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Morphine-6β-glucuronide and morphine-3-glucuronide, opioid receptor agonists with different potencies

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Abstract

Using heterologous expression in *Xenopus laevis* oocytes, we compared the potencies of morphine, morphine-6β-glucuronide (M6G), and morphine-3-glucuronide (M3G) for cloned human μ- (hMOR), κ- (hKOR), and δ-opioid receptors (hDOR). Each receptor subtype was individually co-expressed with heteromultimeric G-protein coupled inwardly rectifying K^+ (GIRK) channels, consisting of GIRK1 and GIRK2 subunits, and RGS4, a regulator of G-protein signaling. The two-microelectrode voltage clamp technique was used to measure the opioid receptor-activated GIRK1/GIRK2 channel responses. Compared with morphine, M6G had higher potency at the hMOR, lower potency at the hKOR, and similar potency at the hDOR, while M3G showed a 1000-fold lower and non-selective potency via opioid receptors. In contrast to naloxone, M3G did not antagonize the effects of morphine at the hMOR. We also investigated whether Trp318 and His319 provide the molecular basis for μ/δ selectivity and μ/κ selectivity of morphinan alkaloids by mutating these residues to their corresponding residues in κ- and δ-opioid receptors. A single-point mutation (W318L) on hMOR completely conferred δ-like potency for morphine and M6G on the mutant μ-receptor. Double mutation at Trp318 and His319 positions (Trp318Y/His319Y) only partially conferred κ-like potency for morphine and M6G; the decrease in potency for M6G was significantly larger than for morphine. The results of our study show that both M6G and M3G are opioid receptor agonists with different potencies and that the potency of morphinan receptor ligands can be changed by selective mutations of hMOR at the Trp318 and His319 positions.

Keywords: Morphine; M6G; M3G; K channel; Opioid receptors; RGS

1. Introduction

Morphine is widely used in clinical practice. The metabolism of morphine *in vivo* at C3 and C6 produces two glucuronides: M3G and M6G [1]. The hydroxyl groups of morphine affect the binding to the opioid-receptor subtypes very differently. Binding profiles of morphine and its glucuronides to opioid receptors have been characterized [2–4]. It has been demonstrated that the free phenolic hydroxyl group is an essential requirement for opioid receptor binding [5–7], while the chemical substitution at the 6-position could affect the receptor selectivity of morphine and related compounds [8].

Quantitative and qualitative differences in the binding of morphine and morphine glucuronides have been identified [9]. Glucuronidation at the 6-hydroxyl group did not change the affinity toward μ-opioid receptors, but increased the affinity at δ receptors and decreased the affinity at κ receptors. Masking of the 3-hydroxyl group decreased the selectivity toward opioid receptors [8]. Comparison of morphine with M6G showed no difference in binding to μ_1 and μ_2 receptors [9]. The affinity of M6G for the δ receptor was 1.2 to 1.9 times that of morphine [6,10]. M3G was reported to be poorly bound to opioid receptors [10–12], and the lower binding of M3G to opioid receptors in the brain may have been due to residual contamination of M3G with morphine [12]. The binding of M6G to opioid receptors is more complex. Löser *et al.* [12] suggested that M6G binds to two binding sites for agonists at the μ-opioid receptor. Another M6G-specific binding site in cerebral membranes was proposed [13–15]. The antisense mapping studies have dem-

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Abbreviations: M3G, morphine-3-glucuronide; M6G, morphine-6β-glucuronide; MOR, μ-opioid receptor; KOR, κ-opioid receptor; DOR, δ-opioid receptor; GIRK channel, G-protein coupled inwardly rectifying K^+ channel; RGS, regulator of G-protein signaling; GAP, GTPase-activating protein; TM, transmembrane domain, and HK, high potassium.

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onstrated differences between the M6G site and the two μ receptor subtypes in their regional distribution in the brain [16].

Identification of clones encoding δ- [17,18], μ-, and κ- [19–22] opioid receptors has provided opportunities to study their pharmacology. Rossi *et al.* [23] demonstrated the role of cloned μ-opioid receptors in morphine pharmacology. Cloned opioid receptors μ (MOR-1) [23], κ (KOR-1) [24], and δ (DOR-1) [25] are functionally active when transfected into cells lacking opioid receptors [16]. In this study, cloned human μ-, κ-, and δ-opioid receptors, co-expressed with GIRK1/GIRK2 and RGS4 in *Xenopus* oocytes, were used as an experimental model, as previously described [26,27]. GIRK channels, consisting of GIRK1 and GIRK2 subunits, mimic the probable heteromultimeric state of native neuronal GIRK channels [28] and represent important effectors by which opioids exert their actions at the cellular level [29,30]. Signaling via the G-protein-mediated pathway is regulated by a recently identified gene family, known to encode RGS proteins [31]. These regulators act as GAPs, which resolve the existing discrepancy for GIRK channel gating kinetics when co-expressed in a heterologous expression system. Co-expression of the RGS4 protein, which is highly expressed in the brain, strongly accelerates GIRK deactivation, thereby reconstituting the native gating kinetics [32].

