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Motilin and erythromycin-A share a common binding site in the third transmembrane segment of the motilin receptor

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Abstract

The motilin receptor (MTLR) represents a clinically useful pharmacological target, as agonists binding to the MTLR have gastroprokinetic properties. In order to compare the molecular basis for interaction of the MTLR with motilin and with the non-peptide motilin agonist, erythromycin-A (EM-A), the negatively charged E^{119} located in the third transmembrane (TM_3) region was mutated to D (E119D) and Q (E119Q), respectively, and changes in activity of the mutant receptors were verified.

Methods: Each mutant receptor was stably transfected in CHO-cells containing the Ca^{2+} indicator apo-aequorin. Receptor activation in response to motilin, EM-A and their analogues was assessed by Ca^{2+}-luminescense.

Results: In the E119Q mutant, the Ca^{2+} response to motilin and EM-A was abolished while in the E119D mutant it was reduced with 62% (motilin) and 81% (EM-A). The pEC_{50} values were shifted from 9.65 ± 0.03 to 7.41 ± 0.09 (motilin) and from 6.63 ± 0.12 to 4.60 ± 0.07 (EM-A). Acetylation of the N-terminal amine group as in [*N*-acetyl-Phe]^1 mot (1–14), decreased the potency 6.3-fold (WT-MTLR) and 148-fold (E119D). Acetylation of EM-A enol ether induced a more pronounced shift in potency: 7943-fold (WT-MTLR) and 1413-fold (E119D).

Conclusion: The comparable loss of affinity of the mutant receptors for motilin and EM-A indicate that these agonists both interact with the TM_3 domain of the MTLR. The results with acetylated derivatives support an ionic interaction between E^{119} of the MTLR with the N^+ of the desosamine sugar in EM-A, but not with the N^+ of the free amine group in motilin.

Keywords: Motilin receptor; Mutagenesis; Erythromycin-A; Ca^{2+} luminescence; Motilin fragments

1. Introduction

Gastrointestinal motility is a coordinated neuromuscular process that transports nutrients through the digestive system. Impaired gastrointestinal motility, which can lead to gastroesophageal reflux disease, gastroparesis (diabetic and post-surgical), irritable bowel syndrome and constipation, is one of the largest health care burdens of industrialized nations. Motilin has long been recognized as an important endogenous peptide regulator of gastrointestinal motor function and there is considerable interest into the therapeutic applications of motilin agonists [1]. This interest arose after the demonstration that the antibiotic erythromycin accelerates gastric emptying in patients with diabetic gastroparesis by interacting with the motilin receptor [2,3]. Pharmaceutical companies have developed erythromycin derivatives without anti-bacterial activity but with good gastrointestinal motor stimulating properties. These compounds are currently under investigation for the treatment of hypo-motility disorders. Chemically, these compounds, now called motilides, show little structural resemblance with motilin, raising the question of how they interact with the motilin receptor.

Abbreviations: CHO-cell line, Chinese hamster ovary cell line; EM-A, erythromycin-A; GHS-R, growth hormone secretagogue receptor; GPCR, G-protein coupled receptors; MTLR, motilin receptor; PCR, polymerase chain reaction; TM, transmembrane; TRH-R, thyrotropin releasing hormone receptor; WT, wild type

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In 1999, an orphan receptor, originally isolated from the thyroid gland, was identified as the motilin receptor [4]. The amino acid sequence of the motilin receptor is for 44% identical with the human growth hormone secretagogue receptor (GHS-R), even for 86% in the predicted transmembrane regions. Both receptors stimulate pulsatile biological activity and are now considered a new family within class A of G-protein coupled receptors (GPCRs). The full-length human motilin receptor complementary DNA encodes a polypeptide of 412 amino acids (type 1a). Alternative splicing may produce a truncated version of the motilin receptor (type 1b) of 386 amino acids which is biologically inactive. In a CHO-cell line expressing the cloned motilin receptor (type 1a) and the Ca^{2+} indicator apo-aequorin, the potencies of motilin agonists to induce Ca^{2+} fluxes strongly correlates with their potencies to induce contraction in rabbit intestinal tissue, suggesting that the cloned motilin receptor is indeed the receptor responsible for the contractile effects [5].

While extensive structure–activity studies have been performed to delineate the pharmacophore of motilin and erythromycin [6–10], mutagenesis studies to reveal the receptor domains involved in the interaction of the ligands with the motilin receptor are scarce. A better understanding of the relationship between the molecular structure and function of the receptor may provide important insights for drug development. They are also of fundamental importance to clarify key concepts of receptor biology. With the development of non-peptide agonists for peptide receptors, it is becoming increasingly clear that the simple lock-and-key concept may be insufficient to explain structural changes leading to receptor activation.

In several GPCRs, there is a growing body of evidence implicating that transmembrane (TM) helixes 3, 5 and 6 and extra-cellular loops 2 and 3 are involved in ligand binding [11]. Alignment of the sequences of the receptors belonging to the same subclass as the motilin receptor (thyrotropin-releasing hormone and secretagogue) within the class A rhodopsin like family of GPCRs, shows that the glutamic acid in the TM_3 region is well conserved. Mutagenesis studies revealed that this is an important binding site for activation of the GHS-R to which motilin is most related [12].

The aim of the present study was to evaluate the effect of mutating the negatively charged glutamic acid (Glu, E) at position 119 of the TM_3 to aspartic acid (E119D mutant), which puts the negative charge closer to the receptor backbone, and to glutamine (E119Q mutant), which removes the negative charge, on the response of the mutant receptors stably expressed in CHO-cells to motilin and EM-A. In addition we aimed at delineating the structural elements of motilin and EM-A involved in the interaction with E^{119} by measuring the Ca^{2+} luminescent response upon stimulation with motilin analogues and motilides in the wild-type receptor (WT-MTLR) and in the mutant receptors.

2. Materials and methods

2.1. Materials

Norleucine^{13}-porcine-motilin (1–22) was purchased from Eurogentec, Namur, Belgium. The N-terminal (1–14) fragment of porcine [leu^{13}]motilin and the analogues of the (1–14) fragments were synthesized as previously described [7,8]. A Chinese hamster ovary-K1 (CHO-K1) cell line stably expressing the wild-type human motilin receptor and the mitochondrially targeted apo-aequorin was obtained from Euroscreen (Belgium). The human WT-MTLR coding region, inserted into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and the rabbit anti-human peptide antibody raised against the C-terminal 373–385 amino acid fragment of the WT-MTLR was a gift from Dr. A. Howard from Merck Research Laboratories (NY, USA).

EMA and all of its derivatives used in this study were obtained from Prof. J. Hoogmartens (Laboratory of Pharmaceutical Chemistry, University of Leuven, Belgium).

2.2. Methods

2.2.1. Construction and expression of the motilin receptor mutants

The plasmid vector pcDNA3.1 containing human WT-MTLR was submitted to 22 cycles of PCR (95 °C for 30 s, 53 °C for 1 min and 68 °C for 14 min) using the PfuTurbo^{TM} DNA polymerase (Stratagene, La Jolla, CA, USA) (2.5 U/μl) in a 50 μl reaction volume. The forward and reverse primers were complementary and contained the desired nucleotide changes, flanked on either side by 15 perfectly matched nucleotides:

HuMotR E119Q forward: tccctctacgtgggcCAGggctgcacctacgcc
HuMotR E119Q reverse: ggcgtaggtgcagccCTGgcccacgtagtggga
HuMotR E119D forward: tccctctacgtgggcGACggctgcacctacgcc
HuMotR E119D reverse: ggcgtaggtgcagccGTCgcccacgtagtggga

Following PCR, 10 μl were analyzed by 0.8% agarose gel electrophoresis and the remaining 40 μl were digested for at least 3 h by 1 μl *Dpn*I restriction enzyme (10 U/μl) (Stratagene, LaJolla, CA, USA) to remove the parental methylated DNA. The digested PCR products were transformed into TOP10 One Shot competent *E. coli* bacterial cells (Invitrogen Corp., CA, USA). Of several colonies, verified by agarose gel electrophoresis of miniprep plasmid DNA, three were retained and the mutations checked by DNA sequencing on an ABI automated sequencing apparatus, using the BigDye Terminator Sequencing Prism Kit from ABI (Perkin-Elmer, CA, USA). Twenty

micrograms plasmid DNA from one clone for each mutation, containing the correct nucleotide substitutions, were electroporated into CHO-K1 cells stably expressing the apo-aequorin gene (Euroscreen SA, Brussels, Belgium). Selection of 24 clones was carried out after culturing in medium (50% HamF12, 50% DMEM, 10% fetal calf serum, 1% penicillin (10 mU/ml), 1% streptomycin (10 g/ml) and 1% glutamine (200 mM)), supplemented with 600 μg/ml geneticin (G418). Isolated colonies were transferred to 24-well plates and grown until confluence, trypsinized and further expanded in 6-well plates, from which cells were prepared for screening by an aequorin luminescence assay in presence of 10^{-5} M motilin.

2.2.2. Cell culture

The CHO-K1 cells stably expressing either the WT-MTLR or the mutant constructs and the Ca^{2+} indicator apo-aequorin were cultured at 37 °C in Ham's F12 containing 10% FBS, 100 μg/ml penicillin/streptomycin, 2.5 μg/ml amphotericin B and 400 μg/ml G418 and spliced once a week with 5 mM EDTA in PBS.

2.2.3. Aequorin based Ca^{2+} luminescence assay

Suspended cells (5×10^6 cells/ml) were loaded with coelenterazine h (5 μM) (Molecular Probes, Leiden) for 4 h at room temperature in order to reconstitute active aequorin and afterwards diluted 10-fold in DMEM/HAM's F12 (with Hepes, 0.1% protease free BSA, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml)). Aliquots (100 μl) of the desired dilutions of the compounds to be tested were placed in 96-well plates, and in each well 100 μl of cells (i.e. about 50,000 cells) were injected. The emitted light was measured using the 'Microlumat plus' luminometer (Berthold, Bad Wildbad, Germany) during 20 s. The intensity of the emitted light was integrated using the Winglow Software (Berthold). After subtraction of the blank obtained from injection of cells into saline, the response to a compound was expressed as a percentage of the response towards Triton X-100 (0.9%) which induces maximal Ca^{2+} luminescence. The negative logarithm of the concentration producing half of the maximal response (pEC_{50}) was calculated from the dose response curves by the Graphpad Prism Software (San Diego, CA, USA). All experiments were performed in duplicate and each compound was tested at least three times. Results are represented as mean ± standard error of the mean (S.E.M.).

