J. Pharm. Pharmacol. 1994, 46: 491–496
Received April 27, 1993
Accepted October 25 1993

Analysis of Neurogenic Contractions Induced by ML-1035 and Other Benzamides in the Guinea-pig Non-stimulated Isolated Ileum

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Abstract—4-Amino-5-chloro-substituted benzamides have been shown to increase gastric motility in-vivo and enhance field-stimulated and peristaltic contractions in-vitro. The present experiments examined the contractile response to a series of benzamides in the guinea-pig non-stimulated ileum. Four benzamides elicited contractions in the isolated ileum which were expressed as a percentage of the contraction induced by 1 μM acetylcholine (% acetylcholine response = 12 ± 2, 19 ± 3, 26 ± 2, 51 ± 3, n = 13, 8, 17, and 21, with EC50 values of 0·85, 1·8, 5·7, and 14·2 μM for cisapride, zacopride, metoclopramide, and ML-1035 (4-amino-5-chloro-2-((2-methylsulphinyl)-ethoxy)-*N*-(2-(diethylamino)-ethyl)-benzamide hydrochloride), respectively). ML-1035 contractions were completely blocked by atropine and tetrodotoxin, while ganglionic blockade with hexamethonium was ineffective. Metoclopramide has been reported to sensitize postjunctional muscarinic receptors, however, ML-1035 did not enhance acetylcholine-induced contractions. Tropisetron (ICS 205-930, 1 μM), caused a parallel rightward shift in the concentration-response curve for both ML-1035 and zacopride (EC50 = 14·2 ± 1·3 and 1·8 ± 0·8 μM in the absence, and 26 ± 2·7 and 6·9 ± 2·3 μM in the presence of tropisetron for ML-1035 and zacopride, respectively) with apparent pK_B values of 5·9 and 6·0 for the respective compounds. 5-Hydroxytryptaminergic receptor desensitization by 2-methyl-5-hydroxytryptamine (5-HT_3) and 5-methoxytryptamine (5-HT_4), attenuated the response to ML-1035. We also examined the effect of the benzamides on [^{3}H]acetylcholine release from longitudinal muscle myenteric plexus preparations; however, these compounds had little effect on basal [^{3}H]acetylcholine release. Thus, the pharmacological data indicate that the benzamides can elicit neurogenic contractions in the non-stimulated ileum by activating postganglionic, cholinergic neurons which is independent of an effect on smooth muscle.

Metoclopramide, a 4-amino-5-chloro-2-methoxy benzamide, has served as a prototype for a number of compounds which enhance gastrointestinal motility (Sanger & King 1988). The prokinetic effects include facilitated gastric emptying, and enhanced intestinal transit (Pinder et al 1976; Harrington et al 1983). In-vitro, the benzamides enhance field-stimulated contractions which are mediated by intrinsic cholinergic fibres (Buchheit et al 1985; Schuurkes et al 1985; Gunning et al 1986; Linnik et al 1991). The ability to enhance cholinergic contractions in the gut has been implicated as the mechanism underlying the motility-promoting effects of these compounds. One factor which makes this class of compounds particularly intriguing is that the apparent increase in gastrointestinal cholinergic activity occurs in the relative absence of peripheral cholinergic side-effects (Harrington et al 1983; Sanger & King 1988).

Most in-vitro studies have employed field-stimulation to investigate the mechanism of action of the benzamides on the guinea-pig isolated ileum. The present experiments extend these studies by analysing the direct effect of the benzamides on the isolated ileum in the absence of field-stimulation in order to determine the mechanisms that contribute to these contractions. The majority of studies were conducted with ML-1035 (MDL 201,035, 4-amino-5-chloro-2-(2-(methylsulphinyl)-ethoxy)-*N*-(2-(diethylamino)-ethyl)-benzamide hydrochloride), a substituted prokinetic benzamide which lacks the dopaminergic activity of metoclopramide (Monkovic et al 1989; Linnik et al 1991; Butler et al 1992).

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Materials and Methods

All experiments were conducted in accordance with guidelines established by the Marion Merrell Dow Animal Care Committee.

