C-terminal Splice Variants of the Mouse μ-Opioid Receptor Differ in Morphine-induced Internalization and Receptor Resensitization*

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The main analgesic effects of the opioid alkaloid mor-
phine are mediated by the μ-opioid receptor. In contrast to endogenous opioid peptides, morphine activates the μ-opioid receptor without causing its rapid endocytosis. Recently, three novel C-terminal splice variants (MOR1C, MOR1D, and MOR1E) of the mouse μ-opioid receptor (MOR1) have been identified. In the present study, we show that these receptors differ substantially in their agonist-selective membrane trafficking. MOR1 and MOR1C stably expressed in human embryonic kidney 293 cells exhibited phosphorylation, internalization, and down-regulation in the presence of the opioid peptide [D-Ala²,Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) but not in response to morphine. In contrast, MOR1D and MOR1E exhibited robust phosphorylation, internalization, and down-regulation in response to both DAMGO and morphine. DAMGO elicited a similar desensitization (during an 8-h exposure) and resensitization rates have suggested that endocytozed receptors are predominantly recycled to the cell surface in a reactivated state (8, 9).

We have shown previously that two C-terminal splice variants (rMOR1 and rMOR1B) of the rat μ-opioid receptor differ in their DAMGO-mediated internalization and resensitization rates (10, 11). The rapid internalizing variant rMOR1B revealed a faster resensitization and consequently a slower desensitization as compared with rMOR1. These results clearly show that receptor recycling after internalization affects the rate of agonist-induced desensitization of the μ-opioid receptor. Interestingly, morphine, which is a highly addictive opioid, can activate μ-opioid receptors but does not induce receptor internalization (12, 13). Replacement of the C-tail of the μ-opioid receptor by the C-tail of the δ-opioid receptor led to a receptor chimera that is internalized after morphine treatment, indicating that the C terminus of the μ-receptor can influence the agonist selectivity of endocytosis (14).

Recently, three novel splice variants (MOR1C, MOR1D, and MOR1E) were identified for the mouse μ-opioid receptor (15). The differences between MOR1 and the new variants are restricted to the terminal portion of the intracellular tail (MOR1, 387LENLEATAPLP398; MOR1C, 387PTLAVSVAQIFTGYPQHPV401; MOR1D, 387RNEEPSS393; and MOR1E, 387KKKLDSQRGC-SPTHVEKPCKSCMDRGLNLLPDDGPQESGEGQLGR438; MOR1D, 387RNEEPSS393; and MOR1E, 387KKKLDSQRGC-VQHPV401). Immunohistochemical studies revealed marked differences in the regional distribution of the μ-opioid receptor splice variants in the central nervous system (16–18). In the present study, we investigated whether these splice variants differ in their DAMGO- and morphine-induced phosphorylation, internalization, desensitization, and receptor resensitization. Our results provide strong evidence that DAMGO treatment resulted in a rapid internalization and similar desensitization and resensitization rates of all receptor splice variants, whereas after morphine treatment only the splice variants MOR1D and MOR1E showed receptor phosphorylation and internalization, a faster resensitization, and a slower desensitization as compared with MOR1 and MOR1C.

EXPERIMENTAL PROCEDURES

Epitope Tagging and Cloning of cDNA—The mouse μ-opioid receptor and the three splice variants MOR1C, MOR1D, and MOR1E were cloned into the pEAK10 expression vector (Edge Bio Systems, Gaithersburg, MD).
Internalization of MOR1 Splice Variants

Gaithersburg, MD). The MOR1 splice variant was provided kindly by Dr. Y. Pan (New York, NY). To introduce a HindIII restriction site and an N-terminal HA epitope tag, the forward primer 5’-CGT GAAC TCG TAT CAA TCA GAC TTC GCA GAC TAT GCT GAC AGC-3’ was synthesized and used for polymerase chain reaction amplification (PCR) to produce the HA-tagged receptor splice variants. For confirmation of the mouse µ-opioid receptor splice variants MORID and MORIE and introduction of an XhoI restriction site, polymerase chain reaction primers were designed based on the published DNA sequences of the variants (15). The sequence for the reverse MORID and MORIE primers was 5’-TCC TCT TGA GCA CCA TCA GGA AGA TTC ATG CAC TGT CTT TGG GTT AG-3’ and 5’-ACA ATG TAC TCA CCC TCT CTG CGA TCG CAC TTT CTG GTT AG-3’, respectively.