2.2.4. Receptor expression

2.2.4.1. Immunocytochemistry. CHO-K1 cells were washed with BSA-medium, fixed with 4% paraformaldehyde (30 min, RT), washed, incubated for 2 h with incubation buffer (0.1 M PBS, 4% goat serum, 0.5% Triton-X 100 and 0.3% NaN_3) and stained with the C-terminal (373–385 amino acid fragment) rabbit anti-human WT-MTLR primary antibody (1:500) for 24 h at 4 °C. After washing with PBS, cells were incubated with a secondary antibody (goat anti-rabbit FITC) for 2 h at 4 °C. Cells were washed, semi-dried, mounted on a glass and visualized under a NIKON microscope equipped with a fluorescence unit and photographed.

2.2.4.2. Motilin receptor binding studies. Membrane preparation: Cells were cultured till 90% confluence was reached. After removal of medium, cells were scraped from the plates in Ca^{2+}/Mg^{2+} free PBS. After centrifugation for 3 min at 1500 × *g*, pellets were resuspended 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 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 resuspended 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.

Motilin receptor binding: A competition binding assay was performed by incubating CHO-MTLR membranes (60–200 μg of protein/tube) in a final volume of 0.1 ml with ^{125}I-motilin (0.03 nM) for 60 min at 31 °C. Unlabeled motilin was used as competitor, at concentrations ranging from 10^{-10} to 10^{-5} M in binding buffer (25 mM Hepes pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% BSA). After incubation, the samples were filtered on GF/B filters, washed and counted in a gamma counter. pIC_{50} values were calculated by non-linear regression using the Graph Pad Prism Software (San Diego). All experiments were performed three times in duplicate. Results are represented as mean ± S.E.M.

2.2.4.3. Western blot. Membranes from CHO-K1 cells were separated by gel electrophoresis (50 μg/lane) on 10% SDS-polyacrylamide gels. The separated proteins were transferred (4 h) to an Immobilon-P membrane (Millipore Corporation, Bedford, USA) at 9 °C, washed with TBS (20 mM Tris; 137 mM NaCl; pH 7.6) and blocked for 1 h at RT with 5% milk powder in TBS/T (TBS, 0.1% Tween-20). After washing, the membrane was incubated overnight with the primary rabbit anti-human motilin receptor antibody (373–385 amino acid fragment) in TBS/T (1:1500) at 4 °C, followed by extensive washes. Blots were incubated with an anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) and a HRP-conjugated anti-biotin antibody (1:1000) to detect the biotinylated protein ladder (Cell Signalling, Beverly, MA, USA) for 1 h at RT. Detection was achieved by incubation of the membrane with Lumi-GLO for 1 min at RT (phototope-HRP Western blot detection system, Cell Signalling) and exposure to X-ray film.

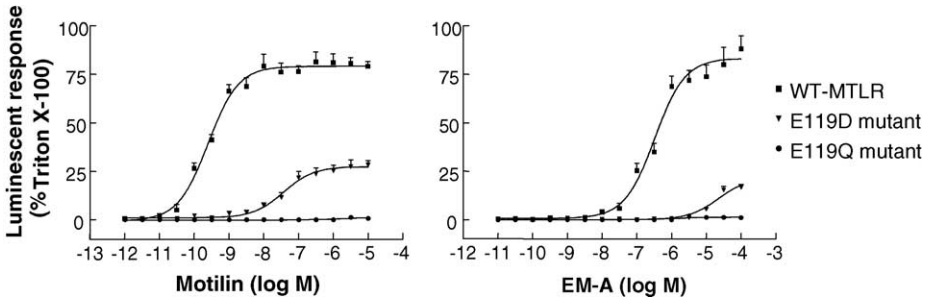

Fig. 1. Dose-dependent effects of motilin (left) and of EM-A (right) on the Ca^{2+}-induced luminescent response in CHO-K1 cells expressing the Ca^{2+} indicator apo-aequorin and either the WT-MTLR, the E119D mutant or the E119Q mutant. The Ca^{2+} response is expressed as percentage of the response to Triton X-100 inducing maximal Ca^{2+} release and is the mean ± S.E.M. of at least three experiments performed in duplicate.

3. Results

3.1. Activity of CHO-cells expressing the WT-MTLR, the E119D mutant or the E119Q mutant

The potency of motilin and of EM-A to mobilize intracellular Ca^{2+} causing concomitant Ca^{2+}-induced aequorin bioluminescence, was measured in CHO-K1 cells expressing either the WT-MTLR, the E119D mutant or the E119Q mutant.

