CLINICALLY SIGNIFICANT DELAY in gastric emptying and small intestinal and colonic transit occurs in a variety of diseases and conditions, including diabetes mellitus, anorexia nervosa, scleroderma, myotonic dystrophy, hypoparathyroidism, postvagotomy, postoperative ileus, and spinal cord injury. Delayed transit may also occur due to functional disorders of no known etiology, such as nonulcer dyspepsia, idiopathic intestinal pseudo-obstruction, irritable bowel syndrome, and anorexia nervosa, which is common to all of them. In contrast, none of the prokinetics stimulated contractions in mucosa-free or mucosa-attached muscle strips, or rings, even though methacholine or electrical field stimulation induced phasic contractions in all of them. The prokinetics also did not release ACh in longitudinal muscle-myenteric plexus preparations. Each prokinetic, however, decreased the length of enzymatically dispersed single cells. In conclusion, each prokinetic may act on a different subset of presynaptic neurons that converge on the postsynaptic cholinergic and nonadrenergic noncholinergic motoneurons. The presynaptic neurons may be impaired in the muscle bath environment.

Enteric locus of action of prokinetics: ABT-229, motilin, and erythromycin

SUSHIL K. SARNA, ASENSIO GONZALEZ, AND ROBERT P. RYAN
Departments of Surgery and Physiology, Medical College of Wisconsin, and Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin 53226

Sarna, Sushil K., Asensio Gonzalez, and Robert P. Ryan. Enteric locus of action of prokinetics: ABT-229, motilin, and erythromycin. Am J Physiol Gastrointest Liver Physiol 278: G744–G752, 2000.—We investigated the in vivo and in vitro locus of actions of prokinetics: motilin, erythromycin, and ABT-229. The test substances were infused close intra-arterially in short segments of the jejunum in the intact conscious state. Each prokinetic acted on a presynaptic neuron and utilized at least one nicotinic synapse to stimulate circular muscle contractions. The final neurotransmitter at the neuromuscular junction was ACh. Motilin and erythromycin, but not ABT-229, also released nitric oxide. Each prokinetic utilized somewhat different subtypes of muscarinic, serotonergic, tachykinergic, and histaminergic receptors, except for the M3 receptor, which was common to all of them. In contrast, none of the prokinetics stimulated contractions in mucosa-free or mucosa-attached muscle strips, or rings, even though methacholine or electrical field stimulation induced phasic contractions in all of them. The prokinetics also did not release ACh in longitudinal muscle-myenteric plexus preparations. Each prokinetic, however, decreased the length of enzymatically dispersed single cells. In conclusion, each prokinetic may act on a different subset of presynaptic neurons that converge on the postsynaptic cholinergic and nonadrenergic noncholinergic motoneurons. The presynaptic neurons may be impaired in the muscle bath environment.

Enteric transit; gastric emptying; constipation; gastroesophageal reflux; enteric nervous system; smooth muscle
Briefly, the circular muscle sheet was cut into 0.5 cm² pieces and incubated at 37°C in 20 ml of Ca²⁺-free Hanks' solution containing 0.38 mg/ml papain and 0.3 mg 1,4-dithiothreitol until the tissue appeared loose and sticky (~10 min). The tissue was washed with Ca²⁺-free HEPES buffer and further digested at 31°C with 0.2 mg/ml collagenase type II (319 U/mg) and 0.1 mg/ml soybean trypsin inhibitor for 30–40 min. The digested tissue was washed three times with enzyme-free HEPES buffer, and the muscle cells were allowed to disperse spontaneously under gentle to-and-fro motion. Cells were harvested by filtration through a 500-µm Nitex mesh and collected by centrifugation at 350 x g for 5 min.

