

JPET #58602

Activation of G-Proteins by Morphine and Codeine Congeners: Insights to the Relevance of O- and N-demethylated Metabolites at μ - and δ -Opioid Receptors¹

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JPET #58602

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Abbreviations: GTP γ S, guanosine-5'-O-(γ -thio)-triphosphate; DAMGO, [D-Ala²,N-MePhe⁴,Gly⁵-ol]enkephalin; P450, cytochrome P450.

JPET #58602

Abstract

Phenotypic differences in analgesic sensitivity to codeine (3-methoxymorphine) results from polymorphisms in cytochrome P450-2D6, which catalyzes O-demethylation of codeine to morphine. However, O-demethylation reportedly is not required for analgesic activity of the 7,8-saturated codeine congeners: dihydrocodeine, hydrocodone and oxycodone. This study determined the potency and efficacy of these compounds and their demethylated derivatives to stimulate μ and δ -opioid receptor-mediated G-protein activation using agonist-stimulated [35 S]GTP γ S binding. Results showed that 7,8-saturated codeine congeners were more efficacious than codeine to activate μ -receptors, but only dihydrocodeine was more efficacious at δ -receptors. Hydrocodone and oxycodone were ~10-fold more potent than codeine and dihydrocodeine at either receptor. Morphine-like compounds with a 3-hydroxy group were ~30-100-fold more potent than their 3-methoxy analogs at the μ -receptor, and these compounds generally exhibited greater efficacy (e.g. morphine produced 2-fold greater maximal stimulation than codeine). Removal of the N-methyl group did not affect efficacy or potency of codeine congeners to activate μ -receptors, whereas this modification generally increased efficacy but decreased potency of morphine congeners. At the δ receptor, morphine congeners showed greater potency and structure-dependent differences in efficacy compared to codeine congeners, whereas removal of the N-methyl group had similar effects to those observed at the μ -receptor. These results demonstrate that 7,8-saturated codeine congeners are more efficacious than codeine, which may explain their lack of requirement for 3-O-demethylation *in vivo*. Nonetheless, because all 7,8-saturated codeine congeners were significantly less potent than their morphine

JPET #58602

derivatives, further research is needed to understand the relationship between metabolism and *in vivo* activity of these compounds.

JPET #58602

The opioid analgesic, codeine (3-methoxymorphine), undergoes several metabolic routes including *N*- and *O*-demethylation, as well as glucuronidation (Dayer et al., 1988; Mikus et al., 1991; Caraco et al., 1996; Yu et al., 2002). It is well established that codeine must be *O*-demethylated to morphine in order to produce analgesia in both humans (Chen et al., 1991) and rats (Mikus et al., 1991; Cleary et al., 1994). In humans, cytochrome P450-2D6 (CYP 2D6) catalyzes this reaction; and individuals with dysfunctional allelic variants of this gene are phenotypically described as poor metabolizers (PM) and are less sensitive to codeine. Individuals expressing multiple copies of active CYP-2D6 are extensive metabolizers (EM) and are more sensitive to codeine. EMs can exhibit the PM phenotype, however, when codeine is coadministered along with a CYP-2D6 inhibitor such as quinidine. The requirement for *O*-demethylation is not surprising given that the reported K_i values for binding to μ -opioid receptors is 200-times higher for codeine than morphine (Chen et al., 1991; Mignat et al., 1995). Large differences in binding affinities are also seen between other codeine congeners and their *O*-demethylated metabolites; for example, the K_i value for hydrocodone is 30-fold greater than hydromorphone (Chen et al., 1991). These data indicate that metabolic conversion of most codeine congeners leads to the formation of metabolites with greater affinity for opioid receptors than their parent compounds.

Although *O*-demethylation of codeine to morphine is required for *in vivo* activity, attempts to demonstrate the importance of this reaction for other codeine derivatives have failed. Oxycodone, for instance, is structurally similar (see Figure 1) to codeine and undergoes similar metabolism (Weinstein and Gaylord, 1979; Ishida et al., 1982; Poyhia et al., 1992), thus it was hypothesized that it too would require *O*-demethylation

JPET #58602

at the 3-position in order to produce analgesia. Studies in humans, however, suggest that oxycodone, and not its *O*-demethylated derivative, is responsible for analgesia (Kaiko et al., 1996). A similar conclusion was drawn from studies on psychomotor effects and subject self-reporting of drug experience following oxycodone administration (Heiskanen et al., 1998). Studies examining *O*-demethylation of hydrocodone to hydromorphone have also shown that the parent compound appears to be responsible for analgesic effects in rats (Tomkins et al., 1997), monkeys (Lelas et al., 1999), and humans (Otton et al., 1993).

Even more surprising is dihydrocodeine, which (like codeine) is reported to have a K_i for the μ -receptor over 100-fold greater than its *O*-demethylated metabolite dihydromorphine (Schmidt et al., 2002). Studies using human liver microsomes indicate that CYP-2D6 is responsible for the *O*-demethylation of dihydrocodeine, and that EM have 10 times the clearance rate of PM (Kirkwood et al., 1997). Research in humans (Wilder-Smith et al., 1998) and rats (Jurna et al., 1997), however, suggest that inhibition of *O*-demethylation has no effect on analgesia. Interestingly, intrathecally injected dihydrocodeine and dihydromorphine displayed similar initial antinociceptive effects, but the action of dihydrocodeine dissipated more rapidly, suggesting that biotransformation was not occurring during the assay (Jurna et al., 1997).

Most clinically relevant opiate agonists, such as morphine, produce biological effects primarily through activation of μ -opioid receptors (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998). However, δ -opioid receptors may also modulate the antinociceptive and reinforcing effects of these opiates (Heyman et al., 1989; Martin et al., 2000), and may play a role in opiate tolerance (Abdelhamid et al., 1991; Nitsche et

JPET #58602

al., 2002). Because μ and δ -opioid receptors activate G-proteins of the $G_{i/o}$ subfamily, agonist efficacy at these receptors can be measured *in vitro* using agonist-stimulated [³⁵S]GTP γ S binding (Traynor and Nahorski, 1995; Selley et al., 1997; Szekeres and Traynor, 1997), which provides a direct measure of receptor-mediated G-protein activation (Asano and Ross, 1984; Hilf et al., 1989). Under conditions where there is no receptor reserve for G-protein activation, such as in rat thalamic membranes, maximal stimulation of [³⁵S]GTP γ S binding by agonist-occupied receptor directly correlates with intrinsic efficacy (Selley et al., 1998). To our knowledge, no one has used [³⁵S]GTP γ S binding to directly measure G-protein activation by codeine congeners and their metabolites that result from P450-mediated biotransformations. The purpose of these studies is to assess whether discrepancies reported between *in vitro* binding data and *in vivo* behavioral studies can be explained at the level of G-protein activation. In addition, these studies will provide information on structure-activity relationships at μ - and δ -opioid receptors. These results will elucidate which structural moieties increase efficacy and potency of codeine-based opioid analgesics, and provide insight into the importance of specific P450-mediated reactions in the actions of these drugs.

