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The rabbit motilin receptor: molecular characterisation and pharmacology

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1 Following identification of the human motilin receptor, we isolated the rabbit orthologue by PCR amplification and found this to be 85% identical to the open reading frame of the human receptor. The protein encoded was 84% identical to the human polypeptide.

2 In HEK293T cells transfected with the rabbit receptor, motilin concentration-dependently increased intracellular calcium mobilisation ($pEC_{50} = 9.25$). After transfection with $G_{o1}\alpha$, motilin similarly stimulated [^{35}S]GTPγS binding ($pEC_{50} = 8.87$). Using both systems, similar values were obtained with the human receptor, with rank-order potencies of motilin = [Nle^{13}]-motilin > erythromycin; ghrelin was ineffective.

3 In circular muscle preparations of rabbit gastric antrum, [Nle^{13}]-motilin 0.1–30 nM concentration-dependently increased the amplitude of electrically-evoked, neuronally-mediated contractions ($pEC_{50} = 8.3$); higher concentrations increased the muscle tension (30–3000 nM). Both responses to [Nle^{13}]-motilin faded rapidly during its continual presence. Rat or human ghrelin 0.01–10 μM were without activity.

4 Erythromycin 30–3000 nM and 10 μM, respectively, increased neuronal activity and muscle tension in rabbit stomach. Unlike [Nle^{13}]-motilin, the increase in neuronal activity did not fade during continual presence of submaximally-effective concentrations of erythromycin; some fade was observed at higher concentrations.

5 We conclude that the pharmacology of the rabbit motilin receptor is similar to the human orthologue and, when expressed as a recombinant, comparable to the native receptor. However, in terms of their ability to increase neuronal activity in rabbit stomach, [Nle^{13}]-motilin and erythromycin are distinguished by different response kinetics, reflecting different rates of ligand degradation and/or interaction with the receptor.

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Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; DEPC, diethylpyrocarbonate; EDTA, ethylenediaminetetraacetic acid; EC_{50}, median effective concentration; EFS, electrical field stimulation; E_{max}, maximally effective concentration; FLIPR, fluorometric imaging plate reader; HBSS, Hanks balanced salts solution; HEK, human embryonic kidney; HEPES, (*N*-[1-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]); [Nle^{13}], norleucine; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SMART, switch mechanism at the 5′ end of RNA transcript

Introduction

There is considerable interest in the possibility that ligands active at the motilin receptor (motilin-R) might play an important role in the treatment of gastrointestinal disorders associated with patterns of hypomotility (see Peeters, 1999). Since the discovery that the antibiotic erythromycin could activate the motilin-R, several derivatives of erythromycin have been identified as being more potent activators of this receptor and effective stimulants of gastric motility. A common feature in the characterisation of these compounds has been the use of rabbit isolated gastrointestinal preparations for radioligand binding or functional pharmacological assays of the receptor (e.g., Sato *et al*., 1997; Clark *et al*., 1999; Tsukamoto *et al*., 2000; Beavers *et al*., 2001; Van Vlem *et al*., 2002). The selection of this species followed the identification of motilin receptors in the gastrointestinal tract of the rabbit (Bormans *et al*., 1986), but its importance as an assay tool may also be at least partly due to the apparent absence of a functional motilin-R in rodent species (Hill *et al*., 2002), commonly used as small laboratory animals. As part of our efforts to identify an animal species in which the motilin-R is both structurally and functionally similar to the human motilin-R, we have isolated, cloned and expressed the rabbit motilin-R, determining its pharmacology using two different functional assays. In addition, we have used a rabbit isolated stomach model of the naturally occurring motilin-R to determine the functional significance of the data obtained using the recombinant receptor, before comparing with published data on the human motilin-R (Feighner *et al*.,

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1999). To achieve this objective, it was first necessary to dissect out and characterise the different neuronal and non-neuronal actions to motilin and erythromycin in the rabbit isolated stomach preparation, to ensure that we compared the correct native tissue responses with those obtained using the recombinant receptor. We conclude that the rabbit motilin-R appears to be a close orthologue of the human receptor, in terms of sequence identity and its functional response to motilin and related compounds. Consequently, this correlation confirms the relevance of the rabbit isolated stomach preparation as a model of the native motilin receptor, to characterise the effects of agonists designed as potential drugs for human use. In addition, the native tissue experiments highlight marked differences in the concentrations of ligands required to stimulate neuronal activity, compared with the higher concentrations which directly contract the muscle, as well as significant differences in the kinetics of the neuronal responses to [Nle^{13}]-motilin and erythromycin. These differences are likely to impact how such agents can be used as therapeutic agents.

