Morphine-related metabolites differentially activate adenylyl cyclase isozymes after acute and chronic administration

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Abstract Morphine-3- and morphine-6-glucuronide are morphine's major metabolites. As morphine-6-glucuronide produces stronger analgesia than morphine, we investigated the effects of acute and chronic morphine glucuronides on adenylyl cyclase (AC) activity. Using COS-7 cells cotransfected with representatives of the nine cloned AC isozymes, we show that AC-I and V are inhibited by acute morphine and morphine-6-glucuronide, and undergo superactivation upon chronic exposure, while AC-II is stimulated by acute and inhibited by chronic treatment. Morphine-3-glucuronide had no effect. The weak opiate agonists codeine and dihydrocodeine are also addictive. These opiates, in contrast to their 3-*O*-demethylated metabolites morphine and dihydromorphine (formed by cytochrome P450 2D6), demonstrated neither acute inhibition nor chronic-induced superactivation. These results suggest that metabolites of morphine (morphine-6-glucuronide) and codeine/dihydrocodeine (morphine/dihydromorphine) may contribute to the development of opiate addiction.

Key words: Morphine; Glucuronide; Codeine; Dihydrocodeine; Opioid receptor; Adenylyl cyclase

1. Introduction

Opiate agonists are the drugs of choice for the treatment of moderate and severe pain, although their use is limited due to side effects, including the development of tolerance, respiratory depression and constipation. Pharmacological studies have defined three types of opioid receptors, termed μ, δ and κ, which differ in their affinity for various opioid ligands, their distribution in the nervous system, and their physiological and behavioral profiles [1,2]. The three opioid receptors are members of the seven-transmembrane domain GTP-binding protein (G protein)-coupled receptor superfamily. Activation of these receptors inhibits the activity of adenylyl cyclase (AC) via the $G_{i/o}$ type of G proteins. Yet, we and others have shown that when these and several other $G_{i/o}$-coupled receptors (e.g. D_2-dopaminergic, m_2- and m_4-muscarinic, α_2-adrenergic, somatostatin) are chronically activated, there is an increase in cAMP accumulation, which is particularly apparent upon withdrawal of the inhibitory agonist [3–11]. This phenomenon has been referred to as AC sensitization, or superactivation, and is believed to represent a possible biochemical substrate for the development of drug tolerance and dependence, commonly observed upon prolonged exposure to opiate drugs [3,12].

The opiate agonist morphine is metabolized in humans to morphine-3- and morphine-6-glucuronides. While it was previously considered that glucuronide metabolites are pharmacologically inactive and that their formation is a mechanism for the detoxification and elimination of the parent compound, it is now recognized that their formation might have important pharmacological implications. In animal experiments, both glucuronides have been shown to influence the analgesic effects of morphine. Morphine-6-glucuronide exhibited both a higher analgesic potency [13–15] and an increased physical dependence [15] compared with morphine. These results appear to be in contrast to binding studies in which morphine-6-glucuronide and morphine displayed comparable binding to the μ-receptor [13,16]. On the other hand, morphine-3-glucuronide showed only a poor affinity to the μ-receptor and even antagonized morphine's analgesic effect [17].

Indeed, recent investigations point to a new binding site for morphine-6-glucuronide, different than the known μ-opioid receptor type 1 (MOR-1) [18,19]. However, the role of MOR-1 in mediating the acute and chronic signaling elicited by opiates, such as morphine-6-glucuronide and heroin, has not yet been systematically investigated. It was therefore of interest to determine whether the acute and chronic activation of the μ-receptor with such compounds in transfected Chinese hamster ovary (CHO) and African green monkey kidney (COS-7) cells would yield results similar to those observed with morphine. Moreover, we have previously shown that not all of the nine isozymes of AC which have been identified to date [20,21] behave identically with respect to regulation by morphine [11]. It was therefore of interest to study the regulation of these AC isozymes by morphine metabolites and derivatives.

Besides the strong opiate morphine, the weak and moderate opiates codeine and dihydrocodeine (see formulas in Fig. 1) are used in the treatment of mild to moderate pain, as well as in cough treatment. Prescription analgesics, including these opiates, are among the major classes of drugs prescribed and used worldwide [22]. Codeine and dihydrocodeine, alone or in combination with other drugs, are among the most

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Abbreviations: AC, adenylyl cyclase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CYP2D6, cytochrome P450 2D6; DMEM, Dulbecco's modified Eagle's medium; FS, forskolin; G protein, GTP-binding protein; IBMX, 1-methyl-3-isobutylxanthine; MOR, μ-opioid receptor; TSH, thyroid-stimulating hormone

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widely abused prescription drugs [22,23]. While these drugs present only weak binding to the μ-receptor (probably due to the blockage of the 3′ OH position), they are metabolized by the enzyme cytochrome P450 2D6 (CYP2D6) to form the strong opiates morphine and dihydromorphine [24,25] (Fig. 1). Consequently, the question arises of whether it are codeine and dihydrocodeine themselves, or rather their 3-*O*-demethylated metabolites morphine and dihydromorphine, that lead to addiction and withdrawal symptoms. We therefore investigated the effects of codeine and dihydrocodeine in comparison to the effects of morphine and dihydromorphine on AC activity in CHO cells stably transfected with the MOR.

2. Materials and methods

2.1. Materials

[^{3}H-2]Adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Morphine and dihydromorphine were obtained from the National Institute of Drug Abuse, Research Technology Branch (Rockville, MD, USA), and naloxone was purchased from Research Biochemical International (Natick, MA, USA). Morphine-3- and morphine-6-glucuronide, codeine and 3-ethylmorphine, as well as forskolin (FS), cAMP and thyroid-stimulating hormone (TSH), were obtained from Sigma (St. Louis, MO, USA). Dihydrocodeine was obtained from Knoll Pharma (Ludwigshafen, Germany). Ionomycin and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (IBMX) and RO-20-1724 were from Calbiochem (La Jolla, CA, USA). Tissue culture reagents were from Life Technologies (Gaithersburg, MD, USA).

2.2. Plasmids

Plasmids containing AC cDNAs (pXMD1-AC-I, pXMD1-AC-II and pXMD1-AC-V), as well as pXMD1-gal, rat TSH receptor in pSG5, and rat MOR in pCMV-neo have previously been described [11].

2.3. Transfected cells

The CHO cell line stably transfected with the rat MOR cDNA (CHO-μ) was previously described [9]. The CHO-μ cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum, 2 mM glutamine, non-essential amino acids, 0.2 mg/ml G418, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

Transient transfection of COS-7 cells was performed as previously described [11]. In brief, COS-7 cells in 10-cm plates were transfected using the DEAE-dextran chloroquine method with 1 μg/plate of plