca\(^{2+}\)/H1\(^{+}\)-activated K\(^{+}\) channels by apamin, blockade of P2X receptors by TNP-ATP, or VIP receptor blockade by VIP6-28 did not suppress the small L-NAME-sensitive component of the IJP (Fig. 5).

**Nonpurinergic IJPs.** Nonpurinergic IJPs made up 4% of the IJPs recorded in the study (Table 1). The nonpurinergic IJPs were suppressed or abolished by L-NAME (Fig. 3). Unlike the

<table>
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<th>Category</th>
<th>Resting Potential, mV</th>
<th>Jejunum Number</th>
<th>Ileum Number</th>
<th>Total Number</th>
</tr>
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<tr>
<td>Purely purinergic</td>
<td>51.4 ± 2.7</td>
<td>18 (17.5%)</td>
<td>9 (8.7%)</td>
<td>27 (26.2%)</td>
</tr>
<tr>
<td>Partially purinergic</td>
<td>50.7 ± 5.4</td>
<td>30 (29.3%)</td>
<td>42 (40.8%)</td>
<td>72 (69.9%)</td>
</tr>
<tr>
<td>Nonpurinergic</td>
<td>49.8 ± 6.6</td>
<td>1 (1.0%)</td>
<td>3 (3.0%)</td>
<td>4 (3.9%)</td>
</tr>
<tr>
<td>Total</td>
<td>49 (47.6%)</td>
<td>54 (52.5%)</td>
<td>103 (100%)</td>
<td>103 (100%)</td>
</tr>
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IJPs, inhibitory junction potentials.

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**Table 1. Purinergic IJPs in guinea pig small intestine**
purely purinergic IJPs, the nonpurinergic IJPs were unaffected by the presence of apamin, TNP-ATP, MRS2179, or VIP6-28 in the bathing solution (Fig. 3).

**IJP-like action of ATP.** IJP-like membrane hyperpolarization was evoked by ATP (10–30 μM) in 49 of 56 preparations (Fig. 6). These hyperpolarizing responses to exogenously applied ATP were concentration dependent and reversible and mimicked the IJPs in the purely purinergic category. The EC50 for the IJP-like action of ATP was 6.6 ± 1.3 μM (Fig. 7). The IJP-like responses to 10-μM ATP were suppressed by 92 ± 15% in the presence of 10 μM MRS2179 in 14 preparations, 95 ± 12% in the presence of 1 μM apamin in 6 preparations, and 76 ± 8% in the presence of 20 μM PPADS in 9 preparations. The IJP-like responses, evoked by ATP, were not influenced by 10 μM TNP-ATP in 14 preparations, 1 μM TTX in 25 preparations, or 200 μM l-NAME in 18 preparations. Exposure to VIP (100 nM –1 μM) did not evoke hyperpolarizing responses in any of 19 preparations (Fig. 6).

**DISCUSSION**

The properties of neuromuscular junction potentials evoked by focal electrical stimulation in the myenteric plexus in our study were generally similar to those reported for several earlier studies in which the potentials were evoked by transmural electrical field stimulation (4, 16, 21, 25, 33, 36, 38, 44). The waveforms of the IJPs evoked by transmural electrical field stimulation in these studies were described as consisting of an initial larger amplitude, rapidly activating hyperpolarizing component (fast IJP) and a smaller and longer-lasting hyperpolarizing component (slow IJP). We found that both components were also evoked by focal electrical stimulation of neurons in the myenteric plexus, where the cell bodies of motor neurons to the circular muscle coat reside (7, 8). Nevertheless, the slow IJP was often obscured by the larger fast IJP and was uncovered after blockade of the fast IJP by MRS2179.

Focal electrical stimulation in the myenteric plexus also activated excitatory motor neurons to the circular muscle, and this was reflected by stimulus-evoked EJPs. These EJPs occurred in the presence of scopolamine and were therefore noncholinergic. Because the simultaneously evoked IJPs usually overlapped the EJPs, the longer-lasting EJPs were seen generally at the end of the IJPs.

The amplitudes of the three kinds of neuromuscular junction potentials were variable and did not lend themselves to quantitative analysis. Several factors might account for the variability. One factor might be the number of motor neurons brought to firing threshold by the stimulus and a direct association between the number of activated neurons and the amplitude of the evoked IJP. A second factor might be the frequency of evoked firing of the motor neurons, because the amount of neurotransmitter released at the neuromuscular junctions occurs in direct relation to the firing frequency. A third factor relates to the possibility for the junction potentials to be occurring in muscle fibers at distances removed from the fiber impaled by the microelectrode. The smooth muscle behaves as a functional electrical syncytium due to electrical coupling between individual fibers. Consequently, junction potentials...
can spread electrotonically with passive decrement from neuromuscular junctions at distant muscle fibers to the fiber from which an electrical record is obtained (2, 6).

