A Single Nucleotide Polymorphic Mutation in the Human μ-Opioid Receptor Severely Impairs Receptor Signaling*

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Large scale sequencing of the human μ-opioid receptor (hMOR) gene has revealed polymorphic mutations that occur within the coding region. We have investigated whether the mutations N40D in the extracellular N-terminal region, N152D in the third transmembrane domain, and R265H and S268P in the third intracellular loop alter functional properties of the receptor expressed in mammalian cells. The N152D receptor was produced at low densities. Binding affinities of structurally diverse opioids (morphine, diprenorphine, DAMGO, and CTOP) and the main endogenous opioid peptides (β-endorphin, [Met]enkephalin, and dynorphin A) were not markedly changed in mutant receptors (<3-fold). Receptor signaling was strongly impaired in the S268P mutant, with a reduction of efficacy and potency of several agonists (DAMGO, β-endorphin, and morphine) in two distinct functional assays. Signaling at N40D and R265H mutants was highly similar to wild type, and none of the mutations induced detectable constitutive activity. DAMGO-induced down-regulation of receptor-binding sites, following 20 h of treatment, was identical in wild-type and mutant receptors. Our data show that natural sequence variations in hMOR gene have little influence on ligand binding or receptor down-regulation but could otherwise modify receptor density and signaling. Importantly, the S268P mutation represents a loss-of-function mutation for the human μ-opioid receptor, which may have an incidence on opioid-regulated behaviors or drug addiction in vivo.

The opioid system controls pain perception and mood and is generally implicated in a wide variety of behaviors that are essential in facing threatening situations (1, 2). Opioid receptors also mediate the strong analgesic and addictive actions of opiate drugs. Pharmacological studies indicate that the prototypic opiate morphine, the main clinically useful opioids such as fentanyl or methadone, and the closely related drug of abuse heroin preferably act by activating the μ-opioid receptors (3–5). In support of this, gene targeting experiments have shown the absence of morphine-induced analgesia (6–10), reward and physical dependence (6), immunosuppression (11, 12), respiratory depression (13), or constipation (14) in MOR-deficient mice, demonstrating unambiguously that the μ-opioid receptor is a main molecular target for morphine action in vivo. The finding of genetic mutations altering the expression or functional activity of MOR is therefore important to understand inter-individual variable responses to the major opioid drugs, both in the clinical management of pain or heroin addiction.

In addition to the direct mediation of opiate-induced euphoria, tolerance, and dependence, μ-opioid receptors have been shown to regulate the effects of other substances with high addictive potential such as cocaine or alcohol (15). As an example, in humans, the μ-receptor antagonists naloxone and naltrexone have been shown not only to reverse heroin overdose but also to alter alcohol consumption (16, 17). Studies using animal models also point at a possible role of the MOR gene in ethanol use. Recently we have reported that MOR-deficient mice do not self-administer alcohol (18), suggesting that μ receptors are essential in mediating the reinforcing properties of this substance. Also a quantitative trait loci (QTL) analysis in mice showed that oral morphine preference is largely mediated by a single locus in chromosome 10 which harbors the MOR gene (19). Thus, among the various genetic components of the opioid system, the μ-opioid receptor gene is an evident candidate in the search for genes potentially involved in the susceptibility to drug abuse.

The cloning of the human opioid receptor gene (20, 21) has prompted studies of DNA sequence variability within the hMOR gene (22–24). We have recently conducted a comprehensive polymorphic study of the MOR gene in 250 patients using large scale multiplex DNA sequencing. We have identified 43 variants within 7-kilobase pair regulatory, exonic, and intronic sequences (25). We have found six mutations in the coding region. Five of these mutations modify the encoded protein sequence, and these include the previously identified A6V and sequence, and these include the previously identified A6V and intronic mutations (25). We have found six mutations in the coding region. Five of these mutations modify the encoded protein sequence, and these include the previously identified A6V and N40D mutations in the N-terminal region of the receptor (22–24), as well as three yet unreported mutations. One of these novel mutations is located in the third transmembrane domain of the receptor and changes an asparagine residue into an aspartic acid residue (N152D). The two other mutations are found in the third intracellular loop of the receptor and replace an arginine and a serine by a histidine (R265H) and a proline (S268P) residue, respectively.

