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Prokineticin-2, motilin, ghrelin and metoclopramide: Prokinetic utility in mouse stomach and colon

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Abstract

The ability of agents described as gastrointestinal prokinetics (prokineticin-2, [Nle^{13}]-motilin, ghrelin), to modulate nerve-mediated contractions of mouse isolated stomach and colon was determined and compared with the prokinetic and 5-HT$_4$ receptor agonist, metoclopramide. Circular muscle preparations were electrically field-stimulated (EFS) to evoke cholinergically mediated contractions. Metoclopramide 10–100 μM facilitated EFS-evoked contractions in forestomach (n=5–11, P<0.05); 1 mM inhibited. Metoclopramide had no effects in colon, apart from 100 μM which reduced contractions. Prokineticin-2 0.001 nM–0.1 μM (n=3–7) or [Nle^{13}]-motilin 0.1 nM–1 μM (n=4–8) had no effects in forestomach or colon. Ghrelin 0.01–1 μM facilitated EFS-evoked contractions in forestomach (n=5–7, P<0.05) but not in colon (n=5–8). We conclude that ghrelin and metoclopramide facilitate excitatory nerve activity because neither affected inhibitory responses to EFS in the presence of atropine, or contractions to carbachol. Further, prokineticin-2 and [Nle^{13}]-motilin are unlikely to exert gastric prokinetic activity in this species, the inactivity of the latter being consistent with an absence of the motilin receptor in rodents.

Keywords: Gastrointestinal motility; Ghrelin; Metoclopramide; Motilin; Prokineticin

1. Introduction

5-HT$_4$ receptor agonists, such as metoclopramide, act within the enteric nervous system (ENS) to accelerate co-ordinated gastric emptying and intestinal propulsion (Sanger, 1998). Studies in vitro have measured the abilities of 5-HT$_4$ receptor agonists to act pre-junctionally and facilitate electrically evoked, cholinergically mediated contractions of gastrointestinal preparations from different species, including guinea-pig, rat and human (Sanger, 1985a,b, 1987; Craig and Clarke, 1990; Tonini et al., 1992). A correlation between the activity observed in isolated tissues and the ultimate therapeutic benefit, suggests that this technique provides a method to assess the gastrointestinal prokinetic potential of novel neuropeptides which act within the enteric nervous system, as well as measure any ability to directly affect muscle tension. Examples of peptides for which gastro-prokinetic activity is associated with an ability to facilitate electrically evoked, cholinergically mediated gastric contractions are motilin (rabbit; Van Assche et al., 1997; Dass et al., 2003a; Depoortere et al., 2003) and ghrelin (rat; Dass et al., 2003b; Edholm et al., 2004). The polypeptide prokineticin-2 (PK2; also known as Endocrine Gland Vascular Endothelial Growth Factor) and its receptors have been detected in the mammalian gut, where PK2 is reported to contract smooth muscle via a mechanism insensitive to tetrodotoxin (Li et al., 2001; Lin et al., 2002). Based on this evidence, the peptide received the name prokineticin and was discussed in terms of a possible involvement in irritable bowel syndrome. However, given that metoclopramide, motilin and ghrelin are proven gastroprokinetic agents and that this activity is more strongly associated with an ability to increase cholinergic contractile activity rather than any direct action on smooth muscle (see above), we have investigated an ability of PK2 to exert such activity in isolated stomach and colon preparations, comparing any activity with that evoked by metoclopramide, [Nle^{13}]-motilin and ghrelin. Mouse isolated gut preparations were developed, thereby also laying a foundation for future

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studies using genetically modified mice. Some of our findings have previously been reported as meeting abstracts (Murray et al., 2002; Bassil et al., 2003).

2. Materials and methods

2.1. Animals

Adult male C57BLK6/J mice (25–30 g) were culled by concussion and cervical dislocation, in accordance with the UK Animals (Scientific Procedures) Act 1986. All efforts were made to minimise the number of animals used. Stomachs and distal colons were blunt-dissected and placed in Krebs solution (NaCl 121.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 5.6 mM) equilibrated with 5% CO_2 in O_2 at room temperature.

2.2. Tissue preparation and pharmacological studies

Mucosa-intact strips (~2×7.5 mm) were cut approximately parallel to the circular muscle of the forestomach and distal colon. These were suspended under 10 mN tension for isometric recording between two platinum ring electrodes, in 5 ml tissue baths containing Krebs solution bubbled with 5% CO_2 in O_2, maintained at pH 7.4±0.1 and 37 °C. This was previously determined as the optimal tension required to maximally detect EFS-evoked responses in both tissues (data not shown). Tension was measured using Pioden dynamometer UF1 force-displacement transducers. Tissues were equilibrated for ~60 min, changing bath solutions every 15 min.

The neurones within the tissues were selectively stimulated by electrical field stimulation (EFS) using biphasic square-wave pulses of 0.5–0.7 ms (forestomach) or 0.2 ms (colon), 5 Hz and a maximally effective voltage (70–90 V, generating a current of approx. 200 mA) applied for 30–60 s every 2 min (forestomach) and 10–30 s every 110–150 s (colon), over 30 min periods. Each period was separated by a 5 min interval and bath solutions were changed. These parameters of EFS consistently evoked nerve-mediated responses with a good signal-to-noise ratio over spontaneous muscle activity and represented a "mid-point" pharmacological phenotype which included the types of responses observed at lower and higher frequencies of EFS (for example, a contraction or relaxation were usually observed during EFS at respectively low or high frequencies of stimulation, but at 5 Hz the response was usually a mixture of both an initial contraction and then a relaxation during EFS).

After obtaining consistent EFS-evoked responses, metoclopramide, ghrelin, PK2, [Nle^{13}]-motilin or vehicle were applied non-cumulatively to separate tissues (20 min contact); changes in resting muscle tension and responses to EFS were measured. In the forestomach, excitatory responses to metoclopramide were observed regardless of the small variations in stimulation parameters described above (data not shown). The effects of treatments on carbachol-induced gastric contractions were performed using 5 min contact times and a submaximally effective concentration of carbachol (0.1 μM; 90 s contact; contractions 43±3% of maximum; n=4). The effects of treatments on non-cholinergic inhibitory EFS-evoked responses were studied in the presence of atropine 1 μM.

2.3. Data acquisition

MP100 hardware and AcqKnowledge® software (Biopac Systems, Inc) was used. Effects on EFS- or carbachol-evoked contractions were expressed as a % change of three successive contractions relative to the mean of three pre-treatment contractions. Effects on baseline muscle tension were measured as an absolute change (g). Data are given as means±standard error and the statistical significance of differences between paired data (drug vs. vehicle) were determined using two-tailed Student's t-test ($P<0.05$=significance); n-values are numbers of animals used.

2.4. Drugs used

Drugs were freshly prepared. Rat ghrelin (Bachem) was prepared in BSA-Saline (0.9% NaCl containing 0.01% bovine serum albumin; Sigma). Metoclopramide, carbachol, atropine (Sigma), [Nle^{13}]-motilin (Bachem), human PK2 (synthesised in-house) and tetrodotoxin (Tocris), were dissolved in dH_2O. Preliminary experiments comparing the effects of each peptide using either BSA-Saline or water as a vehicle were conducted to determine the appropriate vehicles to minimise loss of final peptide concentration caused by any adherence to the apparatus. There was no difference in the effects observed with PK2 and [Nle^{13}]-motilin when dissolved in either vehicle, whereas the

