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Delineation of the motilin domain involved in desensitization and internalization of the motilin receptor by using full and partial antagonists

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ABSTRACT

Studies with fragments of the gastrointestinal peptide, motilin, indicate that the C-terminal region of this peptide plays an important role in the desensitization of the motilin receptor (MTLR).

Aim: To verify this hypothesis we studied the desensitization, phosphorylation and internalization induced by motilin analogues of different chain length with agonistic and antagonistic properties in CHO–MTLR cells.

Methods: We studied motilin [1–22], the [1–14] fragment, the analogues Phe^3[1–22] and Phe^3[1–14], and two putative antagonists, GM-109 and MA-2029 (modified 1–4 and 1–3 fragments). Activation and desensitization (2 h preincubation with the motilin analogues 10 μM) were studied in CHO–MTLR cells by an aequorin based luminescence assay. Phosphorylation was studied by immunoprecipitation and internalization was visualized in CHO–MTLR cells containing an enhanced green fluorescent protein (CHO–MTLR–EGFP).

Results: Motilin [1–22] and [1–14] were more potent than Phe^3[1–22] and Phe^3[1–14] (pEC_{50}: 9.77, 8.78, 7.36 and 6.65, respectively) to induce Ca^{2+} release. GM-109 and MA-2029 were without agonist activity. [1–22] and Phe^3[1–22] decreased the second response to motilin from 78 ± 2% to 11 ± 3% and 34 ± 3% ($P < 0.001$), respectively, whereas [1–14], Phe^3[1–14], GM-109 and MA-2029 had no desensitizing effect (68 ± 5%, 78 ± 3%, 78 ± 6% and 78 ± 5%, respectively, $P > 0.05$). The rank order of MTLR-phosphorylation was: [1–22] > [1–14] > Phe^3[1–22] = Phe^3[1–14] > GM-109 = MA-2029. Only motilin [1–22] and [1–14] induced receptor MTLR–EGFP internalization as shown by a decrease in membrane fluorescence: 20 ± 3% and 7 ± 3%, respectively.

Conclusion: The C-terminus of motilin enhances desensitization, phosphorylation and internalization of the MTLR while modifications of the N-terminus can favor a conformation of the receptor that is less susceptible to phosphorylation and internalization.

1. Introduction

The 22 amino acid peptide, motilin, is an important endocrine regulator of gastro-intestinal motility, involved in the induction of the migrating motor complex during the interdigestive period [1,2]. The finding that the antibiotic erythromycin is a motilin agonist and that motilin and erythromycin accelerate gastric emptying in patients with diabetic gastroparesis has stimulated research for the development of motilin agonists as therapeutic agents in the treatment of hypomotility

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disorders [3–7]. As a result, more potent erythromycin derivatives with motilin agonistic properties were developed without antibacterial activity: the motilides. However the most promising one, ABT-229, failed in clinical trials [8,9].

Desensitization or the loss in the responsiveness of a signaling system, protecting the cell against both acute and chronic overstimulation, significantly limits the therapeutic usefulness of many receptor agonists and tachyphylaxis may have contributed to the failure of the motilide ABT-229 in clinical trials [10,11]. Indeed, using a cellular model of Chinese hamster ovary CHO cells expressing the motilin receptor, Thielemans et al. [12] showed that motilides markedly differ in their ability to desensitize the motilin receptor which was not solely determined by their potency. In particular ABT-229 had a much stronger desensitizing potency than may be expected from its activity. The decline of the contractile response in duodenal segments after stimulation with motilides correlated with the potency to desensitize, validating the model of the CHO cells. Consistent with this finding is a recent study of Lamian et al. [13] on agonist-induced motilin trafficking. These authors clearly showed that intracellular trafficking of identical motilin receptor molecules varies substantially with different ligands according to their distinct agonist properties.

Three distinct regions have been delineated in the interaction of motilin with its receptor: the N-terminal region (amino acids 1–7) constitutes the minimal basic unit of binding and activity. By using motilin fragments and performing a D-amino acid and alanine scan it was shown that the pharmacophore consists of the aromatic rings from Phe^1 and Tyr^7 and the aliphatic side chains of Val^2 and Ile^4 [14–16]. A transition region (amino acids 8–9) links the N-terminal and C-terminal regions; and the C-terminal region (amino acids 10–22) forms an alpha-helix that stabilizes the interaction of the N-terminal residues at the active site [17]. However the alpha-helix may also play another role. Studies with motilin fragments indicated that the C-terminal domain of motilin may play an important role in the desensitization process since C-terminal elongation of the motilin (1–14) fragment promoted full desensitization [18].

In order to confirm the separation between N-terminal activation and C-terminal desensitization we tested the ability of full and partial motilin agonists and antagonists of different chain length to induce desensitization of the motilin receptor expressed in the CHO–MTLR system. We used [Leu^{13}]-motilin (1–22) and [Leu^{13}]-motilin (1–14), which lacks a large part of the C-terminus but is nevertheless a full agonist. It has been shown that substitution of residue 3, proline, by phenylalanine introduces antagonist properties [19,20]. Therefore we also used [Phe^3, Leu^{13}]-motilin (1–22) and [Phe^3, Leu^{13}]-motilin (1–14), as well as two other putative antagonists GM-109 and MA-2029. GM-109 is a modified cyclical analogue of the [1–4] fragment of motilin. MA-2029 is a linear modified analogue of the [1–3] fragment of motilin. The different compounds will further be referred to as [1–22], [1–14], Phe^3[1–22], Phe^3[1–14], GM-109 and MA-2029. By correlating changes in receptor binding affinity with potency to induce Ca^{2+} fluxes, to phosphorylate and internalize (visualized by using a CHO–MTLR–EGFP system), we aimed to elucidate how activation, desensitization and internalization are linked.

2. Materials and methods

2.1. Test compounds

Norleucine^{13}-procine-motilin (motilin) was purchased from Eurogentec (Namur, Belgium). The N-terminal 1–14 fragment of porcine[Leu^{13}]motilin and the antagonists [Phe^3, Leu^{13}]-porcine-motilin (1–14) and [Phe^3, Leu^{13}]porcine-motilin (1–22) were synthesized as previously described [14,16] whereas the antagonists GM-109 (Phe-cyclo[Lys-Tyr(3-tBu)-Ala-.trifluoroacetate) and MA-2029 (*N*-ethyl-(4-fluoro-*N*-methyl-L-phenylalanyl-*N*-methyl-L-valyl)-3-*tert*-butyl-L-tyrosinamide hydrochloride hydrate) were a gift from Dr. H. Takanashi and O. Cynshi (Chugai Pharmaceutical Company, Gotemba, Japan).

2.2. Cell culture

A Chinese hamster ovary cell line (CHO) stably expressing the human motilin receptor (CHO–MTLR) and the mitochondrially targeted Ca^{2+} indicator apoaequorin were obtained from Euroscreen SA (Brussels, Belgium). Construction of CHO cells expressing apoaequorin and the MTLR C-terminally tagged with an EGFP (enhanced green fluorescent protein) (CHO–MTLR–EGFP) was performed as previously described [12]. The cells were cultured at 37 °C in Ham's F12 containing 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 2.5 μg amphotericin B (Invitrogen, Carlsbad, CA) and 400 μg G418 (Sigma, St. Louis, MO, USA) and spliced weekly with 5 mM EDTA in phosphate buffered saline (PBS).