Generation of Cell Lines Expressing µ-Opioid Receptors—Transfection of HEK 293 cells was performed by the calcium phosphate precipitation method (19). Approximately 1.5 x 10⁶ cells were transfected with 20 µg of plasmid DNA. Cells were selected in the presence of 1 µg/ml puromycin (Sigma). Three to six clones were generated for each of the four µ-receptor isoforms. Receptor expression was monitored using saturation ligand binding assays and quantitative Western blot analysis as described below; clones expressing similar numbers of receptors were selected for further study. The Bmax and Kd values are given in Table 1.

Determination of Receptor Desensitization and Resensitization by Measurement of cAMP Accumulation—Approximately 1.5 x 10⁶ cells were seeded in 22-mm 12-well dishes with Dulbecco’s modified Eagle’s medium nutrient F-12 medium containing 10% fetal calf serum. The medium was modified by the addition of 0.5 ml of serum-free media. Agonist stimuli were either 1 µM DAMGO or 1 µM morphine. The cells were incubated with 100 µM of wheat germ lectin-agarose beads (Amersham Pharmacia Biotech) for 90 min at 4 °C with continuous agitation. Beads were washed five times with buffer, and absorbed glycoproteins were either subjected to enzymatic deglycosylation or directly eluted into 200 µl of SDS-sample buffer (25 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 100 mM N-dithiothreitol, and 0.005% bromophenol blue) at 60 °C for 20 min. Deglycosylation experiments were performed using peptide N-glycosidase F according to manufacturer protocol (New England BioLabs, Beverly, MA). After electrophoresis, the membranes were incubated with 1 µg/ml affinity-purified rabbit polyclonal anti-HA-tag antibody for 2 h at 4 °C, followed by detection using an enhanced chemiluminescence detection system. Western blots exposed in the linear range of the x-ray film were densitometrically scanned, and the amount of immuno-reactive material in each lane was quantified using NIH Image 1.57 software.

Whole-cell Phosphorylation Assays—Cells were plated onto 100-mm diameter coverslips down to 80% confluence. The cells were washed twice with supplemented phosphate-free medium and then labeled with 200 µCi/ml carrier-free [32P]orthophosphate (285 Ci/mg P, ICN, Eschwege, Germany) for 60 min at 37 °C. Labeled cells were exposed to either 1 µM DAMGO or 1 µM morphine for 20 min. After incubation, cells were washed with ice-cold PBS and then scraped into 1 ml of radioligand precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM KCl, 10 mM MgCl2, 250 mM sucrose, 10 mM EDTA, 3 mM EGTA, 4 mg/ml β-dodecylmaltoside, 10 mM iodoacetamide, and the proteinase inhibitors listed above) for 1 h on ice. The lysate was centrifuged at 20,000 x g for 30 min at 4 °C. The protein content of the resulting supernatant was determined using the BCA protein assay (Pierce). Samples containing equal amounts of protein (300 µg) were then either subjected to immunoprecipitation or glycoproteins were purified using wheat germ lectin. For enrichment of glycoproteins, 1 ml of the supernatant was incubated with 100 µl of wheat germ lectin-agarose beads (Amersham Pharmacia Biotech) for 90 min at 4 °C with continuous agitation. Beads were washed five times with buffer, and adsorbed glycoproteins were either subjected to enzymatic deglycosylation or directly eluted into 200 µl of SDS-sample buffer (25 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 100 mM N-dithiothreitol, and 0.005% bromophenol blue) at 60 °C for 20 min. Deglycosylation experiments were performed using peptide N-glycosidase F according to manufacturer protocol (New England BioLabs, Beverly, MA). After electrophoresis, the membranes were incubated with 1 µg/ml affinity-purified rabbit polyclonal anti-HA-tag antibody for 2 h at 4 °C, followed by detection using an enhanced chemiluminescence detection system. Western blots exposed in the linear range of the x-ray film were densitometrically scanned, and the amount of immuno-reactive material in each lane was quantified using NIH Image 1.57 software.

