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Allelic Variation S268P of the Human μ-Opioid Receptor Affects Both Desensitization and G Protein Coupling[1]

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ABSTRACT

The decrease in μ-opioid receptor activity after chronic agonist exposure (1 μM [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin) is largely due to kinase-mediated phosphorylation of intracellular receptor domains. We have recently shown that the substitution of two putative Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) phosphorylation sites, S261 and S266, by alanines in the third intracellular loop of the rat μ-opioid receptor (rMOR1) confers resistance to CaMK II-induced receptor desensitization. In the present study, we show that the injection of active CaMK II in *Xenopus laevis* oocytes led to the desensitization of S261A but not S266A receptor mutant, indicating that S266 is the primary CaMK II phosphorylation site of the rMOR1. For the corresponding phosphorylation site in the human μ-opioid receptor (hMOR), an allelic variation S268P has been recently identified. After expression in *X. laevis* oocytes and human embryonic kidney 293 cells, this human S268P receptor and a corresponding rat S266P receptor mutant revealed a loss of CaMK II-induced receptor desensitization and a decreased G protein coupling compared with the wild-type receptors. Our results suggest that serines 266 (rMOR1) and 268 (hMOR) play crucial role in receptor desensitization and signaling and that the allelic variation S268P results in a human receptor type with a weaker but persistent G protein coupling after agonist treatment.

The μ-opioid receptor inhibits adenylate cyclase, activates inwardly rectifying potassium channels, and closes calcium channels via coupling to heterotrimeric G proteins (G_i, G_o). During repeated agonist stimulation, these responses are rapidly desensitized. An important mechanism of desensitization of G protein-coupled receptors is the phosphorylation of serine and threonine residues by two classes of proteinkinases: 1) second messenger-regulated protein kinases, such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II), cAMP-dependent protein kinase, or protein kinase C and 2) the family of G protein-coupled receptor kinases. After the phosphorylation of intracellular receptor domains, binding of β-arrestin molecules led to an uncoupling of the receptor from G proteins. In addition, β-arrestin binding induces receptor internalization via clathrin-coated pits into early endosomes, and after the removal of the bound ligand and dephosphorylation, the receptor is recycled to the cell surface in a reactivated state (Koch et al., 1998).

We have recently shown that the mutation of two putative CaMK II phosphorylation sites, S261 and S266, in the third intracellular loop to alanines delays the time course of agonist-induced desensitization of the rat μ-opioid receptor (rMOR1). Coexpression of constitutively active CaMK II in human embyonic kidney (HEK) 293 cells or injection of active CaMK II into *Xenopus laevis* oocytes led only to a desensitization of the wild-type but not the S261A/S266A receptor mutant (Koch et al., 1997). We have also shown that mutation of S261/S266 lowers agonist-induced rMOR1 phosphorylation and that μ-opioid receptors and CaMK II are frequently colocalized in the rat brain (H. Schmidt and V. Höllt, unpublished data). Although our studies provide strong evidence that the substitution of serines 261 and 266 by alanines decreases agonist-induced desensitization and phosphorylation of the rMOR1, it has not been delineated whether CaMK II phosphorylated either serine residues or only one of them. For the human μ-opioid receptor (hMOR), an allelic variation (S268P) has recently been published in which serine 268 is substituted with proline (Hoehe, 1998; Hoehe and Wendel, 1998). This serine 268 represents one of the two putative CaMK II phosphorylation sites in the third intracellular loop of the hMOR and corresponds to the CaMK II phosphorylation site serine 266 in the rMOR1. Therefore, it was possible that this mutation affects the CaMK II-mediated desensitization of the hMOR.

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ABBREVIATIONS: rMOR1, rat μ-opioid receptor isoform 1; hMOR, human μ-opioid receptor; CaMK II, Ca^{2+}/calmodulin-dependent protein kinase II; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GIRK, G protein-gated, inwardly rectifying K^+ channel; HEK, human embryonic kidney; PCR, polymerase chain reaction.

In the present study, we constructed two rMOR1 mutants consisting of substitutions of either S261 or S266 by alanine and investigated the effect of CaMK II on both receptor mutants. Our results provide strong evidence that primarily S266, and not S261, is involved in the CaMK II-induced desensitization of the rMOR1. To investigate the effect of a proline mutation in this important phosphorylation site on the CaMK II-induced μ-opioid receptor desensitization, we constructed two additional phosphorylation mutants, S268P and S266P, for the hMOR and rMOR1, respectively. After the injection of active CaMK II into *X. laevis* oocytes, both expressed receptors rS266P and hS268P showed a loss of the CaMK II-induced receptor desensitization. In addition, both receptor types revealed a weaker G protein coupling compared with the wild-type receptors. In summary, our results indicate the important role for the serines 266 (rat) and 268 (human) in the third intracellular loop for the CaMK II-induced desensitization and for the G protein coupling of the μ-opioid receptor.