2.3. Receptor binding studies

Confluent (90%) CHO–MTLR cells were scraped from the plates in Ca^{2+}/Mg^{2+} free PBS and centrifuged (3 min, 1500 × *g*). Pellets were re-suspended in a buffer containing 15 mM Tris–HCl pH 7.5; 2 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA and homogenized. The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000 × *g* for 25 min separated by a washing step in the same buffer. The final pellet was re-suspended in a buffer containing 7.5 mM Tris–HCl pH 7.5; 12.5 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA; 250 mM sucrose, and flash frozen in liquid nitrogen.

The protein content was determined by the Folin method [21]. Receptor binding studies were performed by incubating CHO–MTLR membranes (4 μg of protein/tube) in a final volume of 0.1 ml with [^{125}I]-motilin (0.5 nM) for 60 min at 30 °C. Displacement curves were obtained by adding increasing concentrations (10^{-11} to 10^{-5} M) of unlabelled test compound in binding buffer (25 mM Hepes pH 7.4; 1 mM $CaCl_2$; 5 mM $MgCl_2$; 0.5% BSA). After incubation, the samples were filtered on GF/B filters presoaked for at least 1 h in 0.5% BSA. Filters were washed three times with ice cold washing buffer (same composition as binding buffer, supplemented with 0.5 M NaCl), collected and counted in a gamma counter. All values were corrected for non-specific binding determined by addition of an excess (10^{-5} M) of unlabelled motilin. Each compound was tested three times in duplicate. pIC_{50} values were determined by non-linear regression and motilin receptor density (B_{max}) was estimated by analysis of

homologous competition curves using GraphPad Prism 4.0 software (San Diego, CA, USA).

2.4. Ca^{2+} measurements: aequorin based luminescence assay

2.4.1. Receptor activation

Agonist-induced Ca^{2+} fluxes in the CHO–MTLR cell line were determined by measuring the luminescence response emitted after binding of the released Ca^{2+} by aequorin as previously described [22]. Briefly, cells were incubated with 5 μM coelenterazine h (Molecular Probes, Leiden, The Netherlands) for at least 2 h at room temperature in order to reconstitute active aequorin. Cells were then diluted 10-fold and concentration–response curves were obtained by injection of cells (i.e. 50,000 cells) in each well of a 96-well plate, containing 100 μl aliquots of increasing concentrations (10^{-12} to 10^{-5} M) of [1–22], Phe^3[1–22], [1–14], Phe^3[1–14], GM-109 and MA-2029 diluted in BSA-medium. After injection of the cells, the emitted light was recorded during 20 s by using the 'Microlumat plus' luminometer (Berthold Technologies, Bad Wildbad, Germany). To screen the antagonistic properties of the compounds, their ability to inhibit the Ca^{2+} luminescent response to motilin was determined. For this purpose pre-loaded cells were injected in a 96-well plate containing 100 μl aliquots of a dose–response curve to motilin (10^{-12} to 10^{-5} M) and a fixed concentration in each well of 10^{-6} M of Phe^3[1–22], Phe^3[1–14], GM-109 or MA-2029. The Ca^{2+} response was expressed as a percentage of maximal stimulation obtained with Triton X-100 (0.9%). pEC_{50} values were determined by non-linear regression using GraphPad Prism 4.0 software (San Diego, CA, USA). All experiments were performed in duplicate and each compound was tested at least three times.

2.4.2. Receptor desensitization

To induce desensitization, cells were pre-stimulated with the test compound at 10^{-5} M or buffer for 2 h at 37 °C during loading with coelenterazine h, washed and centrifuged before dose–response curves to motilin were established. The degree of desensitization was calculated from the shift and the depression of the dose–response curves to motilin of pre-treated compared to buffer-treated cells.

2.5. Receptor phosphorylation

Before the start of the experiment, confluent CHO–MTLR–EGFP cells were pre-incubated at 37 °C for 24 h with 10 ml phosphate-free DMEM (Invitrogen, Carlsbad, CA). On the day of the experiment, cells were incubated with 200 μCi/ml [^{32}P]orthophosphate (Amersham Biosciences) in phosphate-free DMEM for 2 h at 37 °C while shaking. Loaded cells were then stimulated with 10^{-5} M of the test compound for 10 min at 37 °C and washed three times on ice with ice cold KRH buffer (25 mM HEPES, 124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4 and 8 mM glucose, pH 7.4). Cells were treated with lysis buffer (20 mM Tris, 100 mM $(NH_4)_2SO_4$, 10% glycerol, 1 mM $NaVO_3$, 0.03 μM okadaic acid, pH 7.5), containing protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA). The collected cell suspension was subjected to one cycle of quick freezing, using liquid nitrogen, and subsequent thawing. Cell lysates were cleared by centrifugation at 500 × *g* for 10 min at 4 °C, and the supernatant was subjected to ultracentrifugation at 30,000 × *g* for 25 min at 4 °C. Pellets were re-suspended in lysis buffer with dodecyl maltoside (2 mg/sample) (Boehringer Mannheim, Germany) and incubated for 45 min at 4 °C with gentle agitation, followed by centrifugation at 15,000 × *g* at 4 °C for 20 min. The cleared supernatant was processed for immunoprecipitation for 150 min at 4 °C with protein A-Sepharose beads (Sigma, St. Louis, MO, USA) pre-incubated for 2 h at 4 °C with an EGFP-antibody (1:200 dilution, BD Biosciences, San Jose, CA, USA) and pre-cleared with lysis buffer. The protein A-Sepharose beads were isolated by centrifugation at 15,000 × *g*, washed with lysis buffer, four times with lysis buffer/H_2O (1:1) and once with H_2O. The protein A-Sepharose was then re-suspended in SDS-PAGE sample buffer (4% SDS, 0.125 mM Tris–HCl, pH 6.8, 20% glycerol, 0.02% bromophenol blue, 0.2 M DTT), samples were heated at 60 °C for 10 min and centrifuged at 15,000 × *g* for 10 min. The supernatant was run on a 10% SDS-PAGE gel. Gels were dried and ^{32}P was detected with Storm 860 PhosphorImager (Molecular Dynamics) and quantified by using the ImageQuant software.

2.6. Visualization of CHO–MTLR–EGFP internalization

Internalization of the MTLR during desensitization was visualized by using the EGFP-tagged receptor. CHO–MTLR–EGFP cells were grown on four-well coverglass chambers until 80% confluency. After washing with ice cold BSA medium, digital pictures of the same cells before and after 2 h stimulation at 37 °C with BSA medium containing 10^{-5} M of the test compound were made with an Olympus camera on an inverted NIKON microscope (40× oil) equipped with a fluorescence unit and filtered with filter cube B-2A (EX BP540-580, DM RK595, EM BA 600-610).

The percentage black and white at the membrane and in the cytosol was calculated for eight cells per test compound before and after stimulation using Scion Image (Scion Corporation, Frederick, MD) and compared to non-treated cells. Each test compound was evaluated in three different experiments.

