Desensitization of the Human Motilin Receptor by Motilides

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ABSTRACT

Tachyphylaxis may have contributed to the failure of the motilide ABT-229 (N-ethyl, N-methyl 4′ deoxy erythromycin (EM)-B enolether) in clinical trials. We compared the desensitizing potency of structurally related motilides [EM-A, EM-A enolether (ME4), N-ethyl, N-methyl EM-A (ME36), EM-B enolether (ME67), N-ethyl, N-methyl EM-A enolether (EM523), ABT-229 and 4′ deoxy EM-A enolether (KOS1326)] in a Chinese hamster ovary (CHO)-K1 cell line expressing the human motilin receptor (MTLR) and in rabbit duodenal segments. CHO-MTLR cells were preincubated with motilides prior to stimulation with motilin. The negative logarithm of the preincubation concentration reducing the maximal motilin-induced Ca2+ flux to 50% was calculated (pDC50). Internalization was visualized in CHO-K1 cells containing an enhanced green fluorescent protein (EGFP)-tagged MTLR and quantified in binding experiments. The contractile response of repeated stimulations was measured in duodenal segments. In CHO-MTLR cells, the pDC50 was ABT-229 (8.78) > motilin (7.77) > EM-A (4.78), different from their order of potency to induce Ca2+ release (pEC50): motilin (9.39) > ABT-229 (8.46) > EM-A (7.11). In cells with the EGFP-tagged MTLR, ABT-229 decreased membrane fluorescence by 25 ± 2% compared with 16 ± 2% for motilin and 8 ± 2% for EM-A. Binding studies confirmed that EM-A did not induce MTLR internalization (residual binding 96 ± 4% compared with motilin, 31 ± 3% and ABT-229, 21 ± 1%). Comparison of the pDC50 and pEC50 values of the other motilides ME4 (5.90; 8.08), ME67 (6.03; 8.12), ME36 (3.32; 6.62), EM523 (6.02; 8.22), and KOS1326 (7.32; 8.14) suggested that the strong desensitizing properties of ABT-229 are mostly related to the removal of the 4′-OH of the cladinose sugar. The decline of the contractile response in duodenal segments correlated with the pDC50. The ability to desensitize and internalize the MTLR is not only determined by potency. This may be an important criterion for the development of a clinically useful compound.

The finding that the antibiotic erythromycin-A (EM-A) interacts with the motilin receptor (Peeters et al., 1989) became clinically relevant when it was discovered that EM-A accelerated delayed gastric emptying in patients with diabetic gastroparesis (Janssens et al., 1990). As a result, more potent motor-stimulating EM derivatives were fluorescent without antibiotic activity: the motilides (Omura et al., 1987). Recently the clinical development of the most promising motilide ABT-229 was stopped because of its failure to improve symptoms in patients with functional dyspepsia with normal or delayed gastric emptying (Talley et al., 2000) and in patients with type 1 diabetes mellitus (Talley et al., 2001). The authors of the studies with ABT-229 concluded that there was no future for drugs with the prokinetic profile of the motilides, but others have argued that ABT-229 had undesirable properties that may be avoided in new motilides (Tack and Peeters, 2001; Camilleri, 2002). The most important property in this respect is the development of tachyphylaxis. Previous studies described a decreased contractile response of rabbit colonic myocytes due to down-regulation of motilin receptors after chronic oral treatment (Bologna et al., 1993) and after short-term intravenous treatment with erythromycin A (Depoortere et al., 1991), the parent compound from which ABT-229 was derived. One-month treatment of rabbits with ABT-229 also resulted in a down-regulation of motilin receptors and a reduced contractile response to ABT-229 and motilin (Depoortere et al., 1999). Tachyphylaxis was also observed after treatment of healthy subjects with ABT-229. A single dose of ABT-229 increased gastric emptying after the

ABBREVIATIONS: EM-A, erythromycin-A; CHO, Chinese hamster ovary; MTLR, motilin receptor; EGFP, enhanced green fluorescent protein; ABT-229, N-ethyl, N-methyl 4′ deoxy EM-B enolether; MTLR-EGFP, EGFP-tagged motilin receptor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; Ach, acetylcholine; ME4, EM-A enolether; ME36, N-ethyl, N-methyl EM-A; ME67, EM-B enolether; EM523, N-ethyl, N-methyl EM-A enolether; KOS1326, 4′ deoxy EM-A enolether.
first meal, but emptying of a second meal was not affected, although ABT-229 plasma levels were still high (Verhagen et al., 1997).

