Single Nucleotide Polymorphisms in the Human μ Opioid Receptor Gene Alter Basal G Protein Coupling and Calmodulin Binding*

Received for publication, May 7, 2001, and in revised form, July 5, 2001
Published, JBC Papers in Press, July 16, 2001, DOI 10.1074/jbc.M104083200

Danxin Wang‡, J. Mark Quillan‡, Katharine Winans§, Julie L. Lucas‡, and Wolfgang Sadée¶¶

From the ¶Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry and the Wheeler Center for Neurobiology of Addiction, University of California, San Francisco, California 94143-0446 and the ‡Department of Chemistry, University of California, Berkeley, California 94721

The μ opioid receptor (MOR) plays a central role in mediating acute and chronic effects of narcotic drugs. Three rare single nucleotide polymorphisms in the hMOR gene have been identified that cause amino acid substitutions in the third intracellular (i3) loop of MOR (R260H, R265H, and S268P). Genotyping 252 individuals of the Coriell collection identified one allele encoding the R265H-MOR variant and a new variant encoding D274N-MOR. Variants R260H-, R265H-, and S268P-MOR were constructed and transfected into HEK293 cells. Morphine stimulated G protein coupling of the three receptor variants to a maximal level approaching that of wild type MOR. In contrast, spontaneous, agonist-independent (basal) MOR signaling, proposed to play a role in opioid tolerance and dependence, was significantly reduced for R260H- and R265H-MOR. Moreover, domains within the i3 loop of MOR have been shown to interact with both G proteins and calmodulin (CaM). CaM binding was deficient for variants R265H- and S268P-MOR, suggesting that domains for G protein coupling and CaM binding overlap partially. Morphine pretreatment significantly enhanced basal G protein coupling of wild type MOR, which is thought to result from release of CaM. In contrast basal G protein coupling activity of the three variants was unaffected by morphine pretreatment consistent with diminished CaM regulation, low basal activity, or both. In conclusion, each of the three single nucleotide polymorphisms mapping to the i3 loop of MOR caused substantial changes in basal G protein coupling, CaM binding, or both. Carriers of the mutant alleles might display altered responses to narcotic analgesics.

Opioid analgesics are highly effective in controlling pain elicited by noxious stimuli, but their repeated use causes tolerance and physical dependence and can lead to addiction (1, 2). Individual predisposition toward abuse likely involves genetic factors in addition to behavioral ones (3–7). As the main molecular target of opioid analgesics, the opioid receptor (MOR)3 represents an obvious candidate in the search for genetic variations that affect the response of an individual to opioid analgesics. Genotyping of the human MOR gene has revealed several single nucleotide polymorphisms (SNPs) that exist in the general population (7–9). One SNP mapping to the N-terminal region of the seven-transmembrane structure of MOR has already been shown to alter the binding affinity for β-endorphin (8), but the clinical significance of this genetic variation remains uncertain (10). We hypothesize that by analyzing the effect of additional polymorphisms on the complex signaling behavior of MOR, we might gain further clues as to the molecular basis of opioid drug response.

Opioid receptors are functionally dynamic, seven-transmembrane-spanning proteins known to transmit signals via the GTP-binding proteins, G_i/G_o. However, they cannot be understood adequately in terms of acute G protein activation alone. Activation of MOR also results in long term changes associated with receptor phosphorylation, internalization, and alterations in gene expression (1). Recently, a direct interaction between the ubiquitous calcium-sensitive regulatory protein calmodulin (CaM) and the third intracellular (i3) loop of MOR has been demonstrated (11). The importance of the i3 loop to G protein coupling (12) and CaM binding (11, 13), the presence of multiple phosphorylation consensus sequences (14), and recent observations that this region regulates spontaneous basal G protein coupling (11, 13, 15) all strengthen the rationale for examining i3 loop polymorphisms. A range of functional consequences could emanate from alterations in the i3 loop. At least three allelic, nonsynonymous SNPs that alter the MOR-i3 loop have so far been identified in single individuals, leading to the human variants R260H-, R265H-, and S268P-MOR (8, 9). The allele frequency of these three sporadic variants is unknown. In this study we have genotyped exon 3 of the hMOR gene in 252 subjects (Coriell collection) to obtain more information on allele frequency and discover any further MOR variants. Examination of S268P-MOR had already revealed differences in G protein coupling efficiency and in desensitization kinetics, differences that could stem from structural changes induced by proline insertion and loss of the CaM-kinase II phosphorylation site at Ser268 (14). Moreover, Befort et al. (16) have proposed that the S268P substitution significantly impaired agonist-stimulated G protein coupling of MOR, a finding that we have re-examined in the present study.

Recently, a significant level of basal G protein coupling has been demonstrated for MOR (11, 13, 15). Basal signaling is observable even at relatively low receptor concentrations (~150 fmol of MOR/mg protein) (17), indicating that it could be physiologically relevant. Basal MOR coupling was shown to increase after prolonged morphine stimulation (11, 13, 18), a paradoxical finding that has been confirmed by Liu and Prather (19). We have recently identified a series of neutral antagonists and inverse agonists at MOR in tissue culture (17).
Moreover, neutral antagonists of MOR (agonists that do not affect basal signaling) were shown to elicit substantially less withdrawal jumping than inverse agonists (agonists that suppress basal signaling) in a mouse model of morphine dependence (17, 20). These results suggest that basal signaling activity of MOR plays a key role in narcotic dependence. On the other hand, CaM appears to compete with G protein coupling at the i3 loop and regulates basal MOR-G protein coupling (11, 13). Hence, polymorphisms mapping to the i3 loop of MOR might change the balance between G protein coupling, basal and agonist-stimulated, and CaM interactions. This could affect gene transcription, shown to play a role in opioid dependence (1). For example, up-regulation of the cAMP second messenger system by opioid agonists, considered a biochemical correlate of dependence, is associated with altered transcription of adenyl cyclases and other components of this signaling pathway (1, 21).

In the present study, we have determined functional changes in MOR signal transduction resulting from three previously established SNPs mapping to the i3 loop. For comparison, we have also included a mutant MOR with a K273A substitution in the i3 loop, which disrupts CaM binding to the receptor and leads to elevated basal G protein coupling (11). The results reveal profound changes in G protein coupling, CaM binding, or both, introduced by each of the three i3 loop SNPs examined.

MATERIALS AND METHODS

Cell Cultures and Treatment—Stable human embryonic kidney (HEK 293) cell lines expressing N-terminal FLAG-tagged wild type and R260H-, R265H-, S268P-, and K273A-MOR were maintained as described (11). For morphine pretreatment, the cells were incubated with morphine (1 μM) for 18 h. Then the cells were extensively washed with phosphate-buffered saline to remove the agonist.

Site-directed Mutagenesis of the FLAG-hMOR Receptor—N-terminal FLAG-tagged MOR and K273A-MOR were constructed as described (11). Polymorphic variants R260H-, R265H-, and S268P-MOR were constructed using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. Plasmids were transfected into HEK 293 cells with Superfect reagent (Qiagen, Valencia, CA), and stable cell lines were selected as described (22).

Synthesis of kMOR i3 Loop Peptides—Peptides corresponding to the entire i3 loop of the hMOR wild type receptor (residues 256–286) and i3 loop with R260H, R265H, and S268P substitutions were prepared and purified by high pressure liquid chromatography as described (11).

Gel Mobility Shift Assay—Gel mobility shift assays were performed using 12% nondenaturing polyacrylamide gel electrophoresis. Gel mobility shift assays were performed as described (11, 23). For inverse agonist experiments, the cell membranes (20 μg protein) were incubated with β-CNA (1 μM) and [35S]GTPγS in 500 μl of assay buffer at 30 °C for 20 min as described (13).

Measurement of CaM Levels in Plasma Membranes—The plasma membranes were prepared, CaM was extracted from the plasma membrane fractions, and the CaM levels were determined enzymatically with CaM-dependent phosphodiesterase activity using [3H]cAMP as described (11).

cAMP Accumulation Tested by [3H]Adenine Labeling—Cells cultured in 24-well plates for 3–4 days were labeled with 1 μCi/ml [3,5-3H]adenine (1.11 TBq/nmol, ICN, Costa Mesa, CA) for 16 h, washed, and incubated with 100 μM forskolin in the presence or the absence of drugs at 37 °C for 30 min. Then [3H]cAMP was purified as described (24). The results are expressed as the ratios of [3H]cAMP to total [3H]ATP, [3H]ADP, and [3H]AMP pools. For morphine pretreatment experiments, cells were incubated with 1 μM morphine for 18 h and labeled with 2 μCi/ml [3H]adenine during the last 2 h of the incubation before washing, followed by incubation with forskolin.