2.7. Statistical analysis

Results are represented as mean ± standard error of the mean (S.E.M.). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test (GraphPad InStat Software, San Diego, CA, USA). *P* values lower than 0.05 were considered to be significant.

3. Results

3.1. Characteristics of the motilin analogues

The affinity of the motilin analogues for the motilin receptor was tested in receptor binding studies, and their potency was evaluated by Ca^{2+} luminescence measurements in CHO–MTLR cells (Fig. 1). The binding studies indicated that Phe^3[1–22] and motilin [1–22] had the highest affinity with displacement

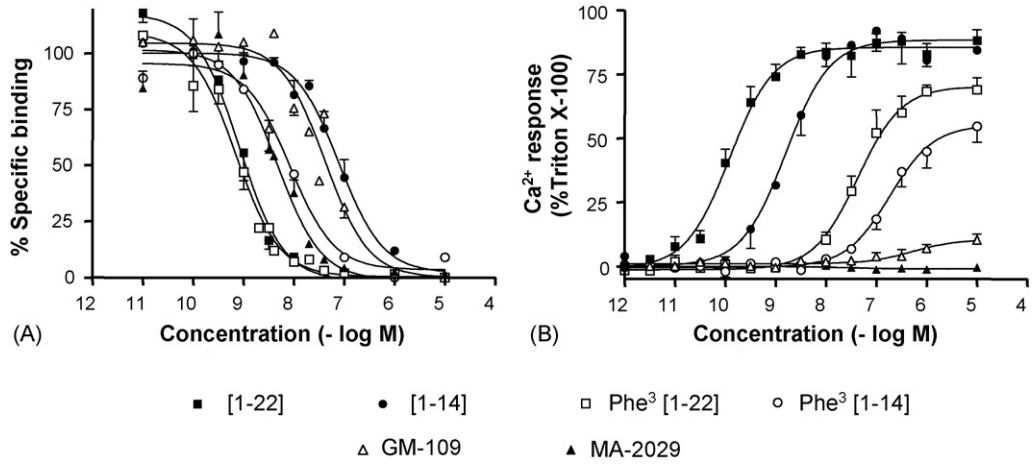

Fig. 1 – (A) Displacement curves obtained by incubating membrane preparations from CHO cells expressing the MTLR with [^{125}I]-motilin and increasing concentrations of unlabeled motilin or motilin fragments/analogues. Membrane bound [^{125}I]-motilin is expressed as percentage specific binding. (B) Concentration-dependent effects of motilin and motilin fragments/analogues on the Ca^{2+}-induced luminescent response in CHO–MTLR cells expressing the Ca^{2+} indicator apoaequorin. The Ca^{2+} response is expressed as percentage of the response to 0.9% Triton X-100 inducing a maximal Ca^{2+} release. Results are the mean ± S.E.M. of three experiments performed in duplicate.

potencies (pIC_{50}) of 9.08 ± 0.07 and 9.02 ± 0.05, respectively, followed by the MA-2029 (8.26 ± 0.07) and Phe^3[1–14] (7.98 ± 0.12) and the more weaker compounds GM-109 (7.55 ± 0.06) and the truncated motilin analogue [1–14] (7.10 ± 0.06). The displacement curves are shown in Fig. 1A and the pIC_{50} values summarized in Table 1.

Pronounced differences were observed in the potencies to induce Ca^{2+} fluxes (pEC_{50}), as is illustrated in Fig. 1B. Full length motilin [1–22] was the most potent compound (9.77 ± 0.09, maximal Ca^{2+} response: 83 ± 2%). Shortening of the peptide to fragment [1–14] reduced the pEC_{50} value (8.78 ± 0.06, $P < 0.05$ versus [1–22]) but did not affect the maximal Ca^{2+} response (87 ± 1%, $P > 0.05$ versus [1–22]). The potency of the modified motilin, Phe^3[1–22] (7.36 ± 0.13, $P < 0.001$ versus [1–22]), and the modified motilin fragment, Phe^3[1–14] (6.65 ± 0.15, $P < 0.001$ versus [1–14]), was 257- and 135-fold lower compared to [1–22] and [1–14], respectively. The maximal Ca^{2+} response to Phe^3[1–22] and Phe^3[1–14] was also lower 63 ± 4% ($P < 0.01$ versus [1–22]) and 46 ± 5% ($P < 0.001$ versus [1–14]), respectively. GM-109, the modified cyclical [1–4] fragment, still had some small agonist activity (pEC_{50}: 5.80 ± 0.38, intrinsic activity: 8 ± 2%) but MA-2029, the modified linear [1–3] fragment, was without any activity. The pEC_{50} values together with the E_{max} values for all motilin analogues tested are summarized in Table 1.

In order to further characterize the agonistic or antagonistic properties of Phe^3[1–14], GM-109 and MA-2029, the dose–response curve to motilin was tested in the presence of 10^{-6} M of these analogues. Fig. 2 shows that MA-2029 and GM-109 shifted the response to full length motilin [1–22] from 9.92 ± 0.10 (pEC_{50}) to 7.48 ± 0.08 ($P < 0.001$ versus buffer) and 8.07 ± 0.25 ($P < 0.001$ versus buffer) respectively while Phe^3[1–14] had a marked intrinsic activity of 67% ± 3 at 10^{-6} M and shifted the response to only 8.21 ± 0.18 ($P < 0.001$ versus buffer). Due to the high intrinsic activity (Ca^{2+} response at 10^{-6} M: 65% ± 3), Phe^3[1–22] could not be tested in this setting. From these data, it can be concluded that the fragments [1–22] and [1–14] are full agonists, the modified fragments Phe^3[1–22] and Phe^3[1–14] are partial agonists and GM-109 and MA-2029 are full antagonists.

Table 1 – Experimental parameters for the different motilin analogues determined from the motilin receptor binding studies and the Ca^{2+} luminescence experiments

Test method	Binding (pIC_{50} ± S.E.M.)	Ca^{2+} luminescence			
		First response		Second response to motilin	
		pEC_{50} ± S.E.M.	E_{max} ± S.E.M.[a] (%)	pEC_{50} ± S.E.M.	E_{max} ± S.E.M.[a] (%)
[1–22]	9.02 ± 0.05	9.77 ± 0.09	83 ± 2	6.65 ± 0.26	11 ± 3
[1–14]	7.10 ± 0.06	8.78 ± 0.06	87 ± 1	8.23 ± 0.15	68 ± 5
Phe^3[1–22]	9.08 ± 0.07	7.36 ± 0.13	63 ± 4	6.52 ± 0.11	34 ± 3
Phe^3[1–14]	7.98 ± 0.12	6.65 ± 0.15	46 ± 5	8.58 ± 0.13	78 ± 3
GM-109	7.55 ± 0.06	5.80 ± 0.38	8 ± 2	8.76 ± 0.14	78 ± 6
MA-2029	8.26 ± 0.07	Inactive	No response	7.50 ± 0.05	78 ± 5

[a] E_{max} is the maximal Ca^{2+} response expressed in % Triton X-100 response.