Isolated tissue experiments

Guinea-pigs, 300–500 g, of either sex were fasted overnight and a 20 cm portion of ileum, approximately 10 cm proximal to the ileocaecal junction was removed. The ileum was cut into 1–2-cm segments and the lumen rinsed by gentle flushing with Tyrode buffer (in mM: NaCl 137, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·05, NaH_2PO_4 0·4, $NaHCO_3$ 11·9, glucose 5·6). The tissues were suspended longitudinally in 30-mL tissue baths containing Tyrode solution maintained at 37°C with oxygenation (95% O_2–5% CO_2), and were allowed to equilibrate for 30 min. The buffer in the tissue bath was replaced at least every 15 min during the duration of the experiments. Tension (1 g) was placed on the tissues and they were allowed to equilibrate for an additional hour. Tension was monitored with Grass FT 03 force displacement transducers and recorded on a Grass 7E polygraph. Drugs were added to the baths at 15-min intervals and exposed to the tissue for 1 min. The tissues were washed twice between each concentration. After the initial concentration-response curve was established the tissues were allowed 1 h to recover before a second curve was generated. Acetylcholine (1 μM) was added to the baths at the end of each dose-response curve and the results were defined as a percent of response to acetylcholine. No more than two dose-response curves were performed on any one tissue. In experiments designed to test the effect of a

compound on agonists, exposure to the first drug was initiated 10 min before challenge with the agonist.

To induce 5-HT_3 and 5-HT_4 receptor desensitization, we utilized a procedure described by Craig et al (1990) for the field-stimulated ileum. Initial dose-response curves were constructed as described above. Tissues were then continuously incubated with 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (10 μM) or 5-methoxytryptamine (5-MT) (10 μM) for 60 min with buffer replacement every 15 min. These agents then remained in the baths during the generation of the second dose-response curve.

[^{3}H]Acetylcholine release

Strips (3 cm) of guinea-pig ileum longitudinal muscle with attached myenteric plexus were isolated and pre-incubated in oxygenated (95% O_2–5% CO_2) Tyrode solution at 37°C for 30 min. [^{3}H]Choline chloride (1 μM, 5 μCi mL^{-1}) was added to the solution and allowed to incubate for an additional 60 min. The tissues were then placed in 900-μL chambers and superfused at 0·9 mL min^{-1} with Tyrode buffer containing 1 μM hemicholinium-3 for 1 h. After the incubation and wash-out period, fractions were collected every 2·8 min and drugs were added directly to the perfusate. At the end of the experiment, tissues were dissolved in Soluene 350 (Packard, Downers Grove, IL, USA) overnight and neutralized with 1 mL 0·5 M HCl before the addition of scintillation fluid (3 mL); samples were counted in a Beckman LS5000 TD liquid scintillation counter.

Data analysis and statistics

Values are expressed as means ± s.e.m. Agonist potencies were calculated by the four-parameter logistic equation with a computerized curve fitting program (Allfit, NIH, Bethesda, MD, USA) and were expressed as the concentration eliciting 50% of the maximal response (EC50). Differences between dose-response curves were evaluated by one-way analysis of variance followed by Bonferroni's test to determine statistical significance; unless otherwise indicated $P < 0.05$ was considered statistically significant. pK_B values for tropisetron against ML-1035 and zacopride were calculated from: $pK_B = \log(\text{concentration ratio} - 1) - \log(\text{antagonist concentration})$.

Materials

Cisapride was generously provided by the Janssen Research Foundation. Tropisetron was either purchased from Research Biochemicals Inc. (Natick, MA, USA) or generously provided by Sandoz Pharma AG. 2-Methyl-5-hydroxytryptamine was purchased from Research Biochemicals Inc. ML-1035 and zacopride (racemate) were prepared by Marion Merrell Dow Inc. All other chemicals and reagents, including metoclopramide, were purchased from Sigma Chemical Co. Inc. (St Louis, MO, USA).

