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Differences in motilin receptor desensitization after stimulation with motilin or motilides are due to alternative receptor trafficking

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ABSTRACT

Backgrounds & aims: The motilin receptor (MTLR) is an important therapeutic target for treatment of hypomotility disorders. The negative outcome in clinical trials with the motilin agonist, ABT-229, indicated that desensitization may limit the therapeutic usefulness of motilides. We therefore compared the mechanisms involved in the intracellular trafficking of the MTLR after stimulation with motilin, erythromycin-A (EM-A) or ABT-229.
Methods: Desensitization was studied by measuring changes in Ca^{2+} rises and by receptor binding studies in CHO cells co-expressing the Ca^{2+} indicator apoaequorin and the MTLR, C-terminally tagged with EGFP. Receptor phosphorylation was studied by immunoprecipitation. MTLR-EGFP trafficking to organelles and translocation of β-arrestins were visualized by fluorescence microscopy.
Results: Agonist-induced desensitization of the MTLR was due to receptor internalization with potencies (p-int_{50}) in the order of: ABT-229 (8.3) > motilin (7.86) > EM-A (4.77) but with no differences in the internalization kinetics ($t_{1/2}$: ~25 min). The percentage cell surface receptor loss was more profound after exposure to ABT-229 (88 ± 1%) than to motilin (63 ± 10%) or EM-A (34 ± 2%). For motilin and EM-A MTLR phosphorylation probably occurs via G protein-coupled receptor kinases while for ABT-229 phosphorylation was also protein kinase C dependent. All agonists translocated cytosolic β-arrestin-2 with greater affinity to the plasma membrane than β-arrestin-1. After internalization the MTLR co-localized with transferrin but not with cathepsin D. After stimulation with motilin and EM-A the $t_{1/2}$ for MTLR resensitization was 3 h and 1 h, respectively but amounted 26 h for ABT-229.
Conclusion: Our results suggest that the resensitization kinetics determine the desensitization properties of the motilin agonists.

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1. Introduction

Identified more than 25 years after the discovery of its natural ligand, the motilin receptor (MTLR) was classified within a new subgroup of Class I of rhodopsin like G-protein-coupled receptors (GPCRs), containing receptors with structural homology to the growth hormone secretagogue receptor, now identified as the ghrelin receptor [1]. Motilin is a gastrointestinal hormone that is involved in the regulation of gastrointestinal motility. By coincidence it was discovered that the antibiotic compound erythromycin mimics the effects of motilin by binding to motilin receptors [2,3]. Erythromycin and motilin itself accelerate gastric emptying in healthy subjects [4,5] and in patients with diabetic gastroparesis [6,7]

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and stimulate interdigestive motility [8,9]. Therefore, the motilin receptor became a potential therapeutic target for the treatment of hypomotility disorders and new erythromycin derivatives with motilin receptor agonist activity devoid of antibacterial properties (the so-called motilides) were developed [10]. Out of several candidate drugs, the most promising one, ABT-229 [N-ethyl, N-methyl 4″deoxy erythromycin (EM)-B enolether], showed no symptomatic benefit in clinical trials of functional dyspepsia [11] and diabetic gastroparesis [12]. This meant a serious drawback for the development of motilin agonists and the therapeutic usefulness of motilides was questioned in general. Nevertheless, some important lessons could be taken from these studies. It appeared that in addition to the study design, the possible strong desensitizing properties of ABT-229 and its possible effect on gastric accommodation may have contributed to the negative outcome [13,14]. These arguments should be taken into consideration when developing new motilin agonists. Sustained efficacy may be a better target to screen for than the potency to stimulate gastrointestinal motility.

Recent in vitro contractility studies with smooth muscle strips preparations from the rabbit duodenum and Ca^{2+} studies in a cell line expressing the MTLR showed that ABT-229 has unusually strong desensitizing properties compared to what can be expected from its activity [15]. A comparison of the effect of structural changes in the motilide on changes in activity and desensitization suggested that removal of a specific hydroxyl group within the ABT-229 molecule contributes most to its strong desensitizing properties and that it is possible to separate agonist potency form the desensitization properties of motilides. Since receptor desensitization may limit the biological effects of the motilin receptor it is crucial to gain insight into the desensitization mechanisms of the motilin receptor.

Desensitization of GPCRs may occur within seconds to minutes of agonist stimulation, and is generally considered to result from receptor uncoupling from downstream effectors due to receptor phosphorylation. Phosphorylation of activated GPCRs by G protein-coupled receptor kinases (GRKs) leads to increased binding of β-arrestins to the receptor complex, which in turn inhibits further receptor-G protein coupling and thus attenuates receptor signaling [16]. β-Arrestins target phosphorylated receptors to clathrin-coated pits for subsequent internalization [17,18] but they can also participate as molecular scaffolds for protein kinase cascade signaling to the cytoplasm and nucleus [19]. Once the receptors are internalized, they are either recycled back to the membrane after dephosphorylation or sorted to the lysosomal pathway for degradation [20,21].

The mechanisms involved in the desensitization process of the motilin receptor have not been completely elucidated, but clarifying its intracellular fate at the molecular level upon agonist stimulation may be helpful in the development of more specific and effective motilin receptor agonists, with less desensitizing properties. Especially motilin receptor phosphorylation and the role of β-arrestins in the desensitization process have not been studied so far. Therefore, the aim of our study was to determine the differences in the molecular and cellular pathways involved in the motilin- and motilide-induced intracellular trafficking of the MTLR. As cellular model we used a Chinese hamster ovary (CHO) cell line which stably expressed the MTLR or the MTLR C-terminally tagged with an enhanced green fluorescent protein (MTLR-EGFP) to study receptor phosphorylation, the role of β-arrestins in the internalization process, the targeting of the receptor to specific organelles and the kinetics of desensitization and resensitization. To clarify the unusually strong desensitizing properties of ABT-229 we compared the intracellular trafficking of the MTLR after exposure to either ABT-229, motilin or erythromycin A (EM-A).

2. Materials and methods

2.1. Test compounds

Norleucine[13]-porcine-motilin (motilin) was purchased from Eurogentec (Namur, Belgium). Erythromycin-A lactobionate (EM-A) was obtained from Abbott Laboratories (Abbott Park, IL, USA). N-Ethyl, N-methyl 4″ deoxy EM-B enol ether (ABT-229) was a gift from Dr. P. Lartey (Abbott Laboratories, Abbott Park, IL, USA). Angiotensin II was obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture and transient transfection

A Chinese hamster ovary cell line stably expressing the human motilin receptor (MTLR) and the mitochondrially targeted Ca^{2+} indicator apoaequorin was 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) (MTLR-EGFP) was performed as previously described [15]. The cells were cultured in Ham's F12 supplemented with 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 split weekly with 5 mM EDTA in phosphate buffered saline (PBS).

For transient transfection, CHO cells were grown to ∼90% confluency on two-well chambered coverglasses. Cells were transfected with Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacture's instructions. In each well, 0.8 μg plasmid containing cDNA encoding the wild type MTLR or HA-AT_{1A}R (hemagglutinin angiotensin II type 1a receptor) was used in addition to 0.6 μg plasmid containing cDNA encoding either β-arrestin-1 fused with EYFP (enhanced yellow fluorescent protein) or β-arrestin-2 fused with EGFP (enhanced green fluorescent protein). Transfected cells were cultured for 48 h to allow expression. The construction of the HA-AT_{1A}R, β-arrestin-1-EYFP and β-arrestin-2-EGFP plasmids has been described previously [22,23] and were a kind gift from Dr. M. Scott (Paris, France).

2.3. Receptor activation: aequorin based Ca^{2+} luminescent assay

Ca^{2+} rises in the MTLR and MTLR-EGFP cell line elicited after stimulation with increasing concentrations (10^{-12} to 10^{-5} M) of motilin, ABT-229 or EM-A were determined by measuring the luminescent response emitted after binding of the released Ca^{2+} with the aequorin complex as previously described [24]. The Ca^{2+} response was expressed as a percentage of maximal

stimulation obtained with Triton X-100 (0.9%) and the concentration which induced 50% of the maximal response (p-EC_{50}) was calculated.