In the E119D mutant, the maximal Ca^{2+} response to motilin and EM-A was reduced from 82 ± 5 and 88 ± 7% of the maximal Triton-induced Ca^{2+} luminescence in the WT-MTLR to 31 ± 2 and 17 ± 1% in the E119D mutant, respectively (Fig. 1). Also, the pEC_{50} values were shifted from 9.65 ± 0.03 to 7.41 ± 0.09 for motilin and from 6.63 ± 0.12 to 4.60 ± 0.07 for EM-A. These results suggest that the negative charge of glutamic acid, which is closer to the receptor backbone when this residue is mutated to aspartic acid, is a binding site for both motilin and EM-A. The requirement of a negative charge at this position was confirmed by the finding that there was a complete loss of Ca^{2+} response to motilin and the motilin agonist, EM-A in the E119Q mutant (Fig. 1).

3.2. Delineation of the residue of motilin and EM-A involved in interaction with E^{119}

3.2.1. Motilin

Previous structure activity studies have indicated that the activity of motilin (1–14) approaches the activity of the full-length motilin [7]. In addition it was shown that the pharmacophore resides in the first seven residues, more particular in Phe^1, Val^2, Ile^4, Tyr^7 [8]. Of these amino acids, only the positive charge of the free N-terminal end of Phe can be identified as a possible candidate for interaction with the negative charge of Glu^{119} in the TM_3 region of the motilin receptor. Therefore, the effect of motilin (1–14) analogues in which the charge of the free amino group was changed by methylation or acetylation was tested on their ability to activate the WT-MTLR and the mutant receptors. The data are summarized in Table 1 and Fig. 2.

Truncation of motilin (1–22) to motilin fragment (1–14) reduced the potency in the WT-MTLR from 9.65 ± 0.03 to 8.33 ± 0.07 and in the E119D mutant from 7.41 ± 0.09 to 5.81 ± 0.19. The potency of motilin (1–14) could be enhanced both in the WT-MTLR and in the E119D mutant by methylation of its N-terminal amine group as in [*N*-Me-Phe]^1 mot (1–14) to, respectively, 9.25 ± 0.06 and 6.25 ± 0.08. However, trimethylation or acetylation of

Table 1
Comparison of the potency (pEC_{50}) of motilin analogues and motilides to induce a Ca^{2+} luminescent response in the WT-MTLR, the E119D mutant and the E119Q mutant

Compound	Structure	Ca^{2+} luminescence (pEC_{50})		
		WT	E119D	E119Q
Mot (1–22)	NH_3^+-FVPIFTYGELQRMQEKERNKGQ	9.65 ± 0.03	7.41 ± 0.09	<4
Mot (1–14)	H-$^+NH_2$-FVPIFTYGELQRMQ	8.33 ± 0.07	5.81 ± 0.19	<4
[*N*-Me-Phe]^1 mot (1–14)	CH_3-$^+NH_2$-FVPIFTYGELQRMQ	9.25 ± 0.06	6.25 ± 0.08	<4
[*N*-tri(Me)-Phe]^1 mot (1–14)	$(CH_3)_3$-$^+$N-FVPIFTYGELQRMQ	7.88 ± 0.06	3.08 ± 0.20	<4
[*N*-acetyl-Phe]^1 mot (1–14)	CH_3-CO-NH-FVPIFTYGELQRMQ	7.53 ± 0.11	3.64 ± 0.40	<4
[β_2 Phe]^1 mot (1–22)	H-$^+NH_2$-CH_2-FVPIFTYGELQRMQ	10.24 ± 0.13	7.98 ± 0.11	<4
EM-A	See Fig. 3	6.63 ± 0.12	4.60 ± 0.07	<4
EM-A enol ether	See Fig. 3	8.85 ± 0.05	6.07 ± 0.07	<4
N-demethyl EM-A enol ether	See Fig. 3	7.55 ± 0.07	5.85 ± 0.05	<4
N-trimethyl EM-A enol ether	See Fig. 3	7.92 ± 0.09	5.74 ± 0.07	<4
N-methyl,acetyl EM-A enol ether	See Fig. 3	4.95 ± 0.13	2.92 ± 0.12	<4

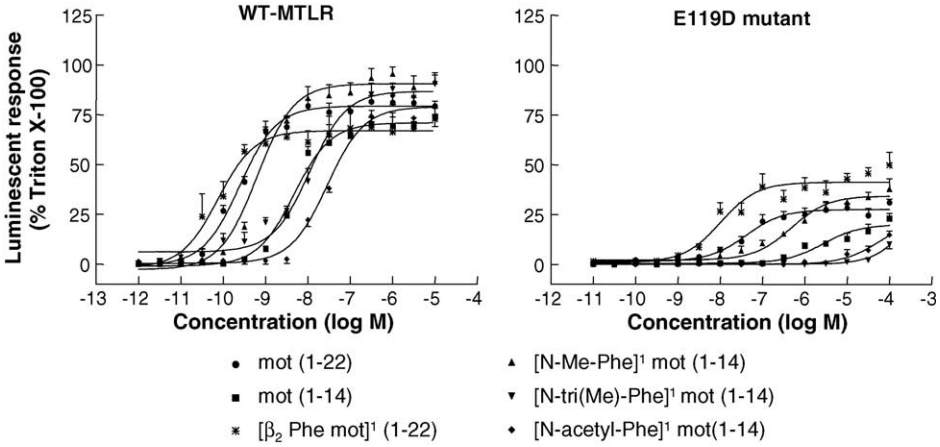

Fig. 2. Effect of increasing doses of motilin and motilin analogues on the Ca^{2+} luminescent response in CHO-K1 cells expressing the WT-MTLR (left) and the E119D mutant (right). The Ca^{2+} response is expressed as percentage of the maximal Ca^{2+} release provoked by Triton-X and is the mean ± S.E.M. of at least three experiments performed in duplicate.