Recently, we have shown that the Ca\(^{2+}\) fluxes induced by peptideyl and nonpeptideyl motilin agonists in a Chinese hamster ovary (CHO) cell line expressing the cloned human motilin receptor and the Ca\(^{2+}\) indicator apoaequorin (CHO-MTLR) correlate strongly with the contractile response of these compounds in rabbit duodenal strips (Thielemans et al., 2002). In the present study, we used the aequorin-based luminescence assay to study the desensitization of the motilin receptor by ABT-229 in more detail. To clarify whether desensitization is a common characteristic of all motilides, the desensitizing properties of several motilides with different structural features were compared. To determine the mechanism underlying desensitization, receptor internalization following application of ABT-229 was quantified by receptor binding studies and visualized by using an enhanced green fluorescent protein (EGFP)-tagged motilin receptor (MTLR-EGFP).

### Materials and Methods

#### Materials

Norleucine\(^1\)porcine-motilin (Nle\(^1\)po-motilin) was purchased at Eurogentec (Namur, Belgium) and will further be referred to as motilin. Erythromycin-A (EM-A), EM-A enol ether (ME4), N-ethyl, N-methyl EM-A (ME36), EM-B enol ether (ME67), and N-ethyl, N-methyl EM-A enol ether (EM-523) were obtained from Prof. J. Hoogmartens (Laboratory of Pharmaceutical Chemistry, University of Leuven, Belgium). N-Ethyl, N-methyl 4\(^\text{thiopropyl} \) enol ether (ABT-229) was a gift from Dr. P. Lartey (Lartey et al., 1995; Abbott Laboratories, Abbott Park, IL). Macrolide KOS1326 was synthesized by Kosan Biosciences, Inc. (Hayward, CA).

#### Cell Culture

A CHO-K1 cell line stably expressing the human motilin receptor and the mitochondrially targeted apoaequorin was obtained from Euroscreen SA (Brussels, Belgium). The cells were cultured in Ham's F-12 containing 10% fetal bovine serum, 100 \(\mu\)g/ml amphotericin B (Invitrogen, Carlsbad, CA), 400 \(\mu\)g/ml G418, 1 mg/ml puromycin (Sigma-Aldrich, St. Louis, MO) and spliced once a week with 5 mM EDTA in PBS.

#### Calcium Measurements: Aequorin Luminescence Assay

Suspended cells (5 \(\times\) 10\(^6\) cells/ml) were loaded with coelenterazine h (5 \(\mu\)M) (Molecular Probes, Leiden, The Netherlands) at room temperature for at least 4 h to reconstitute active aequorin. Cells were then diluted 10-fold with BSA medium (Dulbecco's modified Eagle's medium/Ham's F-12 with Heps, without phenol red, 0.1% BSA, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 2.5 \(\mu\)g/ml amphotericin B) and 100 \(\mu\)l/well was injected (50,000 cells) into a 96-well plate containing increasing concentrations of the test compound diluted in BSA medium.

Immediately after injection of cells, the emitted light was measured using the Lumacratum plus luminometer (Berthold Technologies, Bad Wildbad, Germany) for 20 s. The intensity of the emitted light was integrated using the Winlog software (Berthold Technologies), yielding for each well one value representative of the emitted light and, hence, of the stimulation of the motilin receptor by the agonist present in the well. This value was expressed as a percentage of maximal stimulation obtained with Triton X-100 (0.9%). All values were corrected for background by subtracting the blank value (= BSA medium). The negative logarithm of the concentration producing half of the maximal response (pEC\(_{50}\)) was calculated from the dose-response curves by linear interpolation. All experiments were performed in duplicate, and each compound was tested at least two times. Results are represented as mean ± standard error of the mean (S.E.M.).