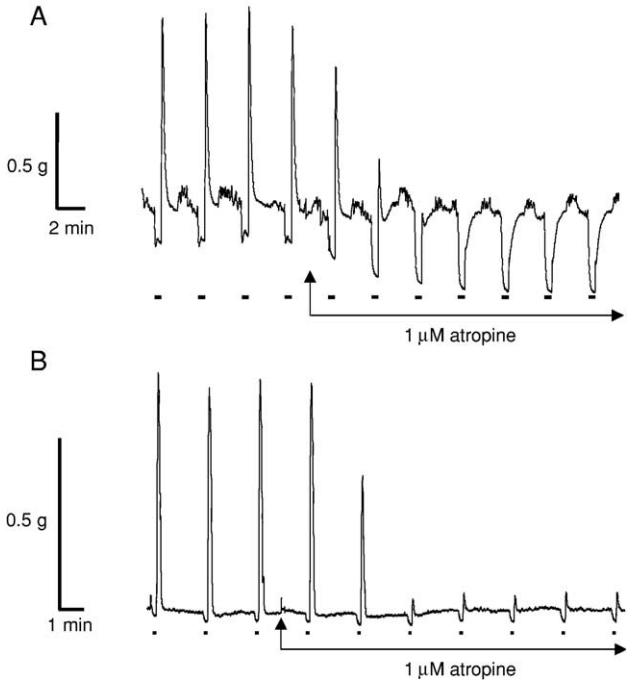

Fig. 1. Typical responses observed during electrical field stimulation of mouse isolated forestomach (A) and distal colon (B), in the absence and presence of atropine 1 μM. Bars indicate periods of EFS, as described in the Materials and methods section.

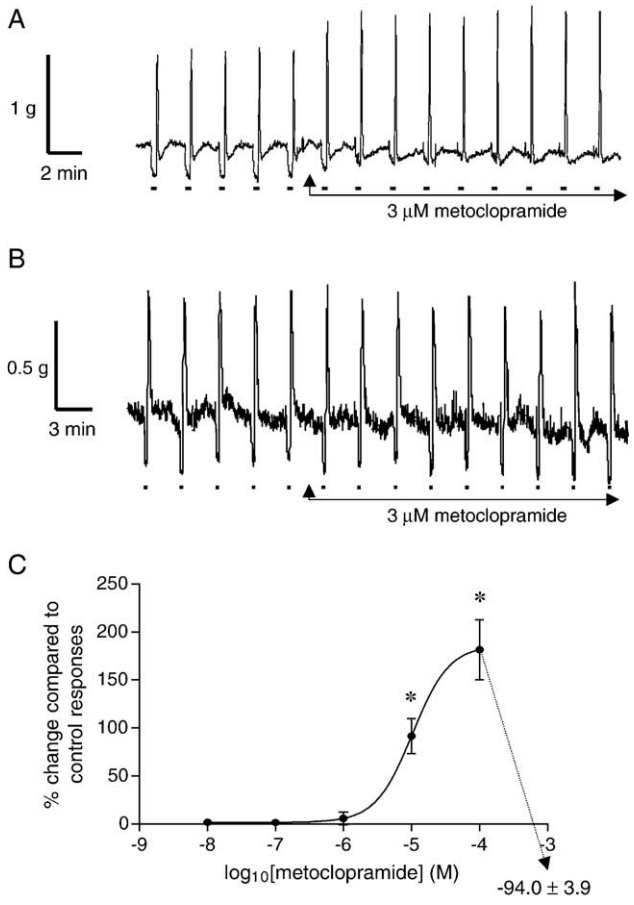

Fig. 2. The effects of metoclopramide 3 μM on EFS-evoked, nerve-mediated contractions in mouse isolated gut. Traces are shown using (A) forestomach and (B) distal colon; the bars indicate the periods of EFS, as described in the Methods. The concentration–response curve for the effects of metoclopramide in the forestomach are shown in panel (C); *$P<0.05$, compared with vehicle controls.

effects of ghrelin appeared to be smaller when the peptide was dissolved in water, compared to BSA/Saline (data not shown).

3. Results

3.1. Responses to EFS in forestomach and distal colon

In the forestomach, EFS-evoked responses were characterised by an initial small contraction (64% of preparations from 43 animals) followed by either a relaxation (84% of preparations) or a further contraction (remaining 16% of preparations) during EFS. Termination of EFS immediately and consistently evoked a large-amplitude 'after-contraction'. In the colon, EFS evoked a small initial contraction which always gave way to a small muscle relaxation; termination of EFS consistently evoked a large amplitude after-contraction. All EFS-evoked responses in stomach and colon were prevented by tetrodotoxin 1 μM (20 min contact, $n=9$ and 4, respectively). Atropine 1 μM (20 min contact) prevented all contractions during EFS, increased EFS-evoked relaxation by 164±61% (forestomach, $n=6$) and 61±13% (colon, $n=7$), and reduced the after-contractions by 63±3% (forestomach, $n=6$) and 81±5% (colon, $n=7$) (Fig. 1). As termination of EFS consistently evoked after-contractions, the effects of treatments were assessed for their ability to modulate this component of the EFS-induced response. By contrast, the inconsistency of the types of responses evoked during the period of EFS itself, meant that treatment effects on these during-EFS responses could not be quantified; nevertheless, visual observations of the general effects were noted.