RESULTS

Functional Expression of µ-Opioid Receptor Splice Variants—The µ-opioid receptor isoforms MOR1, MORIC, MORID, and MORIE were HA-epitope-tagged and stably expressed in HEK 293 cells. Receptor expression was monitored using saturation ligand binding assays. HEK 293 cells stably expressing the four mouse µ-opioid receptor splice variants revealed no

150-mm dishes and grown to 80% confluence. When indicated, the cells were exposed to 1 µM DAMGO, 1 µM morphine, or 1 µM naloxone for 0, 5, 1, 2, 4, or 16 h. The cells were then washed twice with PBS and harvested into ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 10 mM iodoacetamide, and the proteinase inhibitors listed above) for 20 min on ice. The cell pellets were lysed in detergent buffer (200 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 3 mM EGTA, 4 mg/ml β-dodecylmaltoside, 10 mM iodoacetamide, and the proteinase inhibitors listed above) for 1 h on ice. The lysate was centrifuged at 20,000 x g for 30 min at 4 °C. The protein content of the resulting supernatant was determined using the BCA protein assay (Pierce). Samples containing equal amounts of protein (300 µg) were then either subjected to immunoprecipitation or glycoproteins were purified using wheat germ lectin. For enrichment of glycoproteins, 1 ml of the supernatant was incubated with 100 µl of wheat germ lectin-agarose beads (Amersham Pharmacia Biotech) for 90 min at 4 °C with continuous agitation. Beads were washed five times with buffer, and adsorbed glycoproteins were either subjected to enzymatic deglycosylation or directly eluted into 200 µl of SDS-sample buffer (25 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 100 mM N-dithiothreitol, and 0.005% bromophenol blue) at 60 °C for 20 min. Deglycosylation experiments were performed using peptide N-glycosidase F according to manufacturer protocol (New England BioLabs, Beverly, MA). After electrophoresis, the membranes were incubated with 1 µg/ml affinity-purified rabbit polyclonal anti-HA-tag antibody for 2 h at 4 °C, followed by detection using an enhanced chemiluminescence detection system. Western blots exposed in the linear range of the x-ray film were densitometrically scanned, and the amount of immuno-reactive material in each lane was quantified using NIH Image 1.57 software.

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RESULTS

Functional Expression of µ-Opioid Receptor Splice Variants—The µ-opioid receptor isoforms MOR1, MORIC, MORID, and MORIE were HA-epitope-tagged and stably expressed in HEK 293 cells. Receptor expression was monitored using saturation ligand binding assays. HEK 293 cells stably expressing the four mouse µ-opioid receptor splice variants revealed no
subjected to Western blot analysis. However, extracts but also from the remaining cytosolic fraction and glycoproteins were enriched not only from membrane loss of cell surface receptors because of receptor internalization and subsequent down-regulation of the receptor.

The values shown are means ± S.E. from at least four independent experiments performed in triplicate.

| Splice variant | $B_{max}$ | $K_D$ | Reduction of forskolin-stimulated cAMP (fmol/mg) by 1 μM DAMGO | Reduction of forskolin-stimulated cAMP (fmol/mg) by 1 μM morphine |
|---------------|-----------|-------|---------------------------------------------------------------|---------------------------------------------------------------|
| MOR1          | 778 ± 6   | 1.55 ± 0.3 | 59 ± 3                                                        | 53 ± 6                                                        |
| MORIC         | 990 ± 8   | 1.42 ± 0.4 | 65 ± 4                                                        | 68 ± 4                                                        |
| MORID         | 739 ± 9   | 1.33 ± 0.1 | 51 ± 2                                                        | 55 ± 2                                                        |
| MORIE         | 666 ± 7   | 1.54 ± 0.3 | 58 ± 5                                                        | 57 ± 4                                                        |

markedly different numbers of binding sites ($B_{max}$) as assessed by $[^3]H$DAMGO binding. Furthermore, the affinities ($K_D$) for the peptide agonist DAMGO were similar for each of the receptor isoforms (Table I). In addition, the abilities of DAMGO or morphine to inhibit adenylate cyclase activity in HEK 293 cells were similar for all $μ$-receptor splice variants (Table I).