Results

Isolated tissue experiments

The effect of four benzamide prokinetic agents were examined for direct responses on the guinea-pig isolated ileum. ML-1035, metoclopramide, zacopride, and cisapride elicited dose-dependent contractions of the ileum. The rank order of potency of these four benzamides in eliciting contractions in the guinea-pig non-stimulated ileum was: cisapride (EC50 = 0·85 μM) > zacopride (1·8) > metoclopramide (5·7) > ML-1035 (14·2). However, zacopride and cisapride were substantially less efficacious in this model when expressed as a percentage of the contraction induced by 1 μM acetylcholine (51 ± 3, 26 ± 2, 19 ± 3 and 12 ± 2%, n = 21, 17, 8 and 13 for ML-1035, metoclopramide, zacopride and cisapride, respectively) (Fig. 1).

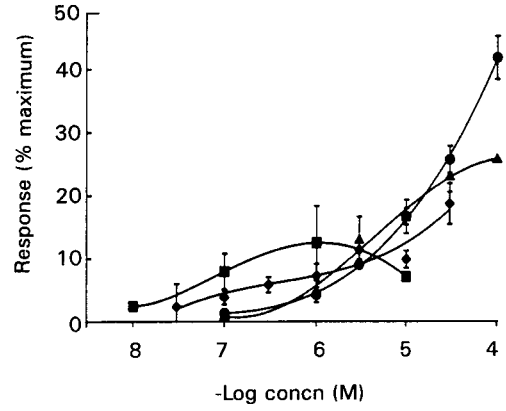

FIG. 1. The effect of ML-1035 (●), metoclopramide (▲), cisapride (■), and zacopride (◆) on the guinea-pig isolated ileum. Drugs were exposed to the tissue for 1 min, with 15 min allowed between concentrations. Results are expressed as a percent of the tissue response to 1 μM acetylcholine (mean ± s.e.m., n = 13–21 tissues).

A series of pharmacological experiments was performed to characterize this contraction. Consecutive noncumulative concentration-response curves were developed for ML-1035 based on a 1-min drug exposure and 1-h recovery period between curves. The tissue response to the second exposure of ML-1035 was diminished relative to the first exposure when expressed as a percent of the response to 1 μM acetylcholine (% maximal response to 1 μM acetylcholine = 51 ± 3 and 42 ± 3% at 100 μM ML-1035 with an EC50 = 9 and 14 μM in the first and second concentration-response curves, respectively, n ≥ 12 tissues from three separate experiments). The attenuated response to ML-1035 during the second exposure did not appear to be mediated postjunctionally as there was no difference in the magnitude of response to 1 μM acetylcholine following the first and second curves (response to 1 μM acetylcholine = 2·5 ± 0·5 and 2·6 ± 0·6 g following the first and second concentration-response curves, respectively, n = 12). Thus, experiments which examined the effect of ligands on the response to ML-1035 were performed by initially establishing the integrity of the preparation relative to ML-1035 and acetylcholine responses in the first concentration response curve, and then comparing ligand-treated tissues with appropriately matched controls in the second concentration-response curve.

Tetrodotoxin (0·1 μM), which blocks voltage-dependent sodium conductance, completely abolished the response to ML-1035, indicating that ML-1035 requires activation of the intrinsic neural fibres (Fig. 2). Atropine, which blocks muscarinic receptors, caused a concentration dependent shift in the ML-1035 dose-response curve (Fig. 3). This verified the cholinergic nature of the contractions and shows that ML-1035 does not compete with acetylcholine for the muscarinic-receptor binding site. The possibility that ML-