3.2. Evaluation of prokinetic-like activity in the forestomach

Metoclopramide 0.01–100 μM had no significant effects on baseline muscle tension ($n=5$–11) but at 1 mM decreased baseline tension by 0.24±0.06 g ($P<0.05$, $n=4$). Metoclopramide 10–100 μM concentration-dependently facilitated EFS-evoked after-contractions, reaching a maximum effect within 5 min after application ($P<0.05$ compared with vehicle, $n=5$–11; Fig. 2); the maximum-effective concentration was 100 μM ($E_{max}=182\pm31\%$, $n=7$). These concentrations of

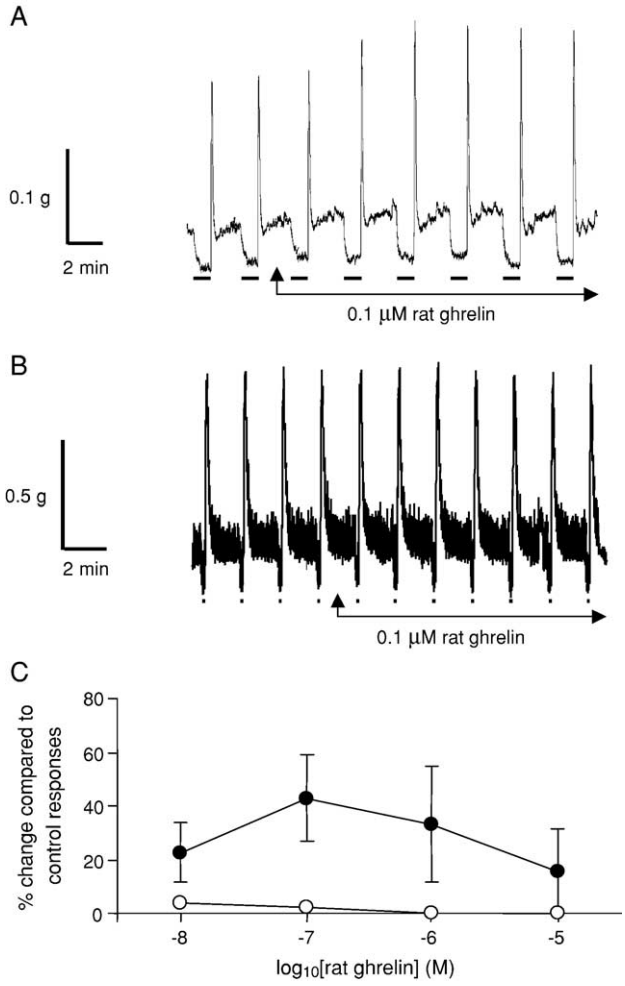

Fig. 3. The effects of ghrelin 0.1 μM on EFS-evoked, nerve-mediated contractions in mouse isolated gut. Traces are shown using (A) forestomach and (B) distal colon; the bars indicate the periods of EFS, as described in the Materials and methods section. The concentration–response curve for the effects of ghrelin (●) in the forestomach are shown in panel (C); *$P<0.05$, compared with vehicle controls (○).