The three latter mutations occur in regions that may be coupled receptors; GTPyS, guanosine 5’-O-(thio)triphosphate; PBS, phosphate buffer saline; SEAP, secreted alkaline phosphatase; WT, wild type.
critical for receptor function, and this has prompted us to determine whether these natural MOR variants indeed exhibit altered pharmacological activity profiles. We have constructed the three novel hMOR variants by site-directed mutagenesis. We also have generated the N40D mutant, some properties of which have been described earlier (24), to extend our knowledge on the functional properties of this frequent polymorphic variant. We have expressed the four mutant receptors in mammalian cells, and we determined binding affinities for four prototypic opioid ligands (morphine, DAMGO, diprenorphine, and CTOP) and the main opioid peptides (Met-enkephalin, β-endorphin, and dynorphin A). We also have investigated agonist-induced functional responses of the receptor using the [35S]GTP-S binding assay, as well as a reporter gene assay. Finally, we have examined receptor down-regulation following chronic exposure to the potent μ-agonist DAMGO. Our results show no obvious modification in receptor binding or down-regulation. However, the data indicate decreased heterologous expression for the N152D mutant receptor and a remarkable decrease of receptor signaling for the S268P variant.

**EXPERIMENTAL PROCEDURES**

**Materials—**DAMGO, naloxone, [Met]enkephalin, β-endorphin, dynorphin A, GDP, and GTP-S were obtained from Sigma; CTOP was from Peninsula Laboratories (St. Helens, Merseyside, UK). Morphine was obtained from Cooper Biomedical (Melun, France), and heroin was from Francopia (Paris, France). [3H]Diprenorphine (specific activity, 58 Ci/mmol), [3H]DAMGO (specific activity, 54.6 Ci/mmol), and [35S]GTP-S (specific activity, 1156 Ci/mmol) were obtained from PerkinElmer Life Sciences. The hMOR cDNA (a kind gift from Pr. Lei Yu, Cincinnati, OH) was subcloned into pcDNA1/Amp (Invitrogen) for site-directed mutagenesis and transient expression in COS cells. The WT and mutant cDNAs were subcloned into pcDNA3 (Invitrogen) for expression in HEK 293 cells. The carrier plasmid used in the electroporation procedure (pBlueScript) was from Stratagene, and the Superfect reagent was from Qiagen. G418 was from Life Technologies, Inc. The reporter plasmid pCRE-SEAP was from CLONTECH, and the Phosphosite detection kit and Enhancer solution were from Trofaz, K., and Bmax values were calculated using the EBDA/Ligand program (G. A. McPherson, Biosoft, UK), and EC50 values were determined using the Bradford assay.

Opioid binding experiments were performed on membrane preparations as described previously (26). For saturation experiments, 5–10 μg of membrane proteins were diluted in 50 mM Tris-HCl, pH 7.4, in a final volume of 300 μl and incubated at 4°C for 2 h with different concentrations of [3H]diprenorphine. Binding analyses were performed on WT and mutant receptors as described previously (27). Briefly, COS cells were transiently transfected, and membranes were prepared as described above (ligand binding section). 5 μg of membrane proteins were incubated for 2 h at 4°C with and without the agonist (10–12 M), in assay buffer containing 50 mM Hepes, pH 7.2, 5 mM MgCl2, 100 mM NaCl, 0.1% bovine serum albumin, 30 μg GDP, and 0.2 μM [3H]GTP-S. For saturation experiments, the membrane preparation was immediately subjected to filtration through H2O-presoaked GF/B filters, followed by three washes with ice-cold 50 mM Tris-HCl, pH 7, 5 mM MgCl2, 50 mM NaCl. Bound radioactivity was determined by scintillation counting. Nonspecific binding was determined in the presence of 10 μM GTP-S, and basal binding was defined as specific [3H]GTP-S binding in the absence of agonist.

**Reporter Gene Assay—**The day before transfection, HEK 293 cells were plated in 96-well plates (Biocat, Packard Instrument Co.) at a density 30,000 cells/well. HEK 293 were cotransfected with the reporter gene pCRE-SEAP (1 μg/well) and hMOR WT or hMOR S268P (0.2 μg/well) using Superfect reagent. 24 h after transfection, cells were stimulated with a final volume of 150 μl. 15 min after substrate addition, luminescence was quantified (Trilux, Wallac).