Recent molecular modeling of opioid receptors has provided new information on key residues contributing to high-binding affinity of opioid ligands, including morphinan alkaloids [33]. Compared to κ- and δ-opioid receptors, key residues involved in favorable interactions are identical to those in the μ-opioid receptor model with the exception of Trp318 (TMVII) and His319 (TMVII). In the δ-opioid receptor, Trp318 is replaced by Leu300 at the corresponding position and the favorable interaction is lost. In the κ-opioid receptor, Trp318 and His319 are both replaced by a Tyr residue (Tyr312 and Tyr313). In our study, we addressed the question of whether Trp318 and His319 could provide the molecular basis for μ/δ selectivity and μ/κ selectivity of morphinan alkaloids. For this reason, we mutated Trp318 and His319 to the residues at the corresponding positions in the κ- and δ-opioid receptors, respectively, and determined the EC_{50} values for GIRK1/GIRK2 channel activation through mutant and wild-type opioid receptors, co-expressed with GIRK1/GIRK2 channels and RGS4 in *Xenopus laevis* oocytes.

2. Materials and methods

2.1. Subcloning and in vitro transcription of cDNA clones encoding GIRK1/2 channels, human μ-, κ-, and δ-opioid receptors, mutant μ-opioid receptors, and RGS4

Plasmids containing the entire coding sequence for the mouse GIRK1 and the mouse GIRK2 channel were subcloned into the vectors pSP35T and pBScMXT, respectively, and designated as pSP/GIRK1 [34] and pBScMXT/GIRK2 [28]. The polylinker in each of these vectors is flanked by *Xenopus* globin 5′ and 3′ untranslated regions, resulting in enhanced protein expression after injection of *in vitro* transcribed cRNA [35]. For *in vitro* transcription, plasmids were first linearized with either *Eco*RI (for pSP/GIRK1) or *Sal*I (for pBScMXT/GIRK2). Next, the cRNAs were synthesized from the linearized plasmids using the Riboprobe combination system (Promega) with SP6 RNA polymerase (for pSP/GIRK1) or T3 RNA polymerase (for pBScMXT/GIRK2) in the presence of a cap analogue diguanosine triphosphate (Boehringer).

The human MOR [36], KOR [37], DOR [38], and rat RGS4 [32] cDNA clones in their original vector, pcDNA3.1(+) (Invitrogen) in the case of MOR, DOR, and RGS4, and pBK-CMV (Stratagene) in the case of KOR, were first subcloned into our custom-made high-expression vector pGEMHE [39]. The cDNAs encoding hMOR, hKOR, and RGS4 were isolated by a double restriction digest with *Hin*dIII, *Xma*I, and *Bam*HI + *Xba*I, respectively. In the case of hDOR, a unique *Xba*I restriction site was introduced in the 3′ untranslated region using the GeneEditor *in vitro* site-directed mutagenesis system (Promega). The cDNA encoding hDOR was subsequently isolated by a double restriction digest with *Bam*HI + *Xba*I. cDNAs were then run on an agarose gel, fragments of interest were cut out, purified using a QIAEX Kit (QIAGEN), and ligated with T4 DNA ligase (Promega), into the corresponding restriction sites of pGEMHE. For *in vitro* transcription, each ligation product, hMOR/pGEMHE, hKOR/pGEMHE, hDOR/pGEMHE, and RGS4/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).

Trp318 and His319 in hMOR were mutated to the corresponding residues in hKOR (Tyr312 and Tyr313, respectively) and hDOR (Leu300 and His301, respectively) as previously described [27]. For *in vitro* transcription, each mutant, hMORW318YH319Y/pGEMHE and hMORW318L/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.

2.2. Experimental model

X. laevis oocytes were isolated as previously described [39]. Oocytes were co-injected with 0.5 ng/50 nL of GIRK1, 0.5 ng/50 nL of GIRK2, and 10 ng/50 nL of RGS4 cRNA, with the addition of 10 ng/50 nL of either hMOR, hKOR, hDOR, hMORW318L, or hMORW318Y/H319Y cRNA. Injected oocytes were maintained in ND-96 solution (composition: 2 mM KCl, 96 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 50 μg/mL of gentamicin sulfate.

2.3. Electrophysiological recordings

Whole-cell currents from oocytes were recorded from 1 to 2 days after injection using the two-microelectrode voltage clamp technique (GeneClamp 500, Axon Instruments). Resistances of voltage and current electrodes were kept as low as possible (approx. 200 kΩ) and were filled with 3 M KCl. Currents were filtered at 10 or 200 Hz, depending on the protocol, using a 4-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath-grounding electrode, the bath potential was actively controlled. All experiments were performed at room temperature [19–23°]. At the start and the end of each experiment, oocytes were superfused with low-potassium (ND-96) solution (composition: 2 mM KCl, 96 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). During application of increasing concentrations of ligands, oocytes were superfused with high-potassium (HK) solution (composition: 96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 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) was used to ensure rapid solution exchanges. Analysis of uninjected cells (N = 3), under the same experimental conditions as injected oocytes, revealed an endogenous current that mounted maximally 1% as compared with the current measured in injected oocytes. 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 7 min and superfused with ND-96 solution. Next, the superfusion solution was switched from ND-96 to HK solution, after which increasing concentrations of morphine or glucuronide 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 it with an HK solution. During this washout period, the channels return to the control current level as a result of a deactivation process that is accelerated dramatically in the presence of RGS4, as previously described [26]. At the end of each experiment, the oocyte was superfused with HK solution containing 300 μM $BaCl_2$, causing a blockage of the net GIRK1/2-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 this study, the expression system was standardized as previously described [27].