Methods

Cloning and sequence analysis

An adult rabbit was killed by CO_2 inhalation overdose followed by cervical dislocation in accordance with Home Office Schedule 1. The entire brain was removed and immediately snap frozen in liquid nitrogen and stored at $-80^{\circ}C$ until required. Total RNA was extracted using the RNeasy Maxi Kit (Qiagen Ltd, U.K.). RNA concentration was measured using an Agilent 2100 bioanalyzer (Agilent Technologies, Germany) and a final concentration of $1\,mg\,ml^{-1}$ was obtained by diluting in diethylpyrocarbonate (DEPC)-treated water. SMART (switch mechanism at the 5′-end of RNA transcript) cDNA was synthesised from $1\,\mu g$ of the isolated total RNA using the SMART RACE cDNA amplification kit (BD Biosciences, U.S.A.). Primers designed to the rabbit (Tan & McKee, 2001) motilin receptor sequence (GPR38) gene (5′ ATGGGCAGCCCCTGGAAC 3′ and 5′ CTATGCAGCCGTCTTTGTGTTAGC 3′) were used to amplify a 1203 base pair fragment in a polymerase chain reaction (PCR) from the SMART cDNA previously synthesised. The fragment generated was cloned into the mammalian expression vector pcDNA3.1/V5/His-TOPO (Invitrogen, U.S.A.), sequenced and confirmed as the gene encoding the rabbit motilin-R.

Cell culture and transfection

The rabbit and human motilin receptors were subcloned into the mammalian expression vector pCDN, and then transiently expressed in HEK293 or HEK293T cells (HEK293 cells stably expressing the SV40 large T-antigen) either alone or in combination with the $G_{i/o}$-family G protein $G_{o1}\alpha$ using Lipofectamine Plus (Life Technologies, U.K.), according to the manufacturer's instructions. $G_{o1}\alpha$ coexpression was used simply to enhance the GTPγS signal output, as previously described by Wise *et al*., (2003).

Measurement of calcium mobilisation

Intracellular calcium assays were carried out as follows. HEK293 cells transiently expressing human or rabbit GPR38 were seeded ($50{,}000\,cells\,well^{-1}$) into poly-D-lysine-coated 96-well black-wall, clear-bottom microtitre plates (Becton Dickinson, U.K.) 24 h prior to assay. Cells were loaded for 1 h with $1\,\mu M$ Fluo-4-AM fluorescent indicator dye (Molecular Probes, The Netherlands) in assay buffer (Hanks balanced salts solution (HBSS), 10 mM HEPES, $200\,\mu M$ Ca^{2+}, 0.1% bovine serum albumin (BSA) and 2.5 mM probenecid), washed three times with assay buffer, and then returned to the incubator for 10 min before assay on a fluorometric imaging plate reader (FLIPR, Molecular Devices, U.K.). Various concentrations of drugs were prepared and added to the cells. The maximum change in fluorescence over baseline was used to determine agonist response. Concentration–response curve data were fitted to a four-parameter logistic equation using GraFit (Erithacus Software Ltd., U.K.).

Measurement of [^{35}S]GTPγS binding

Preparation of membranes Plasma membrane-containing P2 particulate fractions were prepared from cells harvested 48–72 h after transfection. All procedures were carried out at 4°C. Cell pellets were resuspended in 1 ml of 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5 (buffer A), and rupture of the cells was achieved by homogenisation for 20 s with a polytron homogeniser, followed by passage (five times) through a 25-gauge needle. Cell lysates were centrifuged at $1000 \times g$ for 10 min in a microcentrifuge to pellet the nuclei and unbroken cells, and P2 particulate fractions were then recovered by microcentrifugation at $16{,}000 \times g$ for 30 min. P2 particulate fractions were resuspended in buffer A and stored at $-80^{\circ}C$ until required. Protein concentrations were determined using the bicinchoninic acid (BCA) procedure (Smith *et al*., 1985) using BSA as a standard.

High-affinity [^{35}S]GTPγS binding Assays were performed in 96-well format using a method modified from that described in Wieland & Jakobs (1994). Membranes ($10\,\mu g$ per point) were diluted to $0.083\,mg\,ml^{-1.}$ in an assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) and preincubated with $10\,\mu M$ GDP. Various concentrations of the agonist were then added, followed by [^{35}S]GTPγS ($1170\,Ci\,mmol^{-1}$, Amersham, U.K.) at 0.3 nM (total volume of $100\,\mu l$), and binding was allowed to proceed at room temperature for 30 min. Nonspecific binding was determined by the inclusion of 0.6 mM GTP. Wheatgerm agglutinin SPA beads (Amersham, U.K.) (0.5 mg) in $25\,\mu l$ assay buffer were added and the whole was incubated at room temperature for 30 min with agitation. Plates were centrifuged at $1500 \times g$ for 5 min and [^{35}S]GTPγS bound was determined by scintillation counting on a Wallac 1450 microbeta Trilux scintillation counter.