**Down-regulation—**Plots of stable HEK 293 cell lines expressing WT or mutant receptors were produced. HEK 293 cells were grown in minimum essential medium supplemented with 10% fetal calf serum and maintained in a 10% CO2 environment by incubation at 37°C and selected with 0.5 μg/ml G418. Neomycin-resistant colonies from 100-mm diameter cell culture dishes were selected for 2 weeks. For down-regulation studies, the pools of stably transfected HEK cells were cultured for 20 h in the presence or absence of 10 μM DAMGO, washed twice with PBS, once with PBS-2 mM EDTA, and resuspended in 10 mM Tris, pH 7.4 (incubation buffer). Binding reactions were
performed using 4 x 10^6 cells in 0.25 ml of incubation buffer per assay with either 5 nM [3H]DAMGO or 1.5 nM [3H]diprenorphine. Under these conditions, most receptor sites were occupied since Scatchard analysis of [3H]DAMGO and [3H]diprenorphine binding on WT hMOR transfected cell preparations indicates K_d values of 1.36 ± 0.41 and 0.1 ± 0.03 nM, respectively (not shown). Specific binding was determined by the difference between binding measured in the absence (total binding) or presence (nonspecific binding) of 50 nM naloxone. After 1 h of incubation at 25 °C, binding reaction was terminated by rapid filtration over Whatman GF/B filters presoaked with 0.1% polyethyleneimine, and bound radioactivity was counted using a scintillation counter. Data were compared using unpaired Student's t test (Statview).

RESULTS

Expression of Wild-type and Mutants hMOR Receptors in COS Cells—To assess the role of natural protein variants encoded by the hMOR gene, we have substituted the most frequently found nucleotides in the cloned hMOR receptor (GenBank™ accession number L29301) by the nucleotide variants originating from our polymorphism study. Point mutations introduced in the cloned hMOR receptor replaced two asparagines by an aspartate residue (N40D and N152D), an arginine by a histidine residue (R265H), and a serine by a proline (S268P). The localization of these mutations is shown on Fig. 1. The wild-type (WT) and the mutant receptors were transiently expressed in COS cells. We have quantified expression levels of receptors by monitoring the binding of [3H]diprenorphine, a nonselective alkaloid antagonist, to the wild-type and mutant receptors (Table I). Scatchard analysis indicates that WT, N40D, R265H, and S268P mutant receptors are expressed at comparable levels, whereas expression of mutant N152D is significantly lower than WT.

Effect of the Mutations on Opioid Binding—Saturation experiments indicate that [3H]diprenorphine shows identical affinity to WT and N152D receptors and slightly higher affinity to the other mutant receptors. Differences in K_d values were subtle (Table II), and from these data we conclude that the mutations under study do not markedly alter the high affinity interaction of diprenorphine with the receptor. We then used [3H]diprenorphine as the radiolabeled ligand in competition studies to establish whether the mutations modify binding affinity of other opioids.

We tested three prototypic opioid ligands with distinct structural (alkaloid or peptide) and functional (agonist or antagonist) properties. These include the prototypic alkaloid agonist morphine, the peptidic enkephalin-derived agonist DAMGO, and the peptidic somatostatin-derived antagonist CTOP. Again, none of the mutations appeared to modify strongly the binding affinities for these ligands (Table II, top). K_d values tended to be lower at mutant receptors compared with WT but, as for [3H]diprenorphine binding, changes in K_d values between mutant and WT receptors were minimal and never exceeded a 2-fold difference. There was an exception for morphine at the N152D mutant and DAMGO at the R265H mutant, where K_d values were 3.8- and 3.7-fold lower, respectively. In the latter case, however, we were able to use [3H]DAMGO, available as a radiolabeled ligand, to get a direct measurement of DAMGO affinity in a saturation experiment. Scatchard analysis of [3H]DAMGO binding showed no differences in K_d values between WT and mutant receptors (not shown), suggesting that the small modification of binding affinity that was measured from the competition experiment may not represent a real affinity change. Altogether, these data suggest that the polymorphic mutations under study have no major incidence on opioid binding, independently from the chemical nature of the tested compound.

Heroin showed K_d values ranging from 286 (WT) to 466 nM (N40D), confirming the preservation of binding affinity for morphinic compounds at the mutant receptors (not shown). Those affinity values, which are much lower than morphine values, are in accordance with previous data (29, 30) suggesting that heroin metabolites, rather than heroin itself, act in vivo.

Because polymorphic mutations may influence the physiology of the endogenous opioid system in humans, we also tested the major endogenous peptides, β-endorphin, [Met]enkephalin, and dynorphin A. All affinity values were in the nanomolar range (Table II, bottom), as previously reported for hMOR WT.

