Carboxyl-terminal Splicing of the Rat \( \mu \) Opioid Receptor Modulates Agonist-mediated Internalization and Receptor Resensitization

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The rat \( \mu \) opioid receptor is alternatively spliced into two isoforms (MOR1 and MOR1B) which differ in length and amino acid composition at the carboxyl terminus. When stably expressed in HEK 293 cells, both splice variants bind the \( \mu \) receptor agonist \([\text{d-Ala}^2\text{N-Me-Phe}^4\text{-Gly-ol}^5\]enkephalin (DAMGO) with similar affinity and exhibit functional coupling to adenyl cyclase with similar efficiency. However, the shorter isoform, MOR1B, desensitized at a slower rate during prolonged DAMGO exposure (4 h) but resensitized at a faster rate than MOR1 during agonist withdrawal (20 min). Immunocytochemical analysis revealed that DAMGO-induced internalization of MOR1B proceeded much faster than that of MOR1 followed by rapid recycling of the receptor to the cell surface. In addition, the greater resistance of MOR1B to homologous desensitization compared with MOR1 as well as MOR1B resensitization was abolished when receptor reactivation/recycling was blocked with monensin, an inhibitor of endosomal acidification. It is concluded that the sequence at the cytoplasmic tail of MOR1B facilitates clathrin-coated vesicle-mediated endocytosis which, in turn, promotes accelerated receptor reactivation. Taken together, our findings suggest that carboxyl-terminal splicing of the rat \( \mu \) opioid receptor modulates agonist-induced internalization and receptor resensitization.

Prolonged exposure of G protein-coupled receptors to agonists results in a rapid decrease of receptor responsiveness. It is now generally accepted that agonist-induced desensitization involves phosphorylation of intracellular receptor domains. Several kinases have been implicated in opioid receptor desensitization, including cAMP-dependent protein kinase (PKA),\(^1\) protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaM kinase II) (1–5). Specific phosphorylation sites have been localized in the third intracellular loop and at the carboxyl terminus, which play a critical role in homologous desensitization of the opioid receptor (6–10). Following phosphorylation, the receptor is being targeted to the endocytotic machinery. A large body of evidence suggests that the main route of internalization of G protein-coupled receptors is via clathrin-coated pits and vesicles into early endosomes. Within the acidic environment of the endosomes, the ligand is effectively separated from the receptor which becomes dephosphorylated and thus resensitized. As a final step, the receptor recycles back to the cell surface (11).

For the \( \mu \) opioid receptor, desensitization seems to be regulated by CaM kinase II-mediated phosphorylation of two serine residues (Ser\(^{265}\)/Ser\(^{266}\)) in the third intracellular loop (3, 9). Another important phosphorylation site of the \( \mu \) opioid receptor is the threonine at position 394 in the carboxyl terminus. Indeed, we and others have recently observed that site-directed mutagenesis of Thr\(^{394}\) to alanine profoundly delays DAMGO-induced desensitization, suggesting that this site may be a primary target for phosphorylation by GRKs upon agonist binding to the MOR1 (9, 10).

We have previously shown, that the cytoplasmic tail of the rat \( \mu \) opioid receptor undergoes alternative splicing giving rise to two isoforms, MOR1 and MOR1B. The receptor variants share 100% amino acid sequence identity up to amino acid 386 but differ from residue 387 to the carboxyl terminus (MOR1, \( \text{ENLEAETAPLP}^{389}\); MOR1B, \( \text{KIDL}^{391}\)). Both isoforms exhibit similar pharmacological profiles, however, MOR1B which lacks Thr\(^{394}\) appears to be more resistant to agonist-induced desensitization than MOR1 (12).

In the present study, we examined the role of receptor internalization in the desensitization process of the two \( \mu \) opioid receptor isoforms. Evidence will be provided, that the carboxyl-terminal amino acid sequence of MOR1B contains a putative endocytotic motif that leads to an enhanced internalization/reactivation rate and, hence, may explain the delayed desensitization kinetics of MOR1B as compared with MOR1.