Rabbit isolated gastric antrum

Adult male New Zealand White rabbits (1.5–2.5 kg) were culled by pentobarbitone overdose followed by cervical dislocation. All efforts were made to minimise the number of animals used and culling was performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986. Following a midline incision, whole stomachs were blunt-dissected and placed immediately in ice-cold 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) previously equilibrated with 5% CO_2 in O_2. Full wall thickness preparations of gastric antrum ($\sim 15 \times \sim 8$ mm) were cut parallel to the circular muscle, and the mucosa and submucosa were removed by gentle peeling and sharp dissection.

Tissues were suspended under 10 mN of tension for isometric recording between two parallel platinum ring electrodes in 5 ml tissue baths containing Krebs solution bubbled with 5% CO_2/95% O_2, maintained at pH 7.4 ± 0.1 and 37°C. Tension was measured using Pioden dynamometer UF1 force–displacement transducers (Pioden Control Ltd, U.K.). Data acquisition and analysis were performed using MP100 hardware and AcqKnowledge® software (Biopac Systems, Inc., U.S.A.). Tissues were allowed to equilibrate for at least 60 min, during which time the bath solutions were changed every 15 min. Electrical field stimulation (EFS) was achieved using monophasic square-wave pulses of 0.5 ms pulse width and 5 Hz frequency at a maximally effective voltage (70–80 V; Digitimer, U.K.). These parameters of EFS were selected as those which consistently evoked nerve-mediated responses with a good signal-to-noise ratio over background spontaneous muscle activity. EFS was applied for 20 s at 2 min intervals for 30 min periods; each period was separated by a 5 min interval in which the bath solutions were changed. After obtaining consistent EFS-evoked contractions, the effects of pharmacological agents, applied noncumulatively, on both resting muscle tension and on responses to EFS were measured and expressed as a percentage of the mean of at least two pre-drug EFS contractile responses, which was expressed as 100%. Direct muscle contractions were expressed as a percentage of the contractile amplitude induced by a maximally effective concentration of carbachol, 100 μM. $p\mathrm{EC}_{50}$ is the negative logarithm to base 10 of the EC_{50} value, which is the concentration of the agonist that produces 50% of the maximal response. E_{max} denotes the maximal response achieved by the drug. Data are expressed as means ± standard error of the mean; n values are the numbers of animals used. Differences between the means were determined using a Student's t-test for paired data; $P < 0.05$ is considered as statistically significant.

Drugs used

All drugs were freshly prepared prior to use. Human motilin was obtained from Sigma, U.K. The norleucine[13] analogue of porcine motilin, [Nle[13]]-motilin (Calbiochem, U.S.A.), was dissolved in distilled water (dH_2O). Erythromycin (Sigma, U.K.) was dissolved in neat ethanol with subsequent dilutions in 50 : 50 100% ethanol : dH_2O for native tissue studies. Human and rat ghrelin were obtained from Bachem Ltd (U.K.), and were prepared in 0.9% NaCl containing 0.01% BSA (Sigma, U.K.). The muscarinic receptor agonist and antagonist, carbachol and atropine (Sigma, U.K.), respectively, and the nerve toxin tetrodotoxin (Tocris, U.K.) were all also dissolved in dH_2O.

Results

Receptor sequence

Sequence analysis of the fragment generated from the PCR on the rabbit brain cDNA revealed an open reading frame (ORF) of 1203 base pairs. It was found to be 85% identical to the ORF of human motilin receptor and the protein it encoded was determined to be 84% identical to the human receptor polypeptide (Figure 1).

Pharmacology of the recombinant receptor

Rabbit and human motilin receptors, transiently expressed in HEK293 cells, were tested in a calcium mobilisation (FLIPR) assay. In these experiments, the $p\mathrm{EC}_{50}$ values for motilin were, respectively, 9.01 and 9.79. In a second study, the effects of motilin were compared with [Nle[13]]-motilin, erythromycin and ghrelin. A similar rank order of potency was obtained for these ligands at the receptors from both species (motilin = [Nle[13]]-motilin > erythromycin; ghrelin was without activity at the 300 nM concentration tested; Table 1).

Transfection of the rabbit and human motilin receptors into HEK293T cells together with the G protein $G_{o1}\alpha$ led to a concentration-dependent stimulation of [^{35}S]GTPγS binding. The $p\mathrm{EC}_{50}$ values for motilin at the rabbit and human receptors were 8.87 and 8.89, respectively. Similar potencies were obtained for [Nle[13]]-motilin at both species receptors and ghrelin, as in the FLIPR assay, was inactive at the concentration tested (300 nM; Table 1).