Genotyping—Using genomic DNA from 252 individuals in the Corell collection, exon 3 of MOR was first amplified by PCR (Applied Biosys-

![Comparison of CaM binding to synthetic peptides derived from the i3 loop](image-url)

**Fig. 1. Binding of CaM to synthetic peptides derived from the i3 loop residues 256–286 of human wild type and R260H, R265H, and S268P-MOR.** CaM was incubated with peptides in molar ratios of 1:1 and 1:10 in the presence of Ca2+ and separated by nondenaturing polyacrylamide gel electrophoresis.
CaM Depletion from Plasma Membranes—The ability of MOR variants to interact directly with CaM was shown to be correlated with morphine-induced depletion of CaM from plasma membranes (11, 13). Morphine treatment (1 μM, 15 min) significantly reduced CaM content in the plasma membrane of wild type MOR transfected cells (to 68 ± 8% of control, p < 0.01, t test). Morphine stimulation also caused detectable CaM release from plasma membranes of R260H-MOR transfected cells, reducing the CaM content to 85 ± 12% of control (p < 0.05, n = 6, t test). In contrast, morphine had no detectable effect on CaM content in plasma membranes expressing the R265H- and S268P-MOR variants (90 ± 18% of control for R265H-MOR, p > 0.05; 110 ± 30% of control for S268P-MOR, p > 0.05, n = 6, t test).

Activation of G Proteins in Cell Membranes Expressing MOR Variants

Interaction of i3 Loop Peptides with G Proteins—[^35S]GTPγS binding to cell membranes served as a measure of activation of G proteins. Peptides derived from the coupling domains of the receptor can be expected to bind directly to G proteins and affect[^35S]GTPγS binding: Incubation of i3 loop peptides with HEK293 cell membranes (not expressing MOR) increased[^35S]GTPγS binding activity in a concentration-dependent manner (Fig. 3a). Among the four i3 loop peptides tested, wild type i3 loop peptide was most efficacious in eliciting G protein coupling, followed by the S268P, R265H, and R260H mutant peptides. On the other hand, the binding of i3 loop peptides to G protein is expected to inhibit the more efficient agonist-stimulated G protein coupling. Shown in Fig. 3b, preincubation of HEK-MOR membranes for 30 min with wild type and S268P i3 loop peptide decreased morphine-activated[^35S]GTPγS binding, because the peptides compete with MOR for binding to G protein. However, no inhibition was observed with R260H and R265H peptides (Fig. 3b). These results indicate that R260H and R265H substitutions in the peptides impair binding to G proteins and suggest that binding to CaM and G proteins are mediated by different determinants in the i3 loop-derived peptides. However, the structure of the peptides may differ from that present in the intact receptor; therefore, these results do not necessarily reflect the coupling efficiency of the intact receptors.

Basal G Protein Coupling of MOR—MOR has been shown to display a significant level of basal G protein coupling in plasma membranes (11). The effect of i3 loop variation on G protein coupling by MOR was examined by determining GTPγS binding in cell membranes (Table I). The results were compared with K273A-MOR constructed to disrupt a CaM binding motif in the i3 loop (11). Even though each of the five receptor variants tested was able to induce a substantial increase in GTPγS binding of similar magnitude upon exposure to morphine (1 μM), basal levels of GTPγS binding (without agonists) were strongly affected by the i3 loop substitutions. In the absence of agonist, initial basal rates (over 5 min) for nonpre-treated cells varied from a low of 109 ± 6 fmol/mg for the R265H variant to a high of 136 ± 6 fmol/mg GTPγS binding for K273A-MOR, previously shown to display enhanced basal G protein coupling (11). Wild type MOR had an intermediate level of basal activity that was significantly enhanced over the empty vector transfected control cells (102 ± 2 fmol/mg protein) as reported earlier (13). The small elevation in GTPγS binding observed with the R260H and R265H variants did not reach statistical significance. After morphine pretreatment, only the wild type receptor displayed significantly increased basal GTPγS binding over nontreated conditions as shown before (13), whereas basal G protein coupling remained unchanged for each of the receptor variants. Variants differ from the observations by Befort et al. (16), performed under different in vitro conditions, that S268P-MOR is significantly impaired in agonist-stimulated maximal G protein coupling.