JPET #58602

Materials and Methods

Materials. Male Sprague-Dawley rats (150-200 g) were purchased from Harlan. All chemicals were from Sigma (St. Louis, MO) except the following: [³⁵S]GTP γ S (1250 Ci/mmol) was purchased from New England Nuclear Group (Boston, MA), GTP γ S from Boehringer Mannheim (New York, NY), DMEM/F-12 from Fischer (Fair Lawn, NJ), and Ecolite scintillation fluid from ICN Biomedicals (Irvine, CA). All agonists, with the exception of noroxycodone, were obtained from the National Institute for Drug Abuse. Whatman GF/B glass fiber filters were purchased from Fisher Scientific (Pittsburg, PA).

hMOR-low Cell Line Construction. Transfection of CHO-K1 cells with the human μ -opioid receptor cDNA in a pIRES eukaryotic expression vector was performed using Lipofectamine according to the manufacturer's recommended procedure. Stable clones were selected by further incubation in presence of 0.25 mg/ml hygromycin B, and isolated using sterilized cloning wells. Clones were screened for μ -opioid receptor expression levels using [³H]naloxone saturation binding, and G-protein coupling was confirmed by [³⁵S]GTP γ S binding assays with DAMGO (see below).

Membrane Preparations. NG108-15 cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5% fetal calf serum. hMOR-CHO cells were cultured in DMEM/F12 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 mg/ml hygromycin B, and 10% fetal calf serum. Male Sprague-Dawley rats were killed by decapitation followed by thalamic dissection on ice. Membranes were prepared by homogenization of tissue or cells in Membrane Buffer (50

JPET #58602

mM Tris-HCl, 3 mM MgCl₂, 1 mM EGTA, pH 7.4), centrifugation at 50,000 × g for 10 minutes at 4°C, resuspended in the same buffer at 1.5 mg/ml, and stored at -80°C.

[³⁵S]GTPγS Binding Assays. Prior to assays, samples were thawed on ice, centrifuged at 50,000 × g for 10 minutes at 4°C, and resuspended in Assay Buffer (50 mM Tris-HCl (pH 7.4), 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl). Reactions containing 10 μg of membrane protein were incubated for 1.5 hr (NG108-15) or 2 hr (thalamus, hMOR-CHO) at 30°C in Assay Buffer containing 30 μM (thalamus, NG108-15) or 10 μM (hMOR-CHO) GDP, 0.1 nM [³⁵S]GTPγS, and various concentrations of agonist. Nonspecific binding was determined with 20 μM unlabeled GTPγS. For thalamic membranes, an additional 10 min incubation with 4 mU/ml adenosine deaminase was conducted at 30°C prior to adding membranes to the reaction. Reactions were terminated by rapid vacuum filtration through GF/B glass fiber filters, and radioactivity was measured by liquid scintillation spectrophotometry at 95% efficiency for ³⁵S.

[³H]Naloxone Binding Assay. For [³H]naloxone binding, 30-45 μg of hMOR-low cell membrane protein were incubated for 2 hr at 30°C in Assay Buffer with 0.1 to 15 nM [³H]naloxone. Non-specific binding was determined in the presence of 10 μM naltrexone. Reactions were terminated by rapid vacuum filtration through GF/B glass fiber filters, and radioactivity was measured by liquid scintillation spectrophotometry at 45% efficiency for ³H.

Synthesis of Noroxycodone. Noroxycodone was prepared in a synthesis, comprising 3 steps, starting from oxycodone. In the first step, oxycodone was transformed into 14-

JPET #58602

acetoxy-7,8-dihydronorcodeinone using a modified procedure published by Cheng et al. (Cheng et al., 1996): A suspension of 0.9 g (2.9 mmol) oxycodone in acetic anhydride (3.0 mL) was heated at 90 °C for 1 h. The mixture was evaporated and the resulting brown oil was taken up into CHCl₃ (10 mL), washed with NH₄OH solution (pH = 9.5), brine (10 mL), dried over Na₂SO₄ and evaporated to give 14-acetoxy-7,8-dihydronorcodeinone as colorless solid (0.9 g, 84%). This crude product was used without further purification. In the second step, 0.9 g (2.5 mmol) 14-acetoxy-7,8-dihydronorcodeinone was dissolved in CHCl₃ (60 mL) and treated with CNBr (3 g) under reflux conditions for 24 h. Evaporation of the solvent provided crude 14-acetoxy-*N*-cyano-7,8-dihydronorcodeinone as a crystalline solid that was recrystallized from MeOH (0.6 g, 62%), mp (found): 266-269 °C, mp (Currie et al., 1961): 260 °C (dec). In the third step noroxycodone was prepared from 0.5 g (1.4 mmol) 14-acetoxy-*N*-cyano-7,8-dihydronorcodeinone, which was suspended in H₂SO₄ (25%, 20 mL) and stirred under reflux conditions for 4h. During this time the solid gradually dissolved. The solution was cooled to 10 °C and the pH adjusted to 9.5 by addition of NH₄OH. The product was extracted into CHCl₃ (5 x 30 mL). The combined organic layers were dried over Na₂SO₄, filtered through celite and evaporated. The resulting tan foam was dissolved in EtOH (2 mL) and acidified with HI (57%). The product separated upon cooling as colorless solid (0.6 g, 70%, Currie et al., 1961), mp (found): >295 °C (dec), mp (Speyer and Sarre, 1924): >295 °C (dec).

Data Analysis. All data are reported as the means ± SEM of at least three experiments, each performed in triplicate. Nonlinear regression analysis was conducted by iterative fitting using JMP (SAS for Macintosh). Statistical significance was determined by

JPET #58602

ANOVA followed by the Student's t-test. For groups with unequal variances due to large differences in potency (i.e. μM range to nM range), unequal variances were confirmed and the means were then compared using the Welch ANOVA. Percent maximal stimulation is expressed as net stimulated binding by agonist as a percentage of stimulation by DAMGO (30 μM for thalamic and 10 μM for hMOR-CHO membranes) or 5 μM SNC-80 (for NG108-15 membranes).

JPET #58602

Results

Agonist potency and efficacy at the rat μ -opioid receptor. Several studies have provided evidence that biotransformation is required for codeine to exert analgesic properties. In contrast, other codeine congeners such as dihydrocodeine, hydrocodone, and oxycodone, appear not to require biotransformation in order to promote analgesia. To examine the effects of these codeine congeners at the level of μ -opioid receptor-mediated G-protein activation, concentration-effects curves were performed in rat thalamic membranes, which contain a relatively pure population of μ -opioid receptors (Herkenham and Pert, 1982; Sim et al., 1995). Figure 2A shows the concentration-effects curves for the two analogs, codeine and dihydrocodeine, and some of their demethylated derivatives. Despite evidence that only codeine requires O-demethylation to promote analgesia *in vivo*, dihydrocodeine exhibits similar potency characteristics to codeine. This finding was confirmed by non-linear curve fitting analysis of the data, as shown in Table 1. Codeine and dihydrocodeine exhibited 55- and 107-fold higher EC₅₀ values compared to morphine and dihydromorphine, respectively. Interestingly, the relative efficacy (E_{max} value) of dihydrocodeine was significantly greater than that of codeine (66% versus 36%, respectively). Both codeine and dihydrocodeine exhibited significantly lower relative efficacy values compared to their 3-hydroxy derivatives, morphine and dihydromorphine. This difference was greatest with morphine, which exhibited a 1.8-fold greater E_{max} value than codeine.