2.4. *Quantification of MTLR desensitization and resensitization: aequorin based Ca^{2+} luminescent assay*

To induce desensitization, MTLR and MTLR-EGFP cells were pre-stimulated with different concentrations of the agonists or buffer for 2 h at 37 °C during loading with coelenterazine h, washed and centrifuged before concentration–response curves to motilin were established. The average of the maximal plateau values of the concentration–response curve to motilin under desensitizing conditions was expressed as % of maximal response under control conditions (no pre-incubation). From these curves, the pre-treatment concentration which reduces the maximal response to 50% of the control value (p-DC_{50}) was calculated.

For resensitization, MTLR cells were incubated with equipotent doses (1000-fold EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M) for 2 h, washed and kept at 37 °C for 0, 2, 4, 8, 12, and 24 h. The effect of the protein synthesis inhibitor cycloheximide (CHX) on receptor resensitization was measured in the absence or presence of CHX (1 μg/ml) 24 h after pre-incubation of MTLR cells for 2 h with motilin (10^{-7} M) or ABT-229 (10^{-7} M). The Ca^{2+} response of pre-treated cells to a second stimulation with motilin was expressed as % of the Ca^{2+} response of buffer-treated cells at the respective time points and the time at which 50% of the receptors resensitized ($t_{1/2}$) was calculated.

2.5. *Quantification of MTLR internalization: receptor binding studies*

Agonist-induced decreases in the number of receptors located on the cell surface were measured by means of whole cell receptor binding with [^{125}I]-motilin. MTLR-EGFP cells (~80% confluency) were treated with different concentrations of motilin, EM-A or ABT-229 for 2 h at 37 °C. To determine the internalization kinetics, MTLR-EGFP cells were treated with equipotent doses (1000-fold EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M) for 0, 5, 10, 20, 30 and 120 min at 37 °C. At appropriate times, surface-bound ligands were removed with an acid wash (0.5 M NaCl, 5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM HEPES, 1% bovine serum albumin, 0.1% glucose, 0.2 M acetic acid, pH 4.0; 10 min, 4 °C), which does not effect subsequent receptor binding, and then radioligand binding [^{125}I]-motilin (0.3 nM, 4 h at 4 °C) was performed to measure receptors remaining at the cell surface. Membrane bound [^{125}I]-motilin binding was expressed as a percentage of the specific binding obtained with control cells treated with buffer. The pre-treatment concentration which reduces the specific binding to 50% of the control value (p-int_{50}) and the time at which the agonist promoted 50% loss of cell surface receptors ($t_{1/2}$) was calculated.

2.6. *Receptor phosphorylation*

MTLR-EGFP cells were incubated (37 °C, 16 h) in 10 ml phosphate-free DMEM (Invitrogen, Carlsbad, CA) and loaded with 200 μCi/ml [^{32}P]orthophosphate (Amersham Biosciences, Piscataway, NJ, USA) for 2 h at 37 °C to label their ATP pool. Loaded cells were stimulated with different concentrations of motilin, ABT-229 or EM-A for 10 min at 37 °C. Phosphorylation kinetics were determined by stimulation with motilin (10^{-7} M) for 0, 1, 2, 5, 10 and 30 min at 37 °C. Protein kinase A (PKA) and C (PKC) inhibitors were added 30 min prior to addition of equipotent doses (1000-fold EC_{50}) of agonist. Cells were then washed and lysed. Cell lysates were cleared by centrifugation and membrane fragments were immunoprecipitated with an anti-EGFP antibody as previously described [25]. The extent of $^{32}P_i$ incorporation was measured with Storm 860 PhosphorImager and quantified by with the ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA, USA). p-$phos_{50}$ values and the time at which 50% of the receptors are phosphorylated ($t_{1/2}$) were calculated.

2.7. *Visualization of MTLR internalization and resensitization*

Internalization of the MTLR during desensitization was visualized with the EGFP-tagged receptor. MTLR-EGFP cells (~70% confluency) were stimulated with different concentration of agonists for 2 h at 37 °C, washed three times with 0.1 M PBS and fixed for 20 min at room temperature with 2% paraformaldehyde. Finally, cells were washed three times with 0.1 M PBS. To determine the internalization kinetics, MTLR-EGFP cells were stimulated with equipotent doses (1000-fold EC_{50}) of agonist for 0, 5, 10, 20, 30, 60, 90 and 120 min at 37 °C.

For recycling, MTLR-EGFP cells were incubated with equipotent doses of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M) for 2 h at 37 °C. Cells were then washed three times and kept at 37 °C for 0, 2, 4, 8, 12, and 24 h before being fixed and visualized under a fluorescence microscope (see Section 2.10).

2.8. *Intracellular localization of the MTLR*

The localization of the MTLR in endosomes was verified by checking its co-localization with transferrin. MTLR-EGFP cells (~70% confluency) were rinsed three times and incubated with serum-free medium, to deplete endogenous transferrin and growth factors, for 30 min at 37 °C. The cells were then simultaneously incubated with Alexa Fluor 594-transferrin (50 μg/ml; Molecular Probes, Leiden, the Netherlands) and 10^{-7} M motilin, 10^{-4} M EM-A or 10^{-6} M ABT-229 for 0, 5, 30 and 120 min. Cells were rinsed, fixed in 2% paraformaldehyde and visualized under a fluorescence microscope (see Section 2.10).

The localization of the MTLR in lysosomal compartments was verified by checking its co-localization with cathepsin D. MTLR-EGFP cells (~70% confluency) were washed and stimulated with equipotent doses (1000-fold EC_{50}) of agonist for 2 h at 37 °C, washed and fixed in 2% paraformaldehyde for 20 min at room temperature. Subsequently, cells were washed and permeabilized with 0.5% Triton X-100 in a blocking solution (4% donkey serum, 0.3% NaN_3 in 0.1 M PBS) for 2 h at 4 °C and incubated with cathepsin D goat polyclonal primary antibody (1:100; Santa Cruz biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After three washes, cells were incubated

for 2 h at 4 °C with Cy^3-conjugated donkey anti-goat IgG (1:500; Jackson Immuno Research Labs, West Grove, PA, USA) secondary antibody. Finally, cells were washed and imaged (see Section 2.10).

2.9. *Role of β-arrestin-1-EYFP and β-arrestin-2-EGFP in the internalization process of motilin receptor*

Transiently transfected CHO cells co-expressing the MTLR and β-arrestin-1-EYFP (β-arr_1-EYFP) or β-arrestin-2-EGFP (β-arr_2-EGFP) were treated with equipotent doses (1000-fold EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M) for 0, 2, 5, 10 and 30 min at 37 °C. Cells were washed, fixed and processed for immunofluorescence localization with a rabbit anti-MTLR polyclonal antibody (final dilution 1:500; Andy Howard, Merck, 24 h at 4 °C) as primary antibody and Cy^3-conjugated goat anti-rabbit IgG (1:500; Jackson Immunores Laboratories Inc., West Grove, PA, USA, 2 h at 4 °C) as secondary antibody.

As a positive control, we used transiently transfected CHO cells co-expressing the hemagglutinin angiotensin II type 1a receptor (HA-AT_{1A}R) and β-arr_1-EYFP or β-arr_2-EGFP. The intracellular trafficking of the receptor was visualized by means of an "antibody feeding" method. Cell surface receptors were specifically labeled by incubating cells with the primary rat anti-HA monoclonal antibody (3F10; Roche Applied Science, Mannheim, Germany) at a 1:100 dilution for 1 h at 4 °C. Labeled cells were subsequently treated with 10^{-6} M angiotensin II (Sigma, St. Louis, MO, USA) for 0, 2, 5, 10 and 30 min at 37 °C. Cells were washed, fixed, permeabilized and incubated with Cy^3-conjugated goat anti-rat IgG (dilution 1:500; Jackson Immunores Laboratories Inc., West Grove, PA, USA) for 2 h at 4 °C.

2.10. *Fluorescence microscopy and quantification of fluorescence intensity*

Fluorescent images were acquired using a Zeiss Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany) with a 63× H_2O immersion lens and TILLvisION Poly V light source (TILL Photonics, Gräfelfing, Germany) with '*green*' (ex: SPB51, dm: DCLP490, em: BPD525/50) and '*red*' (ex: SP580, dm: DCLP595, em: BP645/75) filter cubes. Images were captured on a cooled CCD camera (IMAGO-QE, Gräfelfing, Germany) with the Image Pro Plus software (Media Cybernetics, Silver spring, MD, USA). Selection of vesicles for co-localization studies were restricted to thinner parts of the cytosol, where, due to absence of out of focus fluorescence, individual vesicles could easily be identified in the two color channels.