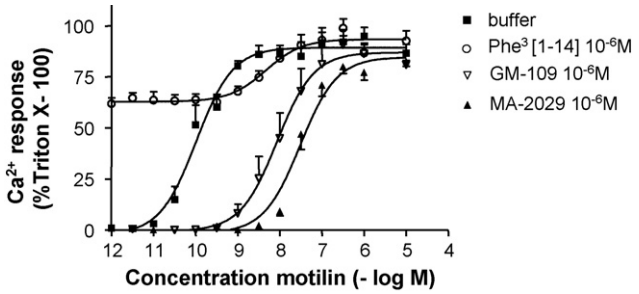

Fig. 2 – Antagonistic properties of motilin analogues tested in CHO–MTLR cells expressing the Ca^{2+} indicator apoaequorin. Cells were injected in a 96-well plate, containing increasing concentrations (10^{-12} to 10^{-5} M) of [1–22] and a fixed concentration of 10^{-6} M of Phe^3[1–14], cyc[1–4], [1–4] or buffer. The Ca^{2+} response is expressed as percentage of the response to 0.9% Triton X-100 inducing a maximal Ca^{2+} release and is the mean ± S.E.M. of three experiments performed in duplicate.

3.2. *Desensitization of the motilin receptor*

To evaluate the importance of motilin's C-terminus in the desensitization process of the MTLR, cells were desensitized by pretreatment for 2 h with the motilin analogues (10^{-5} M). After removing the compound by washing, a concentration–response curve to motilin was obtained (Fig. 3A). The pEC_{50} values together with the E_{max} values from the second response to motilin for all motilin analogues tested are summarized in Table 1.

Pretreatment of the cells with full length motilin [1–22] induced a profound desensitization of the MTLR as the subsequent maximal Ca^{2+} response to motilin [1–22] was depressed from 78 ± 2% to 11 ± 3% ($P < 0.001$ versus buffer) and the pEC_{50} shifted from 9.33 ± 0.12% to 6.65 ± 0.26% ($P < 0.001$ versus buffer). However, the effect of pretreatment with the truncated motilin [1–14] on the maximal Ca^{2+} response to motilin only reached borderline significance (68 ± 5%, $P = 0.052$ versus buffer) and the shift of the motilin concentration response curve was less pronounced (pEC_{50}: 8.23 ± 0.15, $P < 0.001$ versus buffer). The desensitizing properties of Phe^3[1–22] were lower than those of motilin because the maximal Ca^{2+} response to motilin was 34 ± 3% (after [1–22] 11 ± 3%, $P < 0.05$ versus [1–22]). In contrast after pretreatment with Phe^3[1–14] the maximal Ca^{2+} response to motilin remained unaffected (78 ± 3%, $P > 0.05$ versus buffer). The pEC_{50} values after pretreatment with Phe^3[1–22] (6.52 ± 0.11) and Phe^3[1–14] (8.58 ± 0.13) did not differ significantly from those after pretreatment with [1–22] or [1–14], respectively. Preincubation with the antagonists GM-109 and MA-2029, did not significantly lower the maximal Ca^{2+} response to motilin (GM-109: 78 ± 6%, MA-2029: 78 ± 5%, $P > 0.05$ versus buffer). Only pretreatment with MA-2029 shifted the pEC_{50} value from 9.33 ± 0.12 to 7.50 ± 0.05 ($P < 0.001$ versus buffer) but GM-109 did not have a significant effect (8.76 ± 0.14, $P > 0.05$ versus buffer).

3.3. *Characterization of the CHO cell line expressing the EGFP tagged motilin receptor*

To study the phosphorylation of the receptor and to visualize the internalization process we used an EGFP tagged motilin receptor stably expressed in CHO cells. The receptor binding properties and the potency of motilin [1–22] to induce Ca^{2+} release were compared between the wild-type CHO–MTLR and CHO–MTLR–EGFP (Table 2 and Fig. 4).

Receptor density was evaluated by [^{125}I]-motilin binding studies. Motilin receptor density (B_{max}) was significantly lower in the CHO–MTLR–EGFP cell line than in the CHO–MTLR cell line ($P < 0.05$) (Table 2). Displacement curves showed that motilin bound to its receptor with equal potencies in both cell lines (Fig. 4A and Table 2). The concentration–response curves revealed that the intrinsic activity (E_{max}) and the potency of

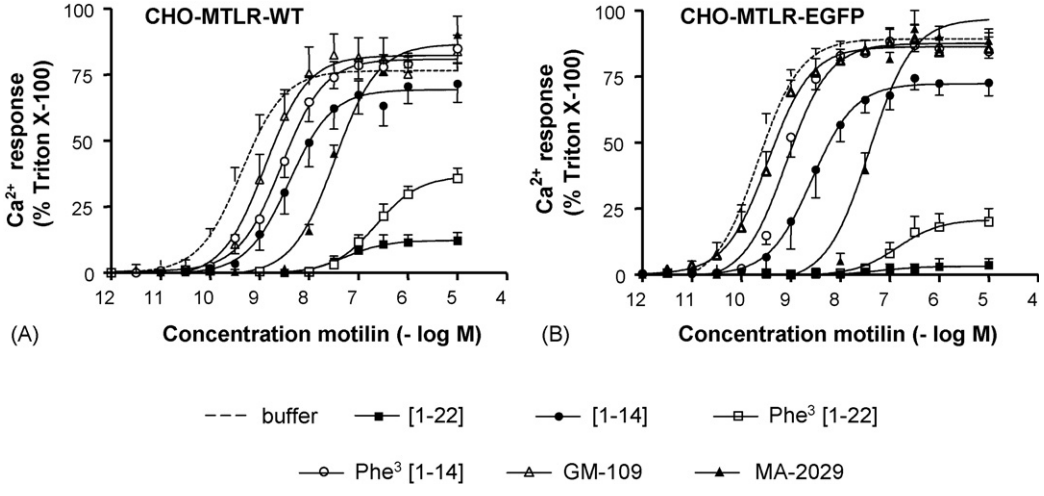

Fig. 3 – Concentration–response curves to motilin in CHO–MTLR cells (A) and CHO–MTLR–EGFP cells (B) pretreated with (—) or without (– –) motilin agonists or partial/full antagonists of different chain lengths at 10^{-5} M for 2 h. The Ca^{2+} response is expressed as percentage of the response to 0.9% Triton X-100 inducing a maximal Ca^{2+} release. The response is the mean ± S.E.M. of at least five experiments performed in duplicate for the CHO–MTLR cells and the mean ± S.E.M. of 1–3 experiments performed in duplicate for the CHO–MTLR–EGFP cells.

Table 2 – Comparison of the binding characteristics and the Ca^{2+} luminescent response of motilin in CHO–MTLR and CHO–MTLR–EGFP cells

Test method	Binding		Ca^{2+} luminescence	
	B_{max} ± S.E.M.[a]	pIC_{50} ± S.E.M.	pEC_{50} ± S.E.M.	E_{max} ± S.E.M.[b]
MTLR-WT	10.2 ± 1.2	8.87 ± 0.08	9.72 ± 0.13	78 ± 3
MTLR-GFP	7.1 ± 0.4*	8.80 ± 0.11	10.09 ± 0.07	86 ± 3

[a] B_{max} is the motilin receptor density expressed as pmol/mg protein.
[b] E_{max} is the maximal Ca^{2+} response expressed as a % of the response to Triton X-100.
* $P < 0.05$ vs. MTLR-WT.

motilin (pEC_{50}) to elicit a Ca^{2+} luminescent response did not differ between both cell lines (Fig. 4B and Table 2).