2.4. Data analysis

The pCLAMP program (Axon Instruments) was used for data acquisition, and data files were directly imported, analyzed, and visualized with a custom-made add-in for Microsoft Excel. The percentage-activated current was calculated using the equation

$$\text{Percentage activation} = \frac{\text{activated current amplitude}}{\text{control current amplitude}} \times 100 - 100$$

and 0% was taken as the control current level. Current percentages were then used for the calculation of concentration–response curves, 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 means ± SEM and were calculated using N experiments, where N indicates the number of oocytes tested. For each experiment, averaged current percentages were normalized to 100%, and an averaged concentration–response curve was drawn using the average EC_{50} values and Hill coefficients of N experiments. 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. SEM of quotients were calculated by taking the square root of the sum of the squares of the relative SEM, multiplied by the quotient of the means [40].

2.5. Compounds

Morphine HCl (Federa), M6G (Sigma), and M3G (Sigma) were dissolved in HK solution, stored at 5°, and diluted appropriately for the experiments.

2.6. Purity analysis of M3G

To verify possible contamination of the M3G with morphine, the purity of the sample obtained from Sigma was verified by means of mass-spectrometric analysis. The apparatus used was an Agilent 6890 Plus (Agilent), equipped with a 5973 Network mass-specific detector, a micro-electron capture detector, a flame ionization detector, and an electronic pneumatic control. The applied column was an HP-5MS (Agilent) capillary column (30.0 m × 0.250 mm i.d., 0.25 μm film thickness –5% phenyl methyl siloxane). The carrier gas was helium, applied at a flow rate of 1 mL/min. The oven temperature was programmed as follows: 50° for 1 min, then 35°/min up to 100°, followed by a temperature change of 10°/min up to 270°. This temperature was held until the end of the run (39.43 min). The electronic pneumatic control was programmed to ensure a constant flow. The injection port was heated up to 250°, and injection occurred splitless. The ionization energy mounted 70 eV. A solution of 5 mg/mL of M3G in MeOH was prepared, and 200 μL of the supernatant was diluted with 1

mL MeOH. One microliter of this solution was injected to screen for morphine, and 1 μL of a solution alkalized with concentrated ammonium was injected to screen for morphine salts. No morphine contaminants could be detected under either of these conditions.

3. Results

3.1. Pharmacological profiles of morphine, M6G, and M3G on cloned human μ-, κ-, and δ-opioid receptors

Using heterologous expression in *X. laevis* oocytes, we compared the potencies of morphine, M6G, and M3G for hMOR, hKOR, and hDOR. Each receptor subtype was individually co-expressed with GIRK1/GIRK2 channels and RGS4, mimicking the native neuronal G-protein-mediated pathway of K^+ channel activation. Functional expression of hMOR, hKOR, and hDOR was confirmed by the electrophysiological measurement of an agonist-gated increase of the K^+ conductance upon application of an opioid receptor subtype-selective agonist (100 nM DAMGO, U50488-H, and DPDPE, respectively [27]). The two-microelectrode voltage clamp technique was used 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. Representative traces of agonist-gated currents, evoked from oocytes expressing either hMOR, hKOR, or hDOR, are shown for morphine in Fig. 1, panels A–C, respectively. For comparison, representative experiments with M6G on each receptor subtype are shown in Fig. 2, panels A–C. Representative traces of M3G-gated currents, evoked from oocytes expressing either hMOR, hKOR, or hDOR are shown in Fig. 3, panels A–C, respectively. M6G and morphine evoked K^+ currents at concentrations from 1 nM to 100 μM, while M3G showed agonistic effects only at concentrations of 1 μM and higher. To confirm that the observed current activation upon application of micromolar concentrations of M3G is mediated through an opioid-receptor pathway, we applied the same concentrations to oocytes expressing GIRK1/GIRK2 channels without an opioid receptor. Under these experimental conditions, no current activation could be observed (data not shown). In addition, application of the same concentrations of M3G to uninjected oocytes did not evoke a current activation. Moreover, the M3G-evoked increase of the opioid-mediated GIRK1/GIRK2 conductance was antagonized by naloxone (Fig. 3D).

Concentration–response relationships, drawn using the average EC_{50} values and Hill coefficients of 4–5 experiments, are shown for morphine (Fig. 4A), M6G (Fig. 4B), and M3G (Fig. 4C). These results show that M6G activates GIRK1/GIRK2 channels via hMOR with a significantly higher potency than morphine. In contrast, the potency of M6G via hKOR was approximately 5-fold lower than that of morphine; M6G and morphine had similar potencies via hDOR. As can be clearly seen in Fig. 4, glucuronidation of the 6α-OH group of morphine changes its potency profile from $\mu > \delta = \kappa$ (Fig. 4A, morphine) to $\mu > \delta > \kappa$ (Fig. 4B, M6G). As compared with morphine, M3G showed a 1000-fold lower and non-selective potency via opioid receptors (Fig. 4C).