Materials and Methods

Cloning of cDNA. For cloning of the hMOR from a cDNA library, polymerase chain reaction (PCR) primers were designed based on the hMOR sequence (Wang et al., 1994). The sequence of forward and reverse primers synthesized is 5′-GTG CTC CTG GCT ACC TCG CAC AGC-3′ and 5′-TAA GCT TGG TGA AGG TCG GAA TGG-3′, respectively. For introducing an *Hin*dIII restriction site upstream of the ATG start codon and an *Xba*I site downstream of the reading frame, we used nested PCR primers 5′-GCT CCT GGC **AAG CTT** GCA CAG CGG-3′ and 5′-TGA AGG **TCT AGA** TGG CAT GAG ACC-3′ in a second PCR step. The resulting 1.2-kb *Hin*dIII/*Xba*I fragment coding for the hMOR was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA). The rMOR1 cDNA obtained from Dr. Lei Yu (GenBank accession number L13069) was subcloned into the pRc/CMV expression vector (Invitrogen).

Dr. H. A. Lester (Pasadena, CA) kindly provided the cDNA from the rat KIR3.1 potassium channel. For cloning of the G protein inwardly rectifying K^+ channel KIR3.4 from a rat cDNA library, PCR primers were designed on the rat KIR3.4 sequence (GenBank accession number X83584). The sequence of forward and reverse primers synthesized is 5′-TGG AAG GAA TCG CCA GAA GTT AGC-3′ and 5′-GCC TGA GAG GCC AGA GAG GCA ATT-3′, respectively. The resulting 1.4-kb fragment was subcloned into pGEM-T vector system (Promega, Madison, WI).

In Vitro Mutagenesis. Point mutations of the μ-opioid receptors were generated by oligonucleotide-mediated site-directed mutagenesis using mutagenesis primers rMOR/S261A (5′-A CTC AAG **GCC** GTT CGC ATG CTA TCG GGC TCC AA-3′), rMOR/S266A (5′-A CTC AAG AGC GTT CGC ATG CTA **GCG** GGC TCC AA-3′), rMOR/S266P (5′-GTT CGC ATG CTA **CCG** GGC TCC AAA-3′), hMOR/S268P (5′-GTC CGC ATG CTC **CCT** GGC TCC AAA-3′), and Altered Sites *In Vitro* Mutagenesis kit from Promega according to the manufacturer's directions. Primers rMOR/S261A and rMOR/S266A match at nucleotides 774 to 806 and rMOR/S266P at positions 784 to 807 of the coding sequence of rMOR1 introducing an alanine or a proline instead of a serine as indicated. The mutagenesis primer hMOR/S268P matches at position 790 to 813 and introduces a proline instead of a serine in amino acid position 268 of the hMOR protein. Nucleotide sequences of all recombinant receptors were confirmed by double-strand DNA sequencing.

Generation of Cell Lines Expressing μ-Opioid Receptors. Transfection of HEK 293 cells were performed according to the calcium phosphate precipitation method (Chen and Okayama, 1988). Approximately 1.5×10^6 cells were transfected with 20 μg of plasmid DNA. Cells were selected in the presence of 500 μg/ml G418 (Life Technologies, Eggenstein, Germany), and resistant cells were grown in the presence of 400 μg/ml G418 without the selection of individual clones.

Measurements of cAMP Levels. Cells (1.5×10^5) were seeded onto 22-mm 12-well dishes with Dulbecco's modified Eagle's medium Nut-F12 containing 10% fetal calf serum. On the day of assay, medium was removed from the individual wells and replaced with 0.5 ml serum-free medium containing 25 μM forskolin or a combination of forskolin (25 μM) and [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin (DAMGO; 1 μM). The cells were then incubated at 37°C for 15 min. The reaction was terminated by removing the medium and sonicating the cells in 1 ml of ice-cold HCl/EtOH (1 volume of 1 N HCl/100 volumes of EtOH). After centrifugation, the supernatant was evaporated, the residue was dissolved in Tris-EDTA buffer, and the cAMP content measured using a commercial radioassay kit (Amersham, Braunschweig, Germany).

Radioligand-Binding Assays. Binding studies were performed on membranes prepared from stable transfected cells. The dissociation constant (K_D) and the number of [^{3}H]DAMGO-binding sites (B_{max}) were calculated by Scatchard analysis using at least seven concentrations of labeled DAMGO in a range of 0.25 to 10 nM as previously described (Koch et al., 1998; Wolf et al., 1999).

[^{35}S]Guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) Binding. [^{35}S]GTPγS binding was conducted as described previously (Befort et al., 1996). Stock [^{35}S]GTPγS was diluted 10-fold in 50 mM Tris, pH 7.4, 10 mM EDTA; aliquoted; and stored at −80°C until use. For each assay, 50 μg membrane protein was incubated in 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 3 μM GDP, 0.05 nM [^{35}S]GTPγS, and the agonist DAMGO in a final volume of 1 ml. Nonspecific binding was determined in the presence of 10 μM GTPγS. After 30 min at 30°C, membranes mixtures were washed three times with ice-cold 50 μM Tris-HCl, pH 7.4, on GF/B filters using an Inotech cell harvester. Bound radioactivity was determined by scintillation counting.