The intracellular fluorescence intensity was quantified by generating random fluorescence intensity profiles (line scans) across cells. The cytosolic area under the curve was quantified with the Image Pro Plus software (Media Cybernetics, Silver spring, MD, USA). The fluorescence intensity of at least 25 individual cells was determined for each set of conditions. The change in fluorescence intensity was expressed as a percentage of the maximal change in fluorescence intensity obtained after 2 h of stimulation with the respective agonist. The fluorescence intensity of buffer-treated cells was subtracted as background.

2.11. *Statistical analysis*

p-EC_{50}, p-DC_{50}, p-int_{50} and p-$phos_{50}$ and $t_{1/2}$ values were determined by non-linear regression (sigmoidal dose–response curve or one phase exponential association) using the GraphPad Prism 4.0 software (San Diego, CA, USA), respectively.

Data were analyzed by means of the Student's *t*-test or one-way ANOVA followed by Tukey's post-hoc test with the GraphPad Prism 4.0 software (San Diego, CA, USA). *P* values lower than 0.05 were considered to be significant.

3. Results

3.1. *Agonist-induced desensitization of the motilin receptor*

We first tested whether fusion of the EGFP protein to the carboxyl terminus of the MTLR altered the functional and desensitizing properties of the MTLR. We already demonstrated in a previous study that changes in the binding characteristics of the MTLR-EGFP cell line were limited to small changes in the B_{max}-values [25]. Functional studies showed that the potency (p-EC_{50}) of motilin and the motilides to induce a Ca^{2+} rise was comparable in both cell lines: motilin > ABT-229 > EM-A (Table 1).

The desensitizing properties of motilin and the motilides were also studied in the MTLR-EGFP by measuring the effect of pre-treatment of the cells with the agonists on motilin-induced Ca^{2+} responses (Table 1). The order of potency to desensitize (p-DC_{50}) MTLR-EGFP cells was: ABT-229 > motilin > EM-A and was similar to those in the CHO-MTLR cell line (Table 1). Thus, these results suggest that fusion of the EGFP protein to the carboxyl terminus of the MTLR is an appropriate tool to study the molecular and cellular pathways of the desensitization process of the MTLR.

3.2. *Agonist-induced phosphorylation of the motilin receptor*

As shown in Fig. 1A, exposure of MTLR-EGFP cells to motilin promoted phosphorylation of a labeled protein band with a molecular mass of approximately 105 kDa similarly to what we observed in a previous report [25]. Kinetic analysis revealed that MTLR-EGFP phosphorylation after exposure to motilin was rapid ($t_{1/2} < 1$ min) and was sustained up to 30 min (Fig. 1A). No labeled protein band was detected in non-transfected cells after exposure with motilin for 10 min (results not shown).

To examine the concentration-dependent effects of the agonists on MTLR-phosphorylation, MTLR-EGFP cells were incubated for 10 min in the presence of buffer or varying concentrations of motilin, EM-A or ABT-229. Fig. 1B demonstrates that motilin, EM-A and ABT-229 induced a concentration-dependent increase in the phosphorylation intensity of the MTLR-EGFP producing maximum values ~7 fold above basal levels. The rank order of potency to induce phosphorylation (p-$phos_{50}$) was motilin (8.10 ± 0.28) ~ ABT-229 (7.94 ± 0.15; $P > 0.05$ versus motilin) > EM-A (4.20) (Fig. 1B).

Table 1 – Comparison of motilin and motilide -induced Ca^{2+} luminescent responses (p-EC_{50}) and desensitization (p-DC_{50}) of Ca^{2+} luminescent responses of MTLR and MTLR-EGFP stably expressed in CHO cells

	Activation p-EC_{50} ± S.E.M.		Desensitization p-DC_{50} ± S.E.M.	
	MTLR-WT	MTLR-EGFP	MTLR-WT	MTLR-EGFP
Motilin	9.73 ± 0.12	10.1 ± 0.07	7.78 ± 0.09	7.77 ± 0.02
EM-A	6.69 ± 0.12	7.13 ± 0.13	4.61 ± 0.08	4.13 ± 0.04
ABT-229	8.93 ± 0.02	9.00 ± 0.15	8.59 ± 0.10	8.47 ± 0.02

We next evaluated the role of the second messenger-dependent kinases, protein kinase A (PKA) and C (PKC), in the phosphorylation process (Fig. 2). Treatment of MTLR-EGFP cells with the PKA activator, forskolin (10^{-5} M), and the PKC activator, phorbol 12-myristate 13-acetate (3×10^{-8} M), did not induce MTLR-EGFP phosphorylation (Fig. 2A, upper panel). As shown in Fig. 2A, a labeled band corresponding to the 105 kDa MTLR-EGFP was detected after stimulation with the agonists (bottom panels). Densitometric analysis of the images (Fig. 2B) revealed that MTLR-EGFP phosphorylation induced by motilin (10^{-7} M) and EM-A (10^{-4} M) was not effected by the PKA inhibitor, H-89 (10^{-7} M), and the PKC inhibitor, GF109203X (10^{-6} M) ($P > 0.05$ versus control), suggesting that receptor phosphorylation after stimulation with motilin and EM-A is not mediated by second messenger-dependent kinases. In contrast, MTLR-EGFP phosphorylation induced by ABT-229 (10^{-6} M) was reduced by 37 ± 4% by GF109203X ($P < 0.01$ versus control) but not by H-89 ($P > 0.05$ versus control) (Fig. 2A and B), indicating that receptor phosphorylation after stimulation with ABT-229 also occurs via the second messenger-dependent kinase PKC.

3.3. Agonist-induced internalization of the motilin receptor

Pretreatment of the MTLR-EGFP cells with increasing concentrations of motilin, EM-A and ABT-229 caused a concentration-dependent loss of cell surface receptors as determined with receptor binding studies (Fig. 3). The order of potency of the agonists to induce receptor internalization (p-int_{50}) was ABT-229 (8.31 ± 0.06) > motilin (7.87 ± 0.03) > EM-A (4.69 ± 0.15).

3.4. Visualization of the agonist-induced motilin receptor internalization

The concentration-dependency of the internalization process was visualized by fluorescence microscopy after pretreatment of MTLR-EGFP cells with buffer or varying concentrations of motilin, EM-A or ABT-229 for 2 h at 37 °C (Fig. 4). In non-stimulated cells, the fluorescent labeling was largely confined to the plasma membrane (Fig. 4A). Exposure of the cells to motilin, EM-A or ABT-229 induced a concentration-dependent increase in MTLR-EGFP accumulation in the perinuclear region together with a decrease in fluorescence at the plasma membrane (Fig. 4A). The order of potency for inducing receptor internalization (p-int_{50}) was ABT-229 (8.24 ± 0.01) > motilin (7.88 ± 0.01) > EM-A (4.86 ± 0.01) (Fig. 4B).

The time-dependency of the internalization process was investigated after pretreatment of MTLR-EGFP cells with an equipotent dose of motilin, EM-A or ABT-229 to achieve maximal receptor activation. The $t_{1/2}$ of MTLR-EGFP inter-

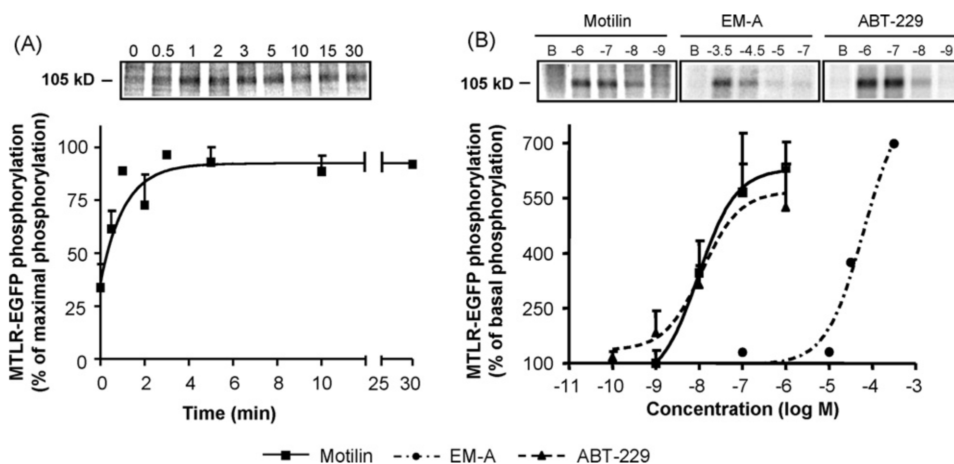

Fig. 1 – Agonist-induced phosphorylation of the MTLR-EGFP. MTLR-EGFP cells were labeled with [$^{32}P_i$] and immunoprecipitated with an anti-EGFP antibody. Samples were subjected to SDS-PAGE and quantified. (A) Time course of motilin-induced MTLR-EGFP phosphorylation. Results are expressed as the percentage of maximal MTLR-EGFP phosphorylation relative to non-treated cells. Results are mean ± S.E.M. from three independent experiments. A representative phosphorimage scan of phosphorylated MTLR-EGFP after electrophoresis is shown. (B) Concentration–response curves of MTLR-EGFP phosphorylation induced by increasing concentrations of motilin, EM-A and ABT-229 10 min after stimulation. Results are expressed as the percentage of basal MTLR-EGFP phosphorylation. Results are expressed as mean ± S.E.M. (motilin: *n* = 6; ABT-229: *n* = 5; EM-A: *n* = 1). A representative phoshporimage scan of phophorylated MTLR-EGFP after electrophoresis is shown (B, basal).

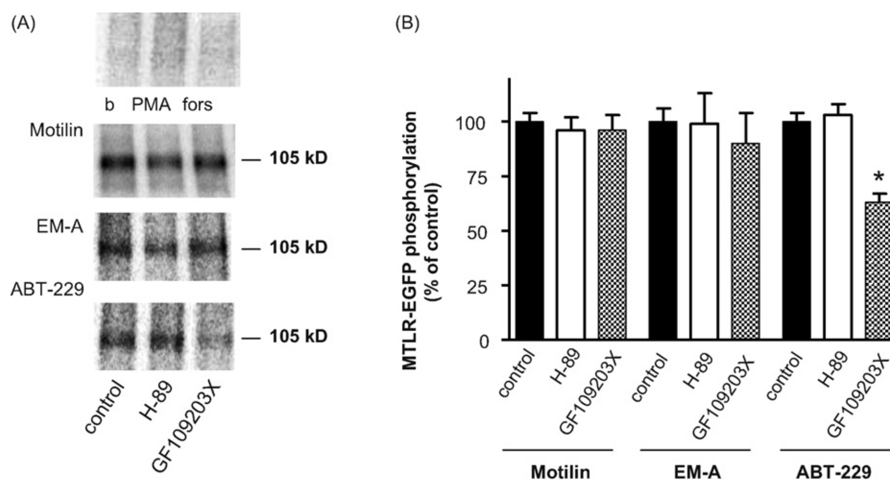

Fig. 2 – The effect of second messenger protein kinase inhibitors and activators on MTLR-EGFP phosphorylation. (A) *Upper panel*, the effect of buffer (b), the PKC-activator phorbol 12-myristate 13-acetate (PMA) (3×10^{-8} M) or the PKA activator forskolin (fors) (10^{-5} M) on MTLR-EGFP phosphorylation. *Bottom panels*, the effect of a 30-min pre-incubation with the PKA inhibitor H-89 (10^{-7} M) or the PKC inhibitor GF109203X (10^{-6} M) on MTLR-EGFP phosphorylation induced by with an equipotent dose (1000-fold of EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M). Shown are representative phosphorimage scans of phosphorylated MTLR-EGFP after electrophoresis. (B) Densitometric analysis of the effect of a 30-min pre-incubation with H-89 (10^{-7} M) or GF109203X (10^{-6} M) on MTLR-EGFP phosphorylation induced by the agonists. The data are means ± S.E.M. and each experiment was repeated three times. *P < 0.01 vs. control.

nalization determined with receptor binding studies was estimated to be ~27 min for all agonists (Fig. 5). A similar $t_{1/2}$ (~23 min) was obtained if the kinetics of motilin receptor internalization were followed by fluorescence microscopy (data not shown). However, the percentage cell surface receptor loss after 120 min of agonist exposure was more for ABT-229 (88 ± 1%) than for motilin (63 ± 10%, $P < 0.05$ versus ABT-229) and EM-A (34 ± 2%, $P < 0.05$ versus motilin).

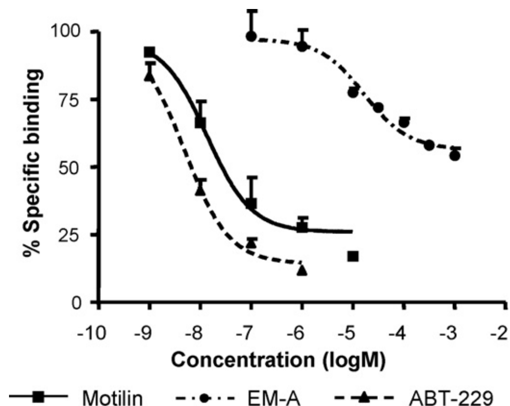

Fig. 3 – Concentration-dependent internalization of the MTLR-EGFP. MTLR-EGFP cells were treated with different concentrations of motilin, EM-A or ABT-229 for 2 h at 37 °C. The remaining receptors at the cell surface were measured after an acid wash by means of whole cell receptor binding studies with [^{125}I]-motilin. Results are expressed as a percentage of specific binding of buffer-treated cells. Mean values ± S.E.M. are plotted from four independent experiments performed in duplicate.

3.5. *Intracellular localization of the motilin receptor*

During the time-course of MTLR-EGFP internalization the intracellular localization of the MTLR was studied in the presence of transferrin, a marker for early and recycling endosomes [26]. Equipotent doses of motilin, EM-A or ABT-229 and Alexa Fluor 594-labeled transferrin (50 μg/ml) were added simultaneously to MTLR-EGFP cells and their co-localization was studied at different time points with fluorescence microscopy (Fig. 6A). After a 5-min incubation period with motilin, EM-A or ABT-229, the MTLR-EGFP (green) was co-localized with transferrin (red) in small vesicles at the periphery of the cells (overlay images, yellow-orange color). After 120 min exposure to motilin, EM-A or ABT-229 the bulk of MTLR-EGFP was distributed into endosomal compartments and overlapped with the transferrin staining (Fig. 6A, overlay images, yellow-orange color).

The possibility that internalized receptors could be targeted to the lysosomes was assessed by co-localization studies with cathepsin D, a lysosomal aspartyl protease marker of the lysosome-directed pathway (Fig. 6B). The MTLR-EGFP did not co-localize with cathepsin D with either of the agonists (Fig. 6B, overlay images, green and red color).

3.6. *Role of β-arrestin-1-EYFP and β-arrestin-2-EGFP in the internalization process of the motilin receptor*

The effect of receptor activation with motilin or the motilides on the recruitment of both β-arrestins-1 and -2 was investigated. In unstimulated MTLR cells co-transfected with plasmids for β-arr_1-EYFP and β-arr_2-EGFP, arrestins were uniformly distributed throughout the cytosol (Fig. 7A). Upon stimulation with 10^{-7} M motilin for 2, 5 and 10 min, cytosolic