Cell length was measured by scanning micrometry as described previously (14, 23). An aliquot (0.45 ml of 5 x 10⁴ cells/ml) was exposed to 50 µl of test agent at 31°C for 40 s. The reaction in each case was terminated by adding acrolein to a final concentration of 1%. In other experiments, the cell samples were incubated with porcine [Phe⁶,Leu⁹]motilin (a motilin antagonist) for 5 min before addition of the agonists. The lengths of 30 consecutive intact healthy cells were measured through a phase-contrast microscope (Nikon), fitted with a video camera (Javelin CCD) and connected to a Macintosh computer. The NIH Image 1.61 program was used to measure the length. The contractile response was expressed as percent cell shortening from vehicle control.

RESULTS

In vivo experiments. Shi and Sarna (22) reported recently that close intra-arterial infusion of 4 µM methacholine for 1 min stimulates nearly the maximum response to this agonist. This response was taken as 100%. All three prokinetics, motilin, ABT-229, and methacholine were infused at the same rate was used as flush. A 2- to 3-cm-long jejunal segment was passed over a glass tube. A dull scalpel blade was used to score the serosal surface longitudinally. The external longitudinal muscle layer along with the myenteric plexus was peeled off by using wet sponges. The longitudinal muscle-myenteric plexus tissue was cut into 2 x 2-mm pieces for stimulation with the prokinetic agents. The tissue pieces were incubated at 37°C with 0.2 µM [³H]choline (80 Ci/mmol, New England Nuclear, Boston, MA) in 3 ml oxygenated Krebs buffer supplemented with 50 µM physostigmine for 1 h. After incubation, the longitudinal muscle-myenteric plexus strips were washed by changing oxygenated Krebs bathing solution every 3 min. The strips were further equilibrated by adding Krebs solution supplemented with 50 µM physostigmine and 10 µM hemicholinium. At this time, the basal release of [³H]JACH reached a plateau.

Materials. ABT-229 (8,9-anhydro-4'-deoxy-3'-N-ethylethryromycin B-6,9-hemiacetal) and erythromycin lactobionate were obtained from Abbott Laboratories. Canine motilin, porcine [Phe⁶,Leu⁹]motilin (a motilin antagonist) porcine [Phe⁶,Leu⁹]motilin was supplied by Dr. Theo Peeters.

Prokinetic agents and motility.
erythromycin, concentration-dependently stimulated phasic contractions (Fig. 1). These contractions resembled those seen during phase II activity of the migrating motor complex and the postprandial state (Fig. 2). The time lags between the arrival of the prokinetic in the gut wall after accounting for the dead space were significantly different for ABT-229 (63 ± 5 s), motilin (19 ± 2 s), and erythromycin (8 ± 1 s). The order of potency was motilin > ABT-229 > erythromycin (EC50, 1.5 × 10^{-10} M, 4.1 × 10^{-9} M, and 9.3 × 10^{-9} M, respectively), and the order of efficacy was motilin = ABT-229 > erythromycin (ECmax, 294 ± 83%, 286 ± 45%, and 88 ± 15% of the response to methacholine for motilin, ABT-229, and erythromycin, respectively). Intravenous infusion of the maximum doses of the agonists given dose intra-arterially did not stimulate contractions in the small intestine.

Enteric locus of action. ABT-229, motilin, and erythromycin infusions at 6, 0.2, and 16 µM, respectively, for 1 min were used to determine their locus of action. These concentrations produced the nearly maximum response (Fig. 1). The previously established effective concentration of atropine (30 µM for 1 min) was used to block muscarinic receptors, hexamethonium (70 µM for 1 min) to block nicotinic receptors, and TTX (25 µM for 1 min) to block Na+ channel enteric neural conduction (22). The infusion of prokinetics was started 1 min after the end of infusion of the antagonists. The response to each prokinetic agent was blocked almost completely by atropine, hexamethonium, and TTX (Fig. 3), indicating that they acted at a presynaptic receptor site, involved at least one nicotinic synapse, and induced release of ACh at the neuroeffector junction to stimulate contractions.

The inhibition of nitric oxide synthase (NOS) by prior close intra-arterial infusion of 10 mM nitro-L-arginine methyl ester (L-NAME) for 1 min had no effect on the response to ABT-229, but it enhanced the response to
motilin and erythromycin (n = 5 each; Fig. 3). The above dose of L-NAME has been reported previously to block NOS (8).