Purely purinergic IJPs. The purely purinergic IJPs in our study appear to be the same as the fast IJPs in the reports of others (16, 21, 22, 25, 33, 36, 44). Occurrence of a purely purinergic IJP is suggestive of a population of inhibitory motor neurons in the myenteric plexus, which is exclusively purinergic. The potent suppression of the purely purinergic IJPs by MRS2179, considered together with the high selectivity and affinity of MRS2179 for the P2Y<sub>1</sub> purinergic receptor subtype, strongly suggests that the purinergic component of IJPs (i.e., fast IJPs) in guinea pig small intestinal circular muscle is mediated by the P2Y<sub>1</sub> receptor subtype. This conclusion was reinforced by our finding that exogenous ATP mimicked the purely purinergic (i.e., fast IJP) and that the IJP-like action of ATP was suppressed by MRS2179.

Partially purinergic IJPs. The IJPs, which we called partially purinergic, probably corresponded to the slow IJPs that have been reported by others. The partially purinergic IJPs might reflect the simultaneous stimulation of one or more inhibitory motor neurons that release ATP and one or more that release another inhibitory neurotransmitter. On the other hand, there might be a population of inhibitory motor neurons in the myenteric plexus that corelease ATP together with a second nonpurinergic neurotransmitter. Our results do not differentiate between these two possibilities. Nevertheless, our results show suppression of the second component of some of the partially purinergic IJPs by L-NAME, which suggests nitric oxide as the inhibitory neurotransmitter. The second component of the partially purinergic IJPs was not always suppressed by L-NAME and was therefore not mediated by nitric oxide. We tested only one possible antagonist (i.e., VIP<sub>0.28</sub>) for the second L-NAME-insensitive component and found no significant suppression of this component by the VIP receptor antagonist.

Nonpurinergic IJPs. The incidence of stimulus-evoked IJPs in our nonpurinergic category was only 4%. When nonpurinergic IJPs occurred, they were always suppressed or abolished by L-NAME, which suggests that they were purely nitricergic.

Purinergic myenteric neurons. At least two functionally distinct populations of purinergic neurons are present in the myenteric plexus of the guinea pig small intestine. One population consists of the inhibitory motor neurons to the circular muscle that were emphasized in the present study. A second purinergic population connects synaptically with secretomotor neurons to the submucosal plexus (24). ATP is the neurotransmitter released at P2Y<sub>1</sub> excitatory postsynaptic receptors on the secretomotor neurons by these projections from the myenteric plexus (18, 24). Focal electrical stimulation in the myenteric plexus activates the purinergic projections and evokes slow synaptic excitation in a population of submucosal secretomotor neurons. Purinergic secretomotor activation in turn stimulates secretion of Na<sup>+</sup>, Cl<sup>−</sup>, HCO<sub>3</sub><sup>−</sup>, and H<sub>2</sub>O from the mucosal secretory glands (18, 19). Elevated glandular secretion is this case is expected to increase the liquidity of the luminal contents.

Inhibitory motor neurons in the myenteric plexus project their axons in the aboral direction to the circular muscle coat along the longitudinal axis of the intestine (7). Activation of these projections during peristaltic propulsive motility relaxes the circular muscle ahead of the advancing luminal contents (8, 43). Accumulating evidence suggests that programmed firing of purinergic inhibitory motor neurons and their release of ATP at P2Y<sub>1</sub> receptors, expressed by the circular muscle, are significant enteric neurophysiological functions in generation of propulsive motility. There is evidence also suggesting that programmed firing of purinergic neurons in the myenteric plexus and the release of ATP at P2Y<sub>1</sub> receptors, expressed on secretomotor neurons, evoke mucosal secretion (15, 18, 19). An important unanswered question is whether the myenteric purinergic projections to the circular muscle and to submucosal secretomotor neurons stem from one and the same neuronal cell body. Do purinergic neurons in the myenteric plexus individually project to the muscle and secretomotor neurons, or do single purinergic neurons project axons that bifurcate to innervate both the muscularis and secretomotor neurons? If the latter were the case, then firing of these neurons would simultaneously inhibit the circular muscle and stimulate secretion in the same segment of intestine. A single purinergic neuronal connection to descending inhibition of the circular muscle and stimulation of secretion is consistent with observations that stroking of the intestinal mucosa reflexly evokes both descending inhibition of the circular muscle and mucosal secretion (15, 35).

ACKNOWLEDGMENTS

Present addresses: X. Fang, Division of Gastroenterology, Peking Union Medical College Hospital, Beijing, Peoples’ Republic of China; H.-Z. Hu, Scripps Research Institute, La Jolla, CA; N. Gao, Senomyx, Inc., La Jolla, CA 92037.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01 DK-37238 and R01 DK-068258 (to J. D. Wood) and K08 DK-60648 (to Y. Xia), a Pharmaceutical Manufacturers of America Foundation Postdoctoral Fellowship (to S. Liu), and an overseas study grant from Peking Union Medical College Hospital, Beijing, China (to X. Fang).

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