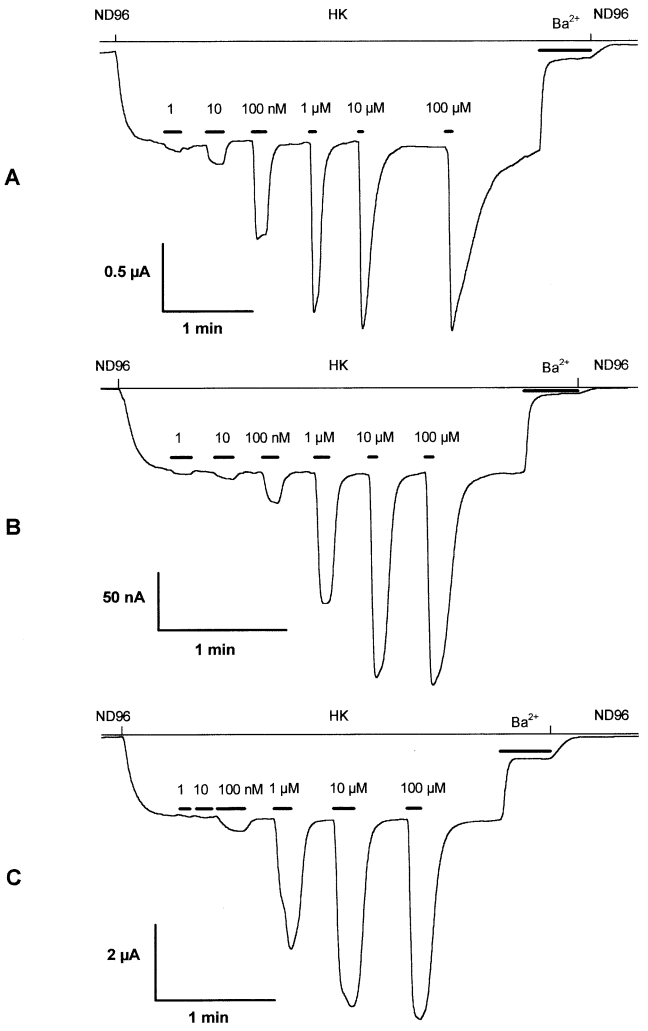

Fig. 1. Representative traces of agonist-gated K^+ currents evoked by morphine from oocytes expressing hMOR (A), hKOR (B), and hDOR (C). The two-microelectrode voltage clamp technique was used to measure the opioid receptor-activated GIRK1/GIRK2 channel response as the increase of the inward K^+ current at –70 mV, evoked by the application of increasing concentrations of opioid ligands.

Table 1 summarizes EC_{50} values calculated for morphine, M6G, and M3G on hMOR, hKOR, and hDOR. The μ/δ selectivity and μ/κ selectivity were calculated as the ratio of $\frac{EC_{50}\ hDOR}{EC_{50}\ hMOR}$ and $\frac{EC_{50}\ hKOR}{EC_{50}\ hMOR}$, respectively [41]. Results shown in Table 1 demonstrate that μ/δ selectivity values for morphine and M6G were not statistically different. However, values calculated for μ/κ selectivity show that M6G (41.5 ± 5.2) was more μ/κ selective than morphine (3.9 ± 0.9).

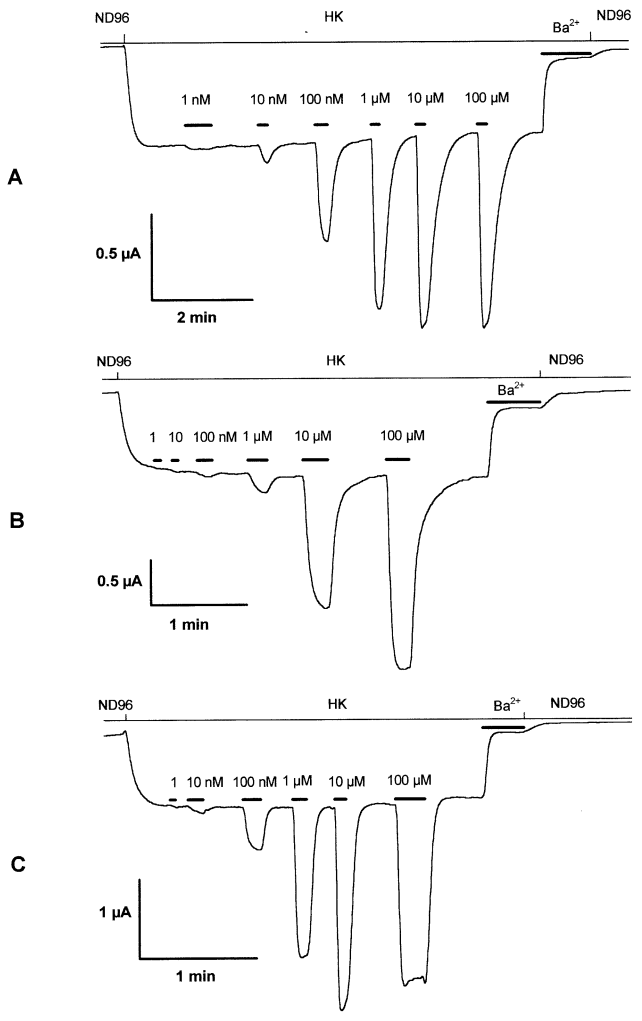

Fig. 2. Representative traces of agonist-gated K^+ currents evoked by M6G from oocytes expressing hMOR (A), hKOR (B), and hDOR (C).

The question of relative efficacies of morphine versus M6G at each receptor subtype was addressed by alternately applying maximally effective concentrations of each drug evoked to the same oocyte, expressing either hMOR, hKOR, or hDOR with GIRK1/GIRK2 channels and RGS4. For each experiment, currents were normalized to the morphine-evoked current set at 100%. Compared with morphine, average current percentages evoked by M6G were 102 ± 3 (N = 6) at hMOR, 100 ± 3 (N = 6) at hKOR, and 104 ± 3 (N = 6) at hDOR. These results suggest that morphine and M6G have identical efficacies at each receptor subtype.