Oocyte Injection and Expression of Receptors and G Protein-Activated K^+ Channel. Oocytes were prepared using standard methods (Gurdon and Wickens, 1983). Mature *X. laevis* females (Nasco, Fort Atkinson, WI) were anesthesized in 0.15% 3-aminobenzoic acid ethyl ester (Tricaine) (Sigma Chemical Co., St. Louis, MO), ovarian lobes were surgically removed, and individual oocytes were isolated manually at room temperature. Stage V and VI oocytes were injected with a total of 50 nl containing 0.5 ng each of KIR3.1 and KIR3.4 mRNA and 1 ng of receptor mRNA and incubated for 3 to 4 days at 20°C in ND96 (96 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES) supplemented with 5% fetal bovine serum, 2.5 mM Na-pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. To generate capped mRNA for injection, T7 RNA polymerase and mCAP mRNA Capping kit (Stratagene, La Jolla, CA) were used. Plasmid templates for all constructs, including rMOR1 and hMOR, were linearized before the cRNA synthesis.

Type II CaMK II (BIOMOL Research Laboratories, Plymouth Meeting, PA) was activated by autophosphorylation to become Ca^{2+}/calmodulin-independent, as previously described (Mestek et al., 1995). At 15 min before recording K16- (82 mM NaCl, 16 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$)- and K16/DAMGO-induced current, oocytes were injected with 50 nl (2 fmol) CaMK II.

Electrophysiology. Oocytes with a membrane resistance (R_m) of >1 MΩ were voltage-clamped at −80 mV at room temperature using a two-microelectrode voltage-clamp (Turbo TEC-05 npi amplifier), and the data were analyzed with Eggworks software (Polder, Tamm, Germany). The clamp electrodes were filled with 3 M KCl and had a tip resistance of 0.5 to 1 MΩ. Clamped oocytes were superfused with either ND96, a potassium solution (K16), K16 containing the μ-opioid specific agonist DAMGO (1 μM), or a K16/$BaCl_2$ solution (K16 with 300 μM $BaCl_2$).

Results

CaMK II-Induced Desensitization of Wild-Type and Mutant μ-Opioid Receptors Expressed in *X. laevis* Oocytes. Mutation of serines 261 and 266 in the rMOR1, representing putative CaMK II phosphorylation sites, to alanines resulted in a S261A/S266A receptor mutant that lacks CaMK II-induced receptor desensitization (Koch et al., 1997). To investigate whether both or only one of these serine residues is a target for the CaMK II, we constructed two receptor mutants consisting of alanine substitutions of either serine 261 or serine 266. To test the effect of an allelic variation (S268P) of the hMOR on the CaMK II-induced receptor desensitization, we constructed the human S268P variant and for comparison the corresponding S266P rat receptor mutant. In *X. laevis* oocytes expressing either the wild types or receptor mutants and G protein-activated K^+ channels (KIR3.1/KIR3.4), DAMGO superfusion led to an inward K^+ current that could be completely blocked by K^+ channel blocker Ba^{2+} (300 μM; Fig. 1A, left). Oocytes injected with KIR3.1/KIR3.4 channel mRNAs alone did not produce the current on DAMGO stimulation (data not shown). Due to variation in mRNA expression among different oocytes, the amplitude of the K^+ current can vary from oocyte to oocyte. Therefore, receptor activity was defined as a ratio of DAMGO-induced K^+ current ($I_{K16/GIRK\text{-}activated}$, where GIRK is G protein-gated, inwardly rectifying K^+ channel) to basal K^+ current through KIR3.1/KIR3.4 channels ($I_{K16/GIRK\text{-}basal}$), both measured under extracellular potassium concentration (K16 superfusing; Fig. 1A, left).

To investigate the influence of the rMOR1 mutations S261A, S266A, or S266P and the hMOR mutation S268P on the CaMK II-induced receptor desensitization, we used direct injection of CaMK II into receptor-expressing oocytes. After the injection of active CaMK II, receptor desensitization was defined as a decrease in the ratio of the DAMGO-induced current ($I_{K16/GIRK\text{-}activated}$) to the GIRK-induced current ($I_{K16/GIRK\text{-}basal}$; Fig. 1A, right). The ratio of the DAMGO-induced current of each receptor type with injection of heat-inactivated CaMK II has been defined as 100%. The absolute current ratios ($I_{K16/GIRK\text{-}activated}/I_{K16/GIRK\text{-}basal}$) were 2.0 ± 0.4, 2.1 ± 0.1, 1.8 ± 0.2, 0.2 ± 0.1, 2.2 ± 0.3, and 0.4 ± 0.2 for rMOR1, rS261A, rS266A, rS266P, hMOR, and hS268P receptors, respectively. Figure 2 shows the effect of the CaMK II on μ-opioid receptor desensitization. For the rMOR1 and hMOR wild types and the rS261A receptor mutant, but not for rS266A, rS266P, and hS268P receptor mutants, coinjection of active CaMK II led to a reduction of the ratio of $I_{K16/GIRK\text{-}activated}/I_{K16/GIRK\text{-}basal}$ from 100% to approximately 30%.