Types of receptors mediating the responses to ABT-229, motilin, and erythromycin. Each of the following antagonists was infused for 5 min at 1 ml/min. The prokinetics were coinfused for 1 min at the beginning of the third minute of infusion of each antagonist. The effective concentration of each of the antagonists has been established previously (8, 21, 22, 27).

Pirenzepine, methoctramine, 4-DAMP, and tropicamide were used to antagonize M₁, M₂, M₃, and M₄ receptors, respectively. Shi and Sarna (22) reported that infusing 4-DAMP at 2 µM for 5 min was effective in blocking M₃ receptors. Other muscarinic receptor antagonists were used at the same concentration. At higher concentrations, M₁, M₂, and M₄ antagonists exhibit nonspecific effects (22).

The responses to all three prokinetics were blocked almost completely by 4-DAMP (Fig. 4). The responses to ABT-229 and motilin were blocked partially but significantly by pirenzepine. In addition, the response to ABT-229 was also partially blocked by methoctramine. The response to erythromycin was not affected significantly by M₁, M₂, and M₄ receptor antagonists (Fig. 4).

NAN-190 hydrobromide (1.2 µM for 5 min), LY-53857 (2 µM for 5 min), tropisetron (2 µM for 5 min), and SDZ-205557 (2 µM for 5 min) were used to block 5-HT₁A, 5-HT₂/5-HT₁C, 5-HT₃, and 5-HT₄ receptors, respectively. The response to ABT-229 was blocked partially by the 5-HT₂/5-HT₁C receptor antagonist LY-53857 but was not affected by other 5-HT receptor antagonists (Fig. 5). The responses to motilin and erythromycin were not affected by any of the 5-HT receptor antagonists.

Fig. 3. Blockade of muscarinic receptors with atropine, nicotinic receptors with hexamethonium, and Na⁺ channel enteric neural conduction with TTX in the infused segment almost completely blocked contractile responses to ABT-229 (A), motilin (B), and erythromycin (C). Blockade of nitric oxide synthase by nitro-L-arginine methyl ester (L-NAME) enhanced the response to motilin and erythromycin but not ABT-229. In each case, the control response to the prokinetic alone was taken as 100%.

Fig. 4. M₃ muscarinic receptor antagonist 4-DAMP almost completely blocked the response to ABT-229 (A), motilin (B), and erythromycin (C). M₁ receptor antagonist pirenzepine blocked the response partially but significantly only to ABT-229 and motilin. M₂ receptor antagonist methoctramine partially blocked the response to ABT-229 only. M₄ receptor antagonist tropicamide had no significant effect on the response to any of the prokinetic agents.
L-703606, L-659877, and [Trp^7, β-Ala^8]neurokinin A 4–10 (each at 1.6 µM for 5 min) were used to block NK1, NK2, and NK3 receptors (27). The response to motilin was not affected by NK1, NK2, or NK3 receptor antagonists (Fig. 6). In contrast, the response to ABT-229 was blocked by almost 50% by each of the three tachykinin receptor antagonists (Fig. 6). The response to erythromycin was only partially blocked by NK2 and NK3 receptor antagonists ($P < 0.05, n = 6$).

Mepyramine (15 µM for 5 min) and ranitidine (150 µM for 5 min) were used to block $H_1$ and $H_2$ receptors, respectively (27). $H_1$ and $H_2$ receptor antagonists had no significant effect on the response to motilin (Fig. 7). However, the response to ABT-229 was blocked partially by the $H_2$ receptor antagonist ranitidine and that to erythromycin by the $H_1$ receptor antagonist mepyramine ($P < 0.05, n = 6$).