the free N-terminal amine group, as in [*N*-tri(Me)-Phe]^1 mot (1–14) and [*N*-acetyl-Phe]^1 mot (1–14), decreased the potency 2.8- and 6.3-fold in the WT-MTLR and even 537- and 148-fold in the E119D mutant.

To further test the hypothesis of an interaction between the N-terminal amino group of motilin and the acidic group on the side chain of residue 119 of the receptor, we tested a motilin analogue in which the terminal amino group was moved in β position, i.e. one CH_2 further removed from the first peptide bond, so as to compensate for the increase in distance between the carboxyl group of the receptor and the peptide backbone. This motilin analogue, [β_2 Phe]^1 mot (1–22), significantly ($P < 0.01$) increased the maximum Ca^{2+} response from 31 ± 2% (mot (1–22)) to 50 ± 6% ([β_2 Phe]^1 mot (1–22)) and shifted the pEC_{50} value from 7.41 ± 0.09 to 7.98 ± 0.11 in the E119D mutant. Also, in the WT-MTLR, there was a comparable shift in potency from 9.65 ± 0.03 to 10.24 ± 0.13. None of the motilin analogues tested induced a Ca^{2+} luminescent response in the E119Q mutant.

3.2.2. EM-A

Previous studies have indicated that the structural requirements of the motilides for interaction with the motilin receptor involve the ring structure, particularly the part that can be transformed in an enol ether, and both attached sugars (Fig. 3) [6]. Especially, modifications at the *N*-dimethyl amino group of the desosamine sugar can affect the potency. This *N*-dimethyl amino group is in its protonated form at the pH of the experiment and is also a likely candidate for interaction with the negatively charged Glu^{119} in TM_3.

When the parent ring of EM-A is converted to an enol ether ring structure, the pEC_{50} value shifts from 6.63 ± 0.12 to 8.85 ± 0.05 in the WT-MTLR and from 4.60 ± 0.07 to 6.07 ± 0.07 in the E119D mutant. Removal or addition of a methyl at the *N*-dimethyl amino group of the desosamine sugar as in *N*-demethyl EM-A enol ether and *N*-trimethyl EM-A enol ether only slightly reduced the potency in the WT-MTLR to 7.55 ± 0.07 (20-fold) and 7.92 ± 0.09 (8.5-fold), respectively. This effect was even less in the E119D mutant, 1.7- and 2.1-fold, respectively (Table 1; Fig. 4). On the contrary, acetylation (*N*-methyl, acetyl EM-A enol ether) dramatically reduced the potency from 8.85 ± 0.05 to 4.95 ± 0.13 (7943-fold) in the WT-MTLR and produced a comparable shift in the E119D mutant. No Ca^{2+} induced luminescence was observed with the motilides in the E119Q mutant (Table 1).

3.3. Expression of the motilin receptor

Competition-binding experiments with membrane preparations from the WT-MTLR revealed a pIC_{50} value of 8.75 ± 0.08 (Fig. 5). In contrast, no significant binding was

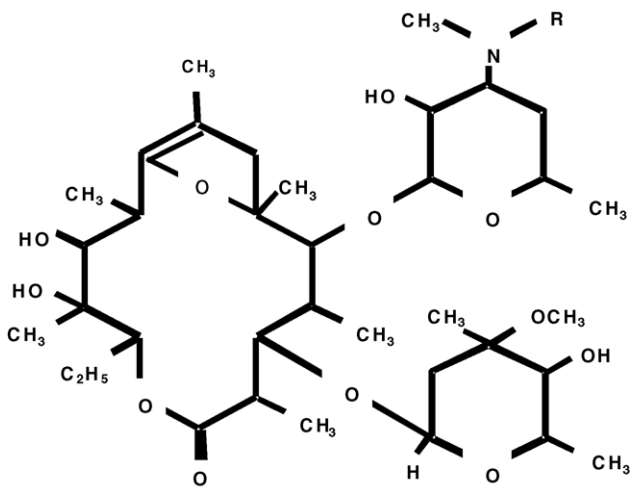

Fig. 3. Chemical structure of EM-A enol ether and its derivatives. It is constructed of three major parts, a 14-membered enol ether lactone ring, an amino sugar (desosamine), and a neutral sugar (cladinose). EM-A (R = CH_3, no enol ether); EM-A enol ether (R = CH_3); *N*-demethyl EM-A enol ether (R = H); *N*-trimethyl EM-A enol ether (R = $2CH_3$); *N*-methyl, acetyl EM-A enol ether (R = $COCH_3$).