Washing of plasma membranes with EGTA pH 8 buffer was used to lower the CaM content of plasma membranes (11). This procedure significantly increased basal GTPγS binding of wild type MOR (from 123 ± 3 to 139 ± 4 fmol/mg protein, p < 0.01, t test), but not that of the CaM-binding deficient K273A mutant, as shown previously (11, 13). Similarly, the three polymorphic MOR variants failed to display significant changes in basal GTPγS binding upon EGTA pH 8 washing (from 118 ± 6 to 117 ± 9, 111 ± 10 to 114 ± 9, 124 ± 10 to 120 ± 14 fmol/mg protein for R260H, R265H, and S268P respectively, p > 0.05, t test, n = 6). This suggested that they either do not interact with CaM, do not have detectable basal activity, or both.

To test basal MOR signaling directly, we used the inverse agonist β-CNA, which reduces basal GTPγS binding to the level seen in control cells (empty vector transfected) (11, 13, 15). Shown in Fig. 4a, β-CNA significantly reduced basal GTPγS binding in membranes expressing the wild type, S268P, and K273A receptor constructs, while having no significant effect on the control cells and cells expressing R260H- and R265H-MOR. Morphone pretreatment significantly enhanced basal GTPγS binding only for wild type MOR, as revealed by the addition of β-CNA (Fig. 4a). In contrast, no significant effect of β-CNA was seen for the R260H and R265H variants even after
The cells were pretreated with or without 1 μM morphine for 16 h, followed by removal of the agonists before membrane preparation. The data are the means ± S.D., n = 6.

| Receptor density | [35S]GTP-γ-S binding | Basal after morphine pretreatment | Decrease in net morphine stimulation after treatment |
|------------------|-----------------------|----------------------------------|-----------------------------------------------|
|                  | No pretreatment | Morphine pretreatment |                      |                                      |
|                  | pmol/mg | fmol/mg | nM | pmol/mg | fmol/mg | nM | % untreated | %                      |
| hMOR             | 3.5 ± 0.5 | 122 ± 3 | 3.2 | 138 ± 4 | 189 ± 18 | 14.4 | 113 ± 3 | 45 ± 9 |
| R260H            | 3.9 ± 0.8 | 111 ± 8 | 3.4 | 116 ± 6 | 170 ± 22 | 7.5 | 105 ± 6 | 27 ± 31 |
| R265H            | 4.2 ± 0.8 | 109 ± 6 | 2.4 | 115 ± 9 | 167 ± 21 | 6.9 | 105 ± 8 | 17 ± 24 |
| S268P            | 4.5 ± 0.9 | 122 ± 6 | 1.5 | 119 ± 9 | 179 ± 15 | 5.6 | 97 ± 8 | 16 ± 11 |
| R273A            | 3.5 ± 0.9 | 136 ± 6 | 2.7 | 137 ± 5 | 232 ± 16 | 3.2 | 101 ± 4 | 15 ± 19 |

The values equal [1 – (E_{max} – Basal)_{after morphine pretreatment} / (E_{max} – Basal)_{no pretreatment}] × 100.

Versus hMOR, p < 0.05.

Versus hMOR, p < 0.01.

Versus hMOR, p < 0.005.

Versus hMOR, p < 0.001.

