The role of the hydrophilic Asn230 residue of the μ-opioid receptor in the potency of various opioid agonists

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1 To investigate the effect of the hydrophilic Asn amino acid at position 230 of the human μ-opioid receptor (hMOR230) on the potency of various agonists, we mutated this residue to Thr and Leu (hMORN230T and hMORN230L respectively).

2 Taking advantage of the functional coupling of the opioid receptor with the heteromultimeric G-protein-coupled inwardly rectifying K$^+$ (GIRK1/GIRK2) channel, either the wild type hMOR or one of the mutated receptors (hMORN230L or hMORN230T) were functionally coexpressed with GIRK1/GIRK2 channels and a regulator of G-protein signalling (RGS4) in *Xenopus laevis* oocytes.

3 The two-microelectrode voltage clamp technique was used to measure the opioid receptor-activated GIRK1/GIRK2 channel responses. The potency of [D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin (DAMGO), remained unaffected as measured *via* hMORN230T and hMORN230L, while the potency of fentanyl and morphine significantly increased *via* these mutated receptors.

4 Our results are indicative for the existence of hydrophobic interactions between a methyl-group of the side chain of Thr or Leu on the one hand and the piperidine-ring of fentanyl and the hexene-ring of morphine on the other. The mutations also had no influence on the potency of morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G).

5 We conclude that the hydrophilic side chain of Asn in position 230 is not involved in the formation of a H-bond with the aliphatic alcohol of morphine and that an enhancement of the potency of morphine and fentanyl can be explained by mutating this residue towards more hydrophobic amino acids.

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Abbreviations: DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; EL, extracellular loop; GIRK channels, G-protein-coupled inwardly rectifying K$^+$ channels; HK, high potassium solution; hMOR, human μ-opioid receptor; IL, intracellular loop; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; ND-96, low potassium solution; RGS, regulators of G-protein signalling; TM, transmembrane domain

Introduction

Opiate and opioid compounds act on opioid receptors to produce their pharmacological action (Xu *et al.*, 1999). Molecular cloning studies have identified cDNAs and genes encoding μ-, δ- and κ-opiate receptors from several species (Kieffer *et al.*, 1992; Knapp *et al.*, 1995; Wang *et al.*, 1993), providing a unique opportunity to examine individual opioid receptor types with regard to pharmacological profile, structure-function analysis, cellular effector coupling, anatomical distribution, regulation and expression.

Deduced amino acid sequences of these clones display the motif of putative seven α-helical lipophilic transmembrane helices (TM's) connected by alternating intracellular and extracellular hydrophilic loops, that is characteristic of G protein-coupled receptors (Xu *et al.*, 1999). A family of 20 endogenous opioid receptor ligands were generated from three precursor proteins, prodynorphin, proenkephalin and proopiomelanocortin. In addition to these endogenous peptide ligands, a large series of alkaloid opiate drugs interact with these receptors. Although the opioid peptides belong to a class of molecules distinct from opiate alkaloids, they all share common structural features including a positive charge juxtaposed with an aromatic ring (Evans *et al.*, 1992).

Opioid receptor activation produces a wide array of cellular responses like inhibition of adenylyl cyclase, inhibition of voltage-dependent calcium channels (N, P, Q and R type) and activation of an inwardly rectifying potassium channel (Ikeda *et al.*, 1995). The μ-opioid receptor serves as the principal physiological target for most clinically important opioid analgesics, such as morphine and fentanyl. δ-agonists are poor analgesic compounds, but exhibit less addictive potential, while κ-agonists should be restricted to the periphery because of the strong dysphoric properties of these compounds (Kieffer, 1999).

In general, two approaches are used to examine issues of selectivity and affinity. First, constructing opioid chimeric receptors provides a powerful tool for mapping the regions involved in drug selectivity. Using receptor chimeras, several groups have reported that the docking sites for the opioid

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peptides and alkaloids are different (Li *et al.*, 1996; Meng *et al.*, 1995; Onogi *et al.*, 1995; reviewed in Law *et al.*, 1999). The second approach involves computational modelling (Filizola *et al.*, 1999; Pogozheva *et al.*, 1998) which predicts possible specific amino acid residues that may be critical for receptor binding and selectivity. Nevertheless, caution must be taken in interpreting the data because these structural modifications must retain the conformation of the parent receptors to a large extent (Mansour *et al.*, 1997).

Structural models and experimental data have indicated Asp147 and His297, which are located in the bottom of the binding pocket, as key anchor residues because they could serve as attachment points for the quaternary nitrogen and hydroxyl-group, respectively, of the tyramine moiety of the opiate ligand (Befort *et al.*, 1996; Li *et al.*, 1999; Spivak *et al.*, 1997). Using this alignment Pogozheva *et al.* (1998) suggested that the preferred binding of morphinans to the μ receptor could be explained by the presence of an additional H-bond between Asn230 and the 6-OH group of morphine. Figure 1 shows a schematic representation of the human μ-opioid receptor with the localization of residue Asn230 together with other residues shown to interact with opiate ligand binding.

The goal of this work was to examine the importance of Asn230 in the μ-opioid receptor. Therefore we mutated Asn230 to Thr and Leu, which are neutral and hydrophobic residues, respectively, and determined the EC_{50}-values for GIRK1/GIRK2 channel activation through consecutive activation of wild type and mutant opioid receptors coupled to G proteins and expressed in *Xenopus laevis* oocytes.

To achieve this, we coexpressed GIRK1- and GIRK2-channels, as the neuronal G-protein-gated inwardly rectifying K^+ channels function as heteromultimers (Kofuji *et al.*, 1995) together with RGS4, a regulator of G-protein signalling. RGS proteins accelerate the GTPase rate of heterotrimeric G_α proteins (De Vries *et al.*, 2000; Doupnik *et al.*, 1997). Coexpression of RGS4, the brain-expressed isoform of RGS

proteins, reconstitutes the native gating kinetics by accelerating GIRK1/GIRK2 channel deactivation (Ulens *et al.*, 2000a).