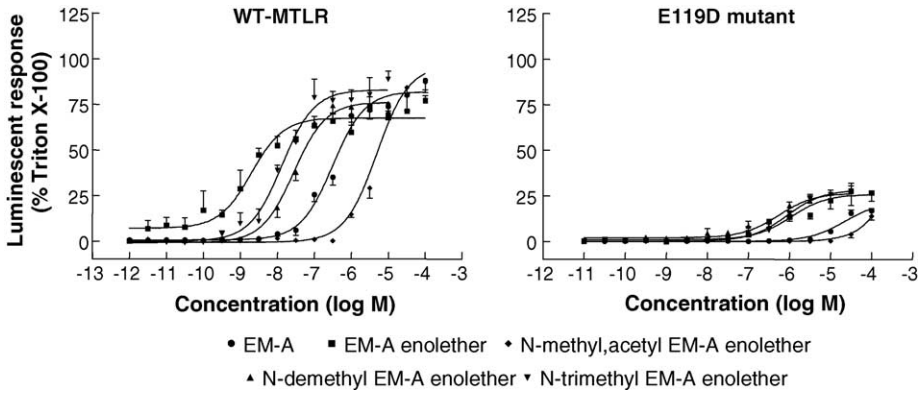

Fig. 4. Effect of increasing doses of EM-A and EM-A derivatives on the Ca^{2+} luminescent response in CHO-K1 cells expressing the WT-MTLR (left) and the E119D mutant (right). The Ca^{2+} response is expressed as percentage of the response to Triton X-100 inducing maximal Ca^{2+} release and is the mean ± S.E.M. of at least three experiments performed in duplicate.

observed with the cells expressing the mutant receptors at protein concentrations up to 200 μg since binding was in the same order of magnitude as the non-specific binding (5–10% of total binding) (Fig. 6A). Actually, this could have been expected based on the reduced potency of the mutant receptors for motilin in the Ca^{2+} luminescent experiments. Therefore, to confirm that differences in receptor function and binding are not due to variable levels of mutant protein synthesis and/or transport to the cell membrane, immunohistochemistry and Western blot was performed.

On the basis of their Ca^{2+} luminescent response to motilin, 3 clones out of the 24 clones from each mutant receptor were selected to determine the expression of the motilin receptor. The immunohistochemistry experiments confirmed that the motilin receptor is stably expressed at the cell membrane of the WT-MTLR, the E119D mutant and the E119Q mutant (Fig. 6B). Western blot studies revealed a band of ±60 kDa in each lane and showed that the expression level of each of the mutant receptors was similar to that of the WT-MTLR (Fig. 6C). No staining was observed when the pre-immune serum was used as primary antibody. Thus, the reduced specific binding with ^{125}I-motilin resulted from a reduced binding affinity rather than a drastic decrease in the binding capacity.

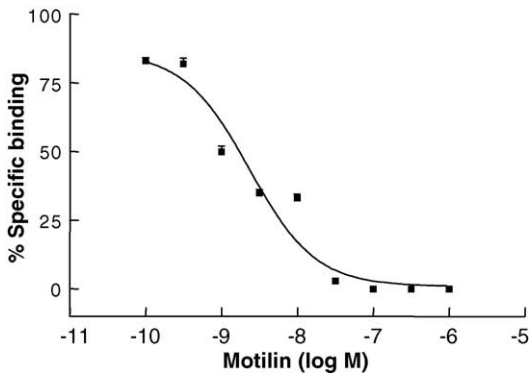

Fig. 5. Displacement curves obtained by incubating membrane preparations from CHO-K1 cells expressing the WT-MTLR with ^{125}I-motilin and increasing concentrations of unlabeled motilin. Results are the mean of three experiments performed in duplicate.

4. Discussion

Our results indicate that motilin and the non-peptide motilin agonist, EM-A, share a common binding site in the third transmembrane region of the motilin receptor. This conclusion is based on the comparable loss of affinity of the mutated receptors for their native ligand and for EM-A. Thus, both motilin and motilides were unable to activate the E119Q mutant, in which there is no negative charge on the side chain of residue 119 and both compounds had a decreased potency in the E119D mutant, in which the negatively charged carboxyl group is moved closer to the receptor backbone. In addition, to ascertain that the effect of substitution of the receptor residue has impact on the binding site directly or indirectly by destroying the conformation of the binding pocket through intra-receptor interactions, the effect of motilin and erythromycin analogues was studied. Acetylation of the amino terminus in motilin and of the *N*-dimethyl amino group of the desosamine sugar in EM-A, which removes the positive charge of the ligand, reduced the potency towards both the WT-MTLR and the E119D mutant. However, only for EM-A evidence for an ionic interaction was obtained.