3.4. *Desensitization of the EGFP tagged motilin receptor*

To determine whether the introduction of the EGFP tag interferes with the desensitization process, desensitization of the wild-type CHO–MTLR and the CHO–MTLR–EGFP by the motilin analogues was compared.

As can be seen from Fig. 3B, the relative order of the Ca^{2+} concentration–response curves to motilin in CHO–MTLR–EGFP cells after pretreatment with buffer, [1–22], Phe^3[1–22], Phe^3[1–14], GM-109 and MA-2029 did not differ from those in CHO–MTLR cells. However, in contrast to the CHO–MTLR cells pretreatment of the CHO–MTLR–EGFP cells with the truncated motilin, [1–14], significantly decreased the maximal Ca^{2+} response to motilin from 90 ± 2% to 72 ± 4% ($P < 0.05$ versus buffer) and shifted the pEC_{50}-value from 9.68 ± 0.09 to 8.40 ± 0.01 to ($P < 0.001$ versus buffer). Also for the other compounds the absolute degree of desensitization was more pronounced than in the CHO–MTLR cells (Fig. 3A).

3.5. *Ligand-dependent phosphorylation of the motilin receptor*

To study phosphorylation of the receptor, cells were incubated with radioactive phosphate and stimulated for 10 min with 10^{-5} M of the different compounds. The phosphorylated receptor was detected by electrophoresis following immunoprecipitation of membrane fragments prepared by ultracentrifugation. Details are given in Section 2.

As is shown in Fig. 5A, a labeled band corresponding to the 105 kDa MTLR–EGFP was detected after stimulation with full length motilin [1–22], the truncated motilin analogue [1–14] and the partial antagonists Phe^3[1–22] and Phe^3[1–14], but not after stimulation with the two full antagonists. Densitometric analysis of the images (Fig. 5B) revealed that the agonists [1–22] and [1–14] caused increased MTLR phosphorylation producing values five- to six-fold above basal levels, respectively. The partial antagonists produced an intermediate effect, two- to three-fold above basal levels. Neither the antagonist, MA-2029 nor the antagonist, GM-109 induced MTLR phosphorylation compared to buffer-treated cells.

3.6. *Visualization of motilin receptor internalization in CHO–MTLR–EGFP cells*

To examine whether the desensitization is due to internalization of the MTLR, the internalization process was visualized by using the CHO cell line expressing the MTLR–EGFP. The changes in fluorescence distribution before and after stimulation (2 h) with the compounds at 10^{-5} M were observed by fluorescent microscopy and the percentage change in fluorescence in the cytosol and membrane was calculated by image analysis.

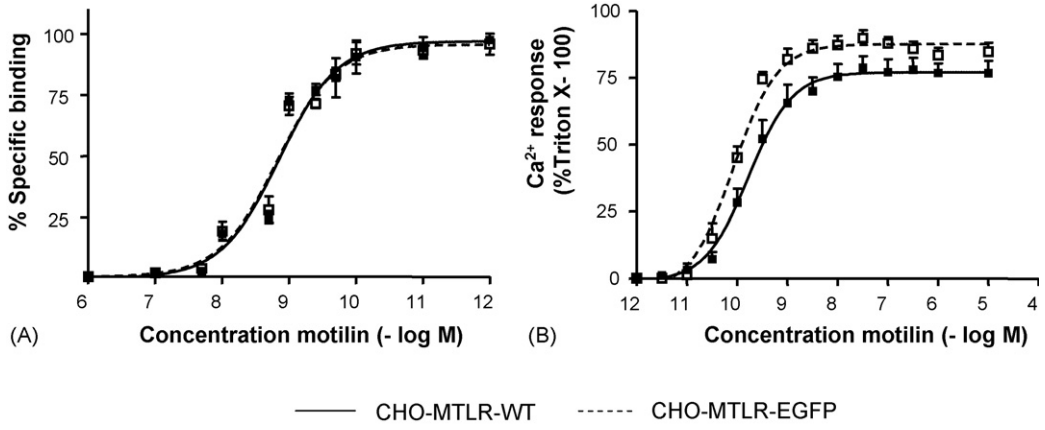

Fig. 4 – (A) Comparison of the displacement curves obtained by incubating membrane preparations from CHO cells expressing the MTLR (—) or the MTLR–EGFP (– –) with [^{125}I]-motilin and increasing concentrations of unlabeled motilin. Membrane bound [^{125}I]-motilin is expressed as percentage specific binding. Results are mean ± S.E.M. of five experiments performed in duplicate. (B) Comparison of the Ca^{2+} release evoked after concentration-dependent activation of the CHO–MTLR and CHO–MTLR–EGFP cell line by motilin [1–22]. The Ca^{2+} response is expressed as percentage of the response to 0.9% Triton X-100 inducing a maximal Ca^{2+} release. Results are mean ± S.E.M. of at least six experiments performed in duplicate.

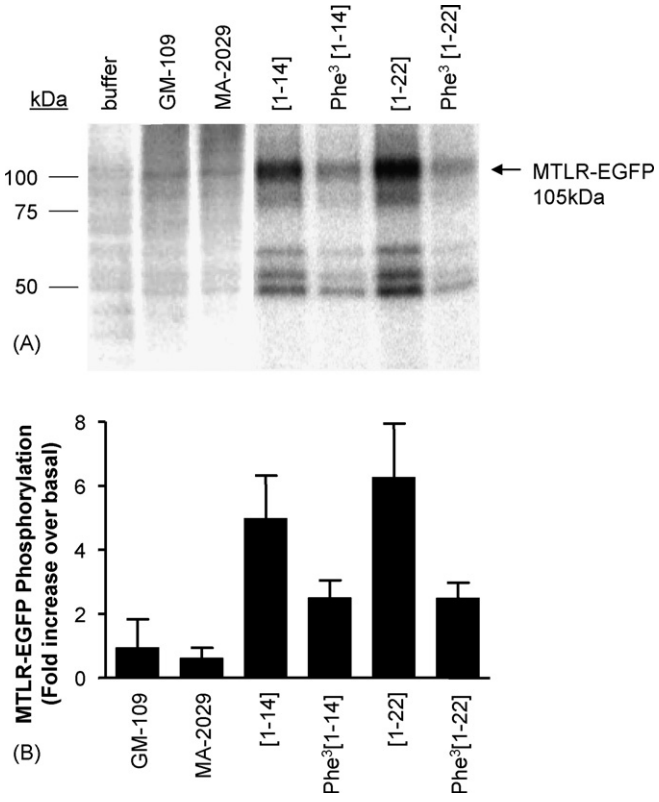

Fig. 5 – Phosphorylation of the MTLR in intact CHO cells expressing the MTLR–EGFP. [$^{32}P_i$]-labeled CHO–MTLR–EGFP cells were treated with buffer or motilin analogues (10^{-5} M) for 10 min. at 37 °C. The receptor was immunoprecipitated with an anti-GFP receptor antibody. (A) A representative phosphorimage scan of phosphorylated MTLR–EGFP after immunoprecipitation and analysis by SDS-polycarylamide gel electrophoresis is shown. The *arrow* indicates a size of 105 kDa corresponding to the phosphorylated MTLR–EGFP. (B) Densitometric analysis of MTLR–EGFP phosphorylation after stimulation with the motilin analogues. Results are expressed as fold increase over basal. The data are means ± S.E.M. from three separate experiments.