Overall, this experimentally created model (Ulens *et al.*, 2000b) provides a defined population of functionally active opioid receptors (wild type receptor or one of the mutant receptors) and an excellent tool for measuring the efficacy and potency of a ligand for a certain receptor.

Methods

Subcloning and in vitro *transcription of cDNA clones encoding GIRK1/2 channels, human μ opioid receptors and RGS4*

Plasmids containing the entire coding sequence for the mouse GIRK1 and the mouse GIRK2 channel were subcloned into the vector pSP35T and pBScMXT, respectively and designated as pSP/GIRK1 (Kobayashi *et al.*, 1995) and pBScMXT/GIRK2 (Kofuji *et al.*, 1995). The polylinker in each of these vectors is flanked by *Xenopus* globin 5′ and 3′ untranslated regions, resulting in an enhanced protein expression after injection of *in vitro* transcribed cRNA (Krieg & Melton, 1984). For *in vitro* transcription, plasmids were first linearized either with *Eco*RI (for pSP/GIRK1), or with *Sal*I (for pBScMXT/GIRK2). Next the cRNAs were synthesized from the linearized plasmids using the large-scale SP6 mMessage mMachine (for pSP/GIRK1) or T3 mMessage mMachine (for pBScMXT/GIRK2) transcription kit (Ambion, U.S.A.).

The human MOR and rat RGS4 were subcloned into pGEMHE by Ulens *et al.* (2000b) as described. For *in vitro* transcription, each clone was linearized with *Nhe*I. Next the capped cRNAs were synthesized from the linearized plasmids using the large-scale T7 mMessage mMachine transcription kit (Ambion, U.S.A.).
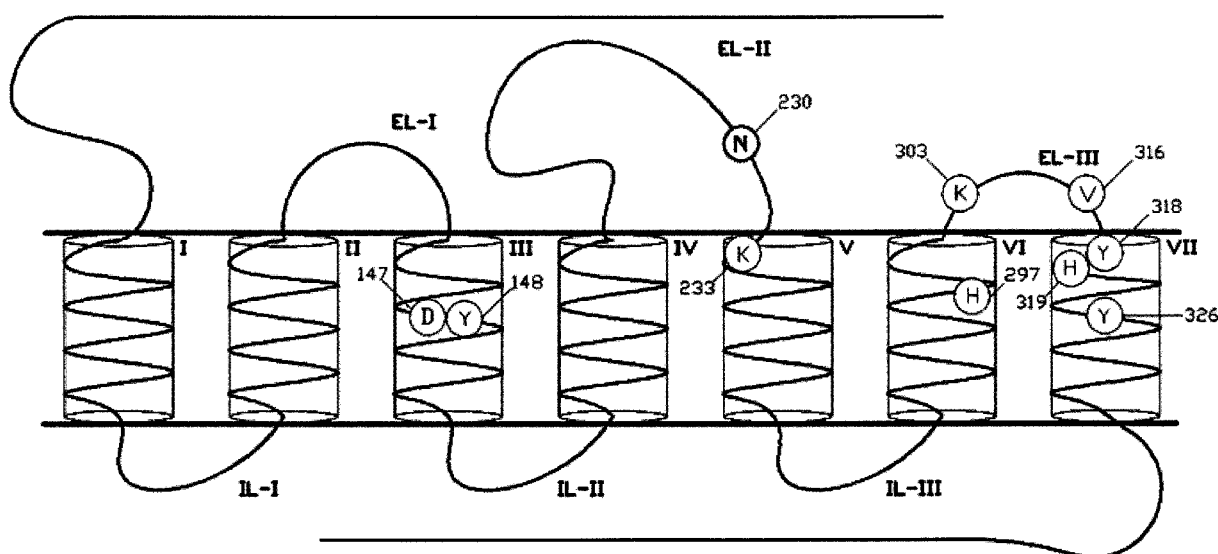

Figure 1 Schematic drawing of the human μ-opioid receptor. Solid black horizontal lines represent the approximate membrane boundaries. The bold circle indicates the position of Asn230. Other residues shown to interact with opiate ligand binding are illustrated and encircled, in their one letter code. The transmembrane helices (TM), the different extracellular loops (EL) and intracellular loops (IL) are denoted by roman numerals.

Construction of mutant human μ-opioid receptors

Asn230 in hMOR was mutated to Leu230 and Thr230 using the Quickchange site-directed mutagenesis kit (Stratagene, U.S.A.). The primers we used were 5′-CCAACTGGTAC-TGGGAG*MYC*CTGCTGAAGATCTGTG-3′ and 5′-CA-CAGATCTTCAGCAG*GRK*CTCCCAGTACCAGGTTGG-3′, with M = A or C, Y = C or T, K = T or G, R = G or A, (codon and complementary codon are underlined). Cycling parameters were set according to the manufacturer's guidelines. A 299 basepair fragment containing the desired mutation was isolated by a double restriction digest with *Msc*I and *Bgl*II. The mutant cDNA was then loaded on an agarose gel, the fragment of interest was cut out, genecleaned (QIAQUICK, QIAGEN, U.S.A.) and ligated with T4 DNA ligase (Promega, U.S.A.) into the corresponding sites of the WT hMOR/pGEMHE. The same mutant fragment was subcloned into pGEM7Zf(+) (Promega, U.S.A.) for DNA sequencing (Eurogentec, Belgium). For *in vitro* transcription, each mutant, hMORN230L/pGEMHE and hMORN230T/pGEMHE was linearized with *Nhe*I. Next the capped cRNAs were synthesized from the linearized plasmids using the large-scale T7 mMessage mMachine transcription kit (Ambion, U.S.A.).

Experimental model

Xenopus laevis oocytes were prepared for injection as described (Liman *et al.*, 1992). Oocytes were co-injected with 0.5 ng 50 nl^{-1} GIRK1, 0.5 ng 50 nl^{-1} GIRK2 and 10 ng 50 nl^{-1} RGS4 cRNA, with the addition of 10 ng 50 nl^{-1} of either hMOR, hMORN230L or hMORN230T cRNA. Injected oocytes were maintained in ND-96 solution (composition in mM: KCl 2, NaCl 96, MgCl$_2$ 1, CaCl$_2$ 1.8, HEPES 5, pH 7.5) supplemented with 50 μg ml^{-1} gentamicin sulphate and incubated at 18°C.

Electrophysiological recordings

Whole-cell currents from oocytes were recorded 1 day after injection using the two-microelectrode voltage clamp technique (Geneclamp 500, Axon Instruments, U.S.A.). Resistances of voltage and current electrodes were kept as low as possible (approximately 200 kΩ) and were filled with 3 M KCl. To eliminate the effect of voltage drop across the bath-grounding electrode, the bath potential was actively controlled. All experiments were performed at room temperature (19–23°C). At the start and the end of each experiment, oocytes were superfused with low-potassium (ND-96) solution (composition in mM: KCl 2, NaCl 96, MgCl$_2$ 1, CaCl$_2$ 1.8, HEPES 5; pH 7.5). During application of increasing concentrations of ligands, oocytes were superfused with high-potassium (HK) solution (composition in mM: KCl 96, NaCl 2, MgCl$_2$ 1, CaCl$_2$ 1.8, HEPES 5; pH 7.5). In HK solution, the K$^+$ equilibrium potential is close to 0 mV and enables K$^+$ inward currents to flow through inwardly rectifying K$^+$ channels at negative holding potentials. A gravity controlled fast perfusion system (Warner Instruments, U.S.A.) was used to ensure rapid solution exchanges. Application of opioid ligands did not evoke an increase of the conductance in uninjected oocytes. In each experiment, oocytes were clamped at a holding

potential of −70 mV for approximately 10 min and superfused with ND-96 solution. Next, the superfusion was switched from ND-96 to HK solution, after which increasing concentrations of an opioid receptor agonist were applied. Each concentration was applied for as long as needed to achieve a steady state GIRK1/GIRK2 current activation. Each ligand concentration was washed out by superfusing with HK solution. During this washout period, the channels return to the control current level as a result of the deactivation process that is dramatically accelerated in the presence of RGS4, as described (Ulens *et al.*, 2000a). At the end of each experiment, the oocyte was superfused with HK solution containing 300 μM BaCl$_2$, causing block of the net GIRK1/GIRK2-gated inward current. Finally, the superfusion was switched back to ND-96 solution to confirm complete reversibility. To avoid the receptor expression level affecting the EC$_{50}$-values of the investigated agonists in the study, the expression system was standardized as previously described (Ulens *et al.*, 2000b).

Data analysis

The pCLAMP program was used for data acquisition and data files (Axon Instruments, U.S.A.) were imported in Microsoft Excel. The percentage activated current was calculated using the equation:

$$\text{Percentage Activation} = \left(\frac{\text{Activated current amplitude}}{\text{Control current amplitude}} \times 100 \right) - 100$$

and 0% was taken as the control current level. Current percentages were used for the calculation of the EC$_{50}$-value, using the Hill equation:

$$I = I_{max}/[1 + (EC_{50}/A)^{n_H}]$$

where I represents the current percentage, I$_{max}$ the maximal current percentage, EC$_{50}$ the concentration of the agonist that evokes the half-maximal response, A the concentration of agonist, and n$_H$ the Hill coefficient. Averaged data are indicated as mean ± s.e.mean and were calculated using *n* experiments, where *n* indicates the number of oocytes tested. Statistical analysis of differences between groups was carried out with Student's *t*-test and a probability of 0.05 was taken as the level of statistical significance.

Compounds

[D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin (DAMGO: Sigma, U.S.A.), fentanyl HCl (kindly provided by NIDA, U.S.A.), morphine HCl (Federa, Belgium), morphine-6-glucuronide (M6G) (Sigma, U.S.A.) and morphine-3-glucuronide (M3G) (Sigma, U.S.A.) were dissolved in high potassium (HK) solution, stored at 4°C, and diluted appropriately for the experiments.

Results

To investigate the role of interactions of opioid agonists with the hydrophilic residue Asn230 in hMOR, we mutated

Asn230 to threonine (hMORN230T) and to leucine (hMORN230L). Using the functional coupling of the opioid receptors to the heteromultimeric GIRK1/GIRK2 channels in *Xenopus* oocytes, we examined the effects of various opioid agonists on the wild type human μ-opioid receptor and on the mutant human receptors, hMORN230T and hMORN230L. GIRK1/GIRK2 channels were coexpressed with RGS4, mimicking the native neuronal G-protein-mediated pathway of K^+-channel activation, together with either the wild type μ-opioid receptor hMORwt or one of the mutant receptors, hMORN230T or hMORN230L. We used the two-microelectrode voltage clamp technique to measure the opioid receptor-activated GIRK1/GIRK2 channel response as the increase of the inward K^+-current at -70 mV, evoked by the application of increasing concentrations of opioid ligands. In our study we examined the potency of DAMGO, fentanyl, morphine, M6G and M3G on hMORwt, and on the mutant receptors, hMORN230T and hMORN230L.

Figure 2 shows representative current traces of agonist-gated currents evoked from oocytes expressing either hMOR wild type (Figure 2A), hMORN230T (Figure 2B) or hMORN230L (Figure 2C) by fentanyl. Analogously, Figures 3 and 4 show representative traces of currents evoked by fentanyl and M6G, respectively. Current traces evoked by DAMGO and M3G are not shown.

Concentration-response relationships (Figure 5A – E) are shown for DAMGO, fentanyl, morphine, M6G and M3G, respectively.

Table 1 summarizes EC_{50}-values calculated for these agonists *via* the receptors and also shows the structure of the amino acid at position 230. Averaged data are indicated as mean±s.e.mean and were calculated using *n* experiments ($n=5-8$).

DAMGO, a μ-opioid-selective ligand showed no difference in potency between the wild type receptor (EC_{50}: 25.6±5.0 nM) and the mutant opioid receptors (25.8±4.7 nM for hMORN230T and 24.7±2.1 nM for hMORN230L).

Fentanyl was approximately two times less potent than DAMGO *via* the wild type receptor. The EC_{50}-value of fentanyl *via* hMORN230T (47.9±3.1 nM) was significantly lower as compared to hMORwt (64.9±7.8 nM). A 3 fold increase in potency was seen when fentanyl was applied to the hMORN230L-receptor (EC_{50}: 20.2±1.9 nM).

Morphine also showed a higher affinity for the mutated receptors than for the wild type receptor (EC_{50}: 148.6±16.3 nM), with no statistical difference between hMORN230T (EC_{50}: 41.2±10.7 nM) and hMORN230L (EC_{50}: 46.3±1.3 nM).

The EC_{50}-value of morphine-6-glucuronide on the wild type receptor (107.4±10.8 nM) was only slightly, albeit significantly, lower as compared to the EC_{50}-value of morphine on the wild type receptor (148.6±16.3 nM). M6G also displayed the same potency *via* hMORN230T (EC_{50}: 97.1±12.9 nM) as *via* hMORN230L (EC_{50}: 106.3±6.3 nM), and this potency was not higher as compared to the wild type receptor.

Table 1 also shows that morphine-3-glucuronide is more than 200 times less potent as compared to morphine and morphine-6-glucuronide. Statistical analysis revealed that there was no difference in potency of morphine-3-glucuronide

on hMORwt (EC_{50}: 34.4±8.1 μM), hMORN230T (EC_{50}: 23.6±6.3 μM) and hMORN230T (EC_{50}: 43.1±15.9 μM).

Discussion

In the present study we demonstrate the importance of the hydrophilic amino acid Asn230 in hMOR in the potency of various agonists on the cloned hMORwt, hMORN230T and hMORN230L receptors co-expressed with the GIRK1/GIRK2 channels and RGS4 in *Xenopus* oocytes. Therefore, Asn230 was mutated to Thr (hMORN230T) and to Leu (hMORN230L), because these residues are neutral and hydrophobic, respectively. The scaled hydrophobicity values of these amino acids are 0.236 for Asn, 0.450 for Thr and 0.943 for Leu (Black & Mould, 1991). These particular mutants were preferred because a Thr (position 211) and Leu (position 224) are present at the corresponding positions in the δ- and κ-opioid receptor, respectively (Lomize *et al.*, 1999). Position Asn230 is situated at the end of the second extracellular loop, near the fifth transmembrane helix (Figure 1) (Xu *et al.*, 2000).

The ligand-binding cavity is partially covered by the extracellular loops (EL). These loops, which have been reported to participate in the binding of opioid ligands, create an almost continuous surface made originating from the β-hairpin formed by EL-2 and the smaller EL-1 and EL-3. These loops appear to act as filters for the ligands, regulating the ability to interact within the core of the receptor (Law *et al.*, 1999). EL-2 also appears to be an absolute requirement for nociceptin-induced receptor activation (Mollereau *et al.*, 1999).

The significance of polar interactions involving Asp147 and His297 residues in the bottom of the binding pocket has been shown by site-directed mutagenesis by different groups (Befort *et al.*, 1996; Li *et al.*, 1999; Spivak *et al.*, 1997). These residues serve as 'attachment points' for the quaternary nitrogen and hydroxyl group, respectively, of the tyramine moiety common to most opioid ligands (Lomize *et al.*, 1999).