Morphine-stimulated Receptor Coupling—Stimulation of wild type and variant MORs with morphine each significantly enhanced GTP-γ-S binding, before and after morphine pretreatment. Although K273A-MOR yielded the highest maximal GTP-γ-S binding, the three polymorphic variants gave somewhat lower maximal stimulation than wild type MOR. The EC_{50} values did not differ significantly between wild type and variant MORs (range of 2.4–3.7 nM for morphine) (Table I). Whereas Befort et al. (16) also found only a small difference in the EC_{50} of morphine between wild type and S268P-MOR, maximal stimulation was drastically reduced for S268P-MOR. Moreover, reported EC_{50} values for the MOR-agonist [α-Ac^2, Nano,Me-Phe^4, Gly-ol^5]enkephalin were unusually high (100–200 nM), possibly because of the low free Mg^{2+} concentrations used by Befort et al. (16).

After morphine pretreatment of the cells, EC_{50} values increased 2–4-fold, an indication of desensitization, but again with no substantial differences observed between wild type and polymorphic MORs (EC_{50} range, 6–14 nM) (Table I). The seemingly greater shift in EC_{50} value for wild type MOR could have resulted from a distortion of the dose-response curve by enhanced basal coupling activity after morphine pretreatment.

Desensitization can also be expressed as a reduction of amplitude of receptor signaling in response to an agonist. Net reduction in stimulated GTP-γ-S binding after pretreatment with morphine was greatest for wild type MOR (45%; Table I). A significant proportion of the net decrease for the wild type receptor can be attributed to a 13 ± 3% increase in basal activity after pretreatment, which is not seen for the MOR variants. The polymorphic variants R265H and S268P displayed a significantly smaller reduction in agonist-stimulated GTP-γ-S binding after morphine pretreatment, indicating diminished desensitization, whereas the R260H variant did not significantly differ from the wild type receptor (Table I).

### Table I

| Basal and morphine-stimulated [35S]GTP-γ-S binding activity in wild-type and variant MOR transfected HEK293 cell membranes |
|----------------------------------------------------------------------------------------------------------------------------------|
| The cells were pretreated with or without 1 μM morphine for 16 h, followed by removal of the agonists before membrane preparation. The data are the means ± S.D., n = 6. |
| Basal | Morphine pretreatment | Basal after morphine pretreatment | Decrease in net morphine stimulation after treatment |
|-------|-----------------------|----------------------------------|-----------------------------------------------|
| pmol/mg | fmol/mg | nM | pmol/mg | fmol/mg | nM | % untreated | %                      |
| hMOR | 3.5 ± 0.5 | 122 ± 3 | 3.2 | 138 ± 4 | 189 ± 18 | 14.4 | 113 ± 3 | 45 ± 9 |
| R260H | 3.9 ± 0.8 | 111 ± 8 | 3.4 | 116 ± 6 | 170 ± 22 | 7.5 | 105 ± 6 | 27 ± 31 |
| R265H | 4.2 ± 0.8 | 109 ± 6 | 2.4 | 115 ± 9 | 167 ± 21 | 6.9 | 105 ± 8 | 17 ± 24 |
| S268P | 4.5 ± 0.9 | 122 ± 6 | 1.5 | 119 ± 9 | 179 ± 15 | 5.6 | 97 ± 8 | 16 ± 11 |
| R273A | 3.5 ± 0.9 | 136 ± 6 | 2.7 | 137 ± 5 | 232 ± 16 | 3.2 | 101 ± 4 | 15 ± 19 |

The values equal [1 – (E_{max} – Basal)_{after morphine pretreatment} / (E_{max} – Basal)_{no pretreatment}] × 100.

Versus hMOR, p < 0.05.

Versus hMOR, p < 0.005.

Versus hMOR, p < 0.001.

Versus hMOR, p < 0.01.

### Fig. 4

(a) Suppression of basal [35S]GTP-γ-S binding activity by 1 μM β-CNA in membranes expressing wild type, polymorphic R260H-, R265H-, and S268P-MOR, and mutant K273A-MOR, with or without morphine pretreatment. Empty-Vec, control cells transfected with vector without MOR cDNA. The data were the means ± S.D., n = 9. a, versus Empty-Vec, p < 0.01; b, versus without β-CNA, p < 0.01; c, versus wild type without β-CNA, p < 0.05, analysis of variance and Dunnett’s Posttest. Blank bar, without β-CNA; filled bar, with β-CNA. b, effect of the inverse agonist, 1 μM BNTX on forskolin (100 μM) stimulated cAMP accumulation (30 min) in wild type and polymorphic MOR transfected cells (in the absence of agonists). The cells were either left untreated or pretreated with 1 μM morphine for 18 h, followed by washout of morphine, means ± S.D., n = 3. c, versus Empty-Vec, p < 0.05; d, versus without BNTX, p < 0.05; e, versus wild type without BNTX, p < 0.05. Blank bar, without BNTX, filled bar, with BNTX.