3.2. Affinities of morphine and M6G on hMORW318L and hMORW318Y/H319Y

To investigate the role of favorable interactions of morphinan alkaloids with Trp318 and His319 in hMOR, we mutated Trp318 to the residue at the corresponding position in hDOR (Leu300). The adjacent His already is present in hDOR (His301). For hMORW318Y/H319Y, a double mutant, Trp318 and His319, were mutated simultaneously to the residues at the corresponding positions in hKOR (Tyr312 and Tyr313, respectively). Each mutant receptor was individually co-expressed with GIRK1/GIRK2 channels and RGS4 in *X. laevis* oocytes. The potencies of morphine and M6G were compared in the same way as for the wild-type receptors. Representative traces of agonist-gated currents, evoked from oocytes expressing either hMORW318L or hMORW318Y/H319Y were evoked under identical conditions as with wild-type opioid receptors for morphine and M6G. For clarification, EC_{50} values on hMOR, hKOR versus hMORW318Y/H319Y and hDOR versus hMORW318L, are compared in the same bar diagram for morphine (Fig. 5A) and M6G (Fig. 5B). Calculated EC_{50} values of each ligand for hMORW318Y/H319Y and hMORW318L are summarized in Table 2. The potency of the ligands via the mutant receptors was compared to the potency via the wild-type (WT) hMOR by calculation of the ratio $\frac{EC_{50}\ \text{MUTANT}}{EC_{50}\ \text{hMOR}}$, yielding a μ/mutant selectivity. To evaluate the efficiency of the mutation to convert μ-potency to its κ-potency (W318Y/H318Y) or δ-potency (W318L), the 'mutation efficiency' was calculated as the percentage $\frac{EC_{50}\ \text{W318Y/H319Y}}{EC_{50}\ \text{hKOR}} \times 100$ or $\frac{EC_{50}\ \text{W318L}}{EC_{50}\ \text{hDOR}} \times 100$ (Table2).

Results shown in Table 2 show that the W318Y/H319Y mutation causes a significantly larger decrease in potency for M6G (5.88 ± 0.65) than for morphine (1.45 ± 0.35). Nevertheless, as compared with wild-type hKOR EC_{50} values, the W318Y/H319Y mutation resulted in better efficiency for morphine (37 ± 7%) than for M6G (14 ± 2%). Thus, a double $\mu \rightarrow \kappa$ mutation only partially confers κ-like potency for morphine and M6G on the mutant μ-opioid receptor.

The W318L mutation (Table 2) caused a significantly larger decrease in potency for M6G (13.56 ± 0.74) than for morphine (3.76 ± 0.76). As compared with the wild-type hDOR EC_{50} values, the W318L mutation showed a complete mutation efficiency (114 ± 25%). A single $\mu \rightarrow \delta$ point mutation confers δ-like potency for morphine on the mutant μ-opioid receptor; the potencies of morphine via hMORW318L (774 ± 56 nM, Table 2) and hDOR (678 ± 142 nM, Table 1) were not statistically different. The W318L mutation efficiency, calculated for M6G (216 ± 62%), demonstrates that a single $\mu \rightarrow \delta$ point mutation increases the EC_{50} value of M6G for hMORW318L (1438 ± 30 nM) to a level significantly higher than the EC_{50} value of M6G for hDOR (665 ± 188 nM, Table 1).

3.3. Investigation of M3G antagonism at the cloned μ-opioid receptor

The effects of M3G on the morphine-gated increase of the GIRK1/GIRK2 channel conductance were investigated

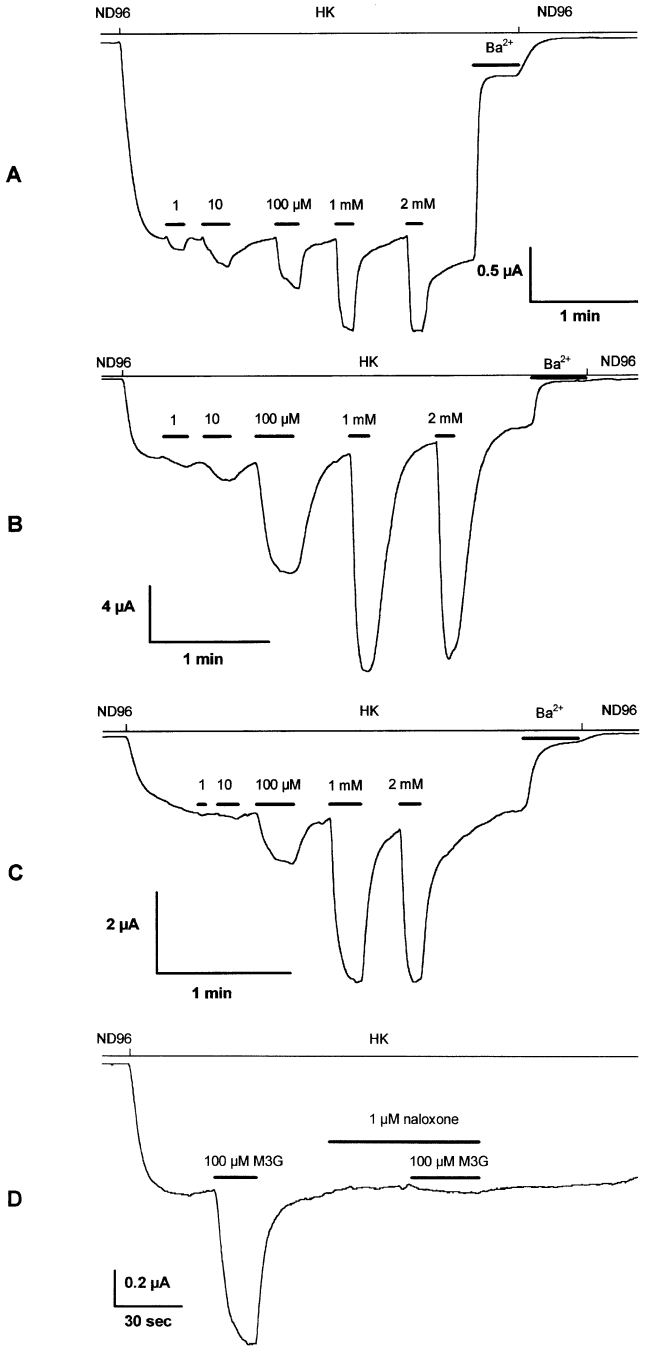

Fig. 3. Representative traces of agonist-gated K^+ currents evoked by M3G from oocytes expressing hMOR (A), hKOR (B), and hDOR (C). Antagonistic effect of naloxone on the M3G-mediated activation of GIRK channels in oocytes expressing hMOR (D).