**Western Blot Analysis of μ-Opioid Receptor Splice Variants**—We then examined whether similar protein levels were expressed for all $μ$-receptor isoforms. When equal amounts of membrane proteins extracted from HEK 293 cells stably expressing the HA-tagged MOR1, MOR1C, MOR1D, or MOR1E receptors were subjected to Western blot analysis using an anti-HA antibody, a similar broad band migrating at 80 kDa was detected for each receptor isoform (Fig. 1A). Interestingly, we also observed an additional band with a higher molecular mass migrating at 160 kDa for MOR1 and MOR1C (Fig. 1A). In contrast, at this molecular mass, only very faint bands were detected for MOR1D and MOR1E (Fig. 1A). As shown in Fig. 1B, enzymatic deglycosylation of the MOR1 receptor protein reduced the size of the 80-kDa protein to 45 kDa and the size of the 160-kDa protein to 90 kDa, suggesting that this additional band may represent a $μ$-opioid receptor dimer. To further characterize the MOR1 homodimer, MOR1-expressing HEK 293 cells were exposed for extended periods (up to 16 h) to either DAMGO or the $μ$-receptor antagonist naloxone. Although DAMGO treatment resulted in a loss of MOR1 dimer and strong reduction of MOR1 monomer (46% of control), naloxone treatment resulted in an increase of both monomeric (153% of control) and dimeric (188% of control) forms of the receptor (Fig. 1B). The DAMGO-induced decrease in MOR1 receptor protein became apparent as soon as after 2 h of continuous exposure (data not shown). To exclude the possibility that this decrease in MOR1 receptor protein is not simply caused by a loss of cell surface receptors because of receptor internalization, glycoproteins were enriched not only from membrane extracts but also from the remaining cytosolic fraction and subjected to Western blot analysis. However, $μ$-receptor proteins were only detectable in membrane extracts and not in the cytosolic fractions, suggesting that the DAMGO-induced decrease of MOR1 dimers and monomers is caused by agonist-induced internalization, monomerization and lysosomal degradation leading to down-regulation of the receptor proteins. In contrast, the antagonist naloxone seems to stabilize MOR1 dimers at the plasma membrane and thus blocks receptor internalization and subsequent down-regulation of the receptor protein.

To test the possibility that the lack of detectable homodimers for MOR1D and MOR1E may be a result of constitutive internalization and subsequent monomerization of these receptors, cells stably expressing MOR1D and MOR1E were either not treated or treated with naloxone for 16 h. Again in untreated cells only monomeric forms of these receptors were detectable.

In contrast, MOR1D and MOR1E homodimers were readily detectable in naloxone-treated cells (Fig. 1C).

**Agonist-selective Endocytosis of μ-Opioid Receptor Splice Variants**—We asked whether the differences in the C-terminal tails of the $μ$-receptor splice variants may influence the agonist selectivity of receptor endocytosis. We therefore incubated HEK 293 cells stably expressing the N-terminal HA-tagged MOR1, MOR1C, MOR1D, or MOR1E receptors with anti-HA-specific antibodies at 4 °C to label cell surface receptors. These cells were then treated with DAMGO or morphine for 30 min at 37 °C. Control cells were incubated at 37 °C for 30 min in the absence of agonist to examine whether these receptors may undergo constitutive or tonic internalization. The cells were subsequently fixed and permeabilized, and bound anti-HA antibody was immunofluorescently detected (Fig. 2). At low temperature (4 °C), which prevents receptor endocytosis, all $μ$-opioid receptor splice variants were exclusively confined to the level of the plasma membrane as assessed by confocal microscopy (Fig. 2, Control 4 °C). During a 30-min incubation at 37 °C in the absence of agonist, only a proportion of MOR1D and MOR1E receptors showed constitutive internalization (Fig. 2, Control 37 °C). After 30 min of DAMGO treatment, all $μ$-opioid receptor isoforms exhibited robust endocytosis. However, only MOR1D and MOR1E appeared to be completely internalized, whereas a proportion of MOR1 and MOR1C receptors could still be detected at the plasma membrane (Fig. 2, DAMGO). After exposure to morphine, only MOR1D and MOR1E receptors revealed receptor endocytosis, whereas the MOR1 receptor was highly resistant to morphine-induced endocytosis. The MOR1C receptor revealed very little endocytosis in response to morphine (Fig. 2, Morphine).