In vitro muscle bath experiments. In contrast to the in vivo experiments, none of the prokinetic agents ($10^{-5}$ M ABT-229, $10^{-6}$ M motilin, and $10^{-5}$ M erythromycin) stimulated a contractile response in mucosa-free or mucosa-attached circular muscle strips from the jejunum of five dogs. Methacholine ($10^{-6}$ M), however, contracted the muscle strips as has been reported previously (22). In one dog, 0.5-cm-wide rings of jejunal segments with mucosa attached also produced no response to $10^{-6}$ to $10^{-4}$ M ABT-229 and $10^{-5}$ M erythromycin. These rings exhibited spontaneous contractions...
and responded to $10^{-6}$ M methacholine. In one additional dog, a dose intra-arterial catheter was implanted in a mesenteric artery, and the perfused area was identified. This perfused segment was removed along with the mesenteric blood vessels and mounted in a 10-ml muscle bath. The segment exhibited spontaneous contractions and contracted in response to methacholine infused for 1 min at $4 \mu M$. However, 5 or $10^{-2}$ M ABT-229 infused for 1 min or $10^{-5}$ M erythromycin infused for 1 min produced no response.

Next, we investigated whether the prokinetic agents would enhance the response when endogenous ACh was already being released by electrical field stimulation (EFS). EFS (75 V, 0.75 ms, 10 Hz for 5 s) consistently and reproducibly contracted jejunal circular muscle strips. Prior addition of $10^{-4}$ M ABT-229 or $10^{-4}$ M erythromycin had no significant effect on the response to EFS (data not shown). Similar results were obtained from antral and duodenal circular muscle strips (data not shown).

Circular muscle strips and segments were also obtained from the jejunum and duodenum of eight rabbits. This tissue exhibited a response to the first dose of $10^{-8}$ M motilin and $10^{-7}$ M to $10^{-5}$ M ABT-229, but subsequent doses after a wash had little or no effect or the response was significantly attenuated. All strips and segments contracted to $10^{-4}$ M methacholine.

Single dispersed cells. Each of the three agonists decreased the cell length concentration dependently in single cells. The $EC_{50}$ values were not different from one another, but the $EC_{\text{max}}$ of ABT-229 was significantly less than that of motilin (Table 1). Motilin antagonist porcine [Phe$^3$,Leu$^{13}$]motilin concentration-dependently inhibited the response to ABT-229, motilin, and erythromycin (Fig. 8). The $IC_{50}$ values of $4.3 \pm 3.2$, $1.7 \pm 0.81$, and $5.1 \pm 0.45 \times 10^{-8}$ M, respectively, were not different from each other. The $IC_{\text{max}}$ values of $81 \pm 12\%$, $80 \pm 5\%$, and $53 \pm 15\%$ were also not different for the three agonists. However, we found that atropine also concentration-dependently reduced the responses to ABT-229, motilin, and erythromycin (Fig. 8). This raised the possibility that, in single cells, the agonists may act on muscarinic receptors and the [Phe$^3$,Leu$^{13}$]motilin antagonist may also partially block the muscarinic receptors.

$[^3H]ACh$ release from longitudinal muscle-myenteric plexus preparations. $10^{-6}$ M ABT-229 or $10^{-6}$ M erythromycin did not significantly increase the release of $[^3H]ACh$ from longitudinal muscle-myenteric plexus preparations (47 ± 25% and 78 ± 28%, respectively). In contrast, Shi and Sarna (21) have reported that $10^{-4}$ M veratridine, an Na$^+$ channel activator, significantly increases $[^3H]ACh$ release from the canine jejunal longitudinal muscle-myenteric plexus preparations by 200 ± 55%.