Representative pictures of cells before and after stimulation are shown in Fig. 6A. It can be seen that only after stimulation with motilin and motilin [1–14] there is a clear change in the fluorescence pattern. This impression was confirmed by image analysis using Scion software (Fig. 6B). After motilin the fluorescence intensity was significantly ($P < 0.001$) decreased at the cell membrane with 20 ± 3% and increased in the cytosol with 19 ± 3% compared to non-treated cells. The effect was much less pronounced after treatment with motilin [1–14] with a decrease of 7 ± 2% at the cell membrane, and an increase in the cytosol of 8 ± 1% ($P < 0.05$ versus buffer) (Fig. 6).

4. Discussion

The aim of the present work was to verify the hypothesis that the C-terminal region of motilin plays an important role in the desensitization of the MTLR. We compared the relationship between activation, desensitization, phosphorylation and internalization of the MTLR after stimulation with full and partial motilin agonists and antagonists.

First we characterized the (ant)agonistic properties of the motilin analogues used in our study. While [1–22] and [1–14] were full agonists, the modified fragments, Phe^3[1–22] and Phe^3[1–14], had the characteristics of partial agonists. The modified [1–4] and [1–3] fragments, GM-109 and MA-2029, respectively, were full antagonists since they had good binding affinities, no or almost no intrinsic activity, and shifted the Ca^{2+} concentration response curve to motilin to the right. The antagonistic properties of Phe^3[1–22], Phe^3[1–14] and of GM-109 on motilin induced smooth muscle responses have also been shown in contractility studies with rabbit duodenal segments [19,20,23].

Ligand binding to the extracellular domain of a G-protein coupled receptor (GPCR) is the first step for both signal transduction and signal termination. In many receptor systems the processes of receptor activation and receptor desensitization are linked [24–26]. Our data seem to confirm the correlation between activation and desensitization since the partial agonists were less potent compared to the full agonists and induced less desensitization. In addition neither of the antagonists were able to induce desensitization of the MTLR.

However, despite the fact that the motilin analogue [1–14] has the full pharmacophore required for activation [16] and was only 10-fold less potent than [1–22] to elicit Ca^{2+} responses, its ability to induce desensitization was much less pronounced than with the full length motilin [1–22]. The same discrepancy between activation and desensitization was observed with the partial antagonists Phe^3[1–22] and Phe^3[1–14] where a five-fold difference in activity resulted in a major difference in ability to desensitize. Vice versa, changes in the N-terminal region of [1–22] and [1–14] by replacement of Pro^3 by Phe^3, profoundly reduced bioactivity (257- and 135-fold, respectively) but affected the desensitizing properties to a lesser extent. These results therefore suggest that the desensitizing effects of motilin are determined by the C-terminal region, while the bioactivity resides in the N-terminal part. Similar findings have been described for substance P where the C-terminus is essential for biological activity and the N-terminal domain is required for complete homologues desensitization [27]. Desensitization of the MTLR after treatment with the partial agonist Phe^3[1–22] was also confirmed in a previous study in TE671 cells [28]. From these data we can conclude that the ability of a peptide to induce desensitization is not solely determined by its ability to act as an agonist for signal activation events. Disparities between activation and desensitization have also been described for dopamine D_{1A} receptors [29], μ-opioid receptors [30], the human tachykinin NK-1 [31] and serotonin 5-HT_{2C} receptors [32].

Fusion of an EGFP protein to the carboxyl terminus of the MTLR did not seem to alter the pharmacological and functional properties of the MTLR. Only the absolute degree of desensitization of the motilin analogues in the wild-type CHO–MTLR cell line was somewhat less pronounced than in the CHO–MTLR–EGFP cell line but the relative order of desensitization of the respective fragments was not different.

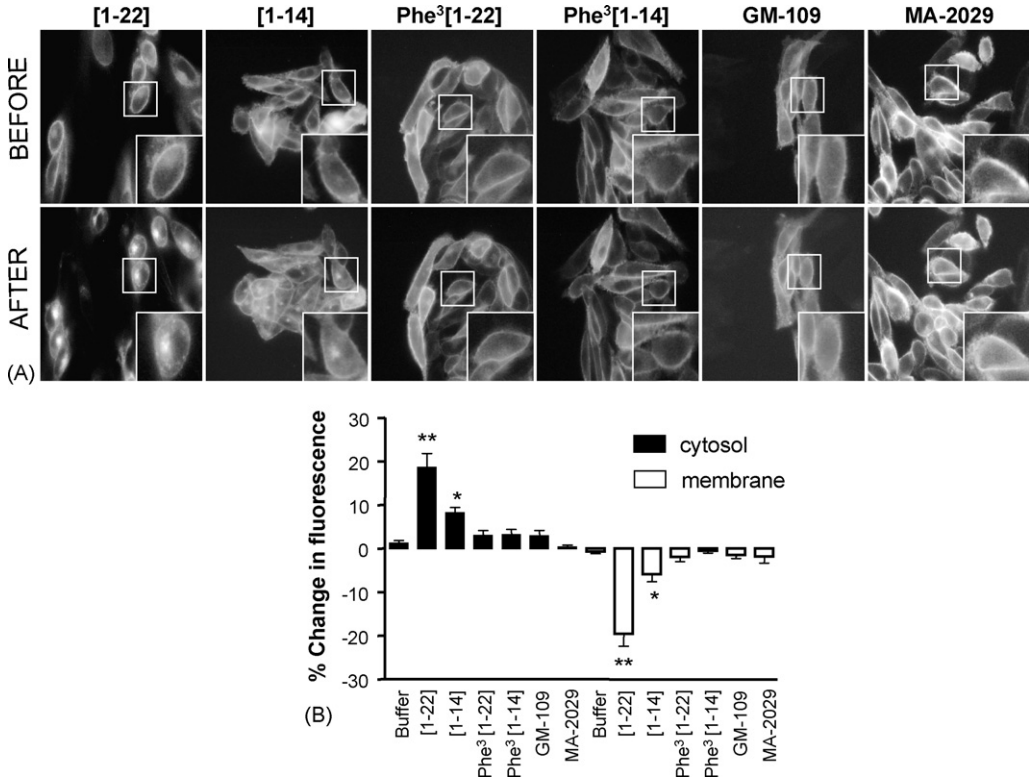

Fig. 6 – (A) Visualization of MTLR–EGFP trafficking expressed in CHO cells by fluorescent microscopy before (*top row*) and after (*bottom row*) stimulation with agonists and full/partial antagonists (10^{-5} M) for 2 h at 37 °C. Before treatment, fluorescent labeling appeared mainly located at the cell surface, while relocation to the cytosol took place after treatment with the agonist [1–22] and, to a lesser extent, with agonist [1–14] but not with the other motilin analogues. (B) Quantification of the change in fluorescent distribution between the cell surface and the cytosol after stimulation with [1–22], [1–14], Phe^3[1–22], Phe^3[1–14], [1–4] and cyc[1–4]. Three different sets of cells were stimulated with the same ligand. The change in fluorescence was measured in eight cells per set. Values were expressed as mean ± S.E.M. $^{**}P < 0.001$ vs. [1–14]; $^{*}P < 0.05$ vs. buffer.