Although the affinity of the N-demethylated metabolite of codeine (norcodeine) for opioid receptors is not different from that of codeine (Chen et al., 1991), it is possible that N-demethylation of dihydrocodeine might increase the potency of this agonist.

JPET #58602

Because such an effect could explain the lack of requirement for *O*-demethylation for *in vivo* activity of dihydrocodeine, the effect of nordihydrocodeine on μ -receptor-mediated G-protein activation was examined. However, results (Figure 2A, Table 1) showed both the efficacy and potency of this derivative were essentially identical to dihydrocodeine.

Oxycodone and hydrocodone are two clinically-relevant opioids that also undergo P450-mediated biotransformation, yet do not appear to require this metabolism in order to exert their *in vivo* effects. Figure 2B shows the concentration-effects curves for these two compounds as well as their *O*- and *N*-demethylated derivatives. The results of curve-fitting analysis (Table 1) show a 30 to 40-fold increase in the potency of the 3-hydroxy derivatives, oxymorphone and hydromorphone, compared to oxycodone and hydrocodone, respectively. In contrast, norhydrocodone and noroxycodone were not significantly different in potency compared to their *N*-methyl analogs, although they showed a trend toward lower potency. The presence or absence of an *O*- or *N*-methyl group generally had no significant effects on the relative efficacies of the codone compounds, with the exception that oxymorphone exhibited a greater relative E_{\max} value compared to its 3-methoxy derivative, oxycodone. Thus, the results of studies in rat thalamus indicate that only codeine and oxycodone are rendered considerably more efficacious by *O*-demethylation, whereas the potency of all codeine congeners is substantially increased (>10-fold) by *O*-demethylation.

Agonist potency and efficacy at the human μ -opioid receptor. To examine whether the findings in rat thalamus are species specific, we studied several of these compounds in CHO-K1 cells stably expressing the human μ -opioid receptor (hMOR-CHO). Using [3 H]naloxone binding to quantify receptor expression (see Methods), a

JPET #58602

clone stably expressing a low level of receptor ($B_{\max} = 0.393 \pm 0.0237$ pmol/mg, $K_D = 1.61 \pm 0.40$ nM, $n=6$) was selected in order to avoid receptor reserve and maintain the ability to discriminate the relative efficacies of partial agonists. Figure 3 shows the concentration-effects curves for several of these opioids at the human μ -opioid receptor, and Table 1 lists the E_{\max} and EC_{50} values of these compounds adjacent to the values observed at the rat μ -opioid receptor. Again, significantly greater potencies of all 3-hydroxy relative to 3-methoxy analogs were observed, as well as a significantly greater relative efficacy of morphine compared to codeine. Interestingly, comparison of several compounds in hMOR-CHO cells and rat thalamus revealed that only codeine and oxycodone exhibited significantly different relative efficacies between the two systems, with approximately 1.5-fold greater relative E_{\max} values in hMOR-CHO cells. Additionally, only dihydrocodeine and dihydromorphine exhibited significant differences in potency between the two systems, with 2-2.5-fold greater EC_{50} values in thalamus compared to hMOR-CHO cells.

Agonist potency and efficacy at the δ -opioid receptor. Another potential explanation for the discrepancies observed between reported *in vivo* effectiveness of dihydrocodeine, hydrocodone and oxycodone relative to their low potency to activate μ -opioid receptors *in vitro* is that these compounds may possess high potency and efficacy to activate δ -opioid receptors. For this reason, efficacy and potency profiles were determined for these codeine based opiates at the δ -receptor using NG108-15 cells as model system, and compared to their *O*- and *N*-demethylated derivatives. Much of the reported receptor binding data indicate that these opioids have much lower affinities for the δ -receptor than the μ -receptor (Mignat et al., 1995; Schmidt et al., 2002). The results

JPET #58602

shown in Figure 4 and Table 2 agree with these binding studies, as evidenced by the higher EC₅₀ values of all compounds tested in NG108-15 cells compared to rat thalamus or hMOR-CHO cells. Despite substantially lower potencies of these compounds at the δ -receptor, certain effects of *O*- and *N*-methylation status are evident. Morphine exhibited 1.4-fold greater relative efficacy and over 30-fold greater potency compared to codeine, whereas dihydromorphine showed greater potency than dihydrocodeine with no difference in relative efficacy (Table 2). Hydromorphone had approximately 6-fold higher potency than hydrocodone, but hydrocodone exhibited greater relative efficacy. In contrast, oxymorphone showed a 1.6-fold greater relative efficacy than oxycodone, and there was also a trend toward greater potency of the 3-hydroxy analog, but this was not statistically significant. Interestingly, the presence or absence of an *N*-methyl group had no significant effect on the potency or relative efficacy of dihydrocodeine, hydrocodone or oxycodone at the δ -opioid receptor. These results indicate that *O*-demethylation generally increases the potency of codeine congeners at the δ -receptor whereas *N*-demethylation does not.

Structure-activity relationships for codeine and morphine congeners. In addition to investigating metabolic effects on G-protein activation by these opioids, the results reported herein can also be examined from a structure-activity relationship perspective. For this purpose, normorphine, norhydromorphone, and noroxymorphone were also included in the analysis. The data shown above indicate that *N*-demethylation of codeine congeners has little effect on relative efficacy and potency at either the μ - or δ -opioid receptor. The presence or absence of an *N*-methyl group on morphine congeners, on the other hand, significantly affected the E_{max} of these compounds.

JPET #58602

Normorphine and norhydromorphone were significantly more efficacious than morphine or hydromorphone, respectively (Table 1). At the δ -receptor, normorphine, norhydromorphone, and noroxymorphone all exhibited greater relative E_{\max} values than their *N*-methyl analogs (Table 2). The potencies of these compounds at the μ -receptor were also significantly affected by *N*-methylation status. Increases of 4-5-fold were observed in EC_{50} values of the nor derivatives of morphine, hydromorphone, and oxymorphone. At the δ -opioid receptor, however, only normorphine exhibited a significantly lower potency (3.2-fold increase in EC_{50} value compared to morphine).

Finally, the effect on relative efficacy of the basic structure of each compound (e.g. 6-hydroxy versus 6-keto and 7,8-saturated versus 7,8-unsaturated) and of *O*- or *N*-methylation status were examined by two-way ANOVA. At the μ -receptor, this analysis revealed a significant interaction (F -ratio = 8.9) for E_{\max} between the basic structure and the presence or absence of an *O*- or *N*-methyl group on each metabolite. At the δ -receptor, however, only the basic structure of the compound significantly effected relative efficacy. At the μ -receptor, compounds with the basic codeine structure (i.e. 7,8-double bond with no keto group) exhibit the widest range in relative efficacy, with the 3-hydroxy-*N*-nor structures reaching near full agonist levels. For 7,8-saturated compounds with a 6-hydroxy group, the *N*-methylation state had less effect, as all such congeners clustered above 60% of maximal stimulation. For 7,8-saturated compounds with a 6-keto group, the methylation state had little effect on relative efficacy unless both the *O*- and *N*-methyl groups were absent, although the 14-hydroxy-6-keto structures exhibited their greatest relative efficacy when the *O*-methyl group was absent.