To assess desensitization, cells were pretreated for at least 1 h with different concentrations of agonist during loading, washed, and centrifuged before dose-response curves were established with motilin. The effect of pretreatment on the maximal response was assigned as the effect of desensitization. The average of the maximal plateau values of the dose-response curve to motilin under desensitizing conditions was expressed as a percentage of maximal response under control conditions. The potency of a compound to induce desensitization was calculated after plotting the remaining maximal responses as a function of the concentration of the compound used to desensitize. A sigmoid concentration-response curve was fitted through the data by nonlinear regression analysis using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA), and a pDC\(_{50}\) value and its standard error of the best-fit value (negative logarithm of the concentration producing half-maximal desensitization) was calculated. For each compound, δ values were calculated by subtraction of pEC\(_{50}\) values with pDC\(_{50}\) values. The square root of the sum of squares of the standard deviation (S.E. for pEC\(_{50}\)) and the standard error of the best-fit value (S.E. for pDC\(_{50}\)) resulted in the standard deviation of these δ values. A pooled standard deviation (\(\delta_p\)) was derived from which a \(\delta\) value was calculated for the comparison of two \(\delta\) values, with \(n_1 + n_2 - 2\) degrees of freedom using:

\[
\delta_p = \sqrt{\frac{\delta_1^2}{n_1} + \frac{\delta_2^2}{n_2}}
\]

#### Receptor Binding Studies

**Membrane Preparation.** CHO-K1 cells expressing the MTLR were cultured until 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, and 1 mM EGTA and homogenized in a glass homogenizer. The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000g 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, and 250 mM sucrose and flash frozen in liquid nitrogen. The protein content was determined by the Folin method (Lowry et al., 1951).

**Cell Membrane Receptor Binding.** Competition binding assays were performed by incubating CHO-MTLR membranes (8 \(\mu\)g of protein/tube) in a final volume of 0.1 ml with \(^{125}\)I-motilin (0.3 nM) for 60 min at 31°C. Unlabeled motilin, motilin fragments, and motilides were used as competitors at concentrations ranging from 10\(^{-12}\) to 10\(^{-4}\) M 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, washed, and counted in a gamma counter. All values were corrected for nonspecific binding determined by addition of an excess (10\(^{-5}\) M) of unlabeled motilin. IC\(_{50}\) values were determined by nonlinear regression using a single-site model (PRISM; GraphPad Software Inc.). Each compound was tested three times in duplicate.

**Construction and Expression of the MTLR Coding Sequence as an N-Terminal Fusion Protein with the EGFP**

The cDNA for the full coding sequence inserted in the pcDNA3.1 vector (provided by Euroscreen SA) was amplified by PCR using the proof reading Taq polymerase Pfu Turbo (Stratagene, La Jolla, CA). The 5’ primer contained the Xhol restriction site followed by a Rozak consensus sequence preceding the ATG initiation codon and 18 gene-specific nucleotides. The 3’ primer contained a 5’ EcoRI restriction site followed by the 26 terminal nucleotides of the coding sequence,
the TGA stop codon being mutated to remove the translation termination signal and provide an in phase sequence with the EGFP coding sequence that follows immediately the multiple cloning site in the pEGFP plasmid (BD Biosciences Clontech, Palo Alto, CA). The PCR product was digested by DpnI restriction enzyme to remove parental target DNA, purified on Qiagen PCR purification columns (Qiagen GmbH, Hilden, Germany), and digested by Xhol and EcoRI. The pEGFP plasmid was digested in the same manner. The digestion products were purified on Qiagen PCR purification columns and an aliquot of the resulting 50 μl was run on a 0.8% Tris acetate EDTA agarose gel. A fraction of the digestion products, i.e., the motilin coding sequence and the pEGFP vector, were mixed and dried in a speed vacuum concentrator. The dried DNA was resuspended, ligated with T4 DNA ligase (Promega, Madison, WI), and transformed into TOP10 One Shot Ultracompetent Escherichia coli bacterial host cells according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). Several colonies were picked; miniprep DNA was prepared using the GFX Micro Plasmid Preparation kit (Amersham Biosciences Inc., Piscataway, NJ), and the presence of an insert was verified by gel electrophoresis. Three clones were checked by automated DNA sequencing on an ABI machine using BigDye Terminator chemistry. One clone was then amplified using the GenElute Endotoxin-Free Midi Prep kit (Sigma-Aldrich). Transfection of 20 μg of purified plasmid DNA was carried out by electroporation (Electroporator II; Invitrogen) according to the manufacturer’s instructions into CHO-PAM28 cells—CHO cells stably expressing the aequorineoporator II; Invitrogen) according to the manufacturer’s instructions in CHO-FAM28 cells—CHO cells stably expressing the aequorine gene (Euroscreen SA). After selection by G418 (Invitrogen), 24 individual clones were picked and further amplified for functional characterization.

**Visualization of Endocytosis of the MTLR-EGFP**

CHO cells containing the MTLR-EGFP were grown on four-well coverglass chambers until 80% confluency. After washes with ice-cold BSA medium, digital pictures 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). BSA medium containing 10^{-5} M compound of interest was added, after which the cells were gently shaken at 4°C for 1 h for equilibration binding, followed by 1 h incubation at 37°C. Internalization was stopped by washing the cells with ice-cold PBS. Pictures of the same cells were taken. For quantification, the fluorescent grayscale pictures were converted to black and white. The percentage black and white at the membrane and in the cytosol was calculated for 10 cells per condition before and after stimulation using Scion Image (Scion Corporation, Frederick, MD) and compared with control. Each condition was evaluated in three different experiments. Means were compared using an unpaired t test (PRISM; GraphPad Software Inc.).

**Quantification of MTLR-EGFP Internalization**

The capacity of different compounds to promote internalization was examined by adding the compound of interest (10^{-5} M) to the CHO cells containing the MTLR-EGFP in 12-well plates for 60 min at 37°C. Surface-bound ligands were removed with a gentle acid wash (50 mM sodium citrate, 0.2 mM sodium phosphate, 90 mM NaCl, and 0.1% bovine serum albumin, pH 5.0; 10 min, 4°C), which does not affect subsequent receptor binding, and then a radioreceptor binding assay was performed (^{125}I-motilin, 0.3 nM, 4 h at 4°C) to measure receptors remaining at the cell surface. Internalized receptors were expressed as a percentage loss of cell surface binding compared with cells not exposed to the compound of interest. Means ± S.E.M. were compared using an unpaired t test (PRISM; GraphPad Software Inc.).

**Contractility Studies**

Integral rabbit duodenal segments (1.5–2 cm) were vertically suspended in tissue baths containing Hepes buffer pH 7.4 (11.6 mM Hepes, 11.5 mM glucose, 137 mM NaCl, 5.9 mM KCl, 1.2 mM CaCl₂, and 1.2 mM MgCl₂) continuously gassed with 100% O₂ and kept at 37°C. Contractions were recorded isotonically using HP 7DCTD 1000 transducers from Hewlett Packard (Palo Alto, CA) and a displacement control unit obtained from Jansen Scientific Instrument Division (Beerse, Belgium). Signal output was connected to a recorder and to a computer and sampled for digital analysis using the WinDaq data acquisition system and a DI-2000 PCH card (Dataq Instruments, Akron, OH).

Strips were equilibrated in the tissue bath until a stable response to acetylcholine (ACh, 0.1 mM) was obtained. Strips were then stimulated with either ABT-229 (69 nM), KOS1326 (0.17 μM), EM-A (14.1 μM), ME4 (0.44 μM), or EM-523 (66 nM) at a concentration corresponding to five times their potency to induce contractions. After 4 min, strips were washed out two times and washed again 2’, 6’, and 10’ after the first wash out. The washing procedure was then repeated every 10 min until 1 h after the application of the agonists. Strips were then stimulated for a second time with the compound of interest and washed again according to the procedure described above. After the third application, strips were not washed out but a supramaximal dose of motilin (1 μM) followed by a supramaximal dose of ACh (0.1 mM) was applied. Desensitization was expressed as a percentage of the response of the agonists obtained after the first application. The response to motilin at the end of the experiment was expressed relative to the response to ACh. All experiments were approved by the Ethical Committee of the University of Leuven.

**Results**

**Desensitization of the Motilin Receptor.** Desensitization of the motilin receptor was induced by prestimulation of CHO-MTLR cells with different concentrations (10^{-12}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M) motilin. This reduced the maximal response in a subsequent concentration response curve to motilin to 97.2 ± 1.3, 83.9 ± 7.8, 64.9 ± 1.3, 3.8 ± 1.3, 2.8 ± 0.9, and 1.6 ± 1.3%, respectively (Fig. 1).