**DISCUSSION**

Our findings show that in the intact conscious state, each of the three prokinetic agents acts on presynaptic neurons to stimulate phasic contractions. These contractions resemble those seen during phase II activity or the postprandial state but not during phase III activity. In our study, we infused the prokinetic agents close intra-arterially in a short segment of the jejunum. The doses were so small that they affected only the infused segment. This method allows investigations of the enteric neural and smooth muscle function in the intact conscious state without the modulating effects of the

| Table 1. $EC_{50}$ and $EC_{\text{max}}$ values in single cells |
|-----------------|------------------|------------------|
| Prokinetic      | $EC_{50}$, M     | $EC_{\text{max}}$, % |
| ABT-229         | $1 \pm 0.18 \times 10^{-9}$ | 20.6 ± 2.4*      |
| Motilin         | $1.2 \pm 0.54 \times 10^{-8}$ | 28.7 ± 2.2      |
| Erythromycin    | $5.3 \pm 1.9 \times 10^{-8}$ | 22.9 ± 3.2      |

Values are means ± SE; $n = 6$. $EC_{\text{max}}$, maximal effective concentration. *$P < 0.05$ vs. motilin.
drugs on other organs, such as the central nervous system and the spinal cord. Also, in the intact conscious state, all elements of the enteric nervous system are fully operational. As noted later, the presynaptic neurons may be impaired in the muscle bath. In previous studies (10, 26, 30), when some of the prokinetic agents were infused intravenously, they acted preferentially on the duodenum and the antrum to stimulate phase III activity, which then migrated distally.

The response to each agent was blocked by atropine and hexamethonium, indicating that it utilizes at least one nicotinic synapse and the release of ACh at the neuroeffector junction. The nicotinic receptor may not be in parallel with any other receptor because its blockade inhibited the response almost completely. There was little or no direct effect of any prokinetic on circular smooth muscle cells in the intact conscious state since the responses were blocked almost completely by TTX. Intravenous infusion of motilin and erythromycin in humans and dogs has been reported also to stimulate phasic contractions or phase III activity by acting at a presynaptic neuronal site and utilizing at least one nicotinic synapse (1, 19).

Our findings also show that molecular modification of erythromycin that eliminates its antibiotic activity significantly alters its potency, efficacy, the time lag for onset of contractions, and the types of receptors that are involved in the stimulation of jejunal contractions by the new molecule, ABT-229. Many of the above characteristics are also different between motilin and erythromycin or ABT-229. It seems that each of the above prokinetic agents may act on a different subset of presynaptic neurons. The synapses in each subset contain different subtypes of serotonergic, tachykinergic, and histaminergic receptors. The subset of neurons for each prokinetic agent converges on postsynaptic cholinergic excitatory motoneurons. M₃ receptors located on smooth muscle cells (7) mediate the final response to all prokinetics, but the involvement of M₁ and M₂ receptors may be specific to each prokinetic agent. These findings explain the previous observations of Haba and Sarna (9) that in intact dogs motilin and erythromycin stimulate different spatial and temporal characteristics of gastro-pyloro-duodenal contractions in the postprandial state by acting on different subsets of presynaptic neurons.

The subset of presynaptic neurons associated with motilin and erythromycin but not ABT-229 also synapse on nitroergic inhibitory neurons. The inhibition of NOS enhanced the response to motilin and erythromycin, but it had no effect on the response to ABT-229.

Whereas all three prokinetic agents consistently and reproducibly contracted the canine jejunal circular muscle when infused close intra-arterially in the intact conscious state, none exhibited a response in circular muscle strips taken from nearly the same location in the jejunum. These muscle strips responded normally to methacholine that acts directly on muscarinic receptors on smooth muscle cells (22) and to EFS that releases ACh from the postsynaptic neurons (21, 22). The above anomaly may be due to the following reasons: 1) the prokinetics act on mucosal sensory neurons that are removed in the preparation of mucosa-free strips; 2) the prokinetics do not diffuse to the ganglia in the muscle bath environment, but with intra-arterial infusion they are transported close to them by the blood vessels; or 3) the presynaptic neurons are impaired in the muscle bath environment. We ruled out the first two possibilities because the prokinetics had no effect on mucosa-attached muscle strips and rings or when they were infused close intra-arterially in ex vivo segments.
Both veratridine and EFS induce the release of ACh from the longitudinal muscle-myenteric plexus preparations of the dog small intestine (21). These responses are not affected by hexamethonium and atropine but are blocked by TTX, indicating that these stimuli release ACh by their action on postsynaptic neurons. In our study, the prokinetic agents did not release ACh in longitudinal muscle-myenteric plexus preparations. This confirms the above findings that the presynaptic neurons that are the site of action of prokinetics may be impaired in the muscle bath environment. However, electrophysiological studies (5, 6, 13, 24, 31), mostly in the guinea pig model, show that these neurons are excitable. However, concurrent recordings of contractile activity and electrophysiological tracings have not been made to show that presynaptic neural excitation results in contraction in a muscle bath environment. The mechanisms of impairment or dysfunction of the presynaptic neurons in the in vitro environment, therefore, remains unknown.