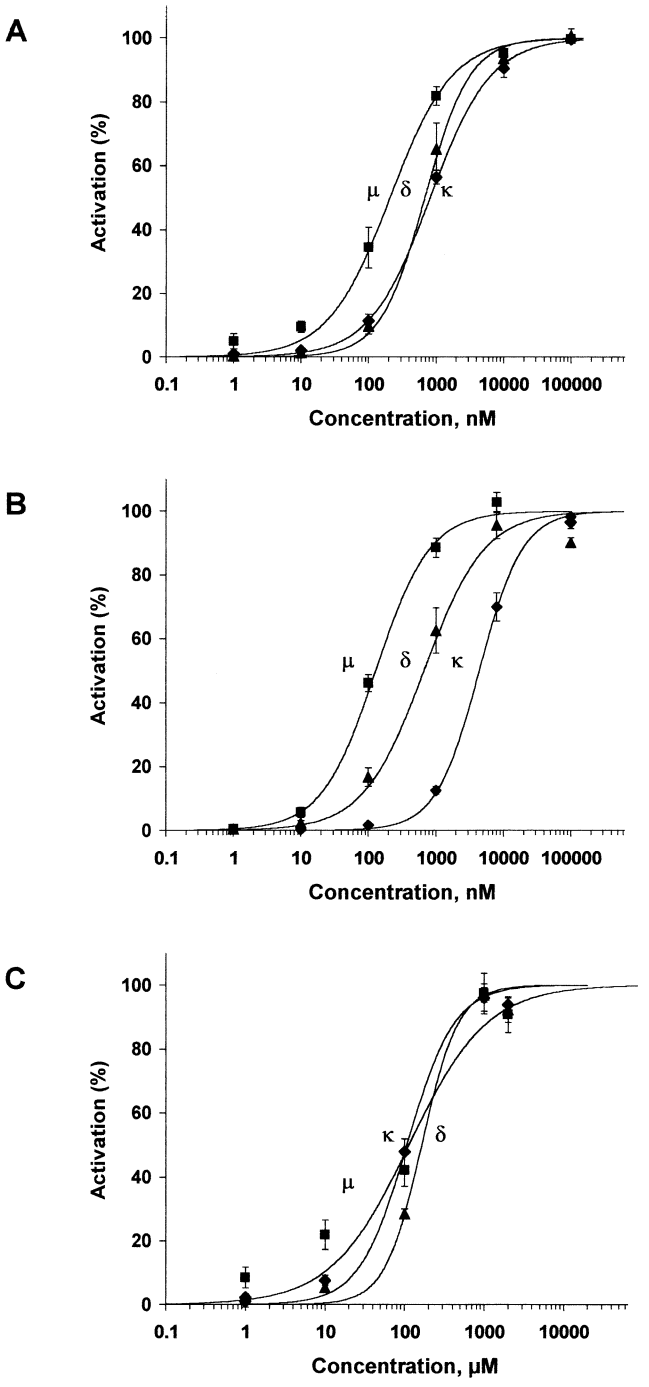

Fig. 4. Concentration–response relationships for morphine (A), M6G (B), and M3G (C) on hMOR, hDOR, and hKOR. Values are means ± SEM, N = 4–6.

in *X. laevis* oocytes, co-expressing hMOR, GIRK1/GIRK2 channels, and RGS4. Representative traces evoked using morphine in combination with either M3G or naloxone are illustrated in Fig. 6. M3G at 100 nM, in contrast to 1 μM naloxone, did not antagonize the effect of morphine. Similar results were obtained in four other experiments. Lower concentrations of M3G (1 and 10 nM) also did not show antagonistic properties.

4. Discussion

Studies exploring binding of morphine and its glucuronides to opioid receptors have provided inconsistent findings on the binding profiles of these two metabolites. The variability of the results may be due to the differences in the experimental models ranging from brain homogenates from