**Agonist-selective Desensitization and Resensitization of μ-Opioid Receptor Splice Variants**—Next, we investigated whether the observed differences in receptor internalization would affect the rate of agonist-induced desensitization and resensitization of the $μ$-receptor splice variants. Receptor desensitization was measured as the decreased ability of the agonist to inhibit forskolin-stimulated adenylylate cyclase activity after extended agonist pretreatment (up to 8 h). Thus, HEK 293 cells expressing MOR1, MOR1C, MOR1D, or MOR1E were pretreated with either 1 μM DAMGO or 1 μM morphine for various time periods followed by the determination of agonist-induced inhibition of forskolin-stimulated adenylate cyclase. Forskolin treatment resulted in a 5-fold increase of intracellular cAMP (up to 4 pmol) as compared with untreated HEK 293 cells. For each splice variant, the maximum agonist-induced inhibition of cAMP accumulation without agonist preincubation has been defined as 100%. Prolonged exposure (8 h) to DAMGO as well as to morphine led to complete desensitization of the four receptor variants (Fig. 3, A and B). Although the time courses of DAMGO-dependent loss of receptor activity were very similar for all $μ$-receptor variants (Fig. 3A), MOR1...
and MOR1C showed a markedly facilitated desensitization in the presence of morphine compared with MOR1D and MOR1E (Fig. 3B). In contrast, the morphine-mediated desensitization rates of MOR1D and MOR1E were nearly identical to those obtained after DAMGO treatment.

We next determined, whether the \( \mu \)-opioid receptor splice variants differ in their resensitization rates after DAMGO- and morphine-induced receptor desensitization. Resensitization was measured as the increased ability of the previously desensitized receptor to inhibit forskolin-stimulated adenylate cyclase activity during a 50-min drug-free interval. Thus, HEK 293 cells expressing MOR1, MOR1C, MOR1D, or MOR1E were pretreated with either 1 \( \mu \)M DAMGO or 1 \( \mu \)M morphine for 8 h, the medium was removed, and after an additional drug-free interval of 0, 10, 20, 30, 40, or 50 min the agonist-induced inhibition of forskolin-stimulated adenylate cyclase was determined. As depicted in Fig. 3C, all splice variants completely resensitized during 50 min of agonist withdrawal after DAMGO-mediated desensitization. After morphine-mediated desensitization, the splice variants MOR1D and MOR1E showed only partial resensitization (~25–30% of the maximum receptor activity) during 50 min of agonist withdrawal (Fig. 3D). Under otherwise identical conditions, however, the \( \mu \)-opioid receptor isoforms MOR1 and MOR1C were not able to gain function after complete morphine-induced desensitization (Fig. 3D). These results suggest that the lack of morphine-induced internalization of MOR1 and MOR1C not only facilitates desensitization but also prevents resensitization of these receptors.

Agonist-selective Down-regulation of \( \mu \)-Opioid Receptor Splice Variants—We then examined whether the observed agonist selectivity of receptor internalization would affect agonist-induced down-regulation of the \( \mu \)-receptor splice variants. Thus, HEK 293 cells expressing MOR1, MOR1C, MOR1D, or MOR1E were pretreated with either 1 \( \mu \)M DAMGO or 1 \( \mu \)M morphine for 16 h, and the levels of the monomeric forms of these receptors were determined using quantitative Western blot analysis. As shown in Fig. 4, treatment with DAMGO for extended time periods resulted in a massive loss of receptor proteins of all \( \mu \)-receptor variants. Specifically, quantitative analysis of three independent experiments revealed that the individual isoforms were down-regulated to 46% for control for MOR1, 58% for MOR1C, 10% for MOR1D, and 6% for MOR1E. In contrast, extended exposure to morphine resulted in a selective down-regulation of MOR1D (36%) and MOR1E (63%). No such down-regulation was observed after prolonged morphine treatment of MOR1 or MOR1C, suggesting that the lack of morphine-induced internalization of MOR1 and MOR1C also