DAMGO is a μ-opioid selective peptidic ligand. Onogi *et al.* (1995) showed the importance of the first extracellular loop (EL-1) for the binding of DAMGO, by the use of μ/δ chimeras. Chimeras that include the first extracellular loop from the δ-opioid receptor lost the high-affinity binding for DAMGO (Wang *et al.*, 1995). A later mutational study suggested that Lys108 in EL-1 of the δ-opioid receptor prevents DAMGO from binding to the δ-opioid receptor (Fukuda *et al.*, 1995; Minami *et al.*, 1996). Studies with μ/κ chimeric receptors revealed the importance of the region around the third extracellular loop to distinguish between μ- and κ-opioid receptors for the high affinity binding of the μ-opioid receptor selective peptide ligand, DAMGO (Minami *et al.*, 1995). Seki *et al.* (1998) demonstrated that Lys303, Val316, Trp318 and His319 around the third extracellular loop of the μ-opioid receptor act as determinants for the discrimination between μ- and κ-opioid receptors by DAMGO. Results from point mutations relatively deep within the binding cavity (e.g. Tyr326), suggest that DAMGO not only interacts with the extracellular loops of the receptor, but likely extends within the binding cavity itself that is formed by the seven TM-helices (Mansour *et al.*, 1997).
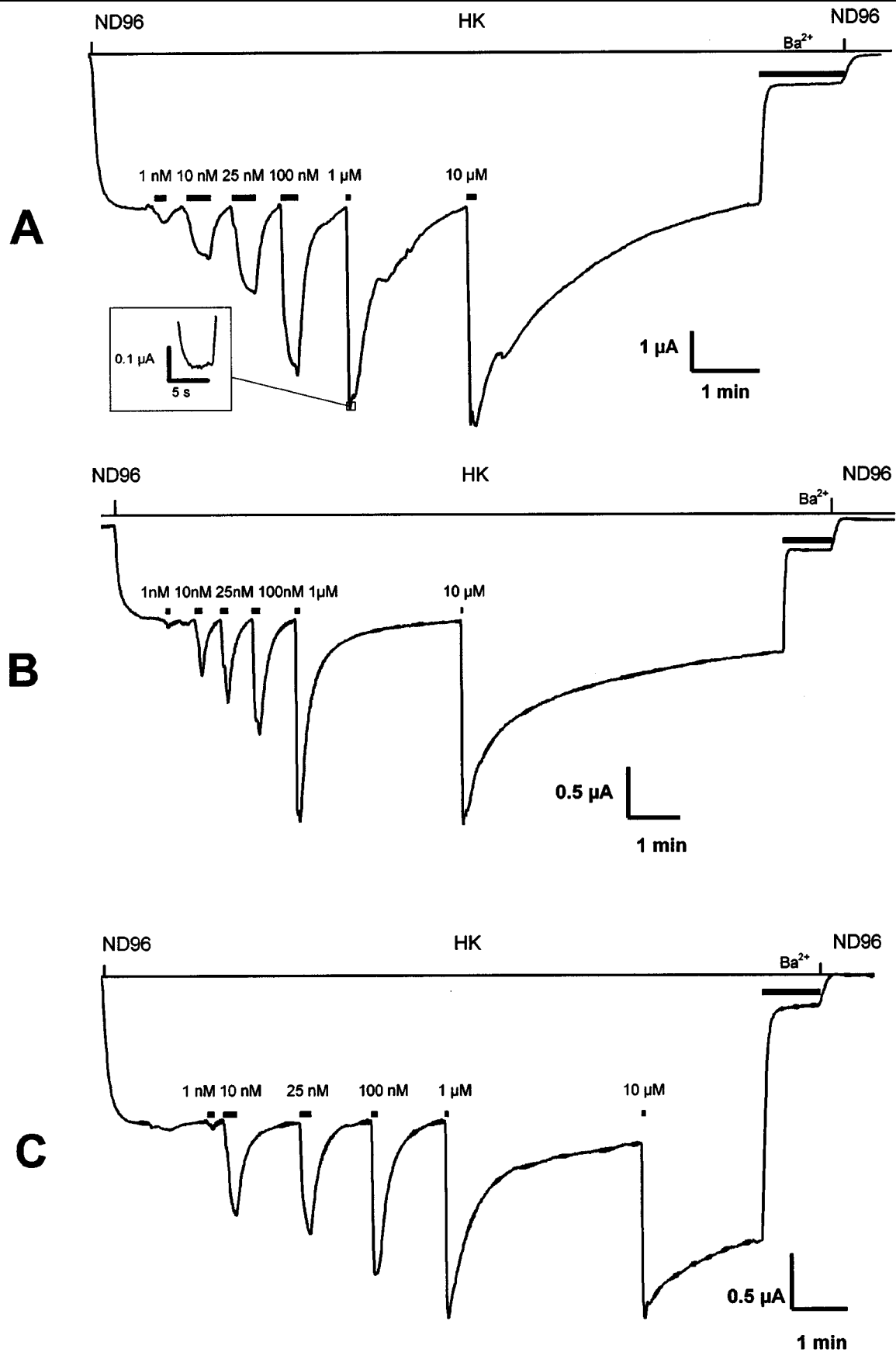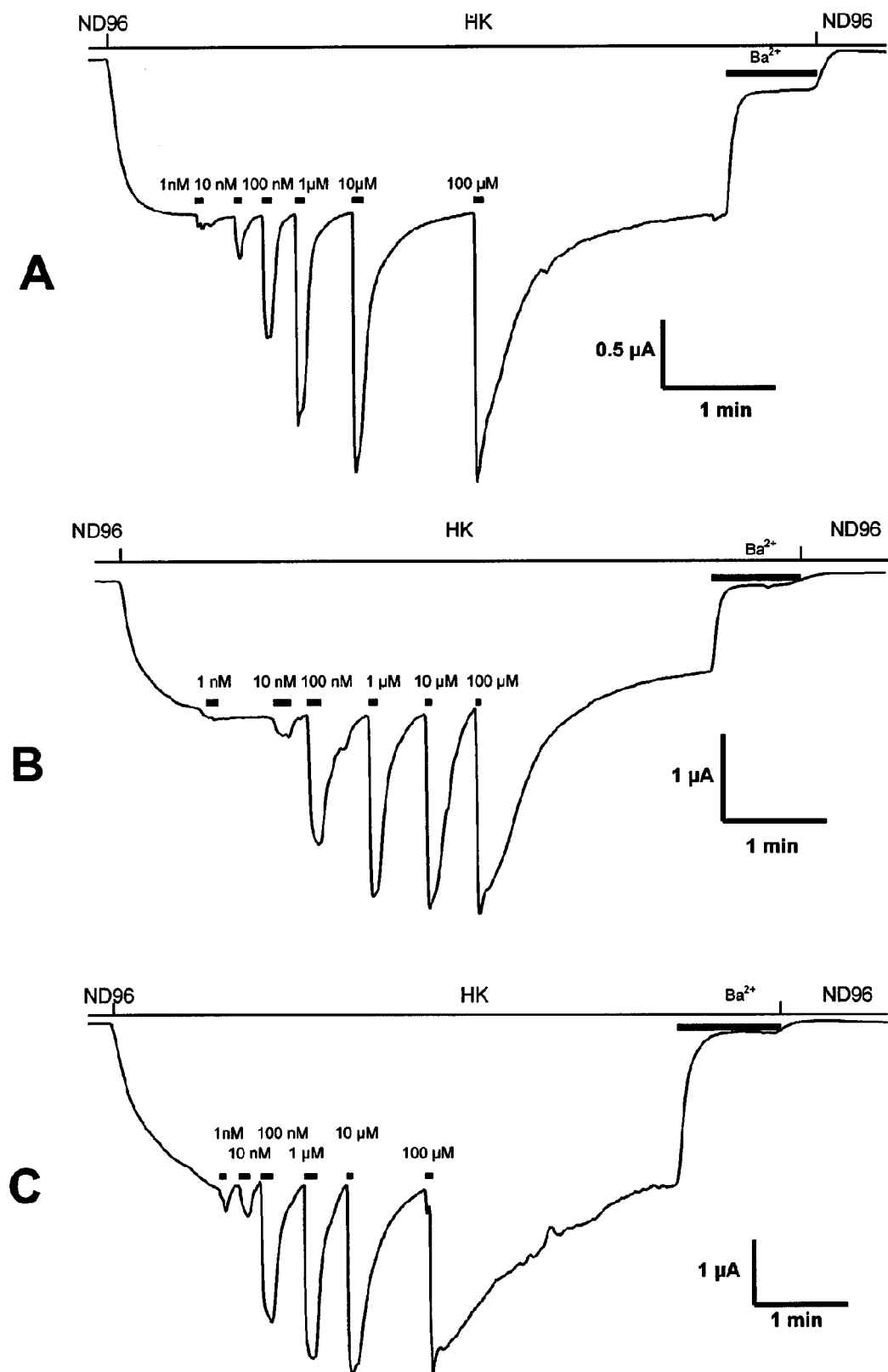
Figure 2 Representative current traces evoked from *Xenopus laevis* oocytes coexpressing GIRK1/GIRK2 channels and RGS4 with hMOR (A), hMORN230T (B) or hMORN230L (C). Agonist gated currents were evoked at −70 mV by application of increasing concentrations of fentanyl. The inset in A shows a trace on an expanded time scale to illustrate that steady state was reached.