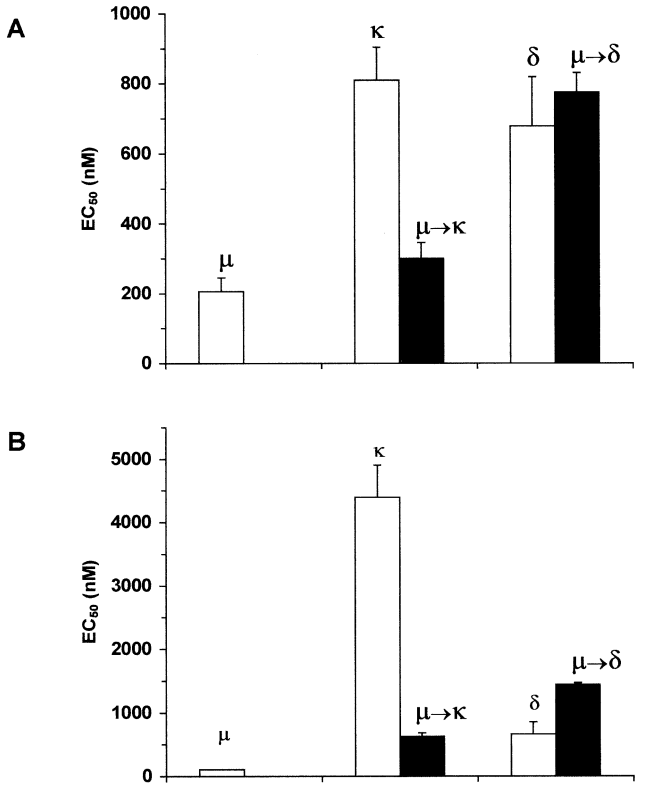

Fig. 5. Effects of hMORW318L and hMORW318Y/H319Y mutations on the affinity of morphine (A) and M6G (B) to opioid receptors hMOR (μ), hDOR (δ), and hKOR (κ). Open bars represent wild-type receptors, and filled bars represent mutant receptors. Values are means ± SEM, N = 4–6.

different species to cell membrane preparations from *in vitro* cell cultures.

Structure-related studies investigating specific ligand binding to guinea pig brain homogenates were performed by Mignat *et al.* [8]. It was shown that the substitution of the 6-hydroxyl group of morphine did not affect the affinity to μ receptors, slightly increased the affinity for δ receptors, and reduced the affinity for κ receptors. Furthermore, the investigators reported that the substitution of the phenolic group (C3) of morphine caused a decrease in binding at opioid receptors without affecting the μ/δ or μ/κ ratio. Binding experiments on whole undifferentiated SH-SY5Y human neuroblastoma cell suspensions, expressing both μ- and δ-opioid receptors, showed that M6G was 4-fold weaker than morphine and M3G was inactive [10]. No specific types of opioid receptors were addressed in this study. Löser *et al.* [12] performed saturation binding experiments of M6G and M3G with the μ-, δ-, and κ-opioid receptors in cerebral membranes from rats (μ receptors), guinea pigs (δ receptors), pigs (κ receptors), and cattle (μ receptors). Their experiments revealed a competitive interaction of M6G with all three opioid receptors and no effects of M3G on any of the opioid binding sites. The investigators did not compare interactions of M6G, M3G, or morphine at any specific receptor site.

In our present study we have explored the pharmacological profiles of morphine, M6G, and M3G in a well-defined system—oocytes expressing cloned human opioid receptors or specific mutants of the hMOR. The hMOR, hDOR, and hKOR were co-expressed with GIRK1/GIRK2 channels and RGS4, thus mimicking the native neuronal G-protein-mediated pathway of K^+ channel activation. This experimentally created model provided a single, defined population of functionally active opioid receptors, overcoming the ambiguity of using membrane preparations.

In *Xenopus* oocytes co-expressing GIRK1/GIRK2 channels and RGS4 with opioid receptors, the agonist-gated currents from morphine, M6G, and M3G were readily observed. M6G activated GIRK1/GIRK2 channels through hMOR with a significantly higher potency than morphine; it did not differ from morphine in its potency via hDOR, but its potency via hKOR was about 5-fold lower than that of morphine. Thus, structure modification of morphine at 6α-OH changed the potency profile from $\mu > \delta = \kappa$ to $\mu > \delta > \kappa$.

Our electrophysiological studies on cloned human opioid receptors show that a substitution of the hydroxyl group at C3 of morphine resulted in a 1000-fold reduction of the potency and similar profiles for hMOR, hDOR, and hKOR. In contrast, glucuronidation at the C6 position increased the potency via the hMOR receptor and significantly reduced the potency via the hKOR receptor. The discrepancies between our results and the results from other studies may be due to different experimental models. The brain and cell preparations may have a wide range of opioid receptors while our model allowed us to investigate a single, defined type of opioid receptors. In a study of M6G binding, Brown *et al.* [16] demonstrated an advantage of using stable Chinese hamster ovary cells transfected with MOR1. Compared to K_d values obtained for morphine and M6G in this cell line

Table 1
EC_{50} Values for morphine, M6G, and M3G on hMOR, hKOR, and hDOR opioid receptors

Ligand	hMOR EC_{50}	hKOR EC_{50}	hDOR EC_{50}	μ/δ Selectively	μ/κ Selectivity
Morphine	206 ± 39 nM	810 ± 93 nM	678 ± 142 nM	3.29 ± 0.93	3.93 ± 0.87
M6G	106.0 ± 5.3 nM	4398 ± 506 nM	665 ± 188 nM	6.27 ± 1.80	41.47 ± 5.20
M3G	113 ± 36 μM	107 ± 18 μM	164 ± 10 μM	1.45 ± 0.0001	0.94 ± 0.0003

Values are means ± SEM (N = 4–6).

Table 2
EC_{50} values for morphine and M6G on mutant opioid receptors, hMORW318L and hMORW318Y/H319Y

Ligand	EC_{50} (nM) hMORW318Y/H319Y	μ/Mutant selectivity	Mutation efficiency (%)	EC_{50} (nM) hMORW318L	μ/Mutant selectivity	Mutation efficiency (%)
Morphine	290 ± 46	1.45 ± 0.35	36.9 ± 7.0	774 ± 56	3.76 ± 0.76	114 ± 25
M6G	623 ± 61	5.88 ± 0.65	14.2 ± 2.1	1438 ± 30	13.56 ± 0.74	216 ± 62

Values are means ± SEM (N = 4–6).