MOR-mediated cAMP Activity in Intact Cells

Demonstration of Basal MOR Activity by Measuring cAMP Levels—MOR is known to couple to G_{i/Go} proteins, inhibit adenylyl cyclase, and decrease cAMP levels. Thus, inverse agonists are expected to block tonic inhibition and increase cAMP levels, opposite to what is observed with GTP-γ-S binding (Fig. 4a). Indeed, β-CNA increased cAMP levels in wild type MOR cells, serving as inverse agonist; however, BNTX was the most effective inverse agonist tested in this assay (17) and was therefore selected for this experiment. Shown in Fig. 4b, 1 μM BNTX had no effect on cAMP levels in control cells (transfected with empty vector) but significantly increased cAMP level in wild type MOR, S268P-MOR, and K273A-MOR transfected cells, being more effective in K273A-MOR than in wild type MOR transfected cells (p < 0.05). In contrast, BNTX had no effect on cAMP in R260H- and R265H-MOR transfected cells. These results are a mirror image of the results from
unpaired showing that it appears to be related to basal MOR signaling of wild type MOR cells with 1
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 Activation of G i/Go-coupled receptors and R265H-hMOR but substantial basal activity for wild type
tions of morphine in the presence of 100 nM forskolin and GTPγS binding. Pretreatment with BNTX for 24 h, followed by washout of the antagonist, reversed the sensitization of cAMP, showing that it appears to be related to basal MOR signaling (forskolin-stimulated cAMP levels (percentage of total \([3H]\)adenosine) decreased from 2.5 ± 0.2% to 1.2 ± 0.06%, p < 0.01, unpaired t test, n = 6).

Upon morphine pretreatment and subsequent removal of the agonist, forskolin-stimulated cAMP increased dramatically for all MOR variants tested, although some differences were observed between the receptor variants (Fig. 4b). When BNTX was added to mask any basal signaling activity of MOR in the morphine pretreated cells, this revealed the presence of substantial basal activity for all MOR variants and the full extent of cAMP up-regulation. This was particularly pronounced for wild type MOR. Even R260H-MOR cells responded to the inverse agonist after morphine pretreatment with a significant increase in cAMP levels (from 4.5 to 10% of total \([3H]\)adenosine; Fig. 4b), indicating the presence of some basal signaling activity.

Maximal morphine (10 µM)-induced cAMP inhibition was not significantly different among wild type and three variant MORs (percentage of inhibition: wild type, 79 ± 3%; R260H-MOR, 72 ± 5%; R265H-MOR, 78 ± 6%, S268P-MOR, 74 ± 7%; means ± S.D., n = 3). Dose-response curves for morphine-induced inhibition of cAMP formation also resulted in similar EC50 values for MOR wild type and the variants, except for S268P-MOR, which displayed a significantly higher EC50 value (Fig. 5). This is in contrast to the results obtained with the GTPγS binding assay.

To assess the incidence of the three reported sequence variants mapping to the i3 loop of MOR, we sequenced exon 3 of MOR in DNA samples from 252 individuals in the Coriell Collection. Exon 3 spans the MOR region from 642 to 1126, encoding 1/2 of the second extracellular loop, transmembrane domains V–VII, and 1/2 of the C-terminal tail. The ethnic distribution in the Coriell collection is as follows: 100 Caucasian, 100 African American, 30 Asian, 10 Mexican, 8 Pacific Islander, and 4 Native American. Of the three polymorphisms tested in this study, we confirmed the R265H variant in one allele of this population. In addition, we detected a new non-synonymous sequence variant (G220 → A) mapping to the i3 loop, yielding a D274N substitution, also in a single allele. Both variants were found in samples from African Americans. The effect of the D274N variant on G protein coupling and CaM binding needs to be further investigated. No other sequence variants were observed in exon 3.