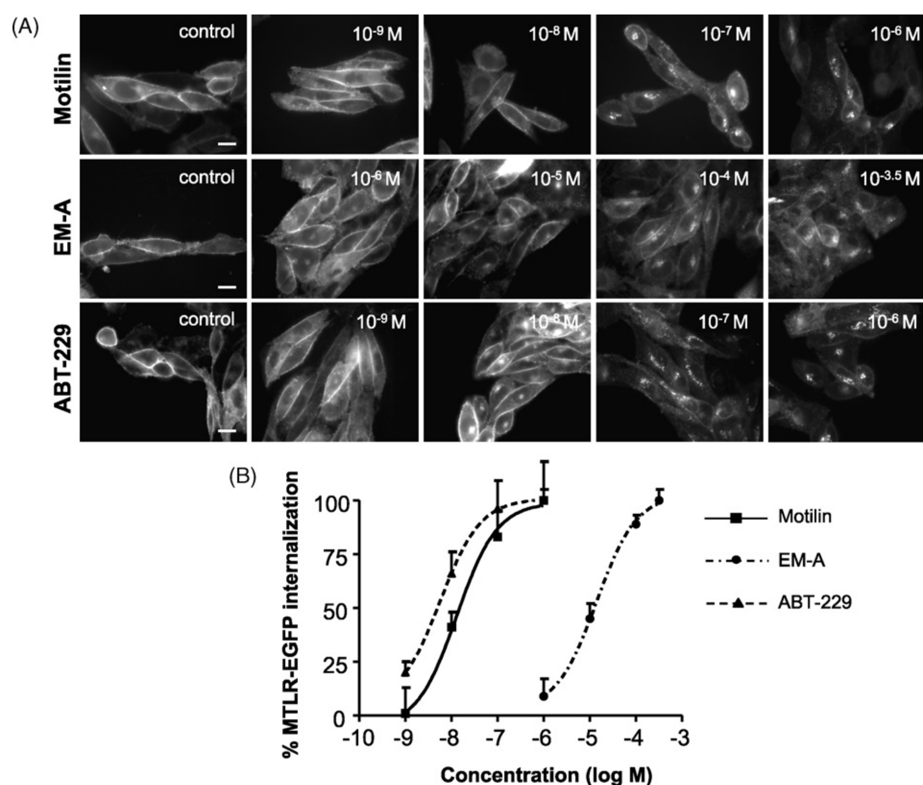

Fig. 4 – Visualization of the concentration-dependency of agonist-induced MTLR-EGFP internalization. MTLR-EGFP cells were treated for 2 h at 37 °C with different concentrations of motilin, EM-A or ABT-229. (A) After fixation cells were visualized by fluorescence microscopy. *Bar*, 15 μm. (B) The intracellular fluorescence intensity was quantified. Results are expressed as a percentage of the change in fluorescent intensity obtained with the highest concentration of each agonist. The data are expressed as mean values ± S.E.M. of 25 cells.

β-arr$_1$-EYFP and β-arr$_2$-EGFP were translocated to the cell membrane. This effect was more pronounced with β-arr$_2$-EGFP than with β-arr$_1$-EYFP. After 30 min of agonist exposure no intracellular vesicles of β-arr$_1$-EYFP and β-arr$_2$-EGFP were observed and the fluorescence was uniformly distributed back throughout the cytosol. Similar results were obtained after stimulation of the cells with EM-A (10^{-4} M) and ABT-229 (10^{-6} M) (data not shown).

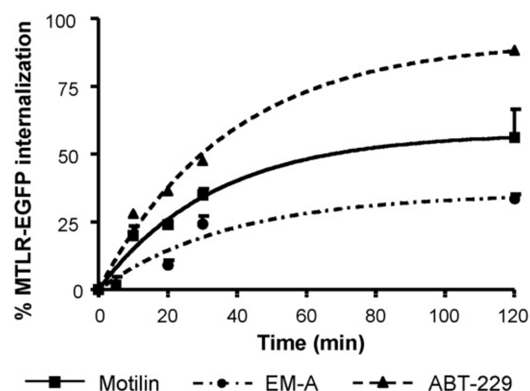

Fig. 5 – The time course of agonist-induced MTLR-EGFP internalization. The loss of cell surface receptors was quantified by measuring the remaining cell surface receptors by means of whole cell receptor binding studies with [^{125}I]-motilin. Results are expressed as percentage of the specific binding obtained with buffer-treated cells. Results are expressed as mean ± S.E.M. from three experiments performed in duplicate.

As a positive control CHO cells were transiently co-transfected with hemagglutinin angiotensin II type 1a receptor (HA-AT$_{1A}$R) and β-arr$_1$-EYFP or β-arr$_2$-EGFP (Fig. 7B) and stimulated with 10^{-6} M angtiotensin II for 0, 2, 5, 10 and 30 min at 37 °C. As expected, in response to agonist stimulation of the HA-AT$_{1A}$R, both β-arr$_1$-EYFP and β-arr$_2$-EGFP translocated with similar affinities to the cell membrane and fluorescent intracellular vesicles were observed after 10 and 30 min of agonist exposure.

Translocation of β-arrestins suggests interaction with the agonist-occupied receptor. In unstimulated MTLR cells, the MTLR (red) was mainly localized at the cell surface (red) and β-arr$_2$-EGFP was uniformly distributed throughout the cytosol (green) (Fig. 8A and B). Exposure to motilin or ABT-229 for 2 min led to membrane targeting of β-arr$_2$-EGFP and the formation of membrane-localized pits, co-localizing with the MTLR (Fig. 8A and B, overlay and line profile). After exposure to motilin or ABT-229 for 30 min, the MTLR was internalized into vesicles which did not co-localize with β-arr$_2$-EGFP (Fig. 8A and B, overlay and line profile). After stimulation with angiotensin II for 2 min a similar translocation was observed with β-arr$_2$-EGFP in cells expressing the HA-AT$_{1A}$R as for motilin (Fig. 8C). In contrast, after stimulation with angiotensin II for 30 min,

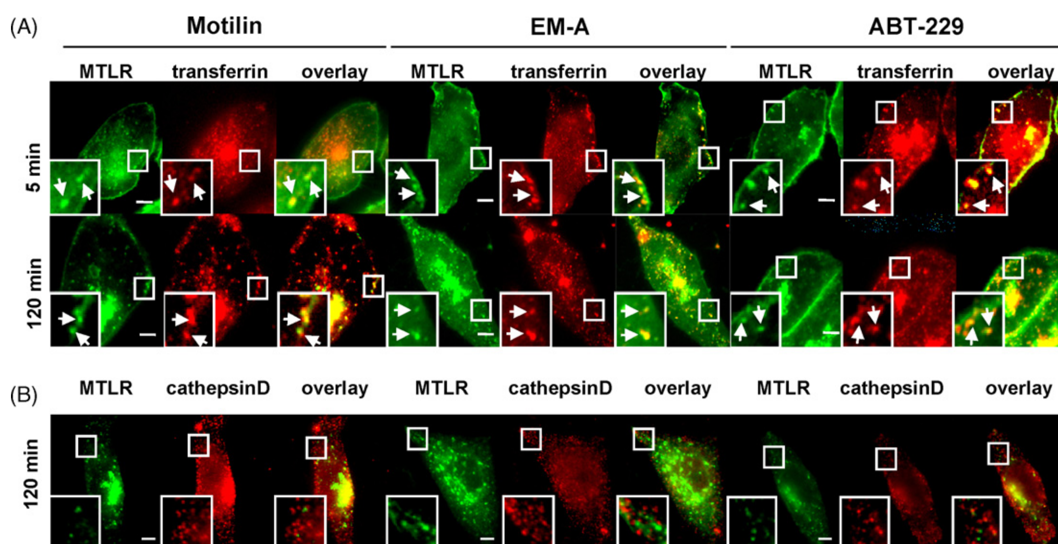

Fig. 6 – Intracellular localization of the MTLR-EGFP after stimulation with agonists. (A) MTLR-EGFP cells (*green*) were simultaneously incubated with an equipotent dose (1000-fold of EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M) and 50 μg/ml Alexa Fluor 594-labeled transferrin (*red*) for 5 or 120 min at 37 °C. The cells were fixed and visualized by fluorescence microscopy. The arrows indicate examples of vesicles containing the MTLR-EGFP and transferrin. *Yellow* colour in the overlap images indicates co-localization of the MTLR-EGFP and transferrin. (B) MTLR-EGFP cells (green) were treated with an equipotent dose (1000-fold of EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M) for 120 min and immunostained for the lysosomal marker, cathepsin D (red). The overlay images show no co-localization of the MTLR-EGFP (*green*) with cathepsin D (*red*). *Bar*, 5 μm. Each experiment was repeated three times.

the HA-AT_{1A}R was still co-localized with β-arr_2-EGFP (green) into to deep core vesicles (Fig. 8C, overlay and line profile). Similar translocation patterns were observed with β-arr_1-EYFP (data not shown). These results suggest that the MTLR is already dissociated from β-arrestin near or at the membrane and not in the endosomes like the HA-AT_{1A}R.

3.7. *Resensitization of the motilin receptor*

Recycling of the receptor to the cell surface was visualized by fluorescence microscopy after internalization of the MTLR-EGFP was triggered by equipotent doses of motilin, EM-A or ABT-229. In contrast to the internalization kinetics, the recycling kinetics were agonist dependent. After maximal receptor activation with motilin or EM-A the half-time for MTLR resensitization was 3 h and 1 h, respectively. In contrast recycling of the MTLR after stimulation with ABT-229 was much slower with a half-time of 26 h (Fig. 9A). Similar results were found if the resensitization kinetics were determined by Ca^{2+} measurements (Fig. 9B). 24 h after pre-treatment with ABT-229, the Ca^{2+} response of the MTLR was still reduced by 87% whereas for motilin and EM-A the Ca^{2+} responses of the MTLR were normalized. The internalized vesicles containing the MTLR-EGFP were still localized in the endosomes 24 h after

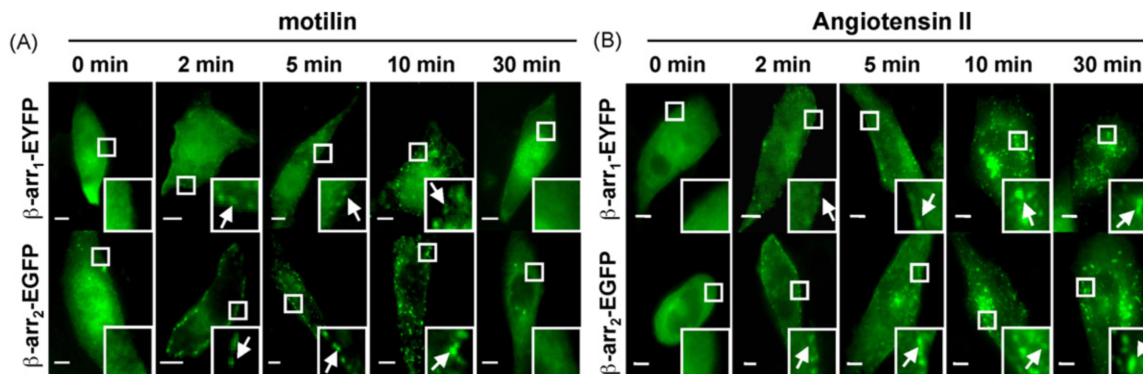

Fig. 7 – Visualization of β-arrestin trafficking in CHO cells transiently expressing the MTLR (A) or HA-AT_{1A}R (B) after agonist stimulation. (A) Cells were transiently transfected with the MTLR and β-arr_1-EYFP or β-arr_2-EGFP, exposed to 10^{-7} M motilin for 0, 2, 5, 10 and 30 min, fixed and visualized by fluorescence microscopy. (B) As a positive control the same experiment was performed on cells transiently expressing the HA-AT_{1A}R and β-arr_1-EYFP or β-arr_2-EGFP. Cells were exposed to 10^{-6} M angiotensin II for 0, 2, 5, 10 and 30 min. The arrows indicate examples of β-arrestin translocation. *Bar*, 5 μm.

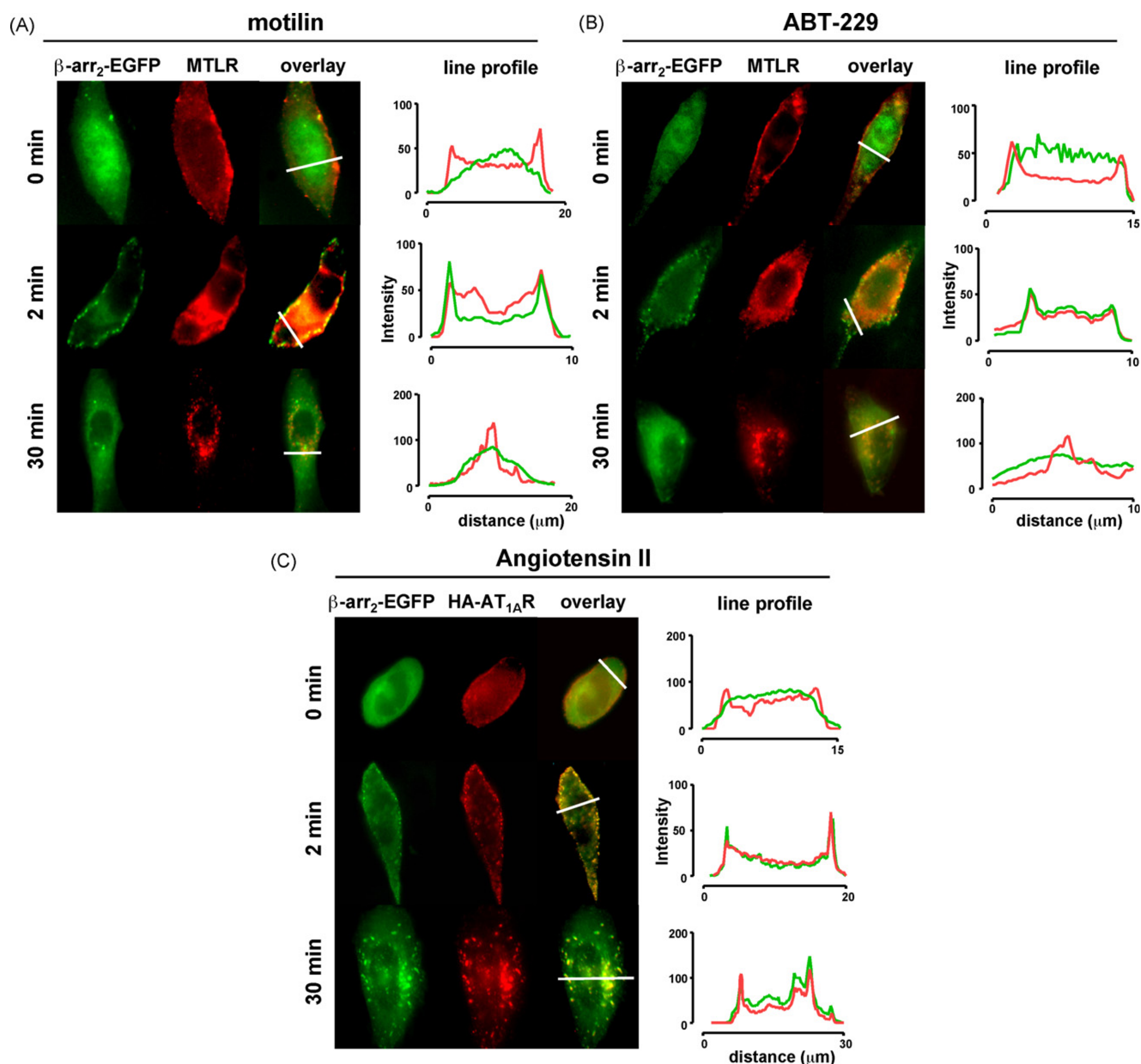

Fig. 8 – Co-localization of the MTLR with β-arr$_2$-EGFP after stimulation with 10^{-7} M motilin (A) and 10^{-6} M ABT-229 (B) and of the HA-AT$_{1A}$R with β-arr$_2$-EGFP after stimulation with 10^{-6} M angiotensin II (C). β-Arr$_2$-EGFP was visualized by its intrinsic fluorescence (*green*) and the MTLR and the HA-AT$_{1A}$R were visualized by immunofluorescence at the respective time points with anti-MTLR or anti-HA as primary antibody and a Cy^3-conjugated secondary antibody (*red*). Profiles of the fluorescence intensity along the lines depicted in the overlap images are shown for MTLR and HA-AT$_{1A}$R. Each experiment was repeated three times.

pre-treatment with ABT-229 since they co-localized with the endosomal marker transferrin (Fig. 10A, left panel) but not with the lysosomal marker cathepsin D (Fig. 10A, right panel). Resensitization of a receptor requires reappearance of the receptor at the cell surface, either by recycling or by new receptor synthesis. As motilin receptor resensitization after stimulation with 10^{-6} M ABT-229 was poor, we used a lower concentration of ABT-229 (10^{-7} M) to study the effect of the protein inhibitor cycloheximide (CHX) on MTLR resensitization (Fig. 10B). Treatment with CHX (1 μg/ml) for 24 h did not significantly alter the response of buffer treated cells. MTLR resensitization after stimulation with motilin or ABT-229 did not differ in the absence or presence of CHX. The possibility that receptor recycling was retarded because the MTLR was still phosphorylated was also investigated. Fig. 10C shows that the internalized MTLR is dephosphorylated 24 h after stimulation with equipotent doses of motilin (10^{-7} M) or ABT-229 (10^{-6} M).