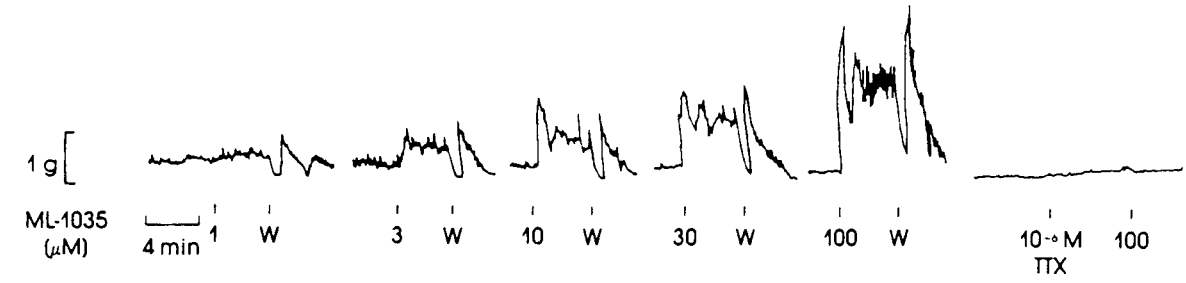

FIG. 2. Chart recording of the contractile response elicited by cumulative addition of ML-1035 in the guinea-pig ileum. The contractions were prevented by tetrodotoxin (TTX) as indicated. The time scale on the figure refers to the paper speed at all times except when the drug was added. During drug exposure, paper speed was increased 4-fold, thus the indicated time scale would be equivalent to 1 min. Drug concentrations are in mol L^{-1} and W indicates wash.

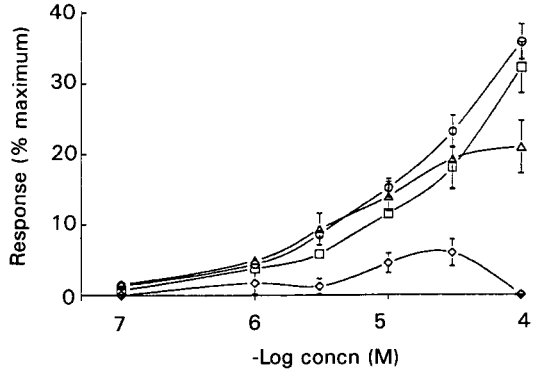

FIG. 3. Effect of increasing concentrations of atropine (□ 0·1, △ 1, ◇ 10 nM, ○ control) on the contractions elicited by ML-1035 in the guinea-pig ileum. Atropine was added to the baths 10 min before each concentration of ML-1035. Data are expressed as a percent of the response to 1 μM acetylcholine (mean ± s.e.m., n = 6–12 tissues).

1035 could have an effect on ganglionic transmission in the ileum was also considered. Hexamethonium (10 μM) had no effect on the ML-1035 concentration-response curve which supports a site of action independent of preganglionic neurons (EC50 = 14 ± 1 and 14 ± 2 μM in the absence and presence of hexamethonium, respectively, n = 6).

Although this data is consistent with a facilitatory effect of ML-1035 on cholinergic transmission, multiple sites of action are possible. One possibility is that ML-1035 sensitizes the postsynaptic muscarinic receptor as has been reported with metoclopramide (Beani et al 1970). Therefore, we examined the effect of ML-1035 on the ileal response to exogenous acetylcholine. Acetylcholine (0·1 μM) produced consistent contractions when administered at 15-min intervals that were sustained throughout the experiment (contraction = 2·6 ± 0·2 g, n = 8). After establishing three control responses, ML-1035 (0·1–100 μM) was added 5 min before acetylcholine and, in some experiments, tetrodotoxin (0·3 μM) was added 5 min before the addition of ML-1035. Low concentrations of ML-1035 had little effect on resting tension or contractions to acetylcholine. Higher concentrations of ML-1035 produced contractions, however, they also had no effect on the ileal response to acetylcholine except at the highest concentration where they caused a decrease in the absolute magnitude of the response (response in the presence of 100 μM ML-1035 = 87·8 ± 6·7% of the control response to 0·1 μM acetylcholine, n = 8). Tetrodotoxin had no effect on the ileal response to acetylcholine (contraction = 2·6 ± 0·2 and 2·5 ± 0·1 g in the absence and presence of tetrodotoxin, respectively, n = 8), but abolished any direct response to ML-1035. In addition, ML-1035 had no effect on the tissue response to acetylcholine in the presence of tetrodotoxin, except at the highest concentration (response to 0·1 μM acetylcholine in the presence of 100 μM ML-1035 and 0·3 μM tetrodotoxin = 92·3 ± 2·6% of the control response to 0·1 μM acetylcholine, n = 8). These data demonstrate that ML-1035 does not cause ileal contractions by sensitizing muscarinic receptors.