MATERIALS AND METHODS

Tissue Culture and Transfections—MOR1 cDNA subcloned into pRc/CMV (kindly provided by Dr. L. Yu, Indianapolis, IN), and MOR1B cDNA subcloned into pCDNA3 expression vectors were used for transfection of human embryonic kidney HEK 293 cells (ATCC). Cells were maintained in Dulbecco’s modified Eagle medium NuF-12 medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO\(_2\). Transfections were performed using the calcium phosphate precipitation method as described by Chen and Okayama (13). Approximately 1.5 \( \times \) 10\(^6\) cells were transfected with 20 \( \mu \)g of plasmid DNA. Cells were selected in the presence of 500 \( \mu \)g/ml G418 (Life Technologies, Inc., Egggenstein, Germany), and the whole pool of resistant cells was used without selection of individual clones.

Determination of Receptor Desensitization and Resensitization by Measurement of cAMP Accumulation—Transfected cells were seeded at a density of 1.5 \( \times \) 10\(^5\) per well in 22-mm 12-well dishes. After 24 h, cells were exposed to 1 \( \mu \)M DAMGO (Bachem, Heidelberg, Germany) for 0, 0.5, 1, 2, or 4 h. When indicated, cells were preincubated either with 0.4 mM sucrose (Sigma, Deisenhofen, Germany), an inhibitor of clathrin-coated vesicle-mediated endocytosis, for 30 min or with 50 \( \mu \)M monensin (Sigma), an inhibitor of endosomal acidification, for 60 min and subsequently maintained under these conditions during agonist exposure.
For resensitization assays, cells were washed after 4 h of DAMGO exposure followed by an additional incubation period of 0, 5, 10, or 20 min in the absence of agonist. For the measurement of cAMP accumulation, medium was removed from individual wells and replaced with 0.5 ml of serum-free RPMI medium (Seromed, Berlin, Germany) containing 25 µM forskolin (Biotrend, Köln, Germany) or 25 µM forskolin plus 1 µM DAMGO. The cells were incubated at 37 °C for 15 min. The reaction was terminated by removing the medium and sonicating the cells in 1 ml of ice-cold HCl/ethanol (1 volume of 1 N HCl/100 volumes of ethanol). After centrifugation, the supernatant was evaporated, the residue was dissolved in TE buffer (50 mM Tris-EDTA, pH 7.5), and the cAMP content was determined using a commercially available radioimmunoassay kit (Amersham, Braunschweig, Germany). Statistical evaluation of results was performed using ANOVA followed by the Bonferroni test.

Radioligand Binding Assay—For whole cell binding, 10⁶ cells treated as described above were incubated with 2.5 nM [³H]DAMGO (NEN, Kols, Germany) for 40 min at 25 °C in 50 mM Tris-HCl, pH 7.8. Cells were collected on GF 10 glass-fiber filters and unbound ligand was removed by extensive washes with 50 mM Tris-HCl, pH 7.8. The radioactivity on the filters was determined by liquid scintillation counting. Specific binding was calculated by subtracting nonspecific binding from total binding. Nonspecific binding was determined as radioactivity bound in the presence of 1 µM unlabeled DAMGO. Results were calculated as bound radioligand per mg protein, measured by Lowry (14). The binding characteristics of MOR1 and MOR1B were determined by saturation binding assays on membranes prepared from transfected HEK 293 cells. The dissociation constant (Kd) and number of [³H]DAMGO binding sites (Bmax) were calculated by Scatchard analysis (15) using at least seven concentrations of labeled DAMGO in a range from 0.25 to 10 nM.