In this context it is important to mention that in the wild-type CHO–MTLR cell line receptor density was more pronounced than in the CHO–MTLR–EGFP cell line. In the former case a larger receptor reserve exists and thus a receptor pool that is incapable of being desensitized probably because accessory molecules (e.g., G-protein-coupled receptor kinases, β-arrestins) involved in GPCR endocytosis become limiting under conditions of excess ligand and maximal rate of MTLR internalization.

Phosphorylation of the GPCR is necessary to initiate both the termination of G-protein signaling and receptor processing through internalization. Our results suggest a correlation between receptor phosphorylation and activation since receptor phosphorylation was more pronounced with the agonists than with the partial agonists while the full antagonists GM-109 and MA-2029, failed to alter phosphorylation. A link between a higher ligand efficacy and enhanced phosphorylation has also been described for the β_2-adrenergic [24] and the μ opioid receptor [33]. These studies demonstrated that partial agonists cause less phosphorylation than full agonists and the amount of receptor phosphorylation generally correlates with agonist's strength.

The rank order of motilin analogues in producing motilin receptor-mediated functional desensitization [1–22] > Phe^3[1–22] > [1–14] > Phe^3[1–14] > MA-2029 > GM-109 generally paralleled their rank order in producing receptor phosphorylation which demonstrates that desensitization is dependent on phosphorylation, except for Phe^3[1–22] which induced less receptor phosphorylation compared to [1–14] but was more efficacious in inducing desensitization. The finding that Phe^3[1–22] only requires a small amount of receptor phosphorylation to induce desensitization suggests that a mechanism other than MTLR phosphorylation is responsible for desensitization. GPCR signalling can also be terminated at the level of the heterotrimeric G protein by "regulator of G-protein signalling" (RGS) proteins which are GTPase accelerating proteins that inactivate G-protein signaling pathways and therefore play a key role in the desensitization process [34,35]. It should also be emphasized that in contrast to the agonists [1–22] and [1–14], desensitization of the motilin receptor by Phe^3[1–22] did not result in internalization. Agonist-induced internalization of GPCRs is a well characterized phenomenon believed to contribute to receptor desensitization [36]. Several studies have demonstrated that partial agonists induce less internalization than full agonists [24,37,38]. In addition, mutations that impaired signaling of GPCRs reduce agonist-induced receptor internalization, whereas mutations that yield constitutively active receptors generate receptors that

are internalized faster than wild-type receptors [39–43]. Therefore, it has been proposed that receptor activation is required for agonist-induced internalization. Nevertheless, studies on the relationship between the activity of agonists and their ability to cause receptor internalization have generated conflicting results. Indeed, it has been reported that an antagonist at the cholecystokinin receptor can stimulate internalization [44]. Certain competitive antagonists of the angiotensin AT1a receptor exhibit similar properties [45]. The difference between activation and desensitization for the full agonists [1–22] and [1–14], can be explained by a difference between activation and internalization. The partial agonist Phe^3[1–14] and the modified [1–4] and [1–3] fragments, GM-109 and MA-2029, respectively, which did not cause desensitization also did not cause internalization. In contrast, internalization is not responsible for the desensitization of the partial agonist Phe^3[1–22].

Several multi-state models have been invoked to explain the complex behaviour of GPCRs in the presence of agonists, antagonists and other binding partners. Fluorescence spectroscopy studies have largely contributed to the 'sequential binding and conformational selection model' [46,47] which assumed that GPCRs spontaneously alternate between different active an inactive conformations and that receptor activation by an agonist is a sequential process. Agonists stabilize a succession of conformational states of the receptor and these states may have distinct cellular functions by interacting with different types of cellular proteins. Each ligand might only stabilize a certain subset of receptor conformations that result in a ligand-specific receptor 'function'. These functions may include the activation of G proteins, desensitization, phosphorylation, internalization, etc., which can act separately [48]. These distinct functions may be dependent upon different domains of the motilin molecule. Our data suggest that the N-terminus of motilin is responsible for activation and binding and the C-terminus causes a conformation change that stabilizes this binding and also makes the receptor more sensitive to phosphorylation and internalization. In addition our data show that modifications in the N-terminus as in Phe^3[1–22] can favor a conformation of the receptor that is less susceptible to phosphorylation and internalization but still induces desensitization of the motilin receptor.

Our data suggest that potent agonists/antagonists may be developed without desensitizing properties. Such compounds may have improved therapeutic potential.