We also examined the possibility that the contractile responses observed for ML-1035 may be due to interactions at either 5-HT_1, opioid, or histaminergic receptors. The concentration-response curves to ML-1035 were not affected by a 1 μM concentration of the following antagonists; methysergide (5-HT_1), naloxone (opioid), or pyrilamine (histamine) (data not shown).

While multiple 5-hydroxytryptamine (5-HT) receptors have been identified in the guinea-pig ileum, the 5-HT_3 and the 5-HT_4 receptor have been most intimately associated with the prokinetic effects of the benzamides. Since ML-1035 has both 5-HT_3 antagonist and 5-HT_4 agonist properties (Linnik et al 1991) we sought to determine which of these receptors may be involved in the contractions induced by ML-1035. Two pharmacological approaches were used to assess potential 5-HT_3 and 5-HT_4 activity. The initial method was an attempt to block the ML-1035 response with tropisetron. At low concentrations tropisetron is a 5-HT_3 antagonist, while at higher concentrations it also functions as a 5-HT_4 antagonist (Richardson et al 1985; Dumuis et al 1988). Tropisetron, which had no intrinsic activity at concentrations up to 10 μM, caused a significant ($P < 0.05$) shift to the right in the EC50 for ML-1035 (EC50 = 14·2 and 26 μM in the presence and absence of tropisetron, respectively, n = 6), with an apparent pK_B of 5·9 (Fig. 4). In addition, 3 μM tropisetron shifted the concentration response curve for zacopride to the right (EC50 = 6·9 and 1·8 μM, in the presence and absence of tropisetron, respectively, n = 8), with an apparent pK_B of 6·0. This compares favourably with dissociation constants established at both the central and peripheral 5-HT_4 receptor (Dumuis et al 1989; Elswood et al 1991).

The second approach for characterizing potential 5-HT-

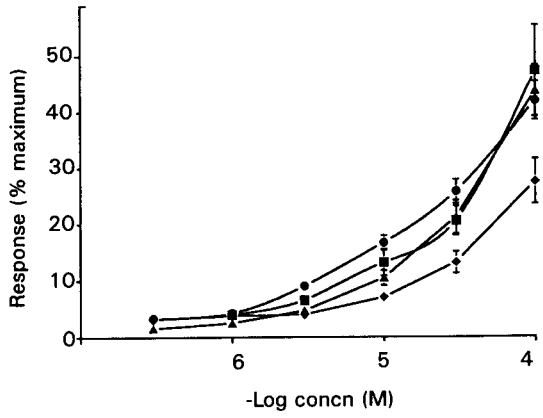

FIG. 4. The effect of increasing concentrations of the 5-HT_3/5-HT_4 antagonist tropisetron (■ 1 nM, ▲ 1 μM, ◆ 10 μM) on the contractions elicited by ML-1035 (●). Tropisetron, which had no intrinsic activity, was added 10 min before each concentration of ML-1035. Data are expressed as a percent of the tissue response to 1 μM acetylcholine (mean ± s.e.m., n = 6 tissues).

ergic receptor interactions utilized selective densensitization of the 5-HT_3 and 5-HT_4 receptors as proposed by Craig et al (1990). In the field-stimulated preparation, sustained exposure to 2-methyl-5-HT (10 μM) desensitized the 5-HT_3 receptors, while exposure to 5-MT (10 μM) selectively desensitized the 5-HT_4 receptors. Tissue exposure to 2-methyl-5-HT and 5-MT for 1 h followed by exposure to ML-1035 in the presence of these agents attenuated the response to ML-1035 (maximal response to 100 μM ML-1035 = 36 ± 3, 9 ± 1, and 17 ± 4% of the response to 1 μM acetylcholine with an EC50 = 14, 29, and 32 μM for control, 2-methyl-5-HT-, and 5-MT-treated tissues, respectively, n = 12) (Fig. 5). 2-Methyl-5-HT and 5-MT treatment also caused a moderate decrease in the tissue responsiveness to exogenous acetylcholine, indicating that the desensitization paradigm may have caused tissue fatigue (tissue response to 1 μM acetylcholine = 2·9 ± 0·2 vs 2·4 ± 0·2 g, and 3·0 ± 0·2 vs 2·3 ± 0·1 g for tissues treated with 2-methyl-5-HT and 5-MT following the first vs the second dose-response curves, respectively, n = 12). In contrast, the tissue response to