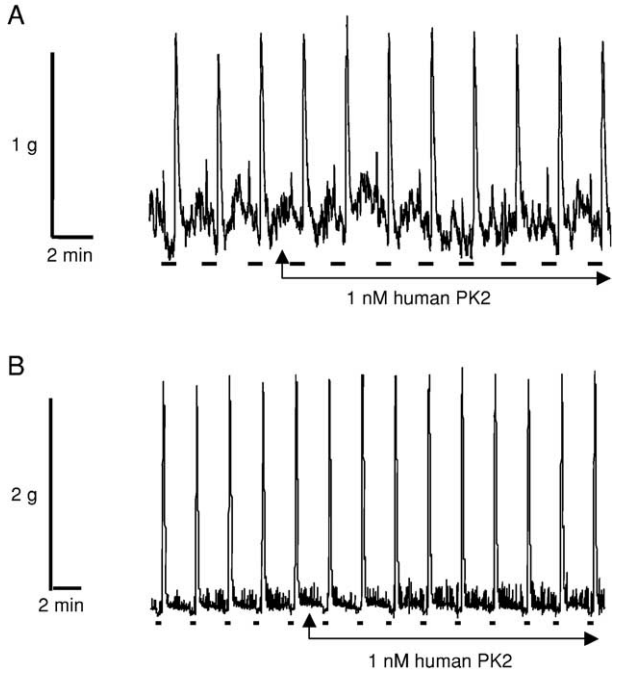

Fig. 4. The effects of human prokineticin 2, 1 nM on EFS-evoked, nerve-mediated contractions in mouse isolated gut. Traces are shown using (A) forestomach and (B) distal colon; the bars indicate the periods of EFS, as described in the Materials and methods section.

metoclopramide also tended to increase any excitatory responses observed during EFS (e.g., Fig. 2). Metoclopramide 1 mM reduced after-contraction amplitudes by 94.0±3.9%, $P<0.001$, $n=4$).

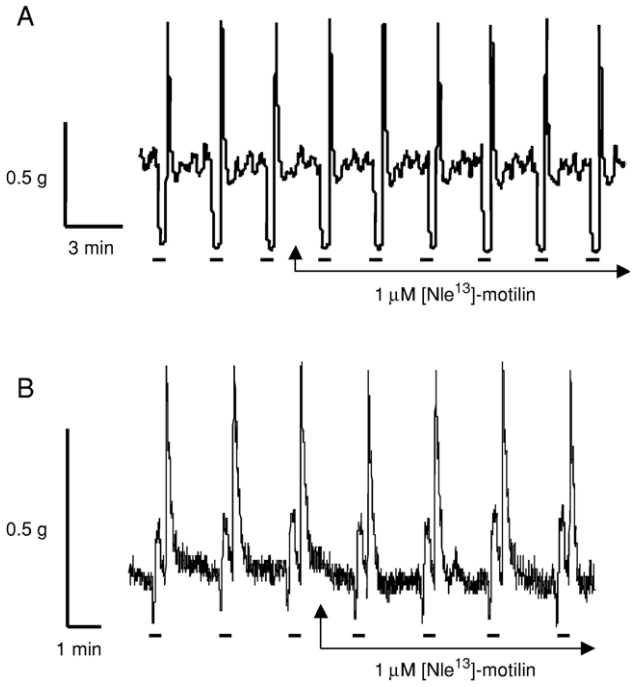

Fig. 5. The effects of [Nle^{13}]-motilin, 1 μM on EFS-evoked, nerve-mediated contractions in mouse isolated gut. Traces are shown using (A) forestomach and (B) distal colon; the bars indicate the periods of EFS, as described in the Materials and methods section.

Ghrelin 0.01–1 μM had no effects on baseline muscle tension ($P>0.05$, $n=5–7$), but concentration-dependently facilitated EFS-evoked after-contractions, the effects reaching a maximum within 5 min after application ($P<0.05$, $n=5–7$; Fig. 3). The maximum-effective concentration was 0.1 μM ($E_{max}=43\pm15\%$, $n=7$). Ghrelin appeared not to affect the responses evoked during EFS (e.g., Fig. 3). Neither PK2 (0.001 nM–0.1 μM) nor [Nle^{13}]-motilin (0.1 nM–1 μM) had any consistent effects on baseline muscle tension ($P>0.05$, $n=3–7$ and 6–8, respectively), EFS-evoked after-contractions ($P>0.05$, $n=3–7$, and 6–8, respectively) or on responses evoked during EFS (Figs. 4 and 5).