Whereas the primary determinants of binding for large glycoprotein hormones appear to lie within the extracellular domains of their GPCRs [13,14] evidence is accumulating that the binding site of GPCRs for small peptides involves both extra-cellular and transmembrane domains. Indeed, mutations similar to those in the present study have been performed with the amine subfamily within class A of rhodospin like GPCR. For the muscarinic 1 (M_1) and M_2 receptors [15], replacement of acidic amino acids, Asp^{105} and Asp^{103} in the TM_3, induced pronounced decreases in affinity. Similarly, the substitution of Asp^{113}

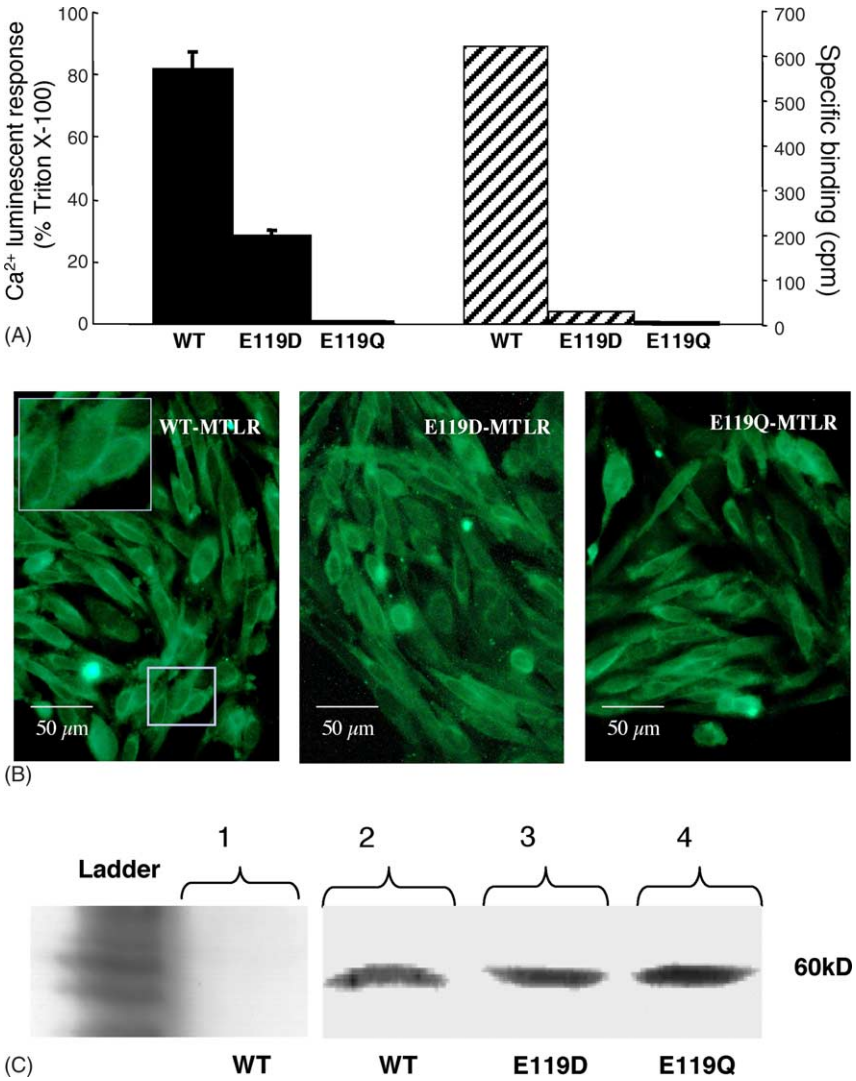

Fig. 6. Comparison of MTLR protein expression in CHO-K1 cells stably expressing the WT-MTLR, the E119D mutant or the E119Q mutant as evidenced by Ca^{2+} measurements, receptor binding studies, immunocytochemistry and Western blot. (A) Ca^{2+} luminescent response to motilin (full bars) and specific binding (empty bars) of ^{125}I-motilin to membranes from WT-MTLR, E119 D and E119Q mutants. (B) Immunohistochemistry experiments performed after culturing, fixation and staining of the CHO-K1 cells expressing the WT-MTLR, E119D, E119Q mutants with a motilin receptor antibody and a FITC labelled second antibody. No difference in intensity was observed. (C) Western blot analysis showing a band of ±60 kDa of similar intensity between the WT-TLR, the E119D and E119Q mutants. No band was observed when pre-immune serum was used as primary antibody.

by Asn or Glu in TM_3 of the β-adrenergic receptor decreased the affinity 8000–40000 and 300–1500 times, respectively [16]. For these receptors, it has been proposed that this Asp is located in a deep pocket formed between TM_3, TM_4, TM_5 and TM_6. The ligands could reach down and touch this trigger area and thereby activate their respective receptors, starting a cascade of conformational alterations down through the TMs, which then eventually would transfer the signal to the G protein. This model could also be applicable to the subfamily of the thyrotropin releasing and growth hormone secretagogue receptors to which the motilin receptor, the GHS-R and the thyrotropin releasing hormone receptor (TRH-R) belong. Thus Glu^{124} in TM_3 of the GHS-R and Asn^{110} in TM_3 of the TRH-R have been identified as part of the binding pocket [12,17]. However the notion that seven transmembrane receptors should be activated only through a ligand which binds to a common lock area located deep within the TM is currently being challenged. Instead, for certain peptides, such as substance P, it was suggested that they activate their receptors merely by stabilising an active conformation through ligand receptor interactions in the exterior portions of the receptor [11]. Thus, although each agonist ‘‘key’’ must have a corresponding ‘‘lock’’ there is probably not a common ‘‘lock’’ for all ‘‘keys’’ in the superfamily of seven transmembrane receptors.