JPET #58602

Discussion

This study examined μ - and δ -opioid receptor-mediated G-protein activation by several codeine congeners and their *O*- and *N*-demethylated derivatives. Apparent discrepancies between *in vivo* studies and *in vitro* binding data on the requirement for metabolic activation of dihydrocodeine, hydrocodone and oxycodone suggested a need to examine whether receptor binding correlates with G-protein activation by these compounds. Interpretation of the efficacy (E_{max}) and potency (EC_{50}) data herein *vis-à-vis* studies involving P450 metabolism is complicated by the fact that concomitant changes in relative efficacy and potency do not occur across metabolic biotransformations or structural moieties. For example, these data indicate that the potency of hydrocodone and oxycodone to activate μ -receptors is increased by *O*-demethylation, yet this reaction only significantly affects the relative efficacy of oxycodone. Similarly, these data indicate that at the δ -receptor, *O*-demethylation of hydrocodone significantly decreases efficacy and increases potency, whereas *O*-demethylation of oxycodone increases efficacy without significantly altering potency (although there was a trend toward an increase). Despite these issues, these results demonstrate that *O*-demethylation of these codeine congeners generally produces metabolites that are significantly more potent than their precursors. These findings suggest that inhibition of P450 enzymes responsible for *O*-demethylation would have discernable effects *in vivo*.

One possible explanation for why inhibition of P450-mediated *O*-demethylation of hydrocodone and oxycodone does not block analgesia is that these two compounds may be potent enough to function without biotransformation. This seems unlikely,

JPET #58602

however, because if the 5-fold difference in EC_{50} values between codeine and hydrocodone results in an observable non-requirement for *O*-demethylation, then one would expect that a 30-fold difference in EC_{50} value (e.g. oxycodone vs. oxymorphone) would result in measurable differences in potency *in vivo* (see Table 1, Rat Thalamus). An alternative explanation for why codeine requires *O*-demethylation for *in vivo* activity, whereas hydrocodone and oxycodone do not, is that both of these codones exhibit greater efficacy to activate G-proteins through the μ -receptor relative to codeine. This finding, in addition to their greater potency at the μ -receptor compared to codeine, may negate the requirement for *in vivo* biotransformation.

Another potential explanation is that different metabolites could be responsible for the *in vivo* activity of hydrocodone and oxycodone. In fact, while it has been shown that inhibition of CYP-2D leads to increased formation of noroxycodone (Heiskanen et al., 1998), Leow and Smith (1994) showed that noroxycodone provides less antinociception than oxycodone. These authors also noted that seizures induced by noroxycodone in rodents are not known to occur in humans, suggesting that this metabolite may not be formed in significant quantities in humans. In the present study, *N*-methylation status of either hydrocodone or oxycodone did not affect potency or efficacy to activate G-proteins through μ or δ -opioid receptors, suggesting that this metabolic pathway is not responsible for their *in vivo* effects. This conclusion is in agreement with studies showing that the binding affinity of norcodeine is not greater than codeine (Chen et al. 1991). Although there was no rationale to examine norcodeine in the present study due to the well established requirement for *O*-demethylation of codeine for *in vivo* effectiveness, the lack of effect of *N*-methylation

JPET #58602

status on the efficacy of other codeine congeners in this study suggest that codeine and norcodeine would possess similar efficacy, and this will be tested in future studies.

While the apparent lack of requirement for *O*-demethylation of hydrocodone and oxycodone may be explained in part by their potency, the same explanation seems implausible for dihydrocodeine. Figure 1 shows the structural similarity between dihydrocodeine and its 7,8-unsaturated form, codeine. Table 1 shows that dihydrocodeine was not more potent than codeine, which supports binding data indicating that, like codeine, dihydrocodeine has about 120-fold less affinity for the μ -receptor compared to its 3-hydroxy analog (Mignat et al., 1995; Schmidt et al., 2002). Again, a possible explanation is that *in vivo* biotransformation such as *N*-demethylation or glucuronidation alter the potency of dihydrocodeine. In a study by Schmidt et al. (2002), however, neither nordihydrocodeine nor dihydrocodeine-6-glucuronide exhibited an increase in affinity for μ , δ , or κ -receptors. Similarly, Table 1 shows that nordihydrocodeine exhibited no change in potency or efficacy at the μ -receptor relative to dihydrocodeine. Similar results were obtained at the δ -opioid receptor, although the relative efficacy of nordihydrocodeine was slightly higher than dihydrocodeine. Taken together, these results suggest that the apparent lack of requirement for *O*-demethylation of dihydrocodeine *in vivo* may be due to the higher efficacy of this compound to activate μ - and possibly δ -opioid receptors relative to that of codeine.

Another possible explanation for the ineffectiveness of P450 inhibitors against dihydrocodeine, hydrocodone or oxycodone may be that sufficient quantities of their *O*-demethylated metabolites are formed within the CNS, where P450 inhibitors are less able to block transformation. P450-mediated reactions within neurons and/or glial cells

JPET #58602

in μ -receptor-rich regions might lead to formation of demethylated metabolites. Furthermore, such hydrophilic metabolites might increase local bioavailability due to sequestration (via the blood-brain barrier) within the CNS. Jurna et al. (1997), however, showed that intrathecal infusion of either dihydrocodeine or dihydromorphine rapidly and naloxone-reversibly inhibits C-fiber firing within 5 min post-infusion, suggesting no difference between the parent compound and metabolite.

An additional potential explanation is agonist-dependent differential activation of G-proteins subtypes by the 7,8-saturated codeine congeners versus codeine. In such a system, the level of total G-protein stimulation may be irrelevant if specific G-protein subtypes and analgesia-relevant effector systems are activated more effectively by certain agonists. This “stimulus trafficking” (Kenakin, 1995) may explain the differences between *in vitro* and *in vivo* studies of codeine versus dihydrocodeine, hydrocodone and oxycodone. Table 1 indicates that only two of the eight congeners studied in rat thalamic membranes and hMOR-CHO cells exhibited significant differences in relative efficacy between the two systems. It is not clear whether these differences are due to species-dependent differences in μ -receptor structure or the $G\alpha$ -protein subtypes present in these two tissues. Although human and rat μ -opioid receptors share significant sequence identity, $G\alpha_o$ is the predominant $G\alpha$ subtype in brain, whereas $G\alpha_i2$ is the predominant subtype in CHO cells (Gettys et al., 1994). The fact that greater relative efficacies of codeine and oxycodone were observed in hMOR-CHO cells suggests that these two compounds might preferentially activate $G\alpha_i2$. Interestingly, neither receptor structure nor $G\alpha$ subtype appeared to affect hydrocodone or dihydrocodeine, although it is possible that these compounds signal preferentially

JPET #58602

through other $G\alpha$ subtypes not present in CHO cells such as $G\alpha_i1$. In any case, there remains a possibility that agonist-dependent differential activation of $G\alpha$ subtypes contributes to the differences in analgesic potency between codeine and the 7,8-saturated codeine congeners, and this question will be addressed in future studies.