Confocal Microscopy—HEK 293 cells stably expressing either MOR1 or MOR1B were grown on poly-L-lysine-treated coverslips overnight. Cells were then exposed to 1 µM DAMGO for 0, 10, 30, or 50 min. When indicated, the medium was supplemented with sucrose or monensin as described above. Cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9, for 30 min at room temperature and subsequently washed several times in TPBS (10 mM Tris, 10 mM phosphate buffer, 137 mM NaCl, 0.05% thimerosal, pH 7.4). After 1 h of preincubation in TPBS containing 0.3% Triton X-100 and 3% normal goat serum, cells were incubated either with anti-MOR1 or anti-MOR1B antibodies at a dilution of 1:500 in TPBS containing 0.3% Triton X-100 and 1% normal goat serum overnight at RT. The antibodies were generated against the following peptide sequences: LENLEAETAPLP, which corresponds to residues 387–398 of MOR1, and VDRTNHIKIDLW, which corresponds to residues 380–391 of MOR1B and have been characterized extensively (16). Bound primary antibody was detected with biotinylated goat anti-rabbit IgG (1:400; Amersham, Germany). Cells were then dehydrated, cleared in xylol and permanently mounted in DPX (Fluka, Neu-Ulm, Germany). Specimens were examined using a Leica TCS-NT laser scanning confocal microscope. Cyanin 3.18 was imaged with 568-nm excitation and 570–630-nm bandpass emission filters. Confocal micrographs were taken by a person blinded to the treatments who was instructed to randomly select one colony of 4–12 cells per coverslip.

RESULTS
Agonist-induced Desensitization of μ Opioid Receptor Isoforms Expressed in HEK 293 Cells—The splice variants MOR1 and MOR1B were stably expressed in HEK 293 cells. First, we compared binding affinities and functional coupling to adenylyl cyclase of the expressed receptors. Saturation binding experiments indicated no major difference between MOR1 and MOR1B with respect to their affinity to [³H]DAMGO. The maximum percentage inhibition of adenylyl cyclase activities for MOR1 and MOR1B was also identical. However, the number of binding sites was higher in MOR1-expressing cells than in MOR1B-expressing cells (Table I).

Next, we studied the time-course of agonist-induced loss of functional coupling to adenylyl cyclase for MOR1 and MOR1B. Forskolin treatment resulted in a five-fold increase in intracellular cAMP levels as compared with untreated HEK 293 cells. DAMGO inhibited forskolin-stimulated cAMP formation by 40% in both MOR1- and MOR1B-expressing cells (Table I).

### Table I

| Splice variant | kD (nM) | Bmax (fmol/mg) | Reduction of forskolin-stimulated cAMP levels by 1 µM DAMGO |
|---------------|--------|---------------|-------------------------------------------------------------|
| MOR1          | 2.4 ± 0.1 | 742 ± 12 | 38 ± 4 |
| MOR1B         | 2.4 ± 0.3 | 200 ± 9 | 37 ± 6 |

When transfected HEK cells were preincubated with 1 µM DAMGO for extended time periods, a time-dependent loss of coupling efficiency, which was complete after 4 h of agonist exposure, was observed for both receptor isoforms (Fig. 1). However, MOR1 and MOR1B did significantly differ in their time-course of DAMGO-induced desensitization. While the ability of MOR1 to inhibit cAMP accumulation was strongly reduced already after 1 h of DAMGO exposure, the MOR1B-mediated inhibition of cAMP formation was retained for longer time periods (Fig. 1). In wild-type HEK 293 cells, no DAMGO-induced inhibition of adenylyl cyclase was observed (data not shown).

Agonist-induced Loss of Cell Surface Binding—To examine the role of the carboxyl-terminal tail in μ opioid receptor internalization, HEK 293 cells expressing MOR1 or MOR1B were treated with 1 µM DAMGO for various time periods, and the number of cell surface binding sites was determined. As depicted in Fig. 2, cell surface binding in MOR1-expressing cells was only slightly reduced to 80% during the 4-h agonist exposure. In contrast, in MOR1B-expressing cells the number of binding sites progressively decreased to 40% within 2 h and
were exposed to 1 mM DAMGO for 0, 10, 30, or 50 min. The culture medium was either not supplemented or supplemented with monensin or sucrose. These cells were subsequently fixed, permeabilized, and fluorescently labeled with antibodies specific for either MOR1 or MOR1B. The subcellular distribution of the receptor proteins was then analyzed by confocal microscopy. The results are depicted in Figs. 3, 4, and 5. Fig. 3 (upper panel) shows that, in the absence of DAMGO, MOR1-like immunoreactivity (Li) was mostly present at the level of the plasma membrane and to a lesser extent in the cytoplasm. After 30 min of agonist exposure, a dramatic loss of MOR1-Li from the plasma membrane with a concomitant accumulation in vesicle-like structures within the cytoplasm was observed. When exposure to DAMGO was continued, MOR1 began to reappear at the plasma membrane while a significant proportion of the receptor remained in the intracellular compartment. When MOR1-expressing cells were cultured in the presence of monensin, an inhibitor of endosomal acidification, the receptor progressively accumulated in the cytoplasm and did not redistribute to the plasma membrane during the 50 min of agonist exposure (Fig. 3, middle panel). When sucrose, an inhibitor of clathrin-coated vesicle-mediated endocytosis, was added to the culture medium, MOR1 internalization was completely blocked (Fig. 3, lower panel).