In separate experiments, neither metoclopramide 10 μM nor ghrelin 0.1 μM changed the amplitude of forestomach contractions evoked by carbachol 0.1 μM (respectively 2.6±1.6%, $P=0.16$, $n=4$; −1.9±5.4%, $P=0.9$, $n=4$). Further, metoclopramide 10 μM did not change the maximum relaxation evoked during EFS in the presence of atropine 1 μM ($n=4$, Fig. 6). However, baseline muscle tension tended to increase slightly and this was associated with an appearance of after-contractions which did not completely relax back to baseline tension between each period of EFS; consequently, the overall magnitude of relaxation observed during EFS was increased by an equivalent amount (Fig. 6). Ghrelin 0.1 μM had no significant effects on baseline tension or on relaxations evoked by EFS in the presence of atropine ($P>0.05$, $n=4$).

3.3. Evaluation of activity in the distal colon

Metoclopramide (0.01–10 μM) or ghrelin (0.1–10 μM) had no effects on baseline muscle tension (respectively $P>0.1$, $n=4–8$; $P>0.8$, $n=5–8$), EFS-evoked after-contractions (respectively $P>0.3$, $n=4–8$; $P>0.1$, $n=5–8$) and did not consistently change the responses evoked during EFS (Figs. 2 and 3). Metoclopramide 100 μM decreased the EFS-evoked contractions by 70.3±9.9% ($P=0.009$, $n=4$). PK2 (0.001 nM–0.1 μM) or [Nle^{13}]-motilin (0.1 nM–1 μM) had no effects on baseline tension ($P>0.3$, respectively $n=3–4$ and 5–6) or EFS-evoked after-contractions ($P>0.3$, $n=3$; $P>0.4$, $n=4–6$), and did not consistently affect the responses evoked during EFS (Figs. 4 and 5).

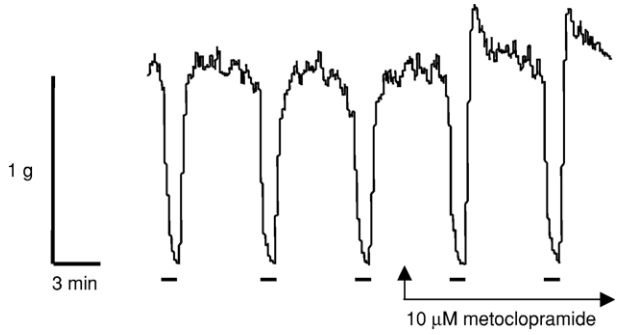

Fig. 6. Trace showing the effects of metoclopramide 10 μM on EFS-evoked, nerve-mediated contractions in mouse isolated stomach in the presence of atropine 1 μM; the bars indicate the periods of EFS, as described in the Materials and methods section.

4. Discussion

In this study the prokinetic potential of several agents was investigated in mouse isolated stomach and colon using a technique previously developed with other species to characterise 5-HT_4 receptor agonists, in which an ability to facilitate electrically-evoked, nerve-mediated contractions is indicative of gastro-prokinetic activity (see Introduction). In the mouse forestomach, metoclopramide 10–100 μM facilitated EFS-evoked after-contractions without affecting baseline muscle tension; higher concentrations reduced the contractions evoked by EFS, an activity which is consistent with local anaesthetic properties of this molecule at high concentrations (Prieto et al., 1977). Since metoclopramide 10 μM, did not reduce inhibitory responses evoked by EFS in non-cholinergic conditions and did not change the amplitude of carbachol-induced contractions, it seems likely that metoclopramide increased the electrically evoked contractions via a pre-junctional cholinergic site. Interestingly, in the presence of atropine, metoclopramide 10 μM induced or facilitated the amplitude of any residual after-contractions. This effect of metoclopramide may be mediated via any cholinergic activity not blocked by atropine 1 μM, or an additional activity of metoclopramide on non-cholinergic excitatory neurones. Regardless, the suggestion that metoclopramide acts mostly via a pre-junctional cholinergic site is consistent with similar experiments performed previously using several species (see Introduction) and has been confirmed by direct measurements of cholinergic transmission and acetylcholine release (Tonini et al., 1989; Kilbinger and Wolf, 1992). Thus, an ability of agents to facilitate excitatory nerve-mediated contractions of the isolated stomach may be described as a 'prokinetic-like' activity, given the corresponding ability of 5-HT_4 receptor agonists to increase gastric emptying in vivo. This is an important conclusion, since it follows that gastric prokinetic activity of novel neuropeptides acting within the ENS could also be detected in vitro.