In Tables 1 and 2, the potency of morphine congeners at the δ -receptor were similar to that of codeine congeners at the μ -receptor. Although this suggests that metabolism of codeine congeners may be even more important for their action at δ - than μ -opioid receptors, behavioral studies concluded that blockage of *O*-demethylation has no measurable effect on analgesia by dihydrocodeine, hydrocodone, or oxycodone. Thus, the data herein suggest that δ -opioid receptors may not play an important role in analgesia by these opioids. This interpretation is in agreement with μ -receptor knockout studies indicating an absolute requirement for this receptor in analgesia and other *in vivo* effects of morphine (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998).

The basic structure and methylation state of the opioids examined in the present study have significant interactions at the μ -opioid receptor but not the δ -receptor, according to two-way ANOVA. These analyses suggest that a 7,8-saturated 14-hydroxy derivative of morphine might exhibit increased efficacy at the μ -receptor, for the following reasons. First, the 7,8-saturated compounds generally exhibit high relative efficacy regardless of methylation state. Comparison of morphine and dihydromorphine in Table 1 suggest that removal of the double bond between C7 and C8 increases relative efficacy without effecting potency. Second, relative to dihydrocodeine and dihydromorphine, the 6-keto compounds have lower relative efficacy, but the 14-hydroxy group of oxymorphone confers a greater E_{max} value relative to hydromorphone.

JPET #58602

Thus, the possibility that 14-hydroxy-dihydromorphine (Garadnay et al., 2001) is the most efficacious μ -agonist of this series will be examined in future studies.

In summary, the G-protein activation data herein generally support previous receptor binding data regarding differences in μ -receptor binding affinities between codeine and morphine congeners. The observation that dihydrocodeine and dihydromorphine exhibit greater potency differences than codeine and morphine is surprising given that *in vivo* data suggests that only codeine requires O-demethylation by P450 to promote analgesia. The non-requirement for O-demethylation of dihydrocodeine, hydrocodone and oxycodone indicates that current understanding of the action of these drugs is incomplete. The present study demonstrates that one factor these three compounds have in common is greater efficacy for μ -receptor-mediated G-protein activation relative to codeine, which may explain the differential requirement for P450 metabolism. Nonetheless, these data also suggest that O-demethylation of these 7,8-saturated codeine congeners would greatly increase their potency for μ -receptor activation, and therefore blockade of this reaction should influence their potency *in vivo*. Thus, further research is needed to fully elucidate the relationship between pharmacokinetic influences and pharmacodynamic activity of these clinically relevant drugs.

JPET #58602

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JPET #58602

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Structural formulas of codeine and morphine congeners. Structures on the right are the 3-hydroxy analogs of those on the left. 17-*N*-Demethylated (nor) metabolites have a methyl group removed from the nitrogen in position 17 of the morphinan backbone.

Figure 2. Agonist-stimulated [³⁵S]GTPγS binding in rat thalamic membranes. Membranes were incubated with 0.1 nM [³⁵S]GTPγS, 30 μM GDP, and varying concentrations of agonist (see Materials and Methods). Data are expressed as the means ± SEM (N ≥ 3) of percentage of the maximal stimulation by 30 μM DAMGO.

Figure 3. Agonist-stimulated [³⁵S]GTPγS binding in hMOR-CHO cell membranes. Membranes were incubated with 0.1 nM [³⁵S]GTPγS, 30 μM GDP, and varying concentrations of agonist (see Materials and Methods). Data are expressed as the means ± SEM (N ≥ 3) of percentage of the maximal stimulation by 10 μM DAMGO.

Figure 4. Agonist-stimulated [³⁵S]GTPγS binding in NG108-15 cell membranes. Membranes were incubated with 0.1 nM [³⁵S]GTPγS, 30 μM GDP, and varying concentrations of agonist (see Materials and Methods). Data are expressed as the means ± SEM (N ≥ 3) of percentage of the maximal stimulation by 5 μM SNC-80.

Table 1. EC₅₀ and E_{max} values for μ-opioid receptor-mediated stimulation of [³⁵S]GTPγS binding in rat thalamus and hMOR-CHO cells

Agonist	Rat Thalamus		hMOR-CHO	
	EC ₅₀ (μM)	E _{max} (%Max)	EC ₅₀ (μM)	E _{max} (%Max)
<u>Codeine Congeners</u>				
Codeine	10.64 ± 0.84 ^a	36 ± 1 ^c	6.59 ± 1.82	55 ± 3 ^{§b}
Dihydrocodeine	20.35 ± 2.99 ^b	66 ± 1 ^a	9.35 ± 2.75 [§]	73 ± 1 ^a
Hydrocodone	2.23 ± 0.28 ^c	51 ± 3 ^b	1.50 ± 0.18	54 ± 6 ^b
Oxycodone	1.46 ± 0.17 ^c	48 ± 2 ^b	1.40 ± 0.24	67 ± 4 ^{§ab}
<u>Morphine Congeners (O-demethylated codeine congeners)</u>				
Morphine	0.194 ± 0.034 ^{†a}	64 ± 1 ^{†,b}	0.19 ± 0.035 [†]	70 ± 4 ^{†ab}
Dihydromorphine	0.190 ± 0.035 ^{†a}	75 ± 3 ^{†,a}	0.071 ± 0.012 ^{†§}	77 ± 4 ^a
Hydromorphone	0.051 ± 0.003 ^{†b}	54 ± 2 ^c	0.030 ± 0.006 [†]	58 ± 4 ^b
Oxymorphone	0.049 ± 0.006 ^{†b}	63 ± 3 ^{†,b}	0.048 ± 0.010 [†]	64 ± 4 ^b
<u>N-Demethylated Codeine Congeners</u>				
Nordihydrocodeine	18.5 ± 0.63 ^a	62 ± 2 ^b	N.D.	N.D.
Norhydrocodone	4.4 ± 0.25 ^b	54 ± 4 ^{ab}	N.D.	N.D.
Noroxycodone	5.2 ± 1.3 ^b	48 ± 3 ^a	N.D.	N.D.
<u>N-Demethylated Morphine Congeners</u>				
Normorphine	0.98 ± 0.24 ^{†a}	81 ± 5 ^{†a}	N.D.	N.D.
Norhydromorphone	0.21 ± 0.03 ^{†b}	69 ± 4 ^{†a}	N.D.	N.D.
Noroxymorphone	0.20 ± 0.02 ^{†b}	53 ± 4 ^b	N.D.	N.D.

Data are means ± SEM (n ≥ 3) obtained from non-linear regression analysis of agonist concentration-effect curves. E_{max} and EC₅₀ values with common letter designations

JPET #58602

were not significantly different from other congeners within subgroup, whereas those without common letter designations were significantly different ($p < 0.05$, Student's *t*-test). [†], $p < 0.05$, significantly different from cognate codeine congener. [‡], $p < 0.05$, significantly different from cognate *N*-methylated congener. [§], $p < 0.05$, significantly different between thalamic and hMOR-CHO membranes. N.D., not determined.

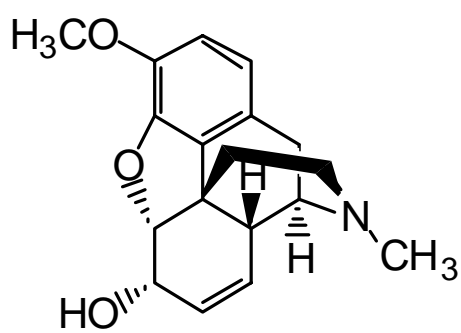
Table 2. EC₅₀ and E_{max} values for δ-opioid receptor-mediated stimulation of [³⁵S]GTPγS binding in NG108-15 cells

Agonist	EC₅₀ (μM)	E_{max} (%Max)
<u>Codeine Congeners</u>		
Codeine	102.8 ± 13.3 ^a	31 ± 4 ^{bc}
Dihydrocodeine	96.2 ± 37.6 ^a	69 ± 4 ^a
Hydrocodone	9.1 ± 1.0 ^b	39 ± 4 ^c
Oxycodone	8.3 ± 1.7 ^b	21 ± 2 ^b
<u>Morphine Congeners (O-demethylated codeine congeners)</u>		
Morphine	3.0 ± 1.2 ^{†a}	43 ± 2 ^{†b}
Dihydromorphine	14.3 ± 7.7 ^a	73 ± 14 ^a
Hydromorphone	1.6 ± 0.37 ^{†a}	29 ± 1 ^{†b}
Oxymorphone	2.2 ± 1.0 ^a	33 ± 4 ^{†b}
<u>N-demethylated Codeine Congeners</u>		
Nordihydrocodeine	153.5 ± 33.6 ^a	80 ± 11 ^a
Norhydrocodone	31.3 ± 11.4 ^b	41 ± 3 ^b
Noroxycodone	18.2 ± 4.0 ^b	27 ± 2 ^b
<u>N-demethylated Morphine Congeners</u>		
Normorphine	9.7 ± 1.0 ^{†a}	87 ± 3 ^{†a}
Norhydromorphone	1.3 ± 0.33 ^b	56 ± 4 ^{†b}
Noroxymorphone	3.8 ± 1.1 ^b	53 ± 2 ^{†b}

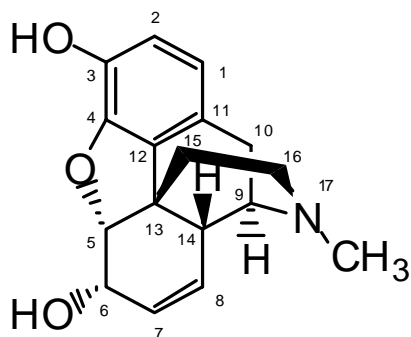
Data are means ± SEM (n ≥ 3) obtained from non-linear regression analysis of agonist concentration-effect curves. E_{max} and EC₅₀ values with common letter designations were not significantly different from other congeners within subgroup, whereas those

JPET #58602

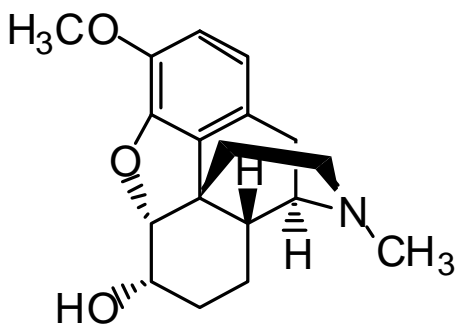
without common letter designations were significantly different ($p < 0.05$, Student's *t*-test). [†], $p < 0.05$, significantly different from cognate codeine congener. [‡], $p < 0.05$, significantly different from cognate *N*-methylated congener.



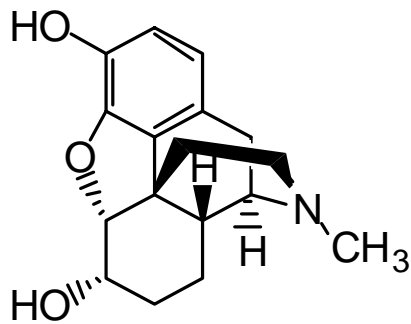
Codeine



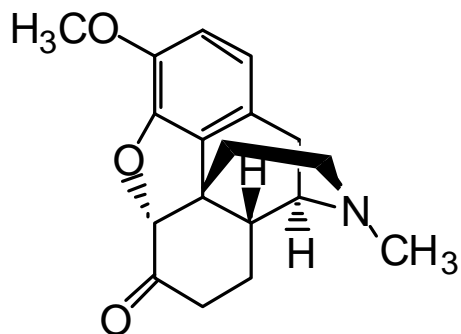
Morphine



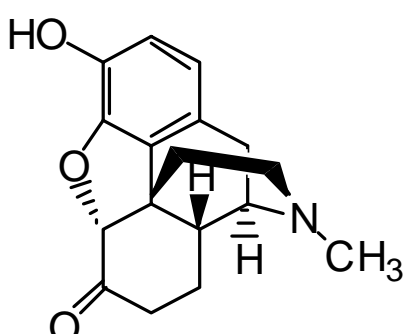
Dihydrocodeine (DHC)



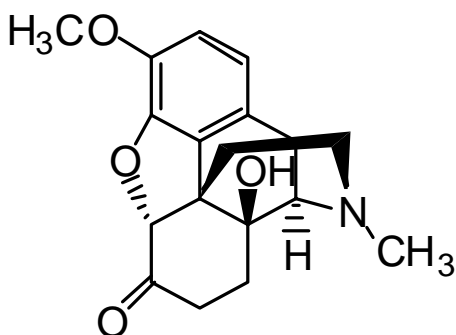
Dihydromorphine (DHM)



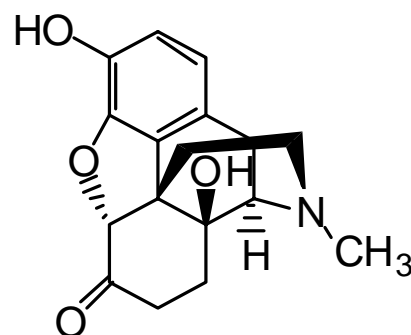
Hydrocodone (HC)



Hydromorphone (HM)



Oxycodone (OC)



Oxymorphone (OM)