Fig. 4 compares the rate and extent of MOR1 and MOR1B internalization in the presence of monensin, which blocks receptor recycling to the plasma membrane. The results clearly show that MOR1B internalization proceeds at a faster rate than that of MOR1. While internalization of MOR1 was not complete before 50 min (Fig. 3, middle panel and Fig. 4, upper panel), in most cells, internalization of MOR1B was complete already after 10 min of DAMGO treatment (Fig. 4, lower panel). In addition, we noted that in MOR1B-expressing cells, a significant proportion of receptor-Li was localized in the intracellular compartment even in the absence of DAMGO. This phenomenon was particularly pronounced when monensin was added to the culture medium (Fig. 4, lower panel, left micrograph, and Fig. 5). In contrast, MOR1B receptor protein was virtually absent from the cytoplasm when the cells were cultured in the presence of sucrose (Fig. 5, right micrograph). These results suggest that the MOR1B receptors are subject to constitutive internalization in the absence of agonist. Monensin effectively trapped internalized receptor proteins in endosomes, resulting in intracellular accumulation of MOR1B-Li. Sucrose prevented internalization of recycled receptor proteins so that MOR1B-Li almost completely disappeared from the cytoplasm. Moreover, binding analyses revealed that addition of the μ opioid receptor antagonist naloxone (12 h before the binding experiment) led to an increase of the \( B_{\text{max}} \) of only MOR1B but not MOR1 receptor type, supporting the idea of the constitutive internalization of the MOR1B receptor (Fig. 6).
Resensitization of MOR1 and MOR1B in HEK 293 Cells—To investigate whether desensitization of the μ opioid receptor isoforms is reversible by agonist withdrawal, HEK 293 cells expressing either MOR1 or MOR1B were treated for 4 h with 1 μM DAMGO, and medium was removed followed by an additional agonist-free incubation interval of 0, 5, 10, 15, or 20 min and determination of cAMP accumulation. Fig. 7A reveals that, after complete receptor desensitization, only MOR1B but not MOR1 resensitized during the 20 min of DAMGO withdrawal. In addition, the rapid resensitization of MOR1B was prevented by monensin (Fig. 7B). These results suggest that the faster rate of internalization of MOR1B is associated with accelerated receptor resensitization and that MOR1B reactivation involves redistribution of receptor proteins to the cell surface.

Effects of Monensin on Agonist-induced μ Opioid Receptor Desensitization—To determine to what extent the faster receptor internalization/reactivation of MOR1B contributes to its greater resistance to homologous desensitization compared with MOR1, we carried out desensitization assays as described above except that monensin was included in all incubations. While agonist-induced desensitization of MOR1 did not dramatically change, MOR1B showed very rapid desensitization which was already complete after 2 h of DAMGO exposure (Fig. 8). These findings suggest that the delayed desensitization of MOR1B involves its rapid targeting to the endocytotic machinery which, in turn, promotes accelerated receptor reactivation and recycling.

DISCUSSION

The μ opioid receptor splice variants, MOR1 and MOR1B, markedly differ in their desensitization kinetics when expressed in HEK 293 cells. Both variants share 100% amino acid sequence identity up to amino acid 386 but differ from residue 387 up to the carboxyl terminus (MOR1, 387LENLEAETAPLP398; MOR1B, 387KIDLF391). Specifically, MOR1B that lacks threonine 394 appears to be more resistant to agonist-induced desensitization than MOR1. It is therefore very tempting to speculate that MOR1B due to its lack of this threonine may be a better substrate for phosphorylation by GRKs and, hence, more resistant to agonist-induced desensitization. However, an alternative explanation emerges from our internalization studies showing that MOR1B undergoes faster endocytosis than MOR1.