Next, motilin’s ability to desensitize was compared with that of the motilides ABT-229 and EM-A. These compounds are structurally related but differ at four positions, as illustrated in Fig. 2.

Both agonists were tested at 10^{-5} M, a concentration sufficient to evoke a maximal Ca^{2+} luminescent response. However, following pretreatment at this concentration, there was
a marked difference in the maximal response to a subsequent stimulation to motilin, which was reduced to 65.8 ± 1.8% for EM-A and to −0.2 ± 0.4% for ABT-229. The desensitizing capacity of ABT-229 but not of EM-A was therefore comparable with that of motilin.

To investigate which structural elements underlie the stronger desensitizing potency of ABT-229 in comparison to EM-A, several EM-A derivatives were tested, containing one or two ABT-229-like modifications (Fig. 2). The modifications include the presence of an enol configuration (ME4, ME67, EM-523, and KOS1326), the presence of an ethyl group at the amine group of the desosamine sugar (ME36, EM-523), and the lack of a hydroxyl at position 12 of the lactone ring (ME67) or at position 4" of the cladinose sugar (KOS1326).

Figure 3 shows that compounds with an enol configuration such as ABT-229, ME4, EM-523, ME67, and KOS1326 induced a more profound desensitization at 10⁻⁵ M than EM-A. In contrast, desensitization was seemingly absent with ME36, which compared with EM-A, contains only a modification at the amine group of the desosamine sugar.

However, the enol configuration also increases the potency of motilides to induce contractions in muscle strips and to induce a Ca²⁺ flux in the CHO-MTLR cells (Depoortere et al., 1989; Thielemans et al., 2002). Therefore, although the pretreatment concentration was sufficient to induce a maximal response for all compounds tested, one could argue that less potent compounds may require higher pretreatment concentrations. To fully discriminate between potency and ability to desensitize, we therefore established a “concentration response curve of desensitization” for each motilide. Figure 4 shows the effect of pretreatment of the CHO-MTLR cells with increasing concentrations of motilin, EM-A, or ABT-229 on the maximal response to a subsequent concentration response curve to motilin.

From these curves, the pretreatment concentration which reduces the maximal response to 50% of the control value was calculated. The negative logarithm of this concentration, pDC₅₀ (−log[desensitizing concentration 50%]) reflects the potency to desensitize. The values for all motilides tested are shown in Table 1. This table also lists the potencies of these compounds to induce Ca²⁺ fluxes (pEC₅₀ values) in untreated cells (Thielemans et al., 2002).

Although ABT-229 is 8.5 times less potent than motilin in activating the cells (pEC₅₀: 8.46 ± 0.08 versus 9.39 ± 0.03), it is 10.2 times more potent in inducing desensitization (pDC₅₀: 8.78 ± 0.03 versus 7.77 ± 0.08). Thus, potency does not seem to solely determine the ability to desensitize. To further illustrate this point, Table 1 shows the difference between the pDC₅₀ and pEC₅₀ values, referred to as δ value.

Five compounds have a similar δ value (motilin, 1.62 ±
Fig. 3. Comparison of desensitization induced by different motilides. Cells were first stimulated with a motilide at 10^-5 M (x-axis), and the maximal response to a subsequent concentration-response curve to motilin was determined and expressed as a percentage of control (no pre-treatment). Each value represents the mean ± S.E.M. of at least two experiments in duplicate.

Fig. 4. Concentration response curves of desensitization induced by motilin, ABT-229, and EM-A. Cells were pretreated with different concentrations of the test compounds (x-axis), and the reduction in maximal response to a subsequent concentration response curve to motilin was calculated and expressed as a percentage of control (y-axis). Each value is mean ± S.E.M. of at least two experiments in duplicate.

Because desensitization involves receptor internalization, an EGFP-tagged motilin receptor was generated and stably expressed in CHO cells to follow receptor trafficking. The cells also expressed the Ca^2+ indicator apoaequorin (MTLR-EGFP), which allowed us to determine whether GFP interfered with signal transduction and desensitization.

For the wild-type receptor and the MTLR-EGFP, the order of potency to induce Ca^2+ release is: motilin > ABT-229 > EM-A (Fig. 5A). The absolute potency (pEC50) of all compounds is 9.17 ± 1.14 (motilin), 8.12 ± 0.17 (ABT-229), and 6.45 ± 0.08 (EM-A) in the MTLR-EGFP and is somewhat lower than in the MTLR.