Our results show that mutation of Asn230 in the second extracellular loop of the μ-opioid receptor did not affect the

EC_{50}-value, neither on hMORN230T mutant nor on hMORN230L. Obviously, this residue is not critical in the
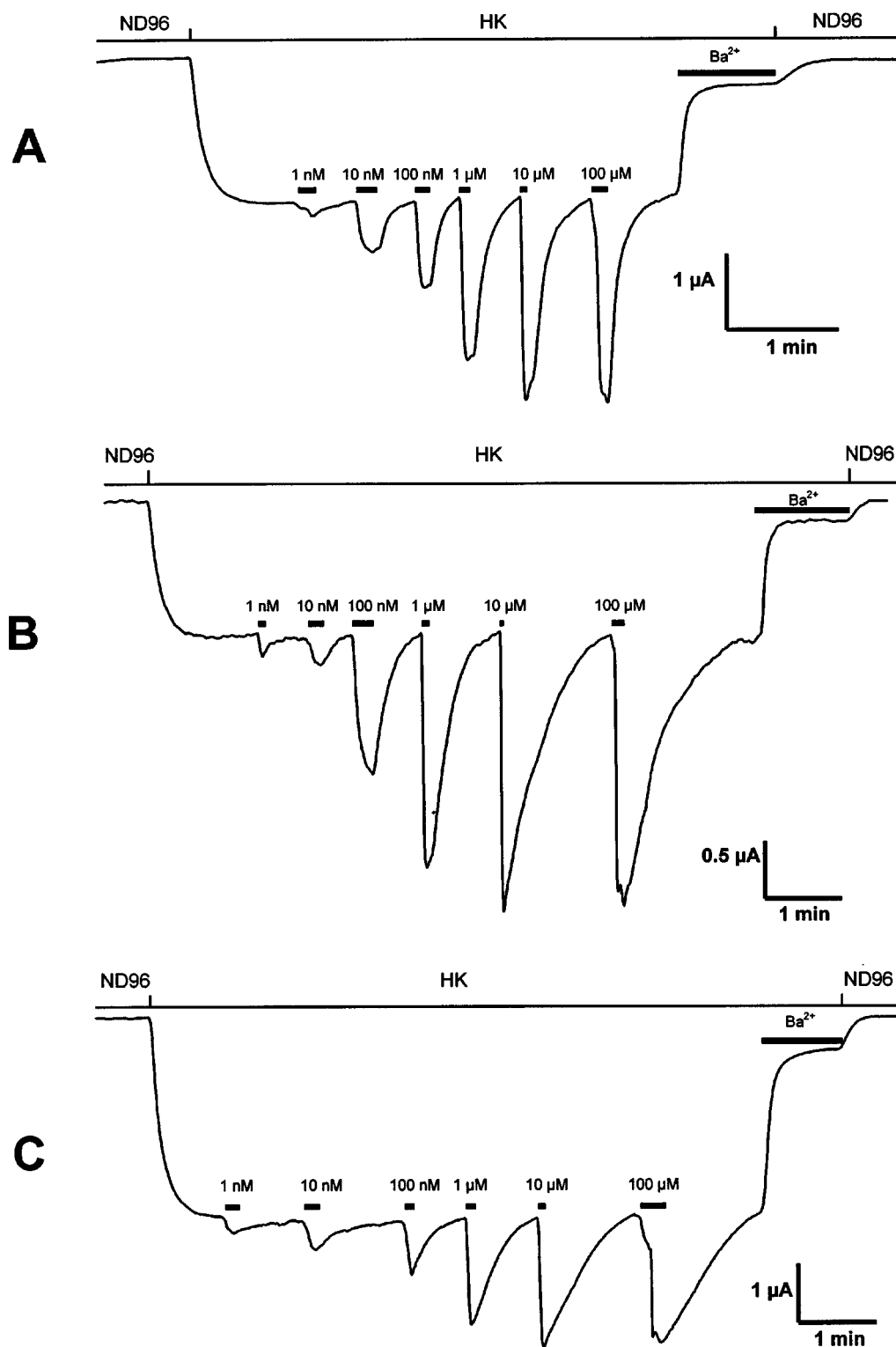

Figure 3 Representative current traces evoked from *Xenopus laevis* oocytes coexpressing GIRK1/GIRK2 channels and RGS4 with hMOR (A), hMORN230T (B) or hMORN230L (C). Agonist gated currents were evoked at -70 mV by application of increasing concentrations of morphine.