![Fig. 1. Western blot analysis of \( \mu \)-opioid receptor splice variants.](http://www.jbc.org/)

A, membrane proteins from HEK 293 cells expressing HA epitope-tagged MOR1, MOR1C, MOR1D, or MOR1E were extracted and immunoblotted using anti-HA antibodies as described under “Experimental Procedures.” B, HEK 293 cells expressing MOR1 were treated with either 1 \( \mu \)M DAMGO or 1 \( \mu \)M naloxone for 16 h, and the membrane proteins were extracted and subjected to Western blot analysis. In addition, an aliquot of membrane proteins extracted from untreated cells was subjected to enzymatic deglycosylation using peptide \( N \)-glycosidase F (PNGase F) and immunoblotted. C, HEK 293 cells expressing MOR1D and MOR1E were either not treated or treated with 1 \( \mu \)M naloxone for 16 h, and the membrane proteins were extracted and subjected to Western blot analysis. Note that 1) MOR1 and MOR1C form stable homodimers, 2) prolonged DAMGO treatment down-regulates MOR1 dimers and monomers, 3) naloxone seems to stabilize MOR1 dimers and prevents down-regulation, and 4) MOR1D and MOR1E homodimers are detectable when constitutive internalization was blocked by naloxone. The positions of molecular mass markers are indicated on the left (in kDa). Two additional experiments gave similar results.

![Fig. 2. Agonist-selective endocytosis of \( \mu \)-opioid receptor splice variants.](http://www.jbc.org/)

HEK 293 cells expressing HA epitope-tagged MOR1, MOR1C, MOR1D, or MOR1E were incubated with anti-HA antibodies at 4 °C to label cell surface receptors (Control 4 °C). The cells were then either not treated (Control 37 °C) or treated with 1 \( \mu \)M DAMGO or 1 \( \mu \)M morphine for 30 min at 37 °C. The cells were subsequently fixed, fluorescently labeled, and examined by confocal microscopy. Note that 1) DAMGO-induced endocytosis of all four \( \mu \)-receptor isoforms, 2) morphine-induced endocytosis of MOR1D and MOR1E but not of MOR1 or MOR1C, and 3) MOR1D and MOR1E underwent constitutive internalization. Representative results from one of four independent experiments are shown. Scale bar, 50 \( \mu \)m.
prevents morphine-mediated down-regulation of these receptors (Fig. 4).

Agonist-selective Phosphorylation of μ-Opioid Receptor Splice Variants—The μ-opioid receptor variants contain various lengths of sequence including serines and threonines in their cytoplasmic tails that may represent phosphoacceptor sites for G protein-coupled receptor kinases. To determine whether the observed differences in agonist-selective internalization and down-regulation may be associated with differences in agonist-induced phosphorylation of the four μ-opioid receptor isoforms, we assessed whole-cell receptor phosphorylation in response to both DAMGO and morphine. DAMGO induced a rapid and robust phosphorylation of all μ-opioid receptor splice variants (Fig. 5A). Specifically, quantitative analysis of three independent experiments revealed that DAMGO promoted an increase in phosphorylation of 4.4-fold for MOR1, 2.0-fold for MOR1C, 4.0-fold for MOR1D, and 2.3 fold for MOR1E above basal levels. In contrast, the μ-opioid receptor isoforms markedly differed in their morphine-induced phosphorylation (Fig. 5B).