Pharmacology in rabbit isolated gastric antrum

In the majority of tissue preparations, EFS evoked a monophasic contractile response. These contractions were prevented during the presence of 3 μM tetrodotoxin ($n = 4$, 30 min contact) and attenuated by 50 ± 7% during the presence of 1 μM atropine ($n = 10$, 30 min contact). [Nle[13]]-motilin (1–30 nM, 15 min contact) had no measurable effect on baseline muscle tension, but concentration-dependently facilitated electrically-stimulated, nerve-mediated contractions in the isolated gastric antrum, with a $p\mathrm{EC}_{50}$ value of 8.3 ± 0.2

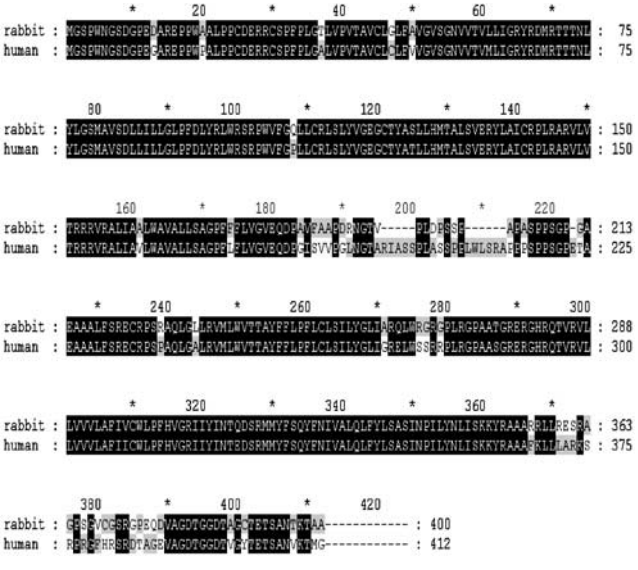

Figure 1 Protein sequence alignment of rabbit and human motilin receptors. Identical residues are shaded in black, differences are shaded in grey and gaps in the alignment are represented by hyphens. The rabbit sequence is from patent WO200132710 and the human sequence is deposited on GenBank, accession number NM_001507.

Table 1 Activation by motilin and related compounds of the recombinant rabbit and human motilin receptors transiently expressed in HEK 293 cells

Ligand	*Rabbit motilin receptor*		*Human motilin receptor*	
(a) Calcium mobilisation				
Motilin	9.01±0.29	$n=5$	9.79±0.14	$n=4$
[Nle^{13}]-motilin	9.07±0.26	$n=6$	9.36±0.14	$n=4$
Erythromycin	6.72±0.15	$n=6$	6.29±0.23	$n=4$
Human ghrelin	>300 nM	$n=5$	>300 nM	$n=4$
(b) [^{35}S]GTPγS binding				
Motilin	8.87±0.03	$n=5$	8.89±0.02	$n=5$
[Nle^{13}]-motilin	8.77±0.06	$n=3$	8.71±0.04	$n=3$
Erythromycin	6.13±0.09	$n=3$	Not determined	
Human ghrelin	>300 nM	$n=3$	>300 nM	$n=3$

Data are expressed as pEC_{50} values±s.e.m. Receptor function was assessed using (a) a calcium mobilisation (FLIPR) assay and (b) [^{35}S]GTPγS binding.

($n=5$–15, each concentration; Figure 2) and an E_{max} of 253±56%. This response to [Nle^{13}]-motilin was not immediate; for example, at 10 nM [Nle^{13}]-motilin (approximate to pEC_{50} value), the time taken for the excitatory response to peak was 6.6±0.6 min ($n=17$). At concentrations ⩾10 nM, this maximal excitatory response subsequently decreased rapidly during the continuous presence of [Nle^{13}]-motilin (Figure 3), in that the time taken for the response, induced by 10 nM, to decline by 50% from maximum ($t_{1/2}$) was 5.0±0.6 min ($n=12$). After washout of the bathing solution containing the ligand (8 s overflow flush, 20 min apart), a repeat application of [Nle^{13}]-motilin, 10 nM, separated by 60 min, potentiated the nerve-evoked contractions by a similar extent (161±34% increase *versus* 166±43%; Figure 4).

In the absence of electrical stimulation, [Nle^{13}]-motilin (30–3000 nM, 5 min contact) concentration-dependently induced monophasic tonic contractions, with a pEC_{50} value of 6.8±0.4 and an E_{max} value of 19±3% ($n=3$–9, each concentration; Figure 5). At 0.1 μM (approximate pEC_{50} value), the time taken for the contraction to peak was 2.1±0.2 min ($n=9$), with a $t_{1/2}$ value of 1.9±0.5 min ($n=8$). The threshold concentration at which [Nle^{13}]-motilin induced direct muscle contraction varied between animals; however, concentrations ⩾30 nM consistently induced contractions in all preparations examined. After washout of the ligand-containing bathing solution, a repeat application of [Nle^{13}]-motilin 1 μM, separated by 60 min, in the absence of electrical stimulation, did not induce muscle contractions of similar amplitude (12.4±2 and 3.6±2%; Figure 4). However, under similar conditions, a submaximally effective concentration of carbachol 10 μM, with a 60 min interval, induced contractions of similar amplitudes (80±3 and 77±4%; Figure 4).