**TABLE I**

| Receptor densities (B_max) for hMOR WT and mutant receptor membrane preparations |
|-------------------------------|-----------------|
|                               | pmol/mg          | n   |
| hMOR WT                       | 5.47 ± 0.65      | 6   |
| N40D                          | 6.06 ± 0.53      | 4   |
| N152D                         | 1.85 ± 0.42      | 3   |
| R265H                         | 4.60 ± 0.10      | 2   |
| S268P                         | 3.58 ± 0.68      | 3   |
Table II

Binding affinities of opioids to WT and mutant receptors expressed in COS cells

| Agonist | Peptide DAMGO | Peptide diprenorphine | Peptide [3H]DAMGO |
|---------|---------------|-----------------------|-------------------|
| hMOR WT | 29.6 ± 5.5    | 10.4 ± 2.04           | 0.38 ± 0.11       |
| N40D    | 20.3 ± 4.3    | 6.70 ± 2.17           | 0.20 ± 0.03       |
| N152D   | 7.71 ± 3.61   | 6.17 ± 0.88           | 0.37 ± 0.15       |
| R265H   | 11.4 ± 2.7    | 2.78 ± 0.11           | 0.15 ± 0.01       |
| S268P   | 10.9 ± 2.4    | 9.49 ± 3.70           | 0.25 ± 0.01       |

Endogenous opioid peptides

| Agonist | Peptide DAMGO | Peptide diprenorphine | Peptide [3H]DAMGO |
|---------|---------------|-----------------------|-------------------|
| β-Endorphin | 2.9 ± 0.5   | 2.2 ± 0.3             | 2.4 ± 0.4         |
| Met-endorphin | 4.15 ± 0.75 | 3.45 ± 1.16           | 3.3 ± 0.8         |
| Dynorphin A | 4.75 ± 1.35 | 3.43 ± 0.13           | 6.5 ± 0.5         |
| hMOR WT | 4.75 ± 1.35  | 3.45 ± 0.13           | 6.5 ± 0.5         |
| N40D    | 4.75 ± 1.35  | 3.43 ± 0.13           | 6.5 ± 0.5         |
| N152D   | 4.75 ± 1.35  | 3.43 ± 0.13           | 6.5 ± 0.5         |
| R265H   | 4.75 ± 1.35  | 3.43 ± 0.13           | 6.5 ± 0.5         |
| S268P   | 4.75 ± 1.35  | 3.43 ± 0.13           | 6.5 ± 0.5         |

(28). Again binding at mutant receptors slightly differed from WT, but variations were even more subtle than for exogenous or synthetic opioids, and changes in $K_i$ values barely exceeded 2-fold. This suggests that natural sequence variations in the coding region of the MOR gene are unlikely to affect significantly the receptor occupancy by endogenous opioids. Of note is the previous report by Bond et al. (24) showing a 3-fold higher affinity of β-endorphin for the N40D mutant. Our experiments do not show this difference, either from [3H]diprenorphine displacement (Table II) or [3H]DAMGO displacement (not shown), perhaps due to different host cells used for receptor expression. Otherwise, both studies showed no marked difference in binding affinities for all other tested opioids at WT and N40D receptors. We cannot exclude that the polymorphic mutations may have a more perceivable influence on opioid binding in a neuronal cellular context.

Receptor Signaling: Effect of the Mutations on Basal or Agonist-induced $[^{35}S]GTP^\gamma$ Binding—To measure agonist-induced stimulation of wild-type and mutant receptors at the G protein level, we used the $[^{35}S]GTP^\gamma$ binding assay. We first tested the two prototypic agonists DAMGO and morphine on membrane preparations expressing the wild-type receptor. We compared agonist-induced stimulation of $[^{35}S]GTP^\gamma$ binding at saturating ligand concentrations ($10^{-5} \text{M}$ for both morphine and DAMGO). In these preliminary assays DAMGO activated the wild-type receptor up to 273 ± 25% above basal level, whereas maximal activation obtained with morphine was 197 ± 18% (not shown), indicating that DAMGO is a full agonist, and morphine is a partial agonist under our expression conditions. This is in accordance with other studies that have used either native (31) or recombinant μ receptors (32). We therefore used DAMGO to examine further the mutant receptors.

In a preliminary experiment, DAMGO-stimulated activation could not be detected for the N152D receptor (not shown). This is presumably due to low expression levels, in accordance with our previous studies showing that functional activity is difficult to quantify when $B_{\text{max}}$ values are below 2 pmol/mg protein in this assay (27). DAMGO significantly increased $[^{35}S]GTP^\gamma$ binding above basal levels for the N40D, R265H, and S268P mutants, demonstrating that those mutant receptors remain functional (not shown). Maximal activation levels, however, were different from wild type, particularly for the S268P receptor. Since the three mutants were expressed at levels comparable to that of the wild-type receptor, altered $[^{35}S]GTP^\gamma$ binding was not due to distinct receptor densities but rather to modifications of agonist potencies or efficacies.

We then established dose-response curves of DAMGO-induced $[^{35}S]GTP^\gamma$ binding at WT, N40D, R265H, and S268P (Fig. 2). For the N40D mutant receptor, maximal activation was increased compared with WT receptor (258 ± 4% versus 228 ± 16% above basal), whereas the EC$_{50}$ value was not significantly modified (Table III). Agonist-induced $[^{35}S]GTP^\gamma$ binding at the R265H mutant was lower than wild type, with a signal of 207 ± 1% above basal. DAMGO potency was not significantly reduced for this mutant (Table III). For the S268P mutant DAMGO efficacy was dramatically reduced. The maximal activation level was 132 ± 2% above basal, indicating that DAMGO-induced receptor activation was 25% from WT. For this mutant also, DAMGO potency was reduced more obviously than for the two other mutant receptors, with an EC$_{50}$ value 2.4-fold higher than wild type (Table III). Altogether, the data suggest that the two mutations in the third intracellular impair receptor-mediated G protein signaling, with a prominent effect of the serine to proline mutation at position 268. In contrast, the N-terminal mutation is not harmful but may rather favor receptor-G protein interactions.

We examined whether the strong impairment of DAMGO-induced $[^{35}S]GTP^\gamma$ binding observed in the S268P mutant receptor was also detectable for other agonists. We compared maximal activation levels using DAMGO, β-endorphin, and morphine at saturating concentrations ($10^{-5} \text{M}$). In this set of experiments $[^{35}S]GTP^\gamma$ binding was 172.3 ± 8.0, 141.7 ± 8.6, and 117.6 ± 4.6% of basal level for DAMGO (n = 12), β-endorphin (n = 7), and morphine (n = 7), respectively, in membranes expressing WT receptors. In membranes with S268P receptors, agonists were ineffective in stimulating $[^{35}S]GTP^\gamma$ binding. Maximal levels were 106.9 ± 5.6% of basal for DAMGO (n = 10), 109.0 ± 6.4% for β-endorphin (n = 7), and 96.3 ± 5.0% for morphine (n = 7, not shown). This suggests that the poor ability of S268P receptors to stimulate $[^{35}S]GTP^\gamma$ binding is not a ligand-dependent phenomenon but rather an intrinsic property of the mutant receptor.

A final observation from this study is that basal $[^{35}S]GTP^\gamma$ binding levels in mutant receptor preparations were comparable (N40D) or lower (R265H and S268P) from WT (Table III), indicating that these mutations do not induce constitutive activity at the receptor.

Receptor Signaling: Effect of the S268P Mutation on Agonist-induced Inhibition of the cAMP Pathway—We further charac-
terized the phenotypic deficiency of the S268P mutant. We verified whether decreased signaling observed at the level of G proteins propagates and translates into decreased cellular response downstream from the signaling cascade. To this objective, we used a reporter gene assay responsive to cAMP levels. In HEK 293 cells, we cotransfected the receptor-encoding plasmid with another plasmid harvesting the alkaline phosphatase promoter. Cells were stimulated with forskolin in the absence or presence of an opioid agonist, and levels of reporter gene activity were compared. Dose-response curves to DAMGO, β-endorphin, and morphine are shown in Fig. 3. In WT receptors, all three agonists potently inhibited forskolin-induced reporter gene activity. Maximal inhibitions were 66.2 ± 3.4, 66.7 ± 3.4, and 71.1 ± 2% for DAMGO, β-endorphin, and morphine, respectively. This indicates similar efficacy for the three agonists in decreasing CAMP levels in the cell and shows that the partial agonist activity of β-endorphin and morphine in the [35S]GTPγS binding assay is less perceptible when measuring cAMP-dependent reporter gene activity, probably due to signal amplification. The rank of order of potencies was DAMGO (EC50 65.8 ± 0.7 nM) > β-endorphin (EC50 290.8 ± 121.6 nM) > morphine (EC50 623.8 ± 44.4 nM). For the S268P mutant, inhibition of forskolin-induced reporter gene activity was much less important than in WT and for all three agonists (Fig. 3). Maximal inhibitions were only 30.5 ± 4.8% for DAMGO, 41.5 ± 6.5% for β-endorphin, and 43.9 ± 4.8% for morphine. Also, agonist potencies were significantly shifted to the right (358.6 ± 24.4 nM for DAMGO, 1325 ± 478 nM for β-endorphin, and 1426 ± 209 nM for morphine). We otherwise verified that the WT and the mutant receptors were expressed at comparable levels under the experimental conditions of the assay (not shown). These results further confirm that the S268P receptor is signaling-deficient and that this property of the mutant receptor is agonist-independent. We show that the impairment of receptor signaling is observable all along the cAMP signaling cascade.

Effect of the Mutations on Agonist-induced Regulation of Receptor Activity—Agonist stimulation of G protein-coupled receptors triggers a number of regulatory processes that limit the cellular response over time. These include short term events occurring at the cytoplasmic face of the receptor, such as phosphorylation, uncoupling, and internalization, followed by longer term regulatory processes that ultimately translate into receptor down-regulation (33, 34). Two polymorphic mutations (R265H and S268P) are located in the third intracellular domain that interacts with intracellular factors, and these mutations may therefore influence adaptive responses to receptor activation. Furthermore, studies of polymorphic variants of the β3-adrenergic receptor have demonstrated alterations of agonist-promoted down-regulation caused by mutations located in the extracellular N-terminal domain of the receptor (35), indicating that mutations in this domain can also influence the regulation of receptor activity. We investigated the influence of opioid treatment on the four hMOR variants, and we chose to examine receptor down-regulation as a prominent and well described regulatory response to chronic opiates. Also the long term response is likely to be most relevant in the context of substance abuse, opioid substitution therapy, or chronic pain treatment.

DAMGO was previously reported to be most efficient in promoting receptor down-regulation in transfected Chinese hamster ovary cells (36) and neuroblastoma (31) cells. In accordance, our stable HEK 293 cells stably expressing the WT receptor underwent 75% down-regulation following 20 h of treatment (Table IV), a time duration that allows complete down-regulation (36). The number of remaining binding sites was identical (25%), independently whether receptors were labeled using the hydrophilic peptidic ligand [3H]DAMGO or the hydrophobic alkaloid compound [3H]diprenorphine. This suggests that the missing receptors have entered the lysosomal pathway and undergone protein degradation, as previously reported (36–38).

Significantly, mutant receptors did not down-regulate differently from WT receptors (Table IV), indicating that no major modification of the various processes that lead to receptor down-regulation has occurred. A tendency to increased down-regulation was observed for the N40D receptor, but the statistical analysis showed no difference from WT. Identical results were obtained when binding sites were labeled with the radiolabeled ligands at nonsaturating concentrations (not shown). This suggests that interindividual variations in hMOR sequence may have no significant consequences on long term responses to chronic opiates. We cannot exclude, however, that some short term regulatory processes could be modified in those mutants.

DISCUSSION

The detection of genetic variation affecting the opioid system is an interesting approach to understand the origin of interindividual differences in response to opiates, in opioid-associated pathophysiology or in diseases of substance dependence. Vulnerability to heroin abuse is thought to result from polygenic influences. It was shown associated with a polymorphism in the D4 dopamine receptor gene (39) or with a mutation in the δ-opioid receptor gene, in one of two studies (40, 41). The μ-opioid receptor has been the focus of several polymorphism studies because this receptor type is the major target for opiate

![Fig. 2. Dose responses for DAMGO-stimulated [35S]GTPγS binding at hMOR WT, N40D, R265H, and S268P mutant receptors. Increasing concentrations of DAMGO (10⁻¹⁰ to 10⁻⁶ M) were used to stimulate [35S]GTPγS binding. Data are means ± S.E. from three to seven independent [35S]GTPγS binding experiments performed in triplicate. DAMGO efficacy and potency from curve-fitting of these data are shown in Table III.](http://www.jbc.org/)

**TABLE III**

| Basal       | Maximal activation (% above basal) | EC50 | n       |
|-------------|-----------------------------------|------|---------|
| hMOR WT     | 26971 ± 4575                      | 228 ± 16 | 95 ± 15 | 7 |
| N40D        | 22193 ± 1737                      | 258 ± 5    | 135 ± 28 | 6 |
| R265H       | 11997 ± 933                       | 207 ± 1    | 127 ± 42 | 3 |
| S268P       | 15260 ± 1466                      | 132 ± 2    | 229 ± 47 | 3 |

This table displays maximal activation levels and EC₅₀ values determined from experiment shown in Fig. 2. 100% is defined as specific [35S]GTPγS binding in the absence of DAMGO. Values from these experiments are means ± S.E. from n independent [35S]GTPγS binding experiments performed in triplicate.
drugs and also is a main modulator of reward pathways in the brain. A number of single nucleotide polymorphisms have now been described for the \( \mu \)-opioid receptor gene. Two mutations that modify the N-terminal sequence of the encoded protein (A6V and N40D) have been found repeatedly. N40D, the most frequent mutation, occurs at an allelic frequency of 10–20%

dependence (24) or alcoholism (43).

The N40D Mutant, Subtle Changes in Receptor Functional-ity—The N40D is the most frequent mutation, and several association studies have been conducted. The analysis of alcohol dependence has led to variable results, depending on the populations under study. Although no association was found in two studies (23, 42), an increased risk factor for the WT allele or genotype was described in another study (43), suggesting a possible protective effect of the mutation. In a group of alcoholics under acute withdrawal, increased dopaminergic sensitivity was found for the variant genotype compared with the WT genotype (44).

The N40D mutation is located in the N-terminal region of the receptor and results in the loss of a putative glycosylation site. One possible consequence is an alteration of the glycosylation status of the receptor that could potentially modify receptor expression (46). However, this was not observed; our data show that receptor densities do not significantly differ from WT when the N40D receptor is transiently expressed in COS cells. This is in accordance with previous studies that have shown that the lack of N-terminal tail (hence the lack of glycosylation sites) does not prevent expression of the receptor in heterologous host cells (47–51).

Modifications of pharmacological properties were hardly detectable for the N40D receptor. First, binding affinities of a large set of opioid ligands were highly similar for N40D and WT receptors. Second, maximal activation of the N40D mutant by the agonist DAMGO was slightly enhanced, but no significant change in potency was observed. Third, down-regulation tended to be more prominent for the N40D receptor, but again the difference was not statistically different from WT. Collectively, the data demonstrate that the N40D polymorphism does not impair receptor activity. On the contrary, we may speculate that, although our expression conditions may not have been optimal to reveal marked modifications of N40D functionality, these subtle differences altogether could contribute in enhancing N40D receptor responsivity under specific circumstances. This would be in accordance with the association studies mentioned earlier (24, 43). These authors have suggested that a potentially enhanced N40D receptor function could translate into a hyperactivity of the endogenous opioid system that would contribute to increase the activity of the hypothalamic-pituitary-adrenal axis and diminish vulnerability to opioid dependence (24) or alcoholism (43).

The N152D Polymorphism in the \( \mu \)-Opioid Receptor—The N152D polymorphism was not described previously and represents a rare mutation (22/50 individuals). In our study this mutant was expressed at lower receptor density compared with wild-type receptor. Another mutation in the transmembrane domain III of the \( \delta \)-opioid receptor was previously shown to alter receptor expression (26), suggesting that structural integrity of transmembrane domain III, believed to be the most buried transmembrane domain within the helical bundle, is important for receptor stability. Otherwise, no obvious affinity change was observed for all the opioid ligands that we have tested. There-

![Fig. 3. Dose responses for opioid agonist activity at hMOR WT and S268P mutant receptors using the reporter gene assay.](http://www.jbc.org/)

The receptor cDNA and the cAMP-responsive element-alkaline phosphatase reporter gene were cotransfected in HEK 293 cells. Cells were incubated with forskolin in the absence or presence of various concentrations (10\(^{-9}\) to 10\(^{-5}\)) of agonist. Reporter gene activity was measured by chemiluminescence, and results were normalized to forskolin-stimulated reporter gene activity in the absence of agonist (100%). Data are means ± S.E. from 5 to 6 independent experiments performed in triplicate. Agonist potency and efficacy values are indicated in the text.

| Remaining receptor sites (%) | \([\text{H}]\text{Diprenorphine}\) | \([\text{H}]\text{DAMGO}\) |
|-----------------------------|-----------------------------|-----------------------------|
| hMOR WT                     | 25.55 ± 1.61               | 24.45 ± 1.66               |
| N40D                        | 23.39 ± 0.89               | 18.37 ± 3.64               |
| N152D                       | 26.94 ± 1.85               | 22.62 ± 3.03               |
| R265H                       | 25.71 ± 1.68               | 31.42 ± 2.57               |
| S268P                       | 29.28 ± 2.28               | 23.39 ± 5.29               |

Comparison of WT with each mutant receptors shows no significant difference (\(p > 0.05\)).
fore, together the data suggest that the general conformation of the N152D receptor is maintained. Whether expression of this receptor variant is modified in vivo remains to be determined.

**S268P, a Loss-of-Function Mutation**—In this paper we have studied two mutations that occur in the third intracellular domain (31), a key region for GPR activity. The amphipathic structures of the membrane-proximal regions of S3 have been shown to be critical for productive interaction of GPRs with the β-subunit of G proteins and therefore receptor signaling (see Ref. 52). This loop is highly conserved across opioid receptor subtypes (53), is involved in their coupling to G/G subunits (54), and may interact with other proteins, as was reported for calmodulin and the μ-receptor (55). This receptor domain also harbors putative phosphorylation sites that could be involved in the regulation of receptor activity (34). The amino acid residue Ser-268 itself represents a putative phosphorylation site for Ca2+/calmodulin-dependent protein kinase II (56). We therefore expected that mutations within this region would alter receptor function.

The R265H mutation has some influence on hMOR activity, which leads to decreased signaling. This modification, as well as changes in opioid binding, remains minor presumably because of the conservative nature of the mutation. Otherwise receptor down-regulation was unchanged. It is therefore unlikely that the R265H polymorphism (rare: 1/250) would drastically alter MOR activity, at least for receptor responses that we have investigated.

In contrast, the S268P mutation strongly impairs receptor signaling, independently from the agonist or the functional assays that are used. Site-directed mutagenesis of this serine residue into alanine was described previously in the rat μ-opioid receptor (56). The authors found no impairment of agonist-induced inhibition of adenylyl cyclase when a double mutant receptor (S261A/S266A) was expressed in HEK cells, as well as assays that are used. Site-directed mutagenesis of this serine residue for Ca2+/calmodulin and the Xenopus oocyte expression system. Very recently, the same authors investigated the human S268P mutant and reported a strong impairment of signaling (57), as we do in this study. A likely explanation for the distinct consequences of the alanine or proline mutations is that the alanine mutation leaves the general structure of the loop intact, and therefore functional coupling to the G protein is maintained. On the contrary, the proline residue of the polymorphic mutant disrupts the tertiary structure of this functionally critical loop, thus severely compromising signal transduction. Down-regulation of hMOR S268P was otherwise unaltered, suggesting that the absence of this putative phosphorylation site has no obvious consequences on long term agonist-induced regulation. We cannot exclude, however, that there may be an impairment of rapid agonist-induced desensitization, as was shown for the S261A/S266A mutant in the rat (56) or recently suggested for the human S268P mutant (57).

Many polymorphic variants have been identified among GPR genes, and some of these mutations account for inherited disorders and diseases (for a review see Ref. 58). Most mutations are loss-of-function mutations, and here we provide an additional example for such a mutation (S268P). Like the R265H mutation described here, the S268P mutation was found in one individual only (1/250). The individual is heterozygous for the mutation, suggesting that he may produce an intact version of the receptor from the WT allele. Although rare and monoallelic, this polymorphic mutation is of considerable interest because the consequence of amino acid replacement strongly impairs receptor signaling. Consequently, the level of fully functional receptors in this individual is most probably close to half of that from most individuals, as observed in heterozygous MOR-deficient mice with one allele inactivated (6). Therefore, future studies will aim at enlarging the DNA sampling to find more examples of the S268P polymorphism. In a preliminary study, we have investigated the possible presence of the mutation in other members of the patient’s family, and DNA sequencing has revealed the existence of the same S268P mutation in one of the siblings (one allele also, not shown). An expanded study of this particular mutation should indicate whether individuals homozygous for the mutation do exist. Finally, the clinical examination of patients expressing the S268P polymorphic receptor from one or the two alleles may provide interesting insights into the functional consequences of decreased μ-opioid receptor function in humans.

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