DISCUSSION

Alternative splicing of the cytoplasmic tail has been observed for a number of G protein-coupled receptors including the sst2A somatostatin receptor (20), the D2 dopamine receptor (21), the EP3 prostaglandin receptor (22), and a number of serotonin receptor subtypes (23). C-terminal splicing is thought to modulate several aspects of G protein-coupled receptor physiology, i.e. cell- and tissue-specific expression, subcellular targeting, and coupling to specific G proteins. We have shown recently that the cytoplasmic tail of the rat μ-opioid receptor undergoes alternative splicing, giving rise to two isoforms, rMOR1 and rMOR1B (24). These isoforms exhibit similar pharmacological profiles; however, they differ in agonist-induced desensitization. Specifically, the shorter isoform rMOR1B desensitized at a slower rate than the longer isoform rMOR1 (10). In contrast, DAMGO-induced internalization and receptor resensitization of rMOR1B proceeded at a faster rate than that of rMOR1 (10). rMOR1B lacks only one phosphorylation site (Thr-394) compared with rMOR1. Changing this Thr-394 into alanine results in a reduction of DAMGO-induced phosphorylation and a slower DAMGO-mediated desensitization of the T394A receptor mutant (11, 25, 26). In addition, the lack of Thr-394 leads to facilitated receptor internalization, enhanced resensitization, and recycling of the T394A receptor mutant (10, 11). Thus,
length and amino acid composition of the C terminus of the µ-opioid receptor seem to play a crucial role in agonist-induced endocytosis and receptor reactivation (10, 11).

It has been reported that individual opioid agonists differ substantially in their ability to induce receptor endocytosis. Although endogenous opioid peptides promote a rapid endocytosis of the µ-opioid receptor, the highly addictive opioid alkaloid morphine fails to stimulate receptor phosphorylation and internalization (12, 13). Interestingly, replacement of the entire µ-opioid receptor tail with the δ-opioid receptor tail results in a receptor chimera that undergoes endocytosis after morphine treatment (27). It was demonstrated further that the µ/δ-tail swapping results in a receptor conformation that facilitates morphine-induced phosphorylation and subsequent β-arrestin binding (27).

Three novel splice variants, MOR1C, MOR1D, and MOR1E, were identified recently for the mouse µ-opioid receptor, MOR1, that differ in the number of potential S/T phosphorylation sites in their cytoplasmic tails (15). Instead of the Thr-394 in the very end of the MOR1 cytoplasmic tail, the C termini of MOR1C, MOR1D, and MOR1E contain seven (Thr-388, Ser-392, Thr-398, Ser-402, Thr-404, Ser-412, and Ser-431), two (Ser-392 and Ser-393), and one (Ser-392) new potential phosphorylation sites, respectively. We therefore addressed the question of whether C-terminal splicing of the µ-opioid receptor may influence agonist selectivity of receptor phosphorylation and internalization. The µ-receptor isoforms showed similar binding profiles and functional properties compared with the MOR1 receptor when expressed in HEK 293 cells (Table I). Our immunocytochemical analysis revealed that all splice variants underwent rapid endocytosis after 30 min of DAMGO treatment, whereas after 50 min of morphine treatment only MOR1D and MOR1E showed marked receptor endocytosis (Fig. 2). MOR1 and MOR1C revealed a faster receptor desensitization under morphine compared with DAMGO treatment, whereas MOR1D and MOR1E showed similar desensitization curves in response to either morphine or DAMGO (Fig. 3, A and B). After complete desensitization, all splice variants resensitized during 50 min of DAMGO withdrawal (Fig. 3C). However, during 50 min of morphine withdrawal, only MOR1D and MOR1E but not MOR1 and MOR1C showed partial resensitization of receptor activity (Fig. 3D). These findings suggest a...
model in which the rapid morphine-induced endocytosis of MOR1D and MOR1E permits accelerated desensitization and recycling of these receptors, thus conferring resistance to agonist-induced desensitization. In contrast, the morphine-desensitized MOR1 and MOR1C receptors cannot gain entrance into the endocytotic-endosomal recycling pathway and are therefore not desensitized after morphine withdrawal. Several lines of evidence support the hypothesis that agonist-induced endocytosis is required for the reactivation of desensitized μ-opioid receptors and hence counteracts the development of physiological drug tolerance: 1) the μ-opioid receptor no longer desensitized when receptor recycling was blocked by monensin, an inhibitor of endosomal acidification (10), 2) the internalization rate of the μ-opioid receptor was increased in the presence of monensin (10), 3) the μ-opioid receptor desensitized faster when receptor recycling was blocked with monensin (10), and 4) the μ-opioid receptor desensitized faster after blocking receptor internalization by sucrose (28).