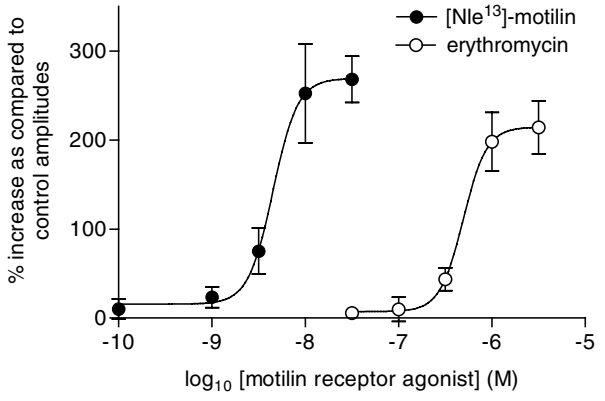

Figure 2 Concentration-dependant potentiation of the electrically-stimulated, nerve-mediated excitatory response in rabbit isolated gastric antrum circular strips by the motilin receptor agonists [Nle^{13}]-motilin and erythromycin. The data were expressed as the percentage increase in the nerve-evoked response as compared to predrug contractile amplitudes. $N=5$–19 at each concentration.

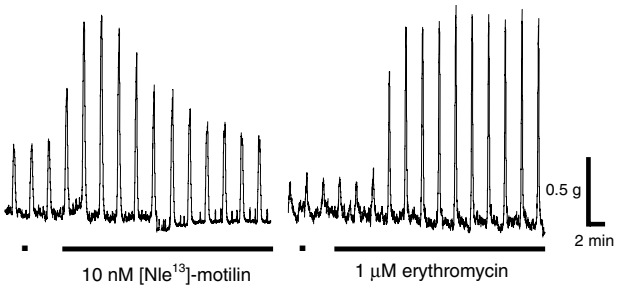

Figure 3 Facilitation of cholinergic motor nerves by [Nle^{13}]-motilin (human/porcine) and erythromycin in rabbit isolated gastric antrum. The response to [Nle^{13}]-motilin decreased rapidly, which was not observed in response to erythromycin. The dots represent EFS: 70–80 V, 20 s duration, 5 Hz, 0.5 ms pulse width every 120 s.

Erythromycin (0.03–3 μM) had no observable effect on baseline muscle tension but concentration-dependently facilitated electrically-stimulated, nerve-evoked contractions; pEC_{50} was 6.3±0.1 and E_{max} was 217±27% ($n=8$–19, each concentration; Figure 2). At 1 μM (approximate pEC_{50} value), the time taken for the maximal response was 9.1±1.2 min ($n=16$) and, unlike [Nle^{13}]-motilin 10 nM, the response to erythromycin did not fade significantly from the maximum response, over a 15 min observation period ($n=13$). However, the response to 3 μM erythromycin, which produced the greatest increase in amplitude of the nerve-evoked contractions, did begin to fade during its continual presence, but $t_{1/2}$ was >30 min observation period ($n=5$). A repeat application of erythromycin 3 μM, separated by 60 min, did not potentiate the nerve-evoked contractions by a similar amount (293±64 *versus* 130±30%, $n=10$, $P=0.02$), whereas repeat application of erythromycin 1 μM did (197±45 *versus* 168±32%, Figure 4). In the continued presence of [Nle^{13}]-motilin 10 nM, and following fade of the response to this application, the subsequent addition of erythromycin 1 μM to the bathing solution did not potentiate the nerve-evoked contractions, 7±21% ($n=5$), as compared to control nerve-evoked amplitudes.