Figure 4 Representative current traces evoked from *Xenopus laevis* oocytes coexpressing GIRK1/GIRK2 channels and RGS4 with hMOR (A), hMORN230T (B) or hMORN230L (C). Agonist gated currents were evoked at -70 mV by application of increasing concentrations of morphine-6-glucuronide.

affinity of DAMGO towards the μ-opioid receptor. This result is in line with a chimeric opiate receptor study which showed that replacing the second extracellular loop of the μ-opioid receptor by the corresponding loop of the κ-opioid receptor did not affect the affinity of DAMGO (Wang *et al.*, 1994).

Nevertheless, these results can serve as a control for the functionality of the mutant receptors. It is therefore very likely that the mutations did not markedly alter the secondary and tertiary structures of the resulting mutants.
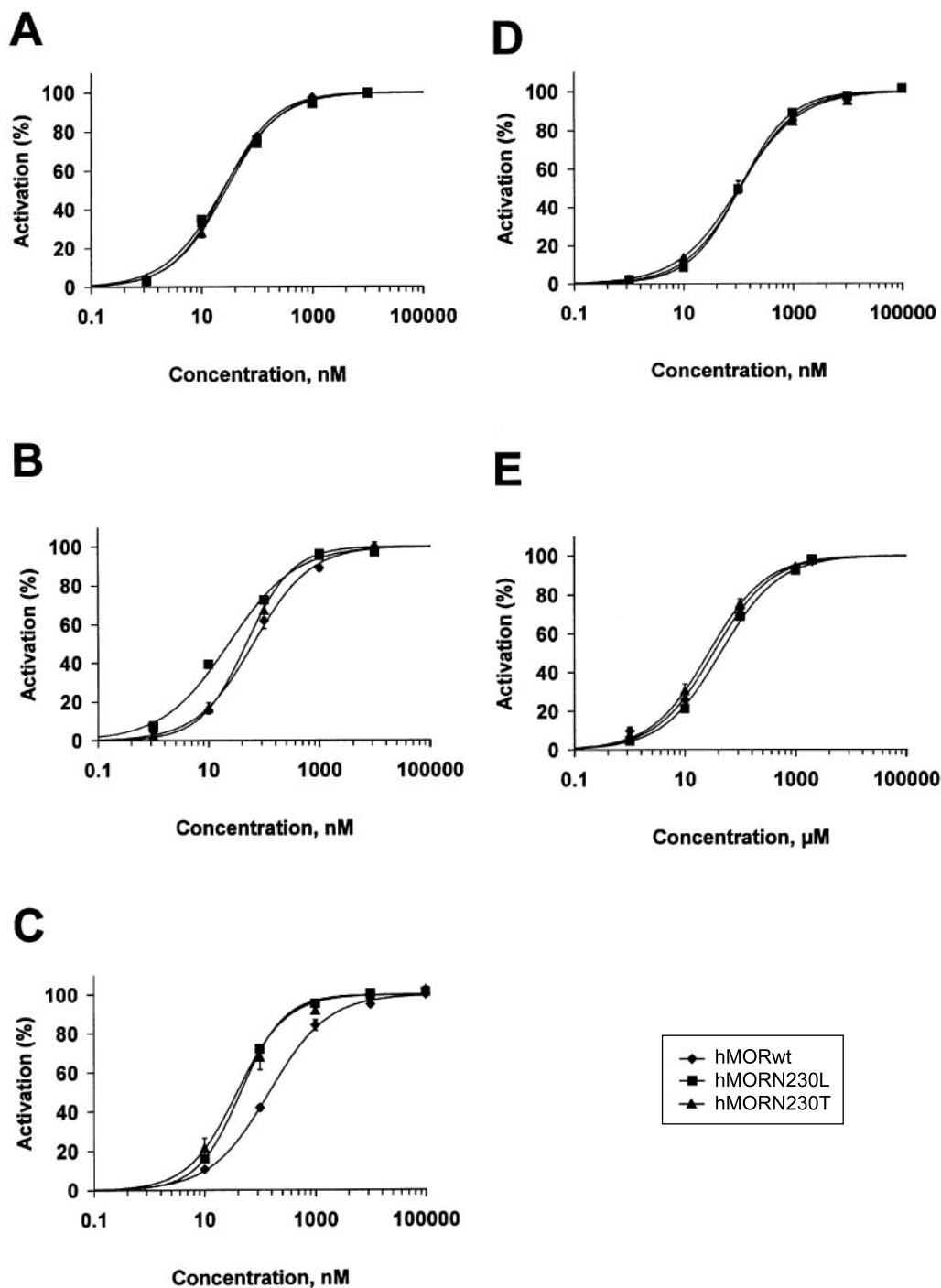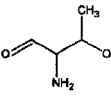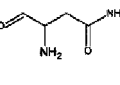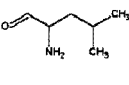
Figure 5 Concentration-response curves for GIRK1/GIRK2 channel activation by increasing concentrations of DAMGO (A), fentanyl (B), morphine (C), M3G (D) and M6G (E). Agonist-gated currents were evoked from *Xenopus laevis* oocytes coexpressing GIRK1/GIRK2 and RGS4 with hMORwt, hMORN230L or hMORN230T. The agonist-gated increase of GIRK current at each concentration was normalized to a maximal response of 100%. Each point represents the average current activation evoked from 5 to 8 oocytes (mean ± s.e.mean).

Fentanyl, a 4-anilidopiperidine, is a highly selective μ-opioid agonist. Substitution of Asn230 to Thr or Leu significantly decreased the EC_{50}-value of fentanyl. A gradual increase in affinity was seen as the amino acid in position 230 became more lipophilic, suggesting the importance of hydrophobic interactions between the receptor and fentanyl. Pogozheva *et al.* (1998) suggested in their receptor structure function study that the $COOCH_3$ group in position 4 of the piperidine ring of lofentanyl forms a H-bond with Lys233 and Asn230. In the case of fentanyl, its piperidine ring could possibly benefit from hydrophobic interactions with the mutant receptors hMORN230T and even more with the hMORN230L receptor, resulting in an increased affinity.