Pretreatment of MTLR-EGFP with 10^-5 M motilin, ABT-229, or EM-A reduced the response to a subsequent stimulation with motilin to 18.7 ± 2.5, 29 ± 1.3, and 99.2 ± 8.5%, respectively (Fig. 5B). These effects are less pronounced than in the wild-type receptor, but the discrepancy between activation and desensitization remains because ABT-229, although less potent than motilin, desensitizes most at 10^-5 M.

**Internalization of the EGFP-Tagged MTLR.** In cells expressing the EGFP-tagged receptor, changes in the fluorescence distribution after stimulation with the ligands at 10^-5 M were studied by fluorescence microscopy. Images obtained before and after stimulation are shown in Fig. 6A.

Before the start of the experiment, MTLR-EGFP was principally localized at the plasma membrane. After 1-h stimulation at 37°C in the presence of the motilin agonists, the intensity of the signal at the cell surface was reduced, especially for motilin and ABT-229 and was concentrated in the cytosol.

The images were analyzed and the percentage change in fluorescence in the cytosol and in the membrane owing to stimulation was calculated (Fig. 6B). All agonists induced a significant cytosolic increase and a significant decrease of membrane fluorescence compared with control (p < 0.005).

The cytosolic increases were similar for motilin, ABT-229, and EM-A (22 ± 2, 25 ± 2, and 20 ± 2%, respectively, p < 0.0001 versus control). In contrast, ABT-229 (25 ± 2%) decreased membrane fluorescence significantly more compared with motilin (16 ± 2%, p < 0.01), which in turn induced a more significant decrease compared with EM-A (8 ± 2%, p < 0.01).

**Radioligand Binding with the EGFP-Tagged MTLR.** To validate the semiquantitative data of the analysis of the fluorescence distribution, the more quantitative method of receptor binding was used to measure ligand-induced endocytosis. Radioligand binding with 125I-motilin was performed after pretreatment of MTLR-EGFP with 10^-5 M motilin, ABT-229, or EM-A for 20 and 60 min at 37°C (Fig. 7). Residual binding after 20-min pre-stimulation was 49.9 ± 2.3% (motilin), 30.8 ± 3.8% (ABT-229), and 124.3 ± 2.0% (EM-A) and was further reduced to 30.8 ± 3.3% (p = 0.0029), 21.3 ± 0.8% (p < 0.0001), and 96.1 ± 4.4% (p < 0.0001), respectively, after pre-stimulation for 60 min.

In line with the desensitization data, internalization after pre-stimulation with ABT-229 was higher (p = 0.029) than after stimulation with motilin or EM-A, although all compounds are equipotent at the concentration used for pre-stimulation. Curiously, pre-stimulation with EM-A for 20 min induced a significant increase in binding (124.3 ± 2.0%; p < 0.0001).

**Contractility Studies.** The desensitizing properties of ABT-229, KOS1326, EM-A, ME4, and EM-523 were also compared in vitro in the tissue bath by determining the contractile response of segments of rabbit duodenum to three appli-

0.15; EM-A, 2.33 ± 0.28; ME4, 2.18 ± 0.42; ME67, 2.09 ± 0.25; and EM-523, 2.20 ± 0.28) indicating that there is no relationship between potency and desensitizing ability (Pearson's r = 0.9054). However, for ABT-229 and KOS1326, the δ value is significantly lower than in these compounds (p < 0.01) and for ME36, it is significantly higher (p < 0.01).

**Binding of Motilides with the MTLR.** Because both activation and desensitization result from the interaction of the ligand with its receptor, we investigated whether the higher than expected ability of ABT-229 and KOS1326 to desensitize could be explained by a higher affinity for the motilin receptor. The pIC50 values (Table 1) calculated from the displacement curves did not reveal an increased affinity for ABT-229 or KOS1326.

**Comparison of MTLR-EGFP and Wild-Type Receptor.** Because desensitization involves receptor internalization, an EGFP-tagged motilin receptor was generated and stably expressed in CHO cells to follow receptor trafficking. The cells also expressed the Ca^2+ indicator apoaequorin (MTLR-EGFP), which allowed us to determine whether GFP interfered with signal transduction and desensitization.