Agonist-Induced Desensitization of μ-Opioid Receptors Expressed in *X. laevis* Oocytes. Next, we determined the time course of the agonist-induced desensitization of wild-type and mutant μ-opioid receptors in *X. laevis* oocytes. The time-dependent DAMGO-induced desensitization was performed by superfusing oocytes with K16 buffer followed by the application of K16 buffer with 1 μM DAMGO. Receptor desensitization was defined as the decrease in agonist-induced potassium currents during 15 min of agonist treatment (Fig. 1B). This decrease in the agonist-induced potassium current is due only to receptor desensitization because the basal currents of KIR3.1/KIR3.4 heteromultimeric potassium channels remained nearly unchanged on prolonged DAMGO treatment in K16 buffer. Oocytes expressing either rMOR1, rS266A, rS266P, hMOR, or hS268P were incubated in K16 buffer with 1 μM DAMGO over a period of 15 min, and the agonist-induced potassium current was determined (Fig. 3, A–C). Our results show that under the prolonged influence of DAMGO, all receptor types showed a decrease in the amount of agonist-induced K^+

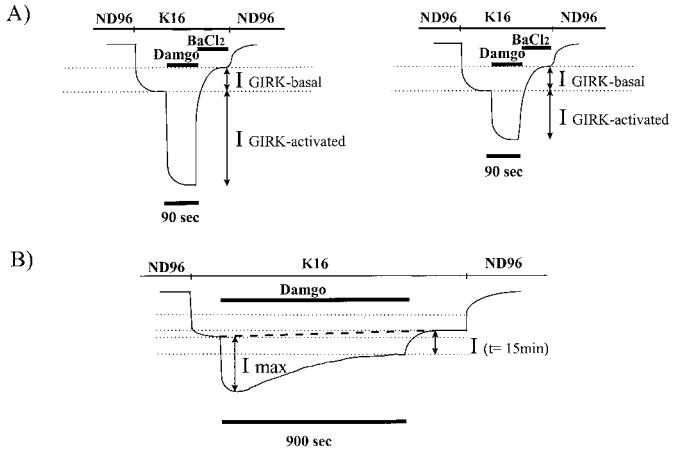

Fig. 1. Coupling of the rMOR1 to the G protein-activated inwardly rectifying K^+ channel, KIR3.1/KIR3.4. Oocytes were injected with mRNAs for the μ-opioid wild-type or mutant receptor and the KIR3.1/KIR3.4 channel. KIR3.1/KIR3.4 complex is represented as GIRK. A, left panel: a representative current trace recorded at a holding potential of −80 mV shows the change in current after solution changes. DAMGO (1 μM) and $BaCl_2$ (300 μM) were applied at the times as indicated. $I_{K16/GIRK\text{-}basal}$ represents the basal KIR3.1/KIR3.4-mediated current in K16 buffer, and $I_{K16/GIRK\text{-}activated}$ represents the KIR3.1/KIR3.4-mediated DAMGO-induced current. Both KIR3.1/KIR3.4-specific currents were completely blocked by $BaCl_2$. Right, after injection of CaMK II, the DAMGO-induced $I_{K16/GIRK\text{-}activated}$ current is reduced, whereas the $I_{K16/GIRK\text{-}basal}$ current is not influenced. B, representative trace from an opioid receptor-expressing oocyte during prolonged agonist treatment. Any change in the $I_{K16/GIRK\text{-}basal}$ after K16 superfusion without agonist treatment was plotted as shown by dashed line. The agonist-induced receptor-activated inward current I_t at any time t was the difference between the total inward current and the inward current value of the baseline. The rate of desensitization was calculated using the formula $(I_{max} - I_t)/t$, where t is the time in minutes after the peak response at which I_t was measured, and the rate was normalized as the percentage of the peak receptor-activated current I_{max}, measured at the beginning of agonist superfusion.

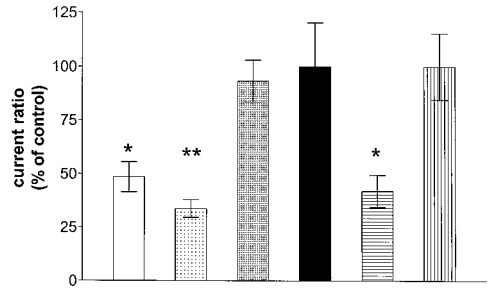

Fig. 2. Modulation of rMOR1 and hMOR coupling to KIR3.1/KIR3.4 in *X. laevis* oocytes by CaMK II. Shown from left to right are values for rMOR1, rS261A, rS266A, rA266P. hMOR, and hS268P. Oocytes were injected with both the μ-opioid receptor wild-type or mutant receptors and the KIR3.1/KIR3.4 mRNAs. Membrane currents were recorded at a holding potential of −80 mV. Data for four separate oocytes are expressed as the percent ratio of $I_{K16/GIRK\text{-}activated}$ to $I_{K16/GIRK\text{-}basal}$. Current ratios of each receptor type with injection of heat-inactivated CaMK II were defined as 100%. Percentage ratios are presented as mean ± S.D. The asterisks indicate a significant difference ($P < .05$) between receptor current ratios in oocytes coinjected with heat-inactivated and active CaMK II (ANOVA followed by Bonferroni test).

current. For the rMOR1 and hMOR wild types, receptor activity decreased from 100% to approximately 30% after 15 min of DAMGO treatment (Fig. 3, A and C). The time course