4. Discussion

The therapeutic potential of motilin receptor agonists for treating hypomotility disorders crucially depends on the

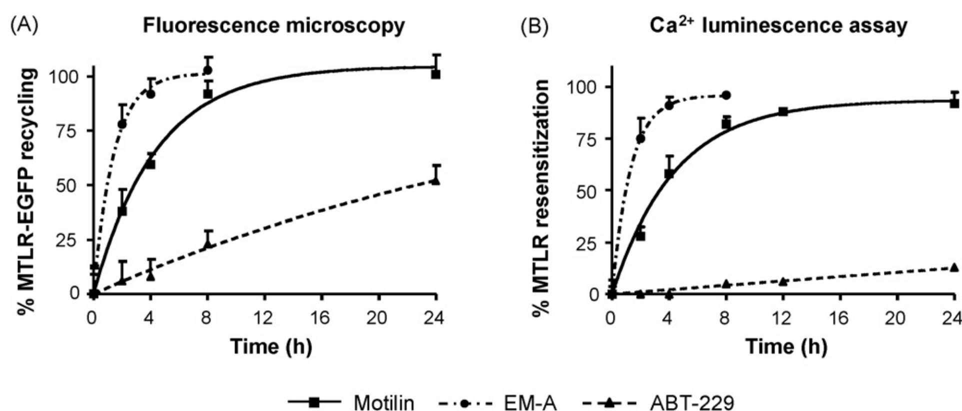

Fig. 9 – Recycling (A) and resensitization (B) of the MTLR after agonist stimulation. (A) MTLR-EGFP cells were treated for 2 h with an equipotent dose (1000-fold of EC_{50}) of motilin (10^{-7} M), EM-A (10^{-4} M) or ABT-229 (10^{-6} M), washed and kept at 37 °C for 0, 2, 4, 8, 12, and 24 h and processed for fluorescence microscopy. For each time point the intracellular fluorescence intensity was quantified. The change in intracellular fluorescence intensity is expressed as a percentage of the intracellular fluorescence intensity measured after 2 h of desensitization which corresponds to time 0 of the recycling kinetics. Results are mean values ± S.E.M. from 48 cells. (B) Resensitization kinetics of the MTLR as determined with Ca^{2+} luminescence measurements under the same experimental conditions as in (A). Results are expressed as a percentage of the Ca^{2+} responses obtained with buffer-treated cells at the respective time points. Mean values ± S.E.M. are plotted from three independent experiments performed in duplicate.

development of motilin receptor agonists with sustained efficacy. A recent study has demonstrated that the MTLR undergoes agonist-dependent desensitization in cell lines expressing the MTLR [15], but less is known about the regulatory mechanisms involved in the desensitization process. Therefore, the aim of the present study was to compare the effect of motilin and the motilides, ABT-229 and EM-A, on the molecular and cellular pathways involved in the intracellular trafficking of the MTLR. The present results suggest that motilin- and motilide-induced desensitization of the MTLR probably involves receptor phosphorylation via G protein-coupled receptor kinases (GRKs) for motilin and EM-A while for ABT-229 phosphorylation is also protein kinase C dependent. The phosphorylated MTLR recruits β-arrestin-2 with greater affinity than β-arrestin-1 and consequently internalizes in transferrin positive vesicle suggesting targeting of the receptor to clathrin-coated pits. The β-arrestins dissociate from the MTLR at the plasma membrane before the receptor is internalized showing that the MTLR belongs to the class A recycling receptor family. The ligand receptor complex is subsequently sorted to recycling endosomes, which traffic the MTLR back to the plasma membrane. Internalization and resensitization of the receptor is agonist-dependent and we found for the first time that the unusual strong desensitizing properties of ABT-229 are not related to a higher degree of receptor phosphorylation, nor to a change in β-arrestin binding or internalization kinetics but to a higher degree of internalization and a slower recycling of the MTLR to the plasma membrane.

For many GPCRs, agonist-induced uncoupling from heterotrimeric G proteins and receptor internalization dependent on receptor phosphorylation. We demonstrated that agonist-induced desensitization of the MTLR is associated with a time and concentration-dependent phosphorylation of the receptor. Phosphorylation of the MTLR occurs within seconds following motilin exposure. The more pronounced ability of ABT-229 to induce receptor desensitization compared with motilin is not reflected in a higher degree of receptor phosphorylation. However it is not unlikely that both ligands phosphorylate different residues. Each ligand may induce a different conformational state of the receptor which may expose different phosphorylation sites within the C-terminal tail and intracellular loops of the receptor. In addition, the types of kinases involved can also play a role. The phosphorylation of GPCRs is catalyzed by two main types of serine/threonine protein kinases: second messenger-dependent protein kinases A (PKA) and C (PKC) [27], or second messenger-independent protein kinases, G-protein coupled receptor kinases (GRKs), for which seven members have been identified [28–30]. GRKs phosphorylate only the agonist occupied or activated form of the receptor and are believed to be critical for agonist-specific or homologous desensitization, whereas PKA and PKC can also phosphorylate receptors in the absence of agonist and lead to heterologous desensitization.

Because in the present study the PKA inhibitor, H-89, and the PKC inhibitor, GF109203X, had no effect on MTLR phosphorylation after exposure to motilin and EM-A, our results suggest that MTLR phosphorylation probably occurs via GRKs. In contrast, ABT-229-induced MTLR phosphorylation was partially inhibited by the PKC inhibitor, GF109203X.

β-Arrestins bind to phosphorylated receptors and disrupt the interaction between the receptor and G-proteins to mediate homologous desensitization. Agonist-induced desensitization of the MTLR was accompanied by translocation of β-arr_1-EYFP and β-arr_2-EGFP from the cytosol to the plasma membrane where it co-localized with the MTLR. In addition, translocation with β-arr_2-EGFP was more profound, suggesting selectivity between the β-arrestin isoforms. The process was very rapid (2 min) and after 30 min the β-arrestins were again redistributed into the cytosol. Thus, β-