For the wild-type receptor and the MTLR-EGFP, the order of potency to induce Ca^2+ release is: motilin > ABT-229 > EM-A (Fig. 5A). The absolute potency (pEC50) of all compounds is 9.17 ± 1.14 (motilin), 8.12 ± 0.17 (ABT-229), and 6.45 ± 0.08 (EM-A) in the MTLR-EGFP and is somewhat lower than in the MTLR.

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cations with a 1-h interval. Figure 8 shows that for all compounds the contractile response decreases, but the effect was most pronounced with ABT-229; the third application resulted in a contraction that was only $8.92/110060.96\%$ of its first application. EM-A is at the other end with $86.50/110064.22\%$ ($n = 3$). Compounds EM-523 and ME4 showed a comparable decrease after the third application ($56.58/110064.71$ and $55.20/110068.86\%$, respectively, $n = 4$).

**Discussion**

Our study shows that motilides desensitize the motilin receptor to different extents in relation to their ability to activate the release of $Ca^{2+}$. However, this does not apply to all motilides since ABT-229 has a much stronger desensitizing potency than may be expected from its activity or from its binding affinity suggesting that for some motilides, activation and desensitization are partially decoupled. The more profound desensitization was also translated in an enhanced internalization of the motilin receptor to the cytosol following stimulation with ABT-229 as was evidenced by radioligand binding and by using an EGFP-tagged MTLR. A comparison of the effect of structural changes on changes in activity and desensitization suggests that the removal of the hydroxyl at position 4 of the cladinose sugar contributes most to the strong desensitizing properties of ABT-229, although a combination with other structural changes may be required. One may question the relevance of the model we developed to study desensitization because CHO cells, although convenient, may have different activation pathways than gastrointestinal smooth muscle. However, the data we obtained in the tissue bath using the classical model to study the potency of motilides, the contractile response of the rabbit duodenum, corroborate the conclusions of the cellular model. We suggest that the strong desensitizing properties of ABT-229 may have contributed to its clinical failure and that a cellular model may be a convenient way to develop motilides without desensitizing properties.

<table>
<thead>
<tr>
<th>Test Method</th>
<th>pEC50 ± S.E.M. Luminescence</th>
<th>pEC50 ± S.E.M. Binding</th>
<th>pDC50 ± S.E. Desensitization</th>
<th>pEC50–pDC50 ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motilin</td>
<td>9.39 ± 0.03</td>
<td>8.55 ± 0.05</td>
<td>7.77 ± 0.08</td>
<td>1.62 ± 0.15</td>
</tr>
<tr>
<td>ABT-229</td>
<td>8.46 ± 0.08</td>
<td>7.23 ± 0.10</td>
<td>8.78 ± 0.03</td>
<td>-0.32 ± 0.16</td>
</tr>
<tr>
<td>EM-A</td>
<td>7.11 ± 0.05</td>
<td>5.51 ± 0.10</td>
<td>4.78 ± 0.26</td>
<td>2.33 ± 0.28</td>
</tr>
<tr>
<td>ME36</td>
<td>6.62 ± 0.02</td>
<td>4.65 ± 0.06</td>
<td>3.32 ± 0.15</td>
<td>3.30 ± 0.16</td>
</tr>
<tr>
<td>ME4</td>
<td>8.08 ± 0.01</td>
<td>6.27 ± 0.13</td>
<td>5.90 ± 0.42</td>
<td>2.18 ± 0.42</td>
</tr>
<tr>
<td>ME57</td>
<td>8.12 ± 0.10</td>
<td>6.49 ± 0.10</td>
<td>6.03 ± 0.10</td>
<td>2.09 ± 0.25</td>
</tr>
<tr>
<td>EM-523</td>
<td>8.22 ± 0.01</td>
<td>6.59 ± 0.06</td>
<td>6.02 ± 0.28</td>
<td>2.20 ± 0.28</td>
</tr>
<tr>
<td>KOS1326</td>
<td>8.14 ± 0.16</td>
<td>7.16 ± 0.08</td>
<td>7.32 ± 0.0002</td>
<td>0.82 ± 0.28</td>
</tr>
</tbody>
</table>