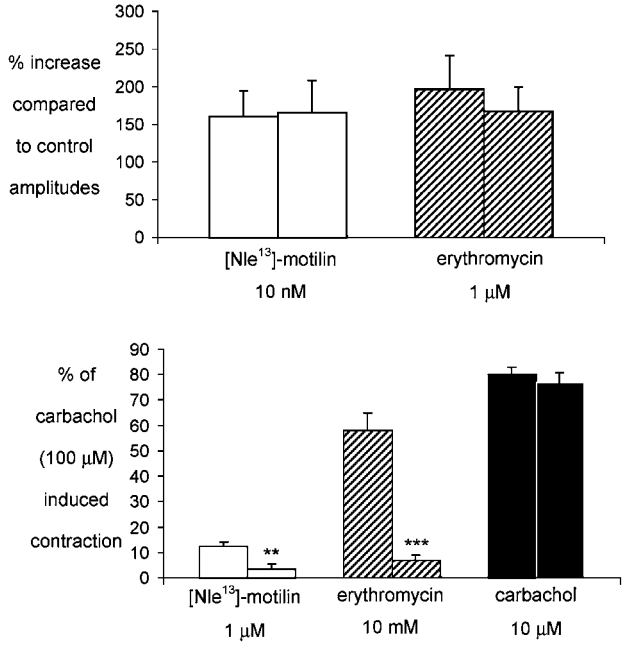

Figure 4 The effects of repeat applications of [Nle^{13}]-motilin or erythromycin in rabbit isolated gastric antrum. (a) Repeat application of [Nle^{13}]-motilin 10 nM and erythromycin 1 μM, separated by 60 min, potentiated the nerve-evoked contractions by a similar extent; [Nle^{13}]-motilin: $n=13$, $P>0.05$; erythromycin: $n=5$, $P>0.05$. (b) Repeat applications of [Nle^{13}]-motilin 1 μM and erythromycin 10 mM, separated by 60 min, in the absence of electrical stimulation, did not induce muscle contractions of similar amplitude; [Nle^{13}]-motilin: $n=5$, $^{**}P=0.01$; erythromycin: $n=3$, $^{***}P=0.001$. However, under similar conditions, a submaximally effective concentration of carbachol 10 μM, with a 60 min interval, induced contractions of similar amplitudes; $n=4$, $P>0.05$. The second column represents the responses on repeat dose, in both the graphs.

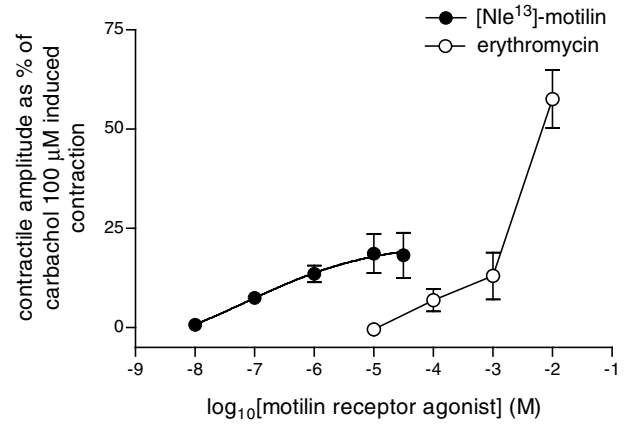

Figure 5 Concentration-dependant contraction of rabbit isolated gastric antrum circular smooth muscle in response to [Nle^{13}]-motilin and erythromycin. The data were expressed as the percentage of the contractile response induced by 100 μM carbachol. $N=5$–19 at each concentration.

In the absence of electrical stimulation, 0.1–10 mM erythromycin concentration-dependently induced monophasic tonic contractions ($n=3$–6, each concentration). Erythromycin, $\geqslant 0.1$ mM, consistently induced muscle contraction in all preparations examined. At 1 mM, the peak contraction was similar to the peak contractions evoked by the highest concentrations of motilin (Figure 5), and this was achieved in 0.9 ± 0.4 min ($n=5$) with a $t_{1/2}$ value of 2.4 ± 0.3 min ($n=5$). A higher concentration of erythromycin (10 mM) evoked a rapid, marked contraction of the muscle, which was considerably larger than that evoked by either motilin or the lower concentrations of erythromycin (Figure 5). After washout of erythromycin from the bathing solution, a repeat application of erythromycin 10 mM, separated by 60 min, in the absence of electrical stimulation, showed a significant reduction in the contractile amplitude (58 ± 7 and 7 ± 5%, Figure 4).

In separate experiments, the effects of [Nle^{13}]-motilin and erythromycin were determined against contractions induced by carbachol, at a concentration previously determined to be submaximally effective (10 μM; 60 s contact time). [Nle^{13}]-motilin 10 nM or erythromycin 0.1 μM had no effects on the amplitude of carbachol-induced contractions (82 ± 4 and 65 ± 5% and 85 ± 3 and 67 ± 4% in their absence and presence, respectively, $P>0.05$). In addition, contractions induced by [Nle^{13}]-motilin 10 μM or erythromycin 10 mM, in the presence of TTX 1 μM, did not vary in their amplitudes (10.2 ± 0.4 and 8.4 ± 0.8% and 57 ± 7 and 56 ± 7%, $n=3$ for each, respectively).