Table 1 EC_{50} values calculated for GIRK1/GIRK2 channel activation *via* hMORwt, hMORN230L or hMORN230T, coexpressed with RGS4 in *Xenopus* oocytes

Receptor	HMOR N230T	HMOR wt	HMOR N230L
Amino acid structure	Threonin (T)	Asparagin (N)	Leucine (L)
Ligand	EC50		
DAMGO	25.8 nM ± 4.7	25.6 nM ± 5.0	24.7 nM ± 2.1
Fentanyl	47.9 nM ± 3.1	64.9 nM ± 7.8	20.2 nM ± 1.9
Morphine	41.2 nM ± 10.7	148.6 nM ± 16.3	46.3 nM ± 1.3
Morphine-6-Glucuronide	97.1 nM ± 12.9	107.4 nM ± 10.8	106.3 nM ± 6.3
Morphine-3-Glucuronide	23.6 µM ± 6.3	34.4 µM ± 8.1	43.1 µM ± 15.9

Much to our surprise, the same tendency was seen with morphine, a rather small alkaloid agonist. The potency of this primary μ-agonist was increased 3.5 fold by mutating the wild type hMOR receptor to hMORN230T or hMORN230L, although a decreased potency was expected. The EC_{50}-values for both mutated receptors did not differ statistically. These findings are not in line with the models of Lomize *et al.* (1999) and Pogozheva *et al.* (1998), who emphasized the importance of the formation of a hydrogen bond between Asn230 and the 6-α-OH group of morphine. The recent alignment of morphine by McFadyen *et al.* (2000) is very similar to previous models, but places the 6-hydroxyl group of morphine outside the binding pocket and postulates that the hexene ring of morphine is surrounded with Lys233, Tyr148, Asn230 and Trp318. Lipophilic methylgroups in the mutated receptors could interact *via* hydrophobic interactions with the hexene ring of morphine and enhance the potency. The finding that Lys233 is involved in covalent bond formation with the fumarate group of β-funaltrexamine provides additional information to determine the site of interaction with Asn230 (Chen *et al.*, 1996).

Based on computer modelling manipulations, yet another model was hypothesized in which Tyr326, in TM-VII, would lie adjacent to the aliphatic alcohol of morphine (Mansour *et al.*, 1997). Furthermore it was suggested in this model that the change of Tyr326 to Phe, thereby removing the possible hydrogen bonding site, decreased the affinity for morphine, as

well as for DAMGO and fentanyl. According to the models of Pogozheva *et al.* (1998) and Subramanian *et al.* (2000) this residue lies in the vicinity of residues that interact with the piperidine ring of the tyramine moiety, like the conserved Asp147.

We suggest that our mutated receptors form hydrophobic interactions with the hexene ring of morphine, which is oriented approximately perpendicular to the tyramine and directed toward the β-hairpin formed by EL-2 (Lomize *et al.*, 1999), and consequently can explain the increase in potency for morphine. In this view, it is highly unlikely that Asn230 forms a hydrogen bond with the 6α-OH group of morphine and that this residue serves as a determinant for the preferred binding of morphinans to the μ receptor as proposed by Pogozheva *et al.* (1998).

Morphine-6-glucuronide activates GIRK1/GIRK2 channels through the wild type μ-opioid receptor with a significantly higher potency than morphine. Analogous results for both ligands were obtained by Ulens *et al.* (2001) who also showed that the potency profile changed from $\mu > \delta = \kappa$ for morphine to $\mu > \kappa > \delta$ for morphine-6-glucuronide. According to McFadyen *et al.* (2000) the 6-hydroxyl group lies outside the binding pocket, so the glucuronide in morphine-6-glucuronide can also lie outside the binding pocket. The enhanced potency could be due to favourable interactions between the hydrophilic glucuronide moiety and residues in the EL-2, EL-3 or in the aminoterminus of the receptor, which was shown to contribute in the affinity for fentanyl, methadone, morphine and other ligands (Chaturvedi *et al.*, 2000). We observed no difference in EC_{50}-value for morphine-6-glucuronide between the mutated receptors and the wild type μ-opioid receptor. Taken together, glucuronidation of the 6-hydroxyl group enhances the potency *via* the wild type hMOR receptor but abolishes the increased potency of morphine *via* the mutated hMORN230T and hMORN230L receptor. The precise reasons for these disparate findings are not clear at present and further investigations are needed to elucidate the responsible residues and interactions.

Our electrophysiological EC_{50}-values for morphine-3-glucuronide, another metabolite of morphine, are situated in the micromolar range, resulting in a 200 fold reduction in potency as compared with morphine on the wild type receptor. This rather big decrease in potency can be explained using the classical alignment of morphine that places the quaternary nitrogen of morphine close enough to Asp147 and the phenolic hydroxyl (3-hydroxyl) group close to His297, rather deep in the binding pocket (McFadyen *et al.*, 2000; Lomize *et al.*, 1999). Substitution of the hydroxyl group at C3 of morphine with a glucuronide moiety disturbs the interaction with these key interaction points. Recently, a revised pharmacophore model was presented by McFadyen *et al.* (2000) where the only required moieties are a quaternary amine nitrogen and a relatively planar, electron-rich region, commonly a phenyl ring. A para-hydroxyl substituent contributes to high-affinity binding but is not critical. The design of 3-deoxyclocinnamox, a high affinity μ-opioid antagonist lacking a phenolic hydroxyl group, confirmed this proposition (Derrick *et al.*, 2000). Morphine-3-glucuronide showed no difference in the affinity for μ-, κ- and δ-receptors (Ulens *et al.*, 2001). Our mutated receptors also displayed the same potency for morphine-3-glucuronide.

In conclusion, the present study sheds new light on the role of Asn230 in the potency of various agonists. Mutation of this residue to the more hydrophobic threonine and leucine resulted in an increased potency for fentanyl and morphine, while the potency of DAMGO, M6G and M3G remained unaffected. Possible hydrophobic interactions between this residue and the piperidine and hexene ring of fentanyl and morphine, respectively, can explain these findings.

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