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REFERENCES

[1] Itoh Z, Honda R, Hiwatashi K, Takeuchi S, Aizawa I, Takayanagi R, et al. Motilin-induced mechanical activity in the canine alimentary tract. Scand J Gastroenterol Suppl 1976;39:93–110.
[2] Vantrappen G, Janssens J, Peeters TL, Bloom SR, Christofides ND, Hellemans J. Motilin and the interdigestive migrating motor complex in man. Dig Dis Sci 1979;24:497–500.
[3] Itoh Z, Nakaya M, Suzuki T, Arai H, Wakabayashi K. Erythromycin mimics exogenous motilin in gastrointestinal contractile activity in the dog. Am J Physiol 1984;247:G688–94.
[4] Peeters T, Matthijs G, Depoortere I, Cachet T, Hoogmartens J, Vantrappen G. Erythromycin is a motilin receptor agonist. Am J Physiol 1989;257:G470–4.
[5] Peeters TL, Muls E, Janssens J, Urbain JL, Bex M, Van Cutsem E, et al. Effect of motilin on gastric emptying in patients with diabetic gastroparesis. Gastroenterology 1992;102:97–101.
[6] Peeters TL. Erythromycin and other macrolides as prokinetic agents. Gastroenterology 1993;105:1886–99.
[7] Janssens J, Peeters TL, Vantrappen G, Tack J, Urbain JL, De Roo M, et al. Improvement of gastric emptying in diabetic gastroparesis by erythromycin. Preliminary studies. N Engl J Med 1990;322:1028–31.
[8] Talley NJ, Verlinden M, Snape W, Beker JA, Ducrotte P, Dettmer A, et al. Failure of a motilin receptor agonist (ABT-229) to relieve the symptoms of functional dyspepsia in patients with and without delayed gastric emptying: a randomized double-blind placebo-controlled trial. Aliment Pharmacol Ther 2000;14:1653–61.
[9] Talley NJ, Verlinden M, Geenen DJ, Hogan RB, Riff D, McCallum RW, et al. Effects of a motilin receptor agonist (ABT-229) on upper gastrointestinal symptoms in type 1 diabetes mellitus: a randomised, double blind, placebo controlled trial. Gut 2001;49:395–401.
[10] Tack J, Peeters T. What comes after macrolides and other motilin stimulants? Gut 2001;49:317–8.
[11] Peeters TL. New motilin agonists: a long and winding road. Neurogastroenterol Motil 2006;18:1–5.
[12] Thielemans L, Depoortere I, Perret J, Robberecht P, Liu Y, Thijs T, et al. Desensitization of the human motilin receptor by motilides. J Pharmacol Exp Ther 2005;313:1397–405.
[13] Lamian V, Rich A, Ma Z, Li J, Seethala R, Gordon D, et al. Characterization of agonist-induced motilin receptor trafficking and its implications for tachyphylaxis. Mol Pharmacol 2006;69:109–18.
[14] Macielag MJ, Peeters TL, Konteatis ZD, Florance JR, Depoortere I, Lessor RA, et al. Synthesis and in vitro evaluation of [Leu^3]porcine motilin fragments. Peptides 1992;13:565–9.
[15] Miller P, Gagnon D, Dickner M, Aubin P, St-Pierre S, Poitras P. Structure–function studies of motilin analogues. Peptides 1995;16:11–8.
[16] Peeters TL, Macielag MJ, Depoortere I, Konteatis ZD, Florance JR, Lessor RA, et al. D-amino acid and alanine scans of the bioactive portion of porcine motilin. Peptides 1992;13:1103–7.
[17] Khan N, Graslund A, Ehrenberg A, Shriver J. Sequence-specific 1H NMR assignments and secondary structure of porcine motilin. Biochemistry 1990;29:5743–51.
[18] Thielemans L, Perret J, Depoortere I, Robberecht P, Peeters TL. The C-terminal domain of motilin is required for complete homologous desensitization and endocytosis. Gastroenterology 2003;124:A-119 [Abstract].
[19] Peeters TL, Depoortere I, Macielag MJ, Dharanipragada R, Marvin MS, Florance JR, et al. The motilin antagonist ANQ-11125 blocks motilide-induced contractions in vitro in the rabbit. Biochem Biophys Res Commun 1994;198:411–6.

[20] Depoortere I, Macielag MJ, Galdes A, Peeters TL. Antagonistic properties of [Phe^3, Leu^3]porcine motilin. Eur J Pharmacol 1995;286:241–7.
[21] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[22] Thielemans L, Depoortere I, Vanden Broeck J, Peeters TL. The motilin pharmacophore in CHO cells expressing the human motilin receptor. Biochem Biophys Res Commun 2002;293:1223–7.
[23] Takanashi H, Yogo K, Ozaki K, Ikuta M, Akima M, Koga H, et al. GM-109: a novel, selective motilin receptor antagonist in the smooth muscle of the rabbit small intestine. J Pharmacol Exp Ther 1995;273:624–8.
[24] January B, Seibold A, Whaley B, Hipkin RW, Lin D, Schonbrunn A, et al. beta2-adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. J Biol Chem 1997;272:23871–9.
[25] Kovoor A, Celver JP, Wu A, Chavkin C. Agonist induced homologous desensitization of mu-opioid receptors mediated by G-protein-coupled receptor kinases is dependent on agonist efficacy. Mol Pharmacol 1998;54: 704–711.
[26] Oppermann M, Mack M, Proudfoot AE, Olbrich H. Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus. J Biol Chem 1999;274:8875–85.
[27] Vigna SR. The N-terminal domain of substance P is required for complete homologous desensitization but not phosphorylation of the rat neurokinin-1 receptor. Neuropeptides 2001;35:24–31.
[28] Thielemans L, Depoortere I, Van Assche G, Bender E, Peeters TL. Demonstration of a functional motilin receptor in TE671 cells from human cerebellum. Brain Res 2001;895:119–28.
[29] Lewis MM, Watts VJ, Lawler CP, Nichols DE, Mailman RB. Homologous desensitization of the D1A dopamine receptor: efficacy in causing desensitization dissociates from both receptor occupancy and functional potency. J Pharmacol Exp Ther 1998;286:345–53.
[30] Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, et al. mu-Opioid receptors: ligand-dependent activation of potassium conductance, desensitization, and internalization. J Neurosci 2002;22:5769–76.
[31] Perrine SA, Bennett VJ, Simmons MA. Tachykinin peptide-induced activation and desensitization of neurokinin 1 receptors. Peptides 2003;24:469–75.
[32] Stout BD, Clarke WP, Berg KA. Rapid desensitization of the serotonin (2C) receptor system: effector pathway and agonist dependence. J Pharmacol Exp Ther 2002;302:957–62.
[33] Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB. Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem 1997;272:28869–74.
[34] Chasse SA, Dohlman HG. RGS proteins: G protein-coupled receptors meet their match. Assay Drug Dev Technol 2003;1:357–64.
[35] Siderovski DP, Willard FS. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci 2005;1:51–66.
[36] Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signalling. Pharmacol Rev 2001;53:1–24.
[37] Szekeres PG, Koenig JA, Edwardson JM. The relationship between agonist intrinsic activity and the rate of endocytosis of muscarinic receptors in a human neuroblastoma cell line. Mol Pharmacol 1998;53:759–65.
[38] Schlag BD, Lou Z, Fennell M, Dunlop J. Ligand dependency of 5-hydroxytryptamine 2C receptor internalisation. J Pharmacol Exp Ther 2004;310:865–70.
[39] Nussenzveig DR, Heinflink M, Gershengorn MC. Agonist-stimulated internalization of the thyrotropin-releasing hormone receptor is dependent on two domains in the receptor carboxyl terminus. J Biol Chem 1993;268:2389–92.
[40] Dhanwada KR, Vijapurkar U, Ascoli M. Two mutations of the lutropin/choriogonadotropin receptor that impair signal transduction also interfere with receptor-mediated endocytosis. Mol Endocrinol 1996;10:544–54.
[41] Barak LS, Caron MG. Modeling of sequestration and down regulation in cells containing beta2-adrenergic receptors. J Recept Signal Transduct Res 1995;15:677–90.
[42] Min KS, Liu X, Fabritz J, Jaquette J, Abell AN, Ascoli M. Mutations that induce constitutive activation and mutations that impair signal transduction modulate the basal and/or agonist-stimulated internalization of the Lutropin/Choriogonadotropin receptor. J Biol Chem 1998;273:34911–9.
[43] Min L, Ascoli M. Effect of activating and inactivating mutations on the phosphorylation and trafficking of the human lutropin/choriogonadotropin receptor. Mol Endocrinol 2000;14:1797–810.
[44] Roettger BF, Ghanekar D, Rao R, Toledo C, Yingling J, Pinon D, et al. Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. Mol Pharmacol 1997;51:357–62.
[45] Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, et al. Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. Mol Pharmacol 2002;61:768–77.
[46] Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H, Kobilka BK. Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. EMBO J 1997;16:6737–47.
[47] Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 2000;21:90–113.
[48] Kenakin T. Drug efficacy at G protein-coupled receptors. Annu Rev Pharmacol Toxicol 2002;42:349–79.