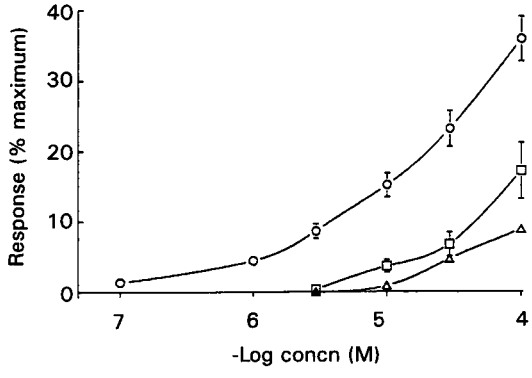

FIG. 5. The effect of pretreatment with 5-MT (5-HT_4 agonist; □ 10 μM) and 2-methyl-5-HT (5-HT_3 agonist; △ 10 μM) on the contractions elicited by ML-1035 (○) in the guinea-pig isolated ileum. Two noncumulative concentration-response curves were generated for ML-1035. Drugs were added to the bath after the first curve, allowed to incubate with the tissue for 60 min, and maintained in the bath during the development of the second curve. Data from the second concentration-response curve are expressed as a percent of the tissue response to 1 μM acetylcholine (mean ± s.e.m., n = 12 tissues).

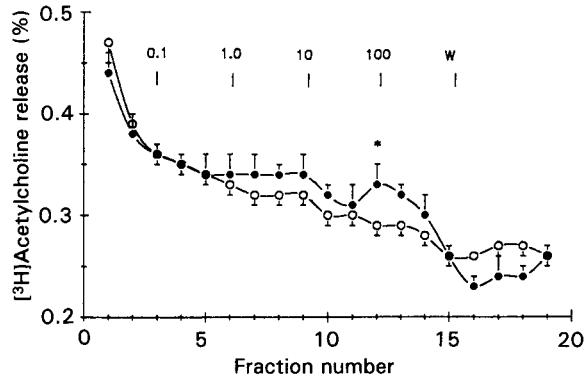

FIG. 6. The effect of cumulative addition of ML-1035 (●; control ○) on the release of [^{3}H]acetylcholine from strips of guinea-pig myenteric plexus with attached longitudinal muscle. Tissues preincubated in [^{3}H]choline chloride were superfused at 0·9 mL min^{-1} with Tyrode solution. The overflow was collected into 2·8 min fractions to determine the amount of tritium released. Data is expressed as percent of total available radioactivity released into the designated fraction (mean ± s.e.m., n = 11 tissues; $*P < 0.05$ using Student's t-test to compare fraction 12 in control vs drug-treated tissues). Additional experiments which address the effect of noncumulative addition of ML-1035 on [^{3}H]acetylcholine release are presented in the Results.

exogenous acetylcholine was reproducible during the duration of the experiment when ML-1035 dose-response curves were repeated in the absence of desensitizing agents (tissue response to 1 μM acetylcholine = 2·5 ± 0·2 and 2·6 ± 0·3 g following the first vs the second dose-response curves, respectively, n = 12).

[^{3}H]Acetylcholine release

The effect of ML-1035 on the release of [^{3}H]acetylcholine from myenteric cholinergic neurons was examined. Strips of longitudinal muscle with attached myenteric plexus were incubated with [^{3}H]choline to label the cholinergic terminals. Preliminary experiments demonstrated that hemicholinium-3 (100 μM) inhibited 54% of the tritium accumulation into the tissue, indicating that approximately half of the choline was incorporated into cholinergic neurons. When the tissues were placed in the release chambers, a rapid ^{3}H wash-out phase lasting 20–30 min was followed by a prolonged, slowly decrementing phase. Thus, all tissues were perfused for 60 min after the [^{3}H]choline incubation before fractions were collected. In addition, all tissues demonstrated an increase in ^{3}H release in response to field stimulation (0·1 Hz, 250 mA, 0·5-ms square waves of alternating polarity) that could be prevented by tetrodotoxin (data not shown).