DISCUSSION

In this study, we investigated G protein coupling and CaM binding of the human MOR variants, R260H-, R265H-, and S268P-hMOR, after transfection into HEK293 cells. These three MOR variants represent previously identified single-nucleotide polymorphisms occurring sporadically in the hMOR gene. Because each SNP had been detected previously in only a single allele in various studies, the allele frequencies of these SNPs remained unclear. By genotyping exon 3 of hMOR in 252 individuals from the Coriell collection (National Institutes of Health), we sought to gain further insight into allele frequencies. Whereas SNPs resulting in R260H- and S268P-MOR were absent in this population sample, one individual carried a single allele encoding R265H-MOR. This suggests that the R265H-MOR allele might occur with a frequency exceeding 0.1%. In addition, a new allele was found (encoding D274N-MOR), also mapping to the i3 loop, but this allele was not further investigated. Taken together, sequence variants affecting the i3 loop structure could occur as single alleles in 0.1–1% of individuals. Because of the low number of subjects, no conclusion can be drawn for allele frequencies among different ethnic groups.

Each of the three MOR variants tested displayed similar or only marginally reduced morphine-stimulated maximal G protein coupling. Only the S268P variant displayed a 4-fold increase in the EC50 value for morphine using the cAMP assay. This result suggested some G protein coupling defect for S268P, but it differs from the findings of Befort et al. (16) that the S268P substitution severely impairs MOR signaling, observed under distinct experimental conditions. We have recently established that the Mg2+ concentration is critical to coupling efficiency measured by [35S]GTPγS binding in cell membranes and needs to be optimized for each cell type studied so as to reflect coupling efficiency in intact cells (17). Whereas in our assay, Mg2+ is present at a calculated concentration of ~150 µM for inverse agonist experiments and 10 mM for agonist experiments, Befort et al. (16) used a high chelator concentration resulting in rather low free Mg2+ concentrations. This difference is critical to observing basal MOR G protein coupling (17), which was undetectable under the conditions used by Befort et al. (16). Moreover, Befort et al. (16) used a reporter gene assay for cAMP performed over an extended time period. Taken together, the results of Befort et al. (16) and those from the present study suggest some impairment of agonist-stimulated G protein coupling, the extent of which remains to be clarified.

Whereas agonist-stimulated maximal MOR G protein cou-
pling was largely unaffected for all three MOR variants in our study, R260H and R265H substitutions significantly impaired basal MOR signaling. On the other hand, the R265H and S268P substitutions decreased affinity of the i3 loop for CaM binding. Moreover, the R265H- and S268P-MOR variants displayed decreased desensitization after morphine pretreatment, possibly as a result of interference with the CaMKII phosphorylation site S268, as reported earlier for S268P-MOR (14). These changes were shown to have marked effects on MOR signal transduction and regulation, with possible relevance to signaling pathways involved in narcotic addiction.

Effects on Basal MOR Signaling—Previous results have suggested a fundamental role of basal MOR signaling in opioid tolerance and dependence (11, 13, 15, 18). The observation that basal MOR signaling increases after morphine pretreatment led to the hypothesis that naloxone may act as an inverse agonist in dependent tissue, thereby causing immediate withdrawal symptoms (18, 20). A recent report by Liu and Frather (19) supported our hypothesis that basal MOR signaling was increased by chronic agonists pretreatment in GH3-MOR cells. The involvement of basal MOR signaling in morphine dependence was supported by our recent finding that neutral antagonists (no effect on basal signaling) are significantly less effective in precipitating withdrawal jumping in acute and chronic mouse models of morphine dependence (17, 20). The presence of basal signaling activity does not appear to be a result of receptor overexpression because it is also detectable in SH-SY5Y neuroblastoma cells with relatively low MOR levels (18) and in cell lines with relatively low MOR expression (17). A relationship between basal signaling and receptor structure rather than expression level has also been demonstrated for the 5HT4 receptor (28).