The motilin and GHS-R family of receptors is rather unique in the way they are activated by their natural ligands. This relates to the location of critical determinants for binding and biological activity at the amino-terminal ends of both motilin [8] and ghrelin [18–20] rather than at the carboxyl terminus, which is most typical of peptide

receptors within the class A GPCRs [21]. Our data in this report also confirm that the positive charge of the N-terminal amine group of motilin is important for interaction with E^{119} which could be located in a binding pocket of the motilin receptor. The highly dynamic ''hinge-like'' region around residue 7 of motilin may flip the α-helix formed between residues 9 and 20 of the C-terminal part of motilin to make other contacts within the receptor, e.g. extra-cellular loops, and stabilise the conformation.

While our data form strong indications for an interaction between a carboxylic function on the receptor and a positive charge on the ligand, the nature of the interaction may differ between the two classes of ligands. Indeed, quantitatively the effect of removing the positive charge in the ligand, as in the acetylated analogues, is dramatic in the motilides, but much smaller in motilin. In GPCRs, like muscarinic and adrenergic receptors, disruption of an ionic interaction induces a shift in potency of 1000–100,000-fold. This is compatible with the loss of potency of the E119Q mutant and with the potency shift of the acetylated motilide, but not of the acetylated motilin in the WT-MTLR and E119D mutant. On the other hand, the basicity of the terminal amine is increased by single methylation as in $[N\text{-Me-Phe}]^1$ mot (1–14), and decreased by trimethylation as in $[N\text{-tri(Me)-Phe}]^1$ mot (1–14) and this is apparently reflected in an increased and decreased affinity for the WT-MTLR and the E119D mutant, respectively, illustrating the importance of the terminal amine. The activity of the E119D mutant is partially enhanced by a complementary change on the basic N^+ group of motilin as in $[\beta_2\ \text{Phe}]^1$ mot (1–22) but the shift in potency was too small to support a charge–charge interaction. Thus, although the direction of changes in potency indicate that the positive charge on the free N-terminal amine group of motilin is important, the shift is not large enough to be compatible with an ionic interaction and might be more easily explained by loss of hydrogen bonds.

If E^{119} is so important for motilin receptor function and if its partner cannot be found in motilin then maybe it is in the receptor. With the Swiss-pdb Viewer Program (ExPASy Molecular Biology Server, Swiss), the positively charged Arg^{318} in TM_6 is in close contact with E^{119} (3.78 Å). Both groups could be important to form a ligand-binding pocket for motilin. Alternatively, acetylated motilin perhaps interacted with the positively charged Arg^{318} residue in the receptor and in this way prevented the profound drop in potency due to loss of interaction with Glu^{119}. Also, for the TRH-R two transmembrane arginines, Arg^{283} in TM-6 and Arg^{306} in TM-7 are important for binding TRH [17].

In contrast to the results with motilin, the magnitude of the shift in potency induced by acetylation of the *N*-dimethyl amino group in EM-A enol ether, supports an ionic interaction between the N^+ group and E^{119}. However, the effect of E–Q mutation is more pronounced as compared to acetylation. Maybe, here too E^{119} forms an additional interaction within the receptor or the acetyl is forming additional bonds with the receptor.

Changing the tertiary amine of the deosamine sugar to a quaternary or secondary amine has little effect on the potency in the WT-MTLR and E119D mutant. In both cases a reduction in potency was noted. Also, muscarinic receptors accept all sorts of amines, including quaternary ones. In this case, the binding site Asp is accommodated in a ''nest'' formed by aromatic side chains (Phe, Tyr or Trp) from neighbouring amino acids. The weaker interaction of the ammonium positive charge with Asp is compensated by a strong cation to aromatic ring binding. In the motilin receptor there are two Tyr (Tyr^{123} and Tyr^{255}) very close to E^{119} ($Tyr^{123} - E^{119} = 8.86$ Å; $Tyr^{255} - E^{119} = 5.48$ Å) which could form an aromatic pocket.

Previous work on the motilin receptor has focussed on the second extra-cellular loop [22]. It was concluded that both ends of this loop, representing domains that are conserved, are functionally important for the binding and action of motilin, whereas the non-conserved residues in the mid-region of the loop are not necessary. In addition, the authors concluded that the regions important for motilin binding are not critical for erythromycin [22]. Matsuura et al. [22] may have detected ''outlying receptor sites'' that may be important for the access of motilin, but not of erythromycin-A into the binding pocket. A similar model has been proposed for the TRH-R. It was suggested that TRH initially interacts with residues in the extra-cellular loops and subsequently moves into the binding pocket that is entirely positioned in the transmembrane domain [23].

In conclusion, our results indicate that motilin and EM-A share a common binding site in TM_3 of the motilin receptor. We, therefore, propose that the ligand-binding pocket in the motilin receptor is disposed in the transmembrane domain similarly to other receptors within its family. Future studies need to elucidate which other amino acid residue within the receptor or motilin are involved.

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