To delineate the mechanistic basis of these observations we carried out whole-cell phosphorylation studies for all splice variants. Previous studies have shown that morphine fails to induce intense μ-opioid receptor phosphorylation. It is believed that morphine restraints the MOR1 receptor in a conformation that is recalcitrant to phosphorylation by G protein-coupled receptor kinases (27, 29). Our data clearly show that DAMGO mediates robust phosphorylation of all splice variants. In contrast, morphine promotes marked receptor phosphorylation only in MOR1D and MOR1E but fails to promote phosphorylation to a similar extent as DAMGO in the MOR1 and MOR1C receptor isoforms (Fig. 5). Thus, the sequence alterations of the cytoplasmic tail caused by C-terminal splicing result in a conformation that makes the morphine-activated MOR1D and MOR1E receptors accessible for G protein-coupled receptor kinase-mediated phosphorylation and permit subsequent receptor sequestration.

However, we also found that morphine activates and desensitizes all four μ-opioid receptor variants. Consequently, the question arises as to which mechanisms guide the morphine-mediated desensitization of MOR1 and MOR1C. Our results show that in HEK 293 cells, morphine elicits phosphorylation of MOR1 and MOR1C to 30 and 55% of DAMGO-induced phosphorylation, respectively. It is therefore not unreasonable to speculate that partial phosphorylation of the MOR1 and MOR1C receptors elicited by morphine is sufficient to terminate signaling but not sufficient to promote receptor endocytosis (29).

After extended exposure (16 h) to DAMGO we observed a marked reduction of monomeric and dimeric forms of the MOR1 receptor (Fig. 1B). In contrast, treatment of MOR1 with naltrexone led to an increase in the amount of receptor monomers and dimers (Fig. 1B). Moreover, among the four μ-opioid receptor splice variants, only MOR1 and MOR1C form homodimers that are stable under denaturing and reducing conditions, whereas only very faint bands were detected at the expected molecular masses for the MOR1D and MOR1C dimers (Fig. 1A). Interestingly, when constitutive internalization of MOR1D and MOR1E was blocked by prolonged exposure to naltrexone, MOR1D and MOR1E homodimers were readily detectable (Fig. 1C). The simplest explanation for these findings is that the DAMGO-induced internalization and subsequent lysosomal degradation leads to down-regulation of the receptor proteins. On the other hand, the antagonist naltrexone blocks receptor internalization and seems to stabilize opioid receptor dimers at the plasma membrane, thereby preventing monomerization and down-regulation of the receptor proteins. This conclusion is supported by the present findings that DAMGO promoted down-regulation of all four μ-opioid receptor splice variants, whereas morphine induced selectively the down-regulation of MOR1D and MOR1E but not of MOR1 or MOR1C (Fig. 4).

In conclusion, our results provide strong evidence that changes in the C-terminal sequence by alternative splicing determine agonist selectivity of phosphorylation and internalization of the μ-opioid receptor isoforms. We show that two splice variants, MOR1D and MOR1E, of the mouse μ-opioid receptor were rapidly phosphorylated and internalized after morphine treatment. The rapid morphine-induced endocytosis of MOR1D and MOR1E confers resistance to desensitization and permits receptor resensitization and down-regulation. In contrast, the lack of morphine-induced phosphorylation and internalization of MOR1 and MOR1C splice variants also prevents receptor resensitization and down-regulation. Together, our findings provide strong evidence that endocytosis is required for the μ-opioid receptor to enter either the endosomal recycling pathway, leading to receptor resensitization, or the lysosomal degradation pathway, leading to receptor down-regulation. Given the emerging evidence of cell- and tissue-specific expression of the four μ-opioid receptor isoforms, it is likely that C-terminal splicing may significantly modulate the development of tolerance to the various effects of morphine.

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C-terminal Splice Variants of the Mouse \( \mu \)-Opioid Receptor Differ in Morphine-induced Internalization and Receptor Resensitization

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