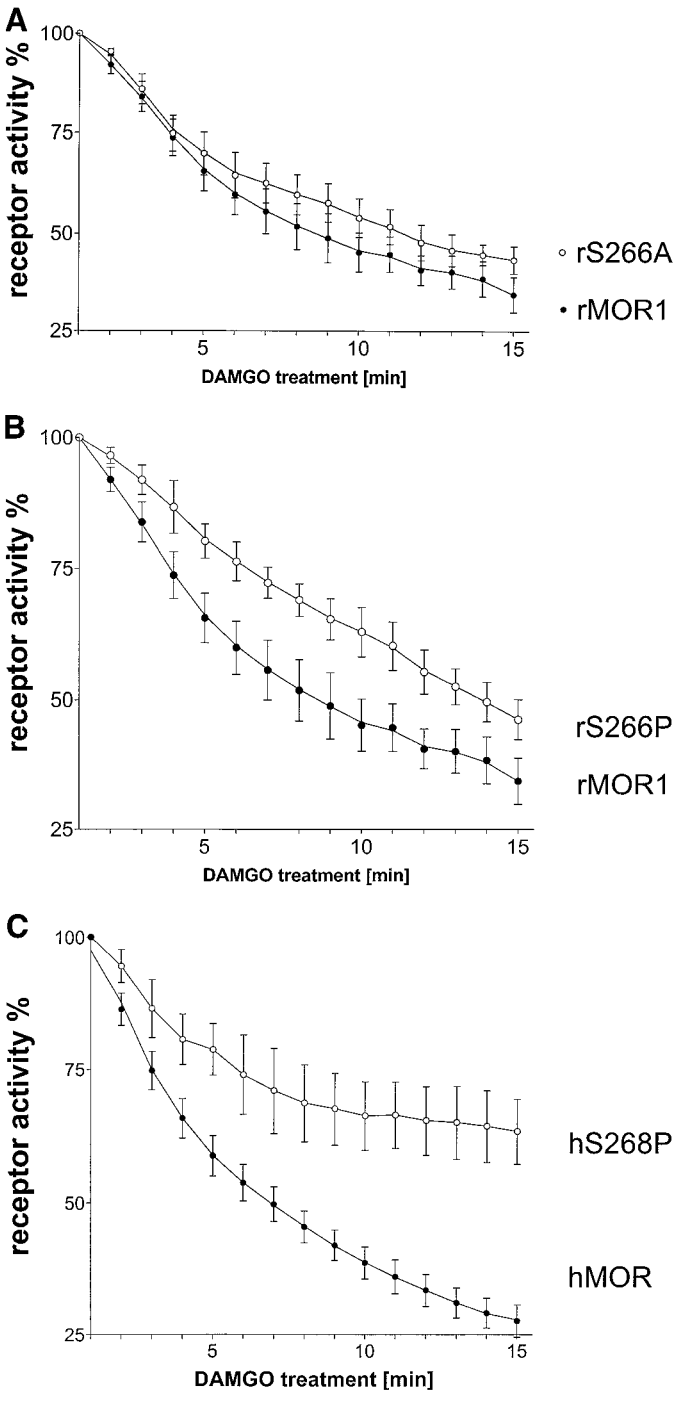

Fig. 3. Effect of rS266A, rS266P, and hS268P mutations on the acute response desensitization of the μ-opioid receptor. Oocytes were injected with KIR3.1/KIR3.4 and μ-opioid receptor wild-type or mutant receptor mRNAs. All recordings were performed 3 to 4 days postinjection, and responses were measured in 16 mM K^+ buffer. After equilibration with K16 buffer, application of the μ-agonist DAMGO (1 μM) increased the inward current response defined as 100%, and prolonged agonist exposure rapidly desensitized the response as shown. Responses were adjusted by baseline subtraction. Values represent mean ± S.E. of at least four separate oocytes. Top, agonist-induced desensitization of the rMOR1 and rS266A receptor. Middle, agonist-induced desensitization of the rMOR1 and rS266P receptor. Bottom, agonist-induced desensitization of the hMOR and hS268P receptor.

of receptor desensitization was markedly slower for the rS266A, rS266P, and hS268P receptor mutants compared with the corresponding wild types (Fig. 3, A and C). These results indicate the importance of the rS266/hS268 CaMK II phosphorylation sites for the DAMGO-induced μ-opioid receptor desensitization.

Expression and Agonist-Induced Desensitization of rMOR1, rS266P, hMOR, and hS268P μ-Opioid Receptors in HEK 293 Cells. In addition to the studies in *X. laevis* oocytes, we examined the agonist-induced desensitization of the rMOR1, rS266P, hMOR, and hS268P receptors in HEK 293 cells. HEK 293 cells stably transfected with both wild-type and mutant receptors revealed similar [^{3}H]DAMGO binding (K_D/B_{max}, Table 1). The affinities of peptide agonist DAMGO were not markedly modified for any receptor constructs. Nevertheless, the abilities to inhibit adenylate cyclase activity in HEK 293 cells were significantly reduced for the rat S266P and the human S268P receptor mutant compared with the corresponding receptor wild types (Table 1).