In the mouse distal colon, metoclopramide did not facilitate EFS-evoked after-contractions. A similar lack of activity has been reported using 5-HT_4 receptor agonists in human isolated colon (Burke and Sanger, 1988; Tam et al., 1994) and when measuring the overflow of [^{3}H]-choline from cholinergic neurones in human *Taenia coli* (Burleigh and Trout, 1985). By contrast, the 5-HT_4 receptor agonist prucalopride may facilitate cholinergically mediated contractions of human isolated *T. coli*, during nitric oxide synthesis inhibition (Prins et al., 2000). Similarly, 5-HT_4 receptor agonists may evoke cholinergically mediated contractions in guinea-pig colon (Wardle and Sanger, 1993), facilitate peristalsis in guinea-pig and rat colon (Craig and Clarke, 1990; Kadowaki et al., 2002) and increase mouse defecation in vivo (Sanger et al., 2000), albeit not in all experimental paradigms or with all 5-HT_4 receptor agonists. Metoclopramide, for example, had no effects on colonic transit or motility in rats (Kishibayashi and Karasawa, 1995), mice (Yamada and Onoda, 1992) or dogs (Yoshida et al., 1991). Nevertheless, further experiments are required to understand these inconsistencies and the reasons why gastric and not colonic prokinetic-like activity can be detected more readily in vitro. Apart from the consideration which must be given to the non-selectivity of some 5-HT_4 receptor agonists (see below for metoclopramide), one possibility is that there may be regional differences in the structure and functions of the ENS, particularly in the distribution of the intrinsic primary afferent neurones (IPANs). These enteric neurones are present in the intestine but not necessarily in the stomach, at least in rats (Grady et al., 1996; Mann et al., 1997); IPANs project circumferentially around the intestine and are believed to be sensitive to mechanical and chemical stimuli (Clerc et al., 1999). Consequently, an ability of metoclopramide and ghrelin to facilitate EFS-evoked contractions in the stomach may reflect a more direct influence of these agents on enteric motor-neurones which project longitudinally along the gut and influence muscle contractility and other functions. However, to exert prokinetic activity in the colon, it may be speculated that 5-HT_4 receptor agonists must indirectly influence enteric motor nerve activity, either by directly changing muscle tension (at least in humans, where 5-HT_4 receptor activation can cause muscle relaxation; Tam et al., 1994; McLean and Coupar, 1996) and/or by operating via IPAN's, the activity of which is obscured by the process of EFS. Further experiments are required to examine these suggestions, using different species.

Ghrelin 0.01–1.0 μM facilitated EFS-evoked after-contractions in the mouse forestomach, without affecting baseline muscle tension or carbachol-evoked contractions. Ghrelin may, therefore, act pre-junctionally to increase excitatory nerve function. This suggestion is consistent with similar in vitro studies (Dass et al., 2003b; Edholm et al., 2004) and with an ability of ghrelin to increase gastric emptying (Masuda et al., 2000; Asawaka et al., 2001; Edholm et al., 2004) and reverse gastric stasis (Trudel et al., 2002, 2003; De Winter et al., 2004). Interestingly, the maximal response to ghrelin was smaller than that to metoclopramide. This may reflect a difference in the prokinetic efficacy of 5-HT_4 and ghrelin receptor agonists, or it may be a function of the non-selective pharmacology of metoclopramide. In particular, concentrations of metoclopramide as low as 1 μM may inhibit acetyl-cholinesterase activity (Graham and Crossley, 1995; Chemnitius et al., 1996), an effect expected to increase cholinergically mediated contractions in the current experiments; even higher concentrations of metoclopramide exert local anaesthetic activity (see earlier), an activity consistent with the abilities of high concentrations of metoclopramide to virtually abolish the EFS-induced contractions in both the stomach and colon. Additionally, lower concentrations of metoclopramide also antagonise at dopamine D_2 or 5-HT_3 receptors, but since selective antagonists at these receptors do not generally affect cholinergic activity of the gut (Sanger, 1985a,b, 1987, 1998), it is unlikely that these additional actions will influence our findings. The absence of an effect of ghrelin in the mouse distal colon accords with findings in the rat and human isolated colon (Dass et al., 2003b) and suggests that ghrelin will not affect colonic motility. However, in the light of the earlier discussion on the colonic activity of 5-HT_4 receptor agonists, further experiments with ghrelin are required to look for any colonic