After internalization, receptors will be dephosphorylated, separated from bound ligands and become subject to endosomal sorting either by passing through a process of receptor degra-
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Our immunocytochemical analysis revealed that MOR1 internalization in HEK 293 cells reached a maximum at 30 min of DAMGO preincubation and that after 50 min, receptors seemed to reappear at the cell membrane. In addition, pretreatment of opioid receptor expressing HEK 293 cells with the recycling blocker monensin led to a complete internalization of both MOR1B and MOR1 within 50 min of DAMGO treatment. It can be assumed that the fast process of receptor reappearance after agonist-induced internalization is due to receptor recycling and not due to receptor desensitization and internalization as determined using ANOVA followed by the Bonferroni test.

We suggest a model whereby rapid endocytosis of MOR1B permits accelerated resensitization and recycling of the receptor. This enhanced rate of resensitization of MOR1B compared with MOR1, in turn, confers a greater resistance to agonist-induced desensitization.

Another difference between MOR1 and MOR1B is that in the absence of agonist a significant proportion of the MOR1B receptor protein seems to be constitutively internalized and recycled to the cell membrane. This assumption is favored by the fact that MOR1B receptor proteins could be effectively trapped in endosomes by monensin incubation, whereas sucrose treatment prevented constitutive MOR1B internalization (Fig. 5).
In addition, our binding analysis showed that preincubation with the antagonist naloxone increased only the receptor number in the MOR1B-expressing cells (Fig. 6), suggesting that antagonist treatment inhibits constitutive internalization by stabilizing the receptor in a conformation that prevents exposing a receptor domain critical to directing it into the clathrin-dependent endocytotic pathway.

It is thus conceivable that the carboxyl-terminal tail of MOR1B contains an endocytotic sequence motif which facilitates targeting of the receptor protein to the endocytotic machinery. Constitutive internalization has previously been observed with a truncation mutant of the μ opioid receptor that lacks a carboxyl-terminal Ser/Thr-rich domain (354Thr-Ser-Thr357). When the truncation spared this Ser/Thr-rich domain, constitutive internalization did not occur (26). These findings confirm the complex role of the carboxyl terminus in opioid receptor endocytosis. While the Ser/Thr-rich domain (354Thr-Ser-Ser-Thr357) which is shared in both opioid receptor splice variants suppresses internalization, the cytoplasmic tail of MOR1B greatly enhances internalization.

Internalization results in a loss of membrane binding sites in intact cells. The binding analyses revealed that 40% of the MOR1B receptor sites have disappeared at 30 min of agonist treatment, without markedly affecting functional activity at this time. The simplest explanation for this finding is that only 60% of intact MOR1B receptors are necessary for maximum inhibition of intracellular cAMP formation. The decreasing pool of receptors in the membrane after agonist treatment may then be balanced by receptor recycling after internalization. Surprisingly, after 4 h of DAMGO preincubation, the MOR1 receptor is completely desensitized, whereas nearly 80% residual MOR1 binding sites could be detected in the cell membrane. This indicates, that phosphorylation and uncoupling of the receptor did not automatically induce massive internalization of the receptor and that only a portion of the residual receptor binding sites seem to represent active receptors coupling to intracellular G-proteins.

In summary, we have established that receptor internalization and resensitization is differentially affected by the divergent cytoplasmic tails of the μ opioid receptor isoforms, MOR1 and MOR1B. It appears that the sequence motif at the cytoplasmic tail of MOR1B facilitates clathrin-coated vesicle-mediated endocytosis and, in turn, promotes accelerated receptor reactivation and redistribution. The comparison between MOR1 and MOR1B provides strong evidence that receptor internalization is a primary rate-limiting step for receptor re-sensitization, and that enhanced resensitization also confers apparent resistance to agonist-induced desensitization. Thus, alternative carboxyl-terminal splicing of G-protein-coupled receptors may be a physiologically relevant mechanism that determines rate and extent of receptor internalization and resensitization.

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