Neither the rat nor human form of ghrelin (0.01–10 μM, 15 min contact, $n=4$–8 at each concentration) had an observable effect on baseline muscle tension or nerve-evoked contractions. For example, the changes in the amplitudes of the nerve-evoked contractions in response to 0.1 μM rat and human ghrelin were -4.7 ± 2.5 and 1.3 ± 2.8%, respectively.

Discussion

In our studies with the native motilin receptor expressed within rabbit isolated stomach, we used the more stable and approximately equipotent analogue of porcine motilin, [Nle^{13}]-motilin, to minimise variations caused by degradation of the peptide (see Kitazawa *et al*., 1993; Feighner *et al*., 1999). [Nle^{13}]-motilin has been shown to have the same potency as porcine motilin in the rabbit gastric antrum, with porcine motilin also being approximately equipotent to rabbit motilin (Peeters *et al*., 1986; Van Assche *et al*., 1997). Using [Nle^{13}]-motilin, we identified both neuronal and non-neuronal actions, these findings being consistent with those previously reported by others. Thus, in rabbit isolated gastric and intestinal preparations, motilin, erythromycin or related peptides and compounds appear to be capable of binding to and activating receptors on both the intrinsic neurones of the gut as well as on the smooth muscle itself (Kitazawa *et al*., 1993; Parkman *et al*., 1995; Poitras *et al*., 1996; Van Assche *et al*., 1997; Miller *et al*., 2000a, b). In particular, Van Assche *et al*. (1997) showed that in rabbit isolated stomach, the ability of motilin to increase cholinergic contractile activity was exerted at lower concentrations (pEC_{50} 8.73) than those required to directly contract the muscle itself. This observation was confirmed in the present study in which we demonstrated a similar potent ability of [Nle^{13}]-motilin to increase cholinergically-mediated contractions (pEC_{50} 8.3), compared with the higher concentrations required to induce direct muscle contraction (pEC_{50} 6.8). In separate experiments, a high concentration of motilin had no ability to facilitate contractions of the muscle evoked by carbachol, suggesting that the ability of this peptide to facilitate neuronally-mediated contractions was due to inactivity at a prejunctional locus. For both the nerve and, more

especially, the muscle systems, the responses to motilin faded rapidly during the continuous presence of the peptide. It has been suggested that these two responses to motilin are mediated *via* two different forms of the motilin receptor. Thus, Poitras *et al*. (1996) and Miller *et al*. (2000b) found some variations in the affinities of motilin peptide fragments in neuronal or muscle membrane-enriched fractions of rabbit gastrointestinal tissues. However, to date, only a dramatically truncated variant of the human receptor mRNA has been identified, in which two of the seven transmembrane domains are deleted (Feighner *et al*., 1999). Accordingly, although the rabbit genome has not been sequenced and putative receptor variants searched for, a simpler explanation for our data is that the nerve- and muscle-mediated responses of motilin may be attributed to a differential coupling of the same receptor to different downstream effector mechanisms within the different nerve and muscle cell types.

In our studies with the rabbit stomach, we found that erythromycin behaved similarly to that of [Nle^{13}]-motilin, acting prejunctionally to increase the nerve-mediated contractions (pEC$_{50}$ 6.3) and at higher concentrations (100 μM or above), directly increasing muscle tone. The direct effect of low concentrations of erythromycin on muscle tone was similar in amplitude to that evoked by motilin, but at the highest concentration of erythromycin used (10 mM), a sharp, additional rise in muscle tone was observed. Further cross-desensitisation experiments are now required to see if this 'additional' activity of erythromycin operates *via* the motilin receptor or *via* some other site of action. In terms of overall potency, the effective micromolar concentrations of erythromycin (for both the nerve and muscle responses) were approximately 2-log units less potent than motilin. Similar potency values have been reported by others using ^{125}I-motilin binding studies (Miller *et al*., 2000a) or measurement of smooth muscle tension in rabbit isolated gastrointestinal tissues (Parkman *et al*., 1995; Thielemans *et al*., 2002). Similarly, in humans, higher doses of erythromycin (200 mg) were required to induce atropine-insensitive contractions of the stomach, compared to the lower concentrations (40 mg) which induced responses sensitive to prevention by atropine (Coulie *et al*., 1998).