[16,42], our EC_{50} values obtained in oocytes are approximately 50-fold different. This could be attributed to: (a) fractional occupancy of the agonist by the receptor. Hence, the agonist may bind to and dissociate from the receptor, but perhaps not activate it to cause a physiological response. Therefore, higher fractional occupancy may be required to effectively activate the receptor and, in turn, activate the G protein; (b) inefficient coupling between the receptor and the G protein. In this case, the receptor may be activated in an efficient manner, but the signal may not be transduced to the G proteins properly; and (c) inefficient coupling between the activated G protein and the channel or, alternatively, dilution of the activated G proteins to different effector proteins that are endogenously present in oocytes (e.g. adenylyl cyclase). Finally, because K_d values obtained in mammalian cell lines and EC_{50} values obtained in oocytes are compared, it should be kept in mind that subtle differences in post-translational modifications between both species could account for possible differences in pharmacological properties of the receptor or its coupling to G proteins.

The results of our studies suggest that the interactions of morphine glucuronides with opioid receptors are dependent not only on the structure of the metabolites but also on the structure of the opioid receptors. It has been suggested that morphinan alkaloids interact favorably with Trp318 and His319 of the hMOR [33]. Mutations of these two critical sites on the opioid receptor significantly changed the potency for M6G and morphine. The results suggest that the W318L mutation completely confers δ-like potency to the μ receptor for morphine and M6G. The observation that M6G is affected more than morphine by the W318L mutation suggests that an interaction between M6G and W318 could explain the higher potency of M6G via the hMOR. The higher EC_{50} value for M6G at the W318L mutant may be due to steric hindrance caused by a slightly different orientation of the Leu residue in the mutant μ-opioid receptor than in the δ-receptor. Literature reports show complexity of M6G interactions at the opioid receptor sites. Recently, antisense mapping studies of MOR1 have provided strong evidence for a distinct M6G binding site [14–16,43]. Since we have used a model with a single, defined opioid receptor, the presence of such a distinct receptor site for M6G in our experimental model is unlikely. Nevertheless, the results with mutants indicate that modifications in the opioid receptor structure can be critical for the potency profile of the M6G. Via the hKOR, M6G had about a 5-fold lower potency than morphine and even further reduction in M6G potency was caused by a double mutation on Trp318 and His319 positions. The results of our investigation suggest that the pharmacological profile for morphine and its 6β-glucuronide may be changed not only by ligand structure modifications but also by a selective mutation of the opioid receptor structure.

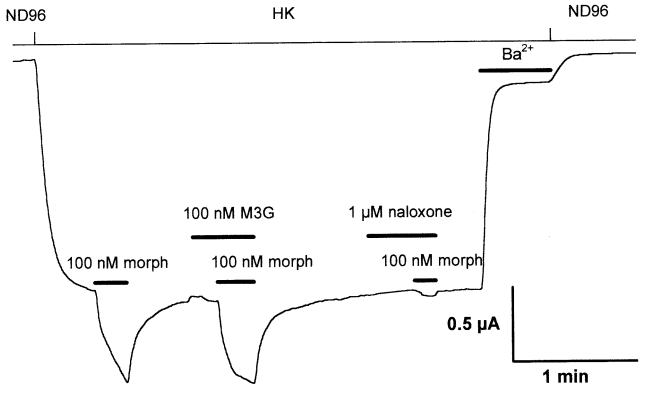

Fig. 6. Effects of M3G and naloxone on morphine-gated K^+ currents in oocytes expressing hMOR. The first step involved applying 100 nM morphine for as long as needed to achieve a steady-state GIRK1/GIRK2 current activation. In a second step, the oocyte was superfused with 100 nM M3G, and 100 nM morphine was co-applied for as long as needed to achieve a steady-state activation. Similarly, in a third step, the oocyte was superfused with 1 μM naloxone, and 100 nM morphine was co-applied. In between each step, ligands were washed out by superfusing with HK solution.

Reports on the effects of M3G at the opioid receptors are contradictory. Previous investigators found that M3G either has no effect [10,12], has effects due to morphine contamination [12], or has a weak effect [9]. In our present study, M3G affected the GIRK1/GIRK2 channel response at higher concentrations than morphine and M6G, but showed no difference in the affinity for hMOR, hDOR, and hKOR. Furthermore, M3G demonstrated no antagonistic effect on the morphine-induced activation of GIRK1/GIRK2 channels through hMOR. These findings support the results of our recent study in which we demonstrated an effect of M3G on the function of opioid receptors at the neuronal level [44]. M3G inhibited cAMP accumulation, and this effect was antagonized by naloxone. Furthermore, in these functional studies we demonstrated a lack of M3G antagonism on the effects of morphine and M6G.

In summary, our present study shows that the two glucuronide metabolites of morphine have agonistic effects at

the cloned human opioid receptors and differ from morphine in their receptor-mediated GIRK1/GIRK2 channel activation. M6G has higher potency than morphine via the hMOR and lower potency via the hKOR. Our results show that a single point mutation (W318L) on the hMOR completely confers δ-like potency for morphine and M6G on the mutant μ receptor. Double mutation at Trp318 and His319 positions only partially confers κ-like potency for morphine and M6G. M3G potency was 1000-fold lower as compared with that of morphine and similar via each cloned opioid receptor type. In addition, M3G did not antagonize the effects of morphine at the hMOR site. Finally, the present study provides electrophysiological evidence that M6G, an agonist with higher potency than morphine via hMOR, may contribute to morphine-induced analgesia, whereas this is less likely for M3G, an opioid receptor agonist with low intrinsic affinity.

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