Addition of ML-1035 to the perfusate in cumulative concentrations had almost no effect on [^{3}H]acetylcholine release (Fig. 6). A significant increase in release was observed in a single fraction (fraction 12) when the tissue was initially exposed to 100 μM ML-1035 (percent release of [^{3}H]acetylcholine into fraction 12 = 0·29 ± 0·01 and 0·33 ± 0·02% for control and ML-1035-treated tissues, respectively, n = 11; $P < 0.05$). Seven of eleven tissues exposed to ML-1035 exhibited an increase in percent release in fraction 12 relative to the preceding fraction, while only 2 of 11 control tissues showed an increase in percent release in this fraction. The percent release in all other fractions was not different from the control tissue.

Table 1. Effect of benzamides on [^{3}H]acetylcholine release*†.

	Tyrode			Tyrode + physostigmine (1 μM)		
	Baseline	Postdrug	n	Baseline	Postdrug	n
Vehicle	0·26 ± 0·03	0·25 ± 0·02	6	0·34 ± 0·03	0·32 ± 0·04	11
ML-1035 (100 μM)	0·25 ± 0·03	0·23 ± 0·02	5	0·41 ± 0·07	0·41 ± 0·08	11
Zacopride (100 μM)	0·26 ± 0·03	0·26 ± 0·02	6	0·28 ± 0·04	0·26 ± 0·03	8
Metoclopramide (100 μM)	nd	nd		0·48 ± 0·06	0·42 ± 0·06	6

* Strips of guinea-pig longitudinal muscle (3 cm) with attached myenteric plexus were incubated with [^{3}H]choline chloride, perfused with Tyrode solution and fractions were collected every 2·8 min. Release is expressed as the percent of available ^{3}H released into a given fraction. † Values presented are the mean ± s.e.m. of the two fractions preceding drug administration (baseline) or immediately after drug exposure (postdrug). nd = not determined.

The possibility that cumulative addition of ML-1035 may have caused tachyphylaxis was evaluated by examining the response to a single concentration of ML-1035 and zacopride (Table 1). No increase in [^{3}H]acetylcholine release was observed for either compound. Kilbinger et al (1982) has reported that physostigmine enhanced the ability of metoclopramide to increase the release of [^{3}H]acetylcholine from myenteric plexus. Thus, we examined the effect of a single concentration of ML-1035, zacopride and metoclopramide on [^{3}H]acetylcholine release from this preparation in the presence of physostigmine (1 μM), but again observed no effect (Table 1).

Discussion

A number of substituted benzamides have been shown to increase gastric motility in-vivo and enhance both field-stimulated and peristaltic contractions in the guinea-pig isolated ileum (Buchheit et al 1985; Craig & Clarke 1991; Linnik et al 1991). The mechanisms underlying the contractile response to a series of substituted benzamides in the guinea-pig in-vitro nonstimulated ileum were examined. ML-1035, metoclopramide, zacopride, and cisapride all elicited concentration-dependent contractions in this model. The present results demonstrate that the benzamides can activate prejunctional postganglionic cholinergic neurons, resulting in direct neurogenic contractions in the ileum.

Metoclopramide exerts a contractile response in the ileum which is similar to that observed with ML-1035 (Beani et al 1970; Okwuasaba & Hamilton 1976; Bury & Mashford 1976; Hay 1977). Contractions elicited by both ML-1035 and metoclopramide were blocked by tetrodotoxin and anticholinergic agents, and were not affected by ganglionic blocking agents (Beani et al 1970; Hay 1977). This supports a postganglionic, neurogenic origin of the contractions. Furthermore, the demonstration of a noncompetitive blockade of ML-1035 contractions by atropine indicates that the response to ML-1035 is dependent on activation of cholinergic neurons, however, it exerts its effect at a site independent of the muscarinic receptor binding site.