Receptor desensitizaton was measured as the decreasing ability of the agonist DAMGO to inhibit forskolin-stimulated adenylate cyclase activity after agonist pretreatment. Therefore, receptor-expressing HEK 293 cells were pretreated with 1 μM DAMGO for various time intervals, followed by determination of the DAMGO-induced inhibition of forskolin-stimulated adenylate cyclase. Forskolin treatment resulted in a 5-fold increase of intracellular cAMP (up to 4 pmol) compared with untreated HEK 293 cells. For each receptor type, maximum agonist-induced inhibition of cAMP accumulation without DAMGO preincubation has been defined as 100%. Prolonged DAMGO exposure (up to 4 h) led to a rapid decrease in the agonist-induced cAMP inhibition for all receptor types. The time course of agonist-dependent loss of receptor activity is illustrated in Fig. 4, A and B. Similar to the results obtained in the *X. laevis* oocytes, rS266P and hS268P receptors revealed a slower desensitzation during 4 h of DAMGO pretreatment compared with the wild-type receptors. These data demonstrated that rMOR1- and hMOR-expressing cells desensitize faster than those expressing the rS266P and hS268P mutant receptors. Our results also revealed that rMOR1 receptor is faster desensitized than the hMOR receptor wild type during 4 h of DAMGO pretreatment. After 1 h of agonist exposure, rMOR1 receptor activity decreased to only 50%, whereas the activity of the hMOR receptor remained nearly unchanged.

G Protein Coupling of Wild-Type and Mutant μ-Opioid Receptors. After expression in HEK 293 cells and *X. laevis* oocytes, the rat S266P and the human S268P receptors revealed a weaker functional coupling compared with the corresponding wild types. DAMGO incubation led to a decrease in the maximum intracellular cAMP level from 100% (for control cells) to 35% for cells expressing the rMOR1 and to 34% for hMOR wild-type receptor and only to 61 and 69% for rS266P and hS268P, respectively (Table 1). In addition, the functional coupling to the KIR3.1/KIR3.4 potassium channel after expression in *X. laevis* oocytes was significantly reduced for the rS266P and hS268P receptor mutants compared with the corresponding wild types. The ratio ($I_{K16/GIRK\text{-}activated}/I_{K16/GIRK\text{-}basal}$) was only 0.2 for the rS266P and 0.4 for the hS268P receptor, whereas both wild-type receptors revealed a current ratio of approximately 2 (Table 1). To confirm these results, we examined directly the coupling between rMOR1 and hMOR wild-type

receptors and rS266P and hS268P receptor mutants and G protein by measuring nucleotide exchange activity in isolated membranes ([^{35}S]GTPγS assay). Under the above-described conditions, [^{35}S]GTPγS specific binding was found to increase severalfold over basal activity on agonist stimulation (Table 1). Maximal activation was lower for rS266P receptor (1897 dpm, or 133% basal activity) compared with rMOR1 (5002 dpm, or 205% basal activity), indicating a greater efficacy of the latter in activating the G_α subunit. Also, the hS268P receptor (1846 dpm, or 132% basal activity) revealed significantly weaker GTPγS binding compared with the hMOR wild type (5428 dpm, or 229% basal activity). These results confirm that the change in the important CaMK II phosphorylation site (rS266 or hS268) in the third intracellular loop to a proline diminishes the G protein coupling of the μ-opioid receptor.

Discussion

Agonist-induced desensitization of μ-opioid receptor signaling appears to require the phosphorylation of intracellular receptor domains (Lohse, 1993; Lefkowitz, 1998). Phosphorylation of G protein-coupled receptors can be accomplished by second messenger-dependent as well as G protein-coupled receptor kinases. CaMK II phosphorylation was shown to involve the regulation of *N*-methyl-D-aspartate, γ-aminobutyric $acid_A$, and μ-opioid receptor activity (McDonald and Moss, 1994; Mestek et al., 1995; Yakel et al., 1995). The third intracellular loop, which has been shown to be important for G protein coupling and signal transduction (Dohlman et al., 1991; Georgoussi et al., 1997), is highly conserved among all opioid receptor types. In this region, there are two strictly conserved serine residues that are localized within consensus sequence motifs (XRXXS/T) for CaMK II phosphorylation. In the rMOR, the putative CaMK II sites were in amino acid positions 261 and 266.

Several lines of evidence suggest that CaMK II-mediated phosphorylation of these serines 261 and 266 in the third intracellular loop may indeed advance μ-opioid receptor desensitization. First, coexpression of constitutively active CaMK II in HEK 293 cells or injection of active CaMK II in *X. laevis* oocytes led to a rapid desensitization of the μ-opioid receptor (Koch et al., 1997). Second, mutation of both serine residues 261 and 266 to alanines resulted in a loss of the CaMK II-mediated desensitization after expression in HEK 293 cells and *X. laevis* oocytes (Koch et al., 1997). Third, the S261A/S266A receptor mutant did not undergo agonist-dependent phosphorylation (H. Schmidt and V. Höllt, unpublished data). In addition, three other points argue for an in vivo function of CaMK II in the desensitization of the μ-opioid receptor: 1) CaMK II is cocontained in virtually all rMOR1-expressing interneurons in pain-processing brain regions and, hence, in a position to phosphorylate the μ-opioid receptor in vivo (H. Schmidt and V. Höllt, unpublished data); 2) agonist-stimulation of the μ-opioid receptor has been found to increase the levels of intracellular Ca^{2+} and to activate CaMK II in vitro and in vivo (Zimprich et al., 1995; Lou et al., 1999); and 3) blocking of the hippocampal increase in CaMK II activity after morphine application by CaMK II inhibitors attenuates morphine tolerance and dependence in rats (Fan et al., 1999).