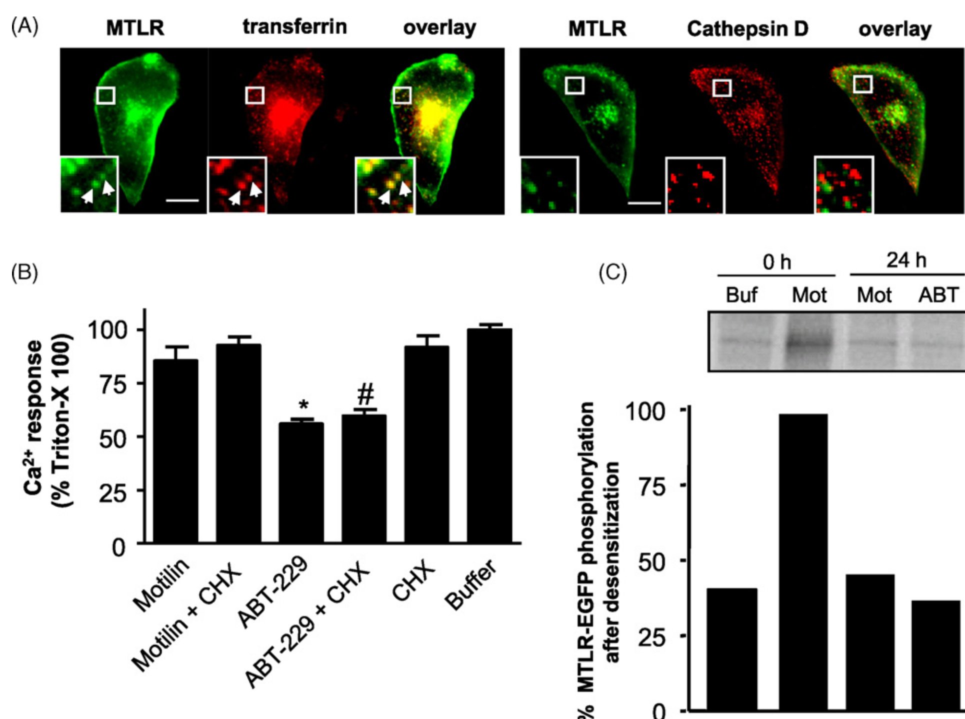

Fig. 10 – Resensitization of the MTLR: intracellular localization, effect of blockade of protein, phosphorylation. (A) Intracellular localization of the MTLR-EGFP 24 h after stimulation with ABT-229 for 2 h. *Left panel* co-localization with transferrin, the arrows indicate examples of vesicles containing the MTLR-EGFP (green) and transferrin (red). Yellow colour in the overlap image indicates co-localization of the MTLR-EGFP and transferring. *Right panel*, co-localization with cathepsin D. The overlap image shows no co-localization of the MTLR-EGFP (*green*) with cathepsin D (*red*). *Bar*, 10 μm. Each experiment was repeated three times. (B) Effect of the protein synthesis inhibitor cycloheximide (CHX) on MTLR resensitization. MTLR-EGFP cells were incubated for 2 h with buffer, motilin (10^{-7} M) or ABT-229 (10^{-7} M), washed and kept at 37 °C for 24 h in the absence or presence of CHX (1 μg/ml) and resensitization was determined with Ca^{2+} luminescence measurements. Results are expressed as a percentage of the Ca^{2+} responses obtained with buffer-treated cells after 24 h. Mean values ± S.E.M. are plotted from three independent experiments performed in duplicate. $^{*}P < 0.0001$ vs. control, $^{\#}P < 0.001$ vs. CHX. (C) MTLR-EGFP phosphorylation after 24 h of resensitization. MTLR-EGFP cells were labeled with [$^{32}P_i$] and 10^{-7} M motilin (Mot) or 10^{-6} M ABT-229 (ABT) were applied to induce maximal receptor phosphorylation (0 h). Cells were then allowed to recover for 24 h without agonist at 37 °C. Cells were immunoprecipitated with an anti-EGFP antibody, subjected to SDS-PAGE and quantified. Basal phosphorylation was measured from cells treated with buffer (Buf). A representative phosphorimage scan after electrophoresis is shown. Results are expressed as the percentage of MTLR-EGFP phosphorylation after desensitization with motilin.

arrestins did not traffic along with vesicles containing the MTLR indicating that the MTLR is already dissociated from β-arrestin near or at the membrane. The different agonists did not alter the pattern of β-arrestin association with the receptor. Although ABT-229-induced MTLR phosphorylation seems to be partially PKC-dependent, phosphorylation of the MTLR was still able to recruit β-arrestin. This proves that other kinases, in particular GRKs, are also involved in receptor phosphorylation. It is also known that PKC can upregulate GRKs [31]. An example of agonist-induced homologous desensitization of receptor signaling involving GRK- and PKC-dependent receptor phosphorylation has also been reported for the human thromboxane A_2 receptor [32]. In addition it has been demonstrated that PKA and PKC can also affect the desensitization of GPCRs by phosphorylating GRK2 that is necessary for its efficient recruitment to the plasma membrane [33,34].

Two major classes of GPCRs have been described according to their different affinities for β-arrestins [35]. Class A receptors, such as β2-adrenergic receptor (β2AR), show preferential binding to β-arrestin-2 compared with β-arrestin-1. β-Arrestin binding to these receptors is transient since β-arrestin dissociates from the receptor shortly after movement of the receptor into clathrin-coated vesicles. This favors rapid receptor dephosphorylation and recycling of the receptor to the membrane [36]. Class B receptors, such as the angiotensin II type 1a receptor (AT1aR) and the vasopressin V2 receptor (V2R), have equal affinity for both β-arrestin-1 and -2, as was also shown in the present study. These receptors form stable complexes with β-arrestin such that the β-arrestin internalizes together with the receptor into endosomes. This favors slow receptor dephosphorylation and targeting of the receptor for degradation [36]. The difference in stability of the β-arrestin-receptor complex for different GPCRs appears to

depend on specific clusters of serine/threonine residues located in the C-terminal tail of the receptor [37]. Such distinct clusters are not present in the C-terminal tail of the MTLR and this may explain why in the present study we detected a higher affinity of the MTLR for β-arr$_2$-EGFP than for β-arr$_1$-EYFP and why no stable β-arrestin-MTLR complexes were formed after agonist stimulation. From these results it can be inferred that the motilin receptor belongs to the class A recycling receptor family and is not internalized via a β-arrestin-independent mechanisms as suggested by Lamian et al. [38]. These authors failed to detect co-immunoprecipitation of β-arrestin with the MTLR after ligand stimulation. The time-periods used to co-immunoprecipitate the receptor with β-arrestin were not specified but we have shown that β-arrestin binding occurs rapidly. Since β-arrestin dissociates from the receptor near or at the membrane and does not form a stable complex with the receptor, co-immunoprecipitation of the receptor with β-arrestin may be difficult, which may explain the lack of co-immunoprecipitation. However, most studies that do report agonist-dependent association of GPCR with arrestin by co-immunopreciptation require the use of prior crosslinking but we cannot exclude whether the authors have done so.

Although we observed similar rates of MTLR internalization after exposure to equipotent doses (1000-fold EC_{50} for receptor activation) of motilin, ABT-229 or EM-A, the extent of receptor internalization differed between the agonists and correlated with their desensitizing properties. Visualization of the agonist-induced intracellular trafficking of the MTLR-EGFP revealed that the receptor is distributed into transferrin positive compartments. Transferrin binds to cell surface transferrin receptors, constitutively internalizes into early endosomes via a clathrin-mediated endocytotic process and then recycles back to the cell surface membrane [39]. These results together with the results of β-arrestin-dependent internalization suggest that the MTLR is internalized via clathrin-dependent pathway into vesicles. Our co-localization studies with the lysosomal marker, cathepsin D, confirmed that the MTLR is not degraded within the lysosomes.

Uncoupling of the ligand and dephosphorylation of the receptor in the endosomes allows subsequent recycling of reactivated receptors to the cell surface [40,41,29]. Receptor recycling was demonstrated by the decrease of MTLR-EGFP vesicles and the increase in Ca^{2+} response of the MTLR over time. Receptor recycling was agonist dependent and strongly related to the desensitizing properties of motilin, ABT-229 and EM-A. In contrast to the internalization kinetics, the resensitization kinetics were agonist dependent and ranking occurred in the order of: ABT-229 (26 h) > motilin (3 h) > EM-A (1 h). Because receptor resensitization after 24 h was unaltered by the protein inhibitor cycloheximide our results suggest that receptor recycling does not require new receptor synthesis. Thus, the unusual strong desensitizing properties of ABT-229 were mediated by the delayed recycling of the internalized receptors back to the cell surface. This may explain why in clinical trials elevated plasma levels of ABT-229 were not able to induce antral contractions and therefore affect gastric emptying after a second meal in healthy volunteers [42]. The stronger desensitization properties of ABT-229 may be related to the long half-life time of ABT-229 (~20 h) [11] in comparison with the half-life time of motilin (3 min) and EM-A (90 min). The observation that motilin receptors were still accumulated in the endosomes, even after 24 h of ABT-229 removal, favor the hypothesis that ABT-229 remains bound to the receptors due to its slow dissociation rate, thereby impairing receptor recycling. Therefore the notion that simple affinity, i.e., the ratio of the ligand's association and dissociation rate, could be responsible for the regulation of MTLR responsiveness should be considered. Different agonist-induced receptor recycling kinetics have also been shown for other GPCRs such as the opioid receptors [43]. However the mechanisms involved for the different recycling kinetics are not known. Although we have established that ABT-229 uses the same internalization pathway as motilin and EM-A, the agonists may be sorted into different endosomal compartments during or after internalization which may influence their recycling kinetics. One can also speculate that the different kinetics in receptor internalization and recycling are dependent on differences in the kinetics of phosphorylation–dephosphorylation of the receptor. Internalization of the MTLR after stimulation with ABT-229 could induce a desensitized MTLR conformation that is less susceptible to serve as a phosphatase substrate thereby delaying but not preventing the translocation of the receptor to the cell surface. The latter hypothesis is rather unlikely since we have shown that the receptor is dephosphorylated 24 h after stimulation with ABT-229. The PKC-dependent receptor phosphorylation by ABT-229 compared with motilin and EM-A could also be responsible for the slow resensitization observed after stimulation with ABT-229. Additional experiments are necessary to elucidate why ABT-229 has this delayed effect.

In conclusion, agonist-induced receptor activation is intimately linked to cellular events that may lead to receptor desensitization and limit the usefulness of GPCRs agonists in long-term therapy. Our results emphasize the utility of studying next to receptor activation also receptor internalization and especially resensitization. This may favor the development of new potent motilin agonists without desensitizing properties for the treatment of hypomotility disorders.

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