A postjunctional site of action has been described for metoclopramide which increases intestinal responses to exogenously administered acetylcholine (Beani et al 1970; Okwuasaba & Hamilton 1976; Bury & Mashford 1976; Hay 1977). Thus, it was suggested that metoclopramide sensitizes postjunctional muscarinic receptors, although support for this hypothesis is not unanimous (Fernandez & Massingham 1985; Sanger & King 1988). In the present studies, ML-1035 elicited larger contractions than metoclopramide, yet it had little effect on acetylcholine-induced contractions. The lack of receptor sensitization has also been observed for other benzamides, including cisapride and BRL 24924, indicating that this property is not shared by this class of compounds (Schuurkes et al 1985; Sanger 1987). These data are consistent with an effect that occurs prejunctionally, rather than at the postjunctional muscarinic receptor.

One apparent common denominator among the benzamides is their ability to enhance cholinergic contractions. This could be explained by inhibition of acetylcholinesterase, but the lack of an effect of ML-1035 on acetylcholine-induced contractions in the presence of tetrodotoxin would indicate that this is not a viable hypothesis. However, the contractile response to metoclopramide depends on maintaining a releasable pool of acetylcholine (Hay & Man 1979). Therefore, the most obvious explanation has been that the benzamides enhance acetylcholine release.

While all the pharmacology is consistent with benzamides enhancing acetylcholine release, a direct demonstration of an increase in release was not readily observed. Other investigators have also had difficulty demonstrating this, leading them to suggest that these drugs may interfere with non-adrenergic, non-cholinergic intramural nervous structures (Beani et al 1970; Okwuasaba & Hamilton 1976). In contrast, Kilbinger et al (1982) examined the effect of metoclopramide on acetylcholine release and found a transient, tetrodotoxin-sensitive, increase in basal [^{3}H]acetylcholine release. In the present experiments, the benzamides had little effect on the basal release of [^{3}H]acetylcholine, which opens the possibility that non-cholinergic neuronal mechanisms may contribute to the contractile response. However, any explanation requires one to account for the complete inhibition of contraction in the presence of atropine. One possible explanation is that the longitudinal muscle/myenteric plexus preparations respond differently when they are placed under tension, however, the cholinergic terminals in these preparations were competent as they increased [^{3}H]acetylcholine release in response to field stimulation (tetrodotoxin sensitive) and exposure to elevated potassium concentrations (data not shown).

There is a large body of evidence which indicates that the benzamides interact with gastrointestinal 5-HT-ergic receptors (Bianchi et al 1970; Fozard & Mobarok Ali 1978; Bradley et al 1986). The most important receptors for contractility appear to be 5-HT$_3$ and 5-HT$_4$ receptors. The

benzamide-induced contractions observed in the absence of field stimulation appeared to be consistent with agonism at the 5-HT_4 receptor as described in the guinea-pig field-stimulated ileum (Craig & Clarke 1990). Tropisetron, a 5-HT_3 and 5-HT_4 antagonist, competitively inhibited the response to ML-1035 and zacopride in the non-stimulated ileum as it does in the field-stimulated ileum (Craig & Clarke 1990; Linnik et al 1991). The inhibition observed in the presence of tropisetron occurred only at micromolar concentrations, consistent with a 5-HT_4 site of action.

The application of specific desensitization paradigms has revealed a potential role for both the 5-HT_3 and the 5-HT_4 receptor (Craig et al 1990). Pretreatment with 2-methyl-5-HT (5-HT_3) was slightly more potent than pretreatment with 5-MT (5-HT_4); however, both paradigms were very effective at shifting the ML-1035 concentration-response curve to the right. These data support the idea that ML-1035 contracts the tissues via a 5-HT-specific mechanism, but does not clearly identify a single receptor associated with this response.

In conclusion, the benzamides elicit contractions in the guinea-pig isolated ileum which depend on activation of the intrinsic cholinergic neurons. The site of action appears to be exclusively neurogenic, as no postjunctional effects could be observed. The contractions may be mediated through a 5-HT-ergic mechanism; however, additional experiments will be necessary to resolve unequivocally the specific receptor subtype.

Acknowledgements

The authors would like to thank Mr Vern Klein for his assistance in designing and manufacturing the equipment used in these experiments.

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