Due to the obviously important role of CaMK II in the μ-opioid receptor desensitization, we investigated whether both or only one of these serine residues in the third intracellular loop is involved in the CaMK II-induced desensitization of rMOR1 by changing either S261 or S266 to alanine. In *X. laevis* oocytes expressing rMOR1, rS261A, or rS266A mutant and G protein-activated K^+ channel (KIR3.1/KIR3.4), DAMGO superfusion led to inward K^+ current. Functional receptor coupling in *X. laevis* oocytes expressing KIR3.1/KIR3.4 and rMOR1, rS261A, or rS266A mutant was measured with the coinjection of heat-inactivated and active CaMK II. The absolute current ratios ($I_{K16/GIRK\text{-}actvated}/I_{K16/GIRK\text{-}basal}$) after the injection of heat-inactivated CaMK II were approximately 2 for rS261A, rS266A, and rMOR1 wild-type receptor, indicating similar functional coupling of these receptors. Desensitization was defined as a decrease in the ratio of the DAMGO-induced current ($I_{K16/GIRK\text{-}activated}$) to the basal KIR3.1/KIR3.4-mediated current in K16 buffer ($I_{K16/GIRK\text{-}basal}$). In rMOR1 and rS261A, but not rS266A, receptor-expressing oocytes, the injection of CaMK II led to desensitization of the receptor coupling (Fig. 2). In receptor-expressing oocytes coinjected with CaMK II, we found no difference in the basal activity of the KIR3.1/KIR3.4 channel ($I_{K16/GIRK\text{-}basal}$) under K16 compared with oocytes injected with heat-inactivated CaMK II (data not shown) and, hence, the decrease in the ratio of $I_{K16/GIRK\text{-}activated}/I_{K16/GIRK\text{-}basal}$ is only due to a decrease in $I_{K16/GIRK\text{-}activated}$. We found that only the mutation affecting the serine 266 revealed a lack of CaMK II-mediated rMOR desensitization (Fig. 2). To test the effect of the S266A mutation on the agonist-induced receptor desensitization, rMOR1 and rS266A receptor-expressing oocytes were superfused with K16 buffer with 1 μM DAMGO over a period of 15 min, and the potassium current was

TABLE 1

Functional properties of wild-type and mutant μ-opioid receptors after expression in HEK 293 cells and *X. laevis* oocytes

The B_{max} and K_D values for the binding of [^{3}H]DAMGO for the wild-type and mutant receptors were determined with membrane preparations from stable transfected HEK 293 cells and calculated by Scatchard analyses. The effect of 1 μM DAMGO on forskolin-stimulated cAMP accumulation in HEK 293 cells and KIR3.1/KIR3.4-mediated potassium current ratio ($I_{K16/GIRK.activated}/I_{K16/GIRK\text{-}basal}$) in *X. laevis* oocytes was determined as described in *Materials and Methods*. Agonist potency and efficacy at the rat and human μ-opioid wild-type and mutant receptors was determined using the [^{35}S]GTPγS binding assay. Maximal activation in dpm is obtained from the difference of specific [^{35}S]GTPγS binding in the presence and absence of 1 μM agonist. Maximal activation is also displayed as a percentage with basal [^{35}S]GTPγS binding defined as 100%. Values shown for the reduction of cAMP level and [^{35}S]GTPγS experiments are mean ± S.E. from at least three independent experiments performed in triplicate, and values shown for the DAMGO-induced current ratios are mean ± S.E. from four separate oocytes.

Construct	B_{max}	K_D	Reduction in Forskolin-Stimulated cAMP Levels	Current Ratio	Specific GTPγS Binding	
					Maximal activation	Activation
	fmol/mg	*nM*	%		*dpm*	%
rMOR1	758	2.5	65 ± 2	2.0 ± 0.4	5002 ± 1500	205
rS266P	570	3.2	39 ± 5	0.2 ± 0.1	1897 ± 600	133
hMOR	643	2.1	66 ± 6	2.2 ± 0.3	5428 ± 950	229
hS268P	340	5.0	31 ± 4	0.4 ± 0.2	1846 ± 300	132

measured. As reported, coexpression of KIR3.1 and KIR3.4 enhanced the agonist-activated response, reduced the amount of channel mRNA required, and greatly attenuated the amount of heterologous desensitization produced on prolonged agonist treatment (Krapivinsky et al., 1995; Kovoor et al., 1997). Therefore, the observed agonist-induced desensitization is first due to a decrease in receptor activity and not to a desensitization of the K^+ channel itself. As shown in Fig. 3A, the S266A mutation revealed a slower agonist-induced desensitization compared with the rMOR wild type. Therefore, in addition to the lack of CaMK II-mediated desensitization, the S266A mutation is also impaired in agonist-induced desensitization. These results clearly indicate that serine 266 is the primary CaMK II phosphorylation site of importance involved in the rMOR1 desensitization.