Figure 1

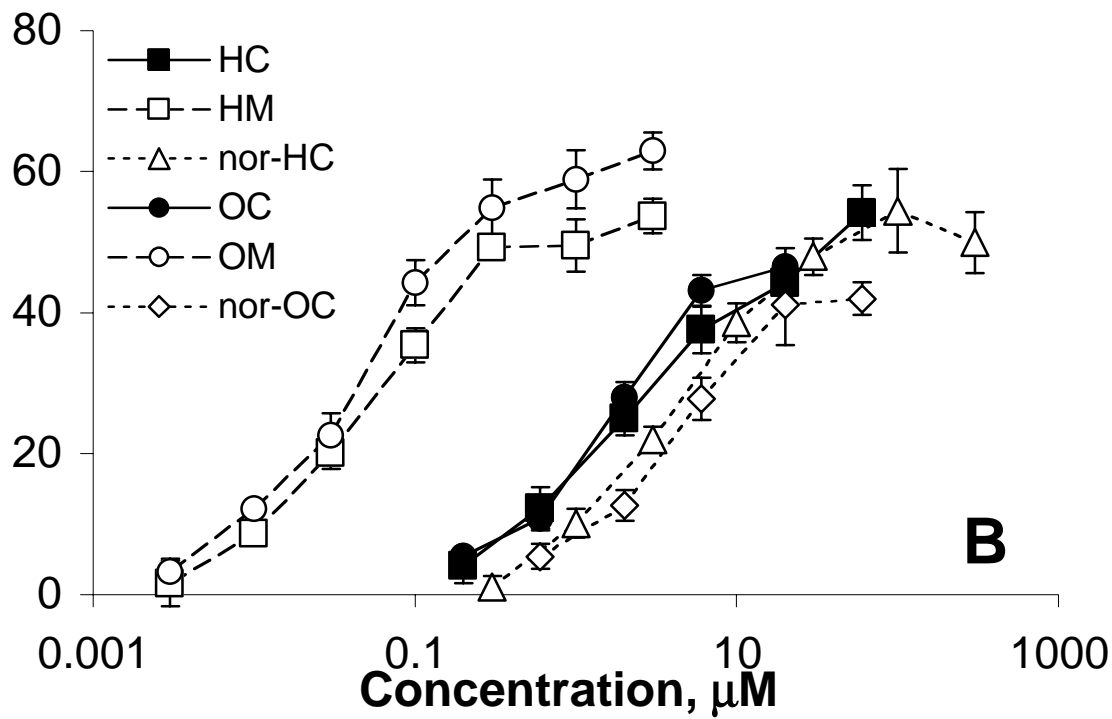
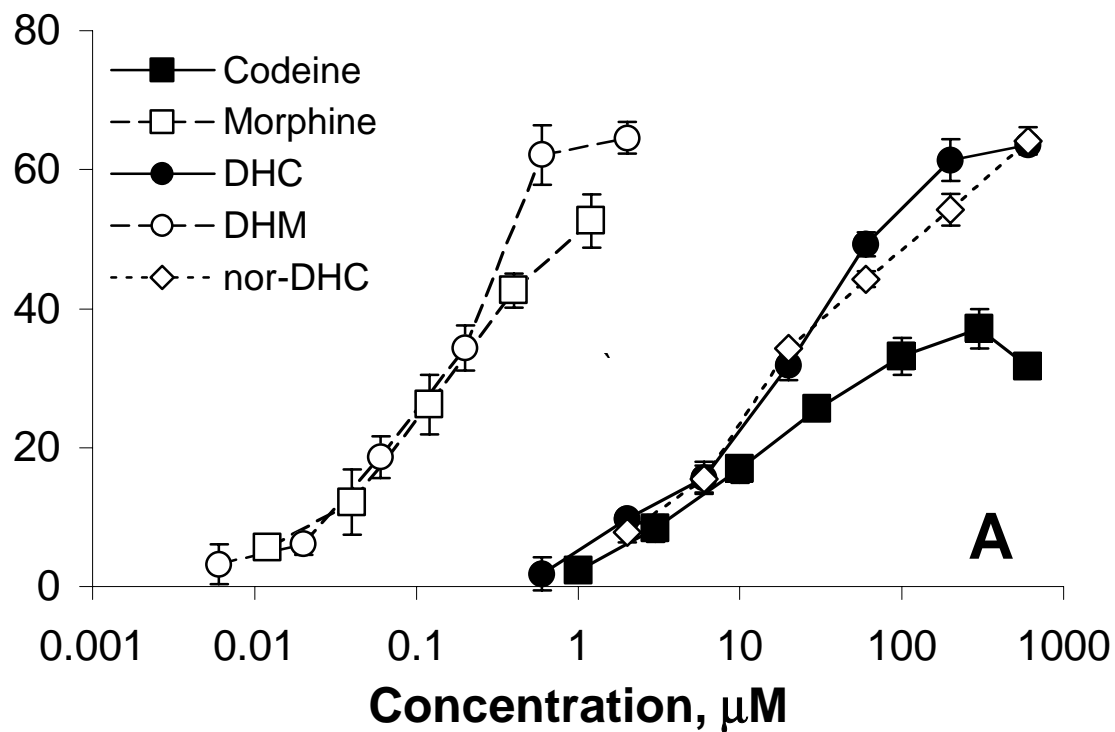