In contrast to the canine jejunal muscle strips, the rabbit jejunal, duodenal, and antral muscle strips did respond to ABT-229. However, the response was a tonic contraction, not phasic contractions, as seen in vivo. In addition, we found that the strips became refractory after the first application of ABT-229. In previous studies (12, 15), the concentration-response curves to motilin in rabbit muscle strips have been determined by cumulative addition of the agonist. Muscarinic agonists did not exhibit such refractoriness. Also, such refractoriness was not observed in intact conscious dogs. Satoh et al. (20) also reported that motilin and another motilide, EM574, stimulate in vitro segments of rabbit small intestine but not those of rat and guinea pig.

The responses in the rabbit muscle strips were not blocked by TTX, hexamethonium, or atropine, indicating that they are mediated by smooth muscle receptors (3, 12, 15). It seems that the direct activation of smooth muscle receptors by these prokinetic agents increases tone, whereas the activation of neuronal receptors stimulates phasic contractions.

Van Assche et al. (29) reported that motilin enhances the twitch responses to EFS in rabbit antral circular muscle strips. The EFS response is mediated by the release of ACh. However, motilin did not enhance the response to carbachol that acts directly on smooth muscle muscarinic receptors. In our study, ABT-229 or erythromycin did not enhance the response to EFS in canine muscle strips. Presumably, this is due to the lack of release of ACh by these agents in the muscle bath environment.

Whereas none of the prokinetic agents indicated a response by direct action on smooth muscle cells in the intact conscious state or in muscle bath, they decreased the cell length concentration dependently in single dispersed cells. There was no difference in potency or efficacy among the three compounds, whereas such differences existed in vivo. The decrease in cell length in response to each agonist was inhibited concentration dependently by motilin antagonist porcine [Phe3, Leu13]-motilin. Farrugia et al. (4) reported also that motilin decreases cell length in single dissociated jejunal circular muscle cells. Surprisingly though, the response to each prokinetic was also inhibited concentration dependently by atropine. This suggested that the prokinetics may act on muscarinic receptors in single cells.

In conclusion, all three prokinetic agents act on presynaptic neurons in the canine jejunum to stimulate phasic contractions. Erythromycin is less potent than ABT-229, which is less potent than motilin. Each prokinetic agent may act on a different subset of presynaptic neurons, and its response is mediated by somewhat different muscarinic, serotonin, tachykinin, and histamine receptors. It may be possible to utilize these differences to design motilides that target specific organs or suborgans of the gastrointestinal tract, such as fundus, gastro-pyloro-duodenal junction, small intestine, and colon, to achieve specific prokinetic activity. The presynaptic neurons on which the prokinetics act converge on postsynaptic cholinergic motor excitatory neurons. The final neurotransmitter at the neuromuscular junction is ACh. Also, the action of all prokinetics involves at least one nicotinic synapse that is not in parallel with another receptor. The presynaptic neurons seem to be impaired in the muscle bath environment.

This study was supported by Abbott Laboratories.
Address for reprint requests and other correspondence: S. K. Sarna, Dept. of Surgery and Physiology, Medical College of Wisconsin, 9200 West Wisconsin Ave., Milwaukee, WI 53226 (E-mail: ssarna@mcw.edu).
Received 15 July 1999; accepted in final form 13 January 2000.

REFERENCES