prokinetic activity in models of peristalsis where the full sensory and motor enteric nerve circuitry is operational.

Neither PK2 nor [Nle^{13}]-motilin facilitated EFS-evoked after-contractions in mouse isolated stomach and are thus unlikely to affect gastric motility via the ENS in this species. The results for [Nle^{13}]-motilin are at variance with the ability of motilin receptor agonists to act pre-junctionally and increase EFS-evoked contractions in rabbit isolated stomach (see Introduction). They are also at variance with a reported ability of motilin to increase rat gastric motility in vivo (Valdovinos et al., 1993). However, most studies have not been able to show an excitatory effect of motilin receptor agonists in different models of rat or mouse upper gut motility (Strunz et al., 1975, 1976; Bertaccini and Coruzzi, 1977; Tani and Muto, 1985; De Winter et al., 1999). Together with the current observations, this lack of activity of motilin in rats or mice is consistent with the reported absence of a motilin receptor in rodents (Hill et al., 2002; Aerssens et al., 2004). The reasons for any species-dependent variations in the presence of the motilin receptor are unknown.

The inability of human PK2 to exert prokinetic-like activity is unlikely to be due to species-dependent variations: human PK2 has nanomolar affinity for both human and rodent PK1 and PK2 receptors (Soga et al., 2002; Lin et al., 2002). In the present experiments, PK2 had no effects on baseline muscle tension, an observation contrasting with a report that PK1 and PK2 contracted guinea-pig gut muscle, via a tetrodotoxin-resistant mechanism (Li et al., 2001). Consequently, our experiments do not support the proposal that prokineticin will increase gastrointestinal motility by acting within the gut itself. Nevertheless, without the presence of peptidase inhibitors in the bathing solution to eliminate peptide degradation we cannot confirm that in our experiments, PK2 had not rapidly degraded. Further, and although not contained within the original proposal, the current experiments cannot rule out any activity mediated by the peptide outside the ENS and contributing to changes in gut motility. Such a possibility is argued by the widespread expression of prokineticin (Li et al., 2001) and PK2 receptor (Soga et al., 2002) mRNA to brain areas such as the nucleus tractus solitarius, which may communicate with the stomach via the vagus nerve. Recently, a preliminary report has suggested that prokineticin may indeed increase the emptying of a liquid meal from rat stomach (Lewis, 2004). If confirmed, the functions of this peptide must, therefore, be studied in vivo, using more complex systems than the gut itself.

In conclusion, the abilities of metoclopramide and ghrelin to facilitate cholinergically-mediated contractions of mouse isolated stomach are consistent with their gastric prokinetic functions and hence, the activity observed in vitro can be regarded as 'prokinetic-like'. The absence of a similar activity for [Nle^{13}]-motilin is consistent with the lack of a rodent motilin receptor. An inability to detect prokinetic-like activity with PK2 suggests that if this peptide does exert prokinetic activity, the locus of action is likely to be outside the gut itself. Finally, the inability of any agent to facilitate EFS-evoked responses in the colon suggests that either these agents do not clearly act within the colon of this species or that this technique cannot readily be used to predict colonic prokinetic activity. The reasons for this discrepancy are complex and not clear, and experiments are required in preparations of intestine where the different excitatory and inhibitory nerve phenotypes are pharmacologically isolated and/or in which the full sensory and motor neuronal circuitry can operate.

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