Figure 2

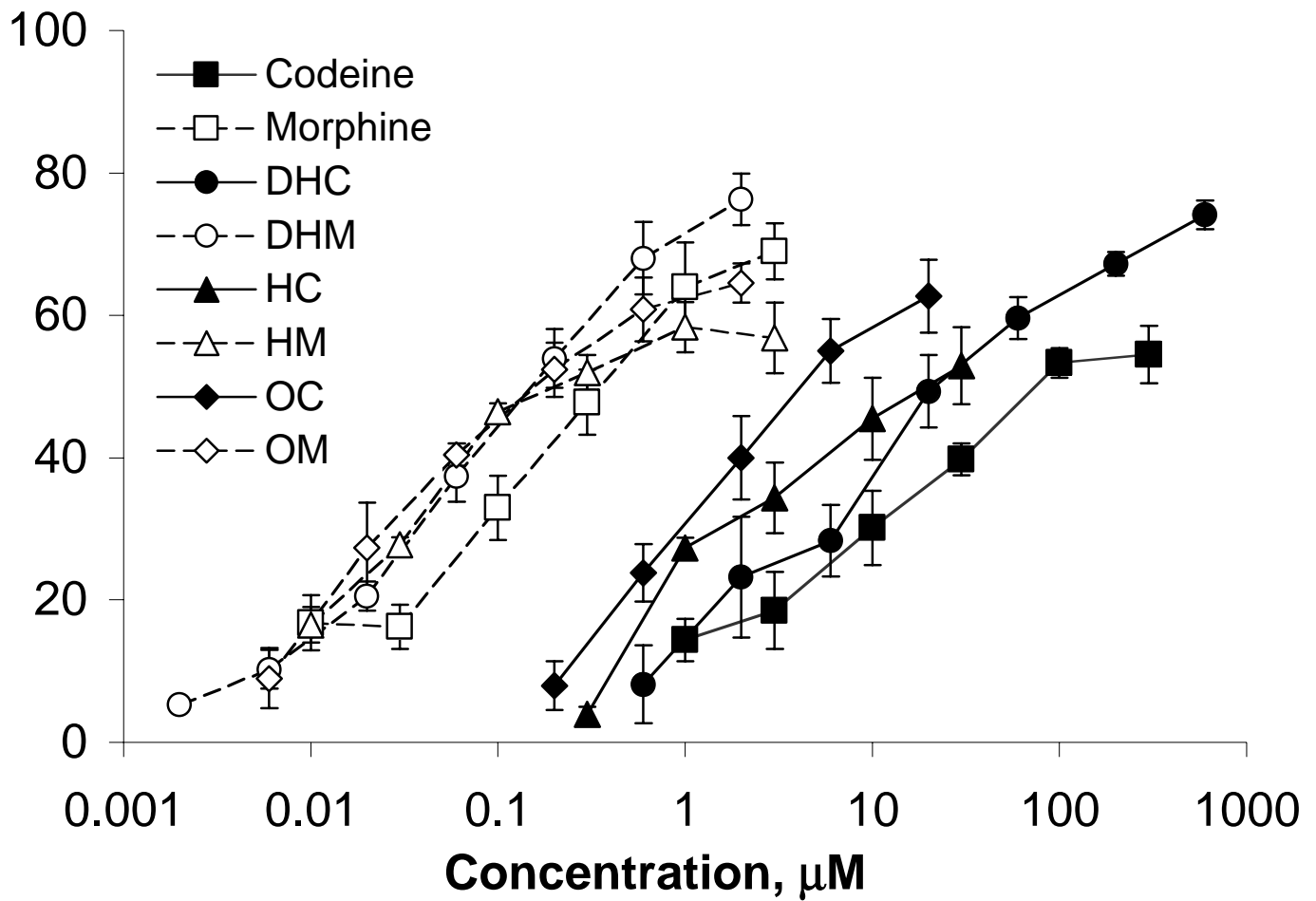


Figure 3

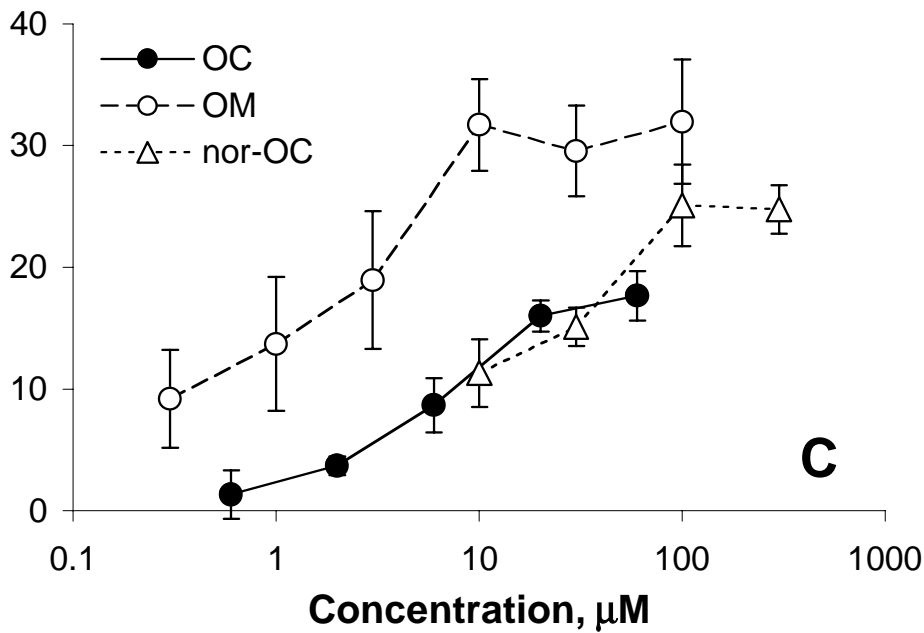
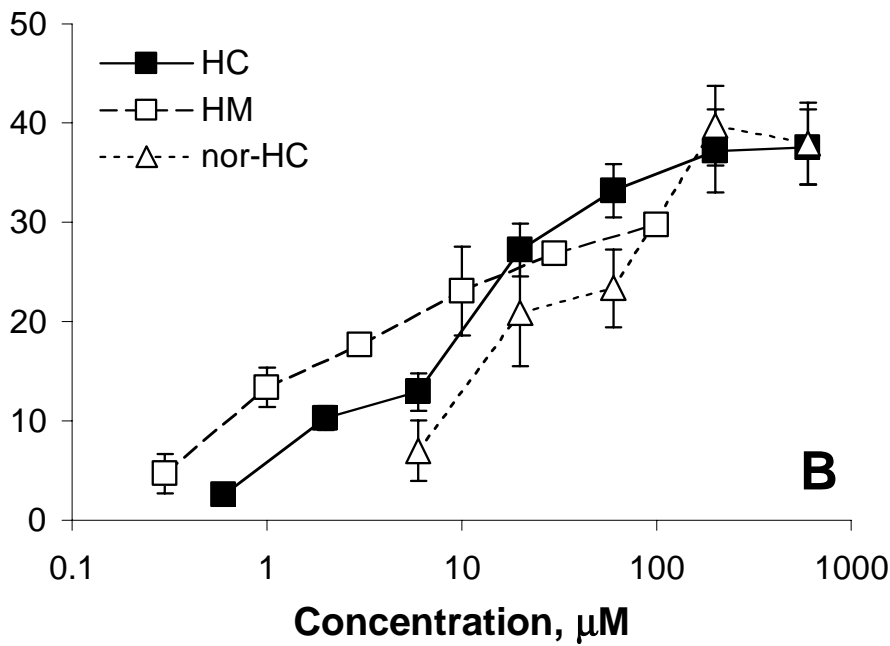
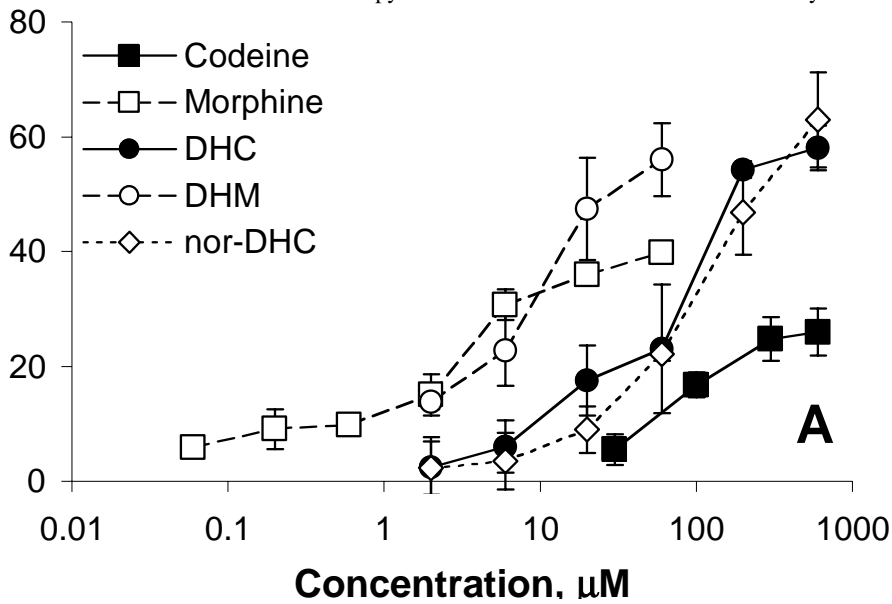


Figure 4