Fig. 5. A, comparison of the $Ca^{2+}$ release evoked after concentration-dependent activation of wild type (top) and MTLR-EGFP (bottom) by motilin and motilides. Concentration-response curves of motilin, ABT-229, and EM-A are expressed as percentage of Triton X-100 values. B, comparison of desensitization of wild-type (■) and EGFP-tagged MTLR (□) by motilin and motilides. The response to motilin after pretreatment with a ligand at $10^{-5}$ M is expressed as percentage versus control (no pretreatment). Values are mean ± S.E.M. of three experiments in duplicate.
As a general rule, ligands cause both receptor-mediated signaling and signal desensitization. In the case of G protein-coupled receptors, a ligand signals by activating its receptor, which in turn activates a second messenger (G protein) inside the cell. G protein-coupled receptor desensitization involves the uncoupling of receptors from their heterotrimeric G proteins, resulting in the internalization (endocytosis) of receptors to endosomes and down-regulation (Ferguson, 2001).

The events of activation and desensitization have been linked in many receptor systems, among which the β opioid receptor has been characterized best (Benovic et al., 1988). Our data confirm that there is indeed a correlation between the ability to activate and desensitize, e.g., EM-A is less potent than the enol ether derivatives ME4, EM-523, and ME67 and also induces less desensitization. However, the results with ABT-229 suggest a decoupling of activation and desensitization. Such discrepancies have also been described for the δ opioid receptor, which is related to the motilin receptor, in the sense that both receptors can be stimulated by a peptide (motilin versus enkephalin) and by a naturally occurring nonpeptide (motilides versus morphine, methadone, etorphine, etc.). The desensitization of the δ opioid receptor mediated by methadone and L-α-acetyl methadone was more pronounced than for morphine and was disproportionate to their efficacies (Yu et al., 1997). Furthermore, functional desensitization of the monkey D1A dopamine receptor cannot be predicted reliably from the agonist potency for stimulating adenylate cyclase (Lewis et al., 1998).

The general paradigm that activation and internalization are coupled is based on studies on β2-adrenergic and muscarinic receptors demonstrating that partial agonists cause less internalization than full agonists and the amount of receptor internalization caused by an agonist generally correlates with coupling efficiency to G proteins (Toews and Perkins, 1984; Thompson and Fisher, 1990; Szekeres et al., 1998). Again, exceptions have been described (Mahan et al., 1985; Barak et al., 1994). Perhaps the best evidence that G protein coupling is not required for receptor endocytosis is provided by the observation that in S49 murine lymphoma cell lines, which either lack Gs or have point mutations preventing receptor/G protein interactions, β2-adrenergic internalization in response to agonist stimulation is normal (Mahan et al., 1985).

In our data, the discrepancy between activation and desensitization is translated into a discrepancy between activation and internalization. Similarly, observations with the δ and μ-opioid receptor in transfected cells and enteric neurons in intact animals show that both peptidyl (enkephalin) and...
nonpeptidyl (etorphine) agonists stimulate receptor desensitization and endocytosis in the expected manner. Remarkably, the high affinity μ-opioid receptor agonist morphine does not cause detectable internalization, even at concentrations that strongly inhibit adenylate cyclase (Keith et al., 1996; Sternini et al., 1996).

Our results indicate that desensitization and internalization of the motilin receptor are coupled in line with the adenylate cyclase (Keith et al., 1996; Sternini et al., 1996). Internalization, even at concentrations that strongly inhibit activation. Indeed, when motilin receptors are not present on ABT-229 as the induction of desensitization rather than activation. Our data also suggest that the presence of an ethyl at the amine group of the desosamine sugar, as in ME36, reduces the desensitizing ability, whereas removal of the hydroxyl at position 4′ of the cladinose sugar, as in ABT-229 and KOS1326 but not at position 12 of the lactone ring as in ME-67, causes an increase.

Internalization was also measured by receptor binding studies. Prestimulation with EM-A induced receptor up-regulation. An increase in receptor density after ligand stimulation has been demonstrated for A1 adenosine receptors after antagonist treatment (Ciruela et al., 1997), and inverse agonists can up-regulate histamine H2 receptors (Alewijnse et al., 1998), cannabinoid receptors (Bouaboula et al., 1999), and opioid receptors (Zaki et al., 2001). Both agonist and antagonist treatments have been shown to up-regulate dopamine receptors (Filtz et al., 1994; Zhang et al., 1994; Geurts et al., 1999).


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