Compared with [Nle^{13}]-motilin, the ratio of the concentrations of erythromycin required to elicit the nerve- and the muscle-mediated responses was approximately similar. However, whereas the increase in muscle tension faded rapidly during the continuous presence of erythromycin, the ability of erythromycin to increase the cholinergically-mediated contractions was sustained during its continuous presence, with some fade being detected only at the highest effective concentration. This type of comparison, between the kinetics of the different responses to the different receptor agonists, has not previously been explored. Further work is required to understand the reason for the different kinetic responses, but two possible explanations are worthy of consideration. Firstly, [Nle^{13}]-motilin is a peptide; so the rapidly fading nature of the responses generated by this ligand might simply be due to its degradation within the tissue. Nevertheless, the abilities of [Nle^{13}]-motilin and erythromycin to directly increase muscle tone faded rapidly at rates that were approximately similar and faster than that recorded for the nerve-mediated response to [Nle^{13}]-motilin. Accordingly, these observations raise the additional possibility that the different kinetics of the nerve-mediated responses to [Nle^{13}]-motilin and erythromycin may be attributed, at least partly, to a different mechanism by which these ligands couple to the receptor. This difference in the kinetics of the response to the two ligands is, nevertheless, likely to operate *via* a common receptor, since the response to erythromycin could be prevented by the continuous presence of motilin, at a time following that in which the response to motilin itself had faded. Further, the observation that during these conditions [Nle^{13}]-motilin was still able to exert significant biological activity lends weight to the arguments that any degradation of [Nle^{13}]-motilin plays only a small part in the mechanism by which the nerve-mediated response to this peptide fades with time, and that important differences exist between the manner by which motilin and erythromycin excite enteric nerve activity.

The structure of the rabbit motilin receptor showed an 84% amino-acid identity with the human motilin receptor, suggesting a high conservation of receptor function across the two species. Such a possibility would be consistent with the findings of Miller *et al*. (2000a), who undertook ^{125}I-motilin binding studies with partially purified nerve membrane fractions from human and rabbit antrum. In these studies, similar pIC$_{50}$ values were obtained in both species with motilin or erythromycin, albeit this similarity was lost when examining the affinities of different peptide fragments of motilin. Similarly, Feighner *et al*. (1999) and Thielemans *et al*. (2002) report similarities between the potency of a range of motilin peptide derivatives using the cloned human motilin receptor and various radioligand-binding or smooth muscle-contractility assays from (undefined) rabbit gastrointestinal tissues.

In our present studies, the different pEC$_{50}$ values of 9.8 and 8.9 for motilin, obtained using the recombinant human receptor and the two assay systems (respectively, calcium mobilisation and [^{35}S]GTPγS binding), may reflect the different levels of response amplification created by the two assay systems. It remains a further possibility that the coupling of the receptor to the G$_{o1}\alpha$ protein does not accurately reflect the coupling of the native receptor. Nevertheless, our data are approximately consistent with that reported in other studies by Thielemans *et al*. (2002) using [Leu^{13}]-motilin, and by Feighner *et al*. (1999) using [Leu^{13}]-motilin and motilin. In addition, for the recombinant rabbit receptor, the values obtained using the same two assays were similar (respectively, 9.0 and 8.9 for motilin, and 9.1 and 8.8 for [Nle^{13}]-motilin), and only an approximately half-log unit higher than that obtained using the rabbit gastric antrum itself, perhaps reflecting at least some susceptibility of the peptide to degradative processes in the native tissue. Overall, the rank orders of the ligands tested against the recombinant rabbit motilin receptor, using both assay systems, were of similar order to that found using the neuronal assay in the isolated stomach.

Sequence analysis of the human motilin receptor suggests that its nearest family member is the growth hormone secretagogue receptor (GHS-R) activated by the peptide ghrelin and exhibiting an overall 50% amino-acid identity between the two receptors (Asakawa *et al*., 2001). This structural relationship extends to at least some of the functions of these peptides, with both motilin and ghrelin being released from the stomach during fasting conditions, and both being capable of stimulating gastric motility and emptying (e.g., Asakawa *et al*., 2001). Nevertheless, in the present study, we found no ability of high concentrations of the rat and/or

human forms of ghrelin to activate either the recombinant or the native rabbit motilin receptor, consistent with the findings of Depoortere *et al*. (2003). Although we cannot rule out the possibility that a hitherto undiscovered rabbit form of ghrelin may have affinity for the rabbit motilin receptor, these data suggest that any apparent relationship between the motilin and ghrelin peptides does not extend to a cross-sensitivity of the motilin and ghrelin receptors for each other's natural ligand.

In conclusion, we have cloned and expressed the rabbit motilin receptor, and have shown that the sequence of this receptor has close similarity with that of the human motilin receptor. Further, the functional potency of ligands active at this recombinant receptor is approximately consistent with that obtained using the native receptor in rabbit gastric antrum, provided care is taken to measure the neuronal response to these ligands. Thus, in the native system, two different responses to motilin and erythromycin are apparent and these are distinguished by the cell type mediating the response, the different effective concentrations and by the different kinetics of the responses generated by the different ligands. Such differences may be critical to the therapeutic use of motilin receptor agonists as gastric prokinetic agents, since they suggest that high doses will directly contract the muscle, exacerbating symptoms of dyspepsia, whereas lower doses would only facilitate neuronal function, increasing gastric motility and exerting therapeutic benefit.

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