Polymorphic variations in the i3 loop of MOR are likely to affect the balance between inactive and active receptor states. The R260H- and R265H-MOR variants failed to show significant basal signaling in the GTP-S binding assay. The basal coupling activity of S268P-MOR was found to be insensitive to regulation by CaM because this variant is deficient in CaM binding. Such changes could profoundly affect the long-term morphine response of an individual carrying these MOR alleles. Whether heterozygosity is associated with altered response remains to be seen; however, the level of basal activity could play a significant role and may be altered by the presence of any of these alleles.

Measuring cAMP levels in intact cells transfected with wild type MOR and its variants revealed remarkable effects of basal receptor activity on sensitization of the cAMP second messenger system, an observation not reported previously. The inverse agonist BNTX reversed enhanced levels of cAMP (forskolin-stimulated) in wild type MOR-transfected cells to those of control cells (transfected with empty vector without MOR cDNA), suggesting that basally active MOR mediated this effect. Commonly observed only after agonist treatment, this sensitization is considered a biochemical marker of opioid dependence (25, 26, 29). cAMP up-regulation was not observed with the R260H- and R265H-MOR variants, consistent with their low basal activity. An alternative explanation of these findings is that HEK293 cells could secrete opioid peptides. However, because R260H- and R265H-MOR respond well to agonists, the absence of cAMP up-regulation with R260H- and R265H-MOR supports basal receptor activity as the underlying cause. Hence, the mere presence of MOR serves to up-regulate the cAMP second messenger system even in the absence of agonist. After morphine pretreatment, a substantial cAMP up-regulation was seen with all MOR variants.

MOR-Calmodulin Interactions—Binding of CaM to the i3 loop could modulate G protein coupling, could result in direct CaM binding by MOR, or both (11, 13). Polymorphic variations in the i3 loop resulted in a rank order of CaM binding affinity of wild type MOR ≧ R260H-MOR > R265H-MOR > S268P-MOR. The CaM binding defect of R265H-MOR suggests that the N-terminal portion of the i3 loop does play a role in the binding motif, as suggested earlier (11). In contrast to the results with a Pro substitution at Ser268. Ala substitution of Ser268 did not appear to affect CaM interaction with MOR (13). This suggests that Ser268 is not required for CaM binding and that the low CaM binding activity of S268P-MOR may have resulted from conformational changes in the i3 loop introduced by Pro268.

Polymorphic changes in CaM binding were also reflected in altered CaM release from the plasma membrane upon stimulation with morphine (11, 13). The amount of CaM in HEK cell plasma membrane is ~45 pmol/mg protein, resulting in a molar ratio of CaM/MOR of ~10.1. Morphine stimulation reduced the CaM content in HEK-hMOR and HEK-R260H-hMOR cell membranes by 15–30%, which is 2–4-fold more than the receptor content. This result implies an iterative process of binding and release or direct release from the membrane rather than from the receptor. This appears to be a specific process, because mutant MOR deficient in CaM binding but active in G protein coupling fails to reduce CaM content in the plasma membrane (11, 13). Similarly, the CaM binding-deficient variants R265H- and S268P-MOR failed to release CaM from the plasma membranes. Moreover, basal G protein coupling of S268P-MOR was unaffected by changes in CaM levels, as previously observed with the CaM-binding-deficient mutant K273A-hMOR (11).

Increasing evidence indicates that Ca2+/CaM play a role in narcotic tolerance and dependence. In separate studies we had already observed changes in nuclear signaling pathways between MOR wild type and K273A-MOR involving the regulation of CREB phosphorylation (30) shown to play a role in narcotic dependence (1, 21). The up-regulation of basal MOR signaling during prolonged morphine treatment has also been attributed to diminished CaM-MOR interactions (13). Therefore, changes in CaM binding to variant MORs are likely to affect the processes underlying tolerance and dependence.

In summary, the examined SNPs mapping to the i3 loop of human MOR alter CaM binding, basal G protein coupling, or both. This results in profound changes in signaling activity and regulation, with the potential to affect the response to opioid analgesics in individuals carrying these alleles.

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\( \mu \) Opioid Receptor Polymorphisms
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Danxin Wang, J. Mark Quillan, Katharine Winans, Julie L. Lucas and Wolfgang Sadée

J. Biol. Chem. 2001, 276:34624-34630.
doi: 10.1074/jbc.M104083200 originally published online July 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104083200

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