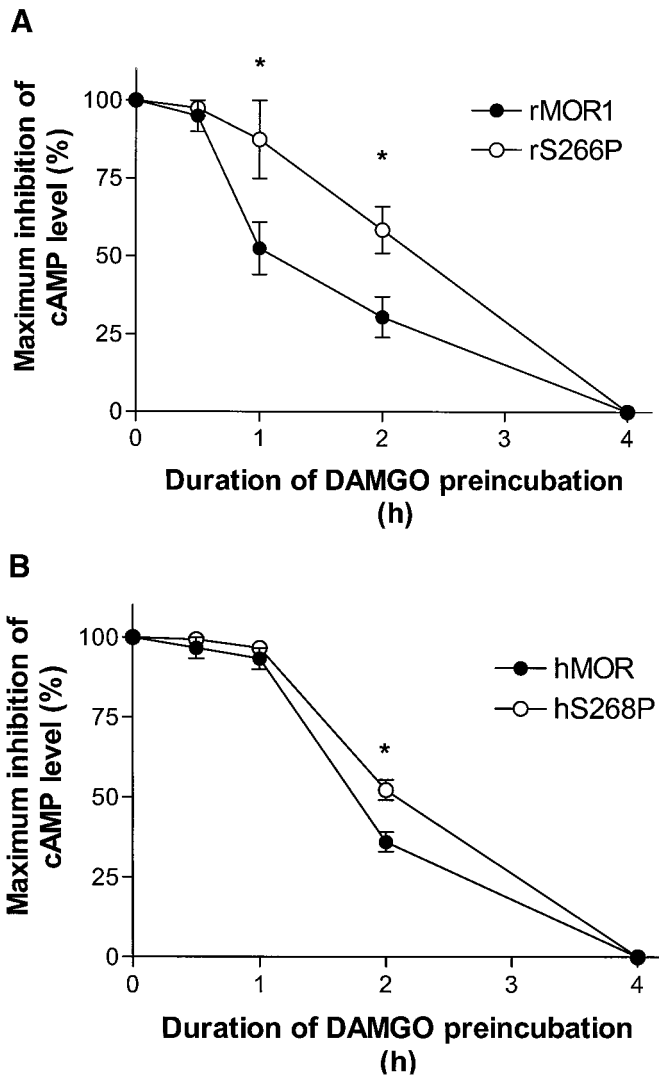

Fig. 4. Time courses of agonist-induced desensitization of rMOR1, hMOR, rS266P, and hS268P receptor mutant. Transfected HEK 293 cells were preincubated at 37°C with 1 μM DAMGO for the indicated time intervals. After removal of the preincubation medium, cells were treated with forskolin (25 μM) or forskolin (25 μM) plus DAMGO (1 μM), and cAMP levels were determined as described in *Materials and Methods*. Values represent mean ± S.E. from four separate measurements performed in triplicate. The asterisks indicate significant difference ($P < .05$) between wild-type and receptor mutant (ANOVA followed by Bonferroni test). A, effect of S266P mutation on the rMOR1 desensitization. B, effect of S268P mutation on the hMOR desensititzation.

Large-scale sequencing of the hMOR gene revealed polymorphic variants (Hoehe, 1998; Hoehe and Wendel, 1998). Some of the mutations occur within coding regions, at locations that may severely affect receptor function: N40D in the N-terminal portion, N152D in the third transmembrane domain, and R265H and S268P in the third intracellular loop. The allele frequency of the latter variants was relatively low; around 1% of heterozygous carriers were found in a sample of 700 U.S. individuals (M. Hoehe, personal communication). Because the S268P mutation affects the putative important CaMK II phosphorylation site in the hMOR, we tested the possibility that the hS268P mutant might be a poor substrate for CaMK II. In fact, the human S268P receptor mutant was resistant to CaMK II-mediated desensitization (Fig. 2) and therefore revealed a slower agonist-induced desensitization compared with the wild type in *X. laevis* oocytes and HEK 293 cells (Figs. 3C and 4B). This finding is consistent with our studies concerning a rat S266P receptor mutant, showing that a change of rS266 to proline also led to a slower agonist-induced desensitization (Figs. 3B and 4A) and no detectable CaMK II-mediated desensitization (Fig. 2). In addition, both proline mutants rS266P and hS268P revealed a weaker functional coupling to the adenylate cyclase in HEK 293 cells and to the inwardly rectifying K^+ channel (KIR3.1/KIR3.4) in *X. laevis* oocytes (Table 1). These observations were confirmed by [^{35}S]GTPγS assays showing a decrease in the direct G protein coupling of rS266P and hS268P receptor mutants. On the other hand, rS266A mutation did not affect the functional coupling compared with the wild-type receptor. A possible explanation for this effect is that in contrast to alanine, the proline insertion causes a conformational change in the secondary structure of the third intracellular loop, interfering with the G protein coupling of the μ receptor. In this connection, it is remarkable that the second polymorphic variant (R265H), concerning the third intracellular loop of the hMOR, also affects the CaMK II consensus site $R^{265}XXS^{268}$. Therefore, it must be elucidated whether this mutation, which probably does not lead to a massive sterical change in the third intracellular loop, is also involved in the regulation of the CaMK II-induced desensitization and coupling of the hMOR.

Our data demonstrate that allelic variation S268P affects the important CaMK II phosphorylation site in the third intracellular loop of the hMOR and results in a receptor type with a weaker but persistent G protein coupling after agonist treatment. These findings indicate that serine 268 in the third intracellular loop plays a crucial role in the functional coupling and signal transduction of the hMOR. Accordingly, the allele frequency of this polymorphic variation was found to be low (see earlier). Because of the clear physiological consequences of this mutation, it will be subjected to considerable selective pressure. For the same reason, the S268P amino acid exchange is likely to produce a detectable phenotype. This was not yet investigated, but it could have implications for diagnostics and it could allow information to be gathered about μ-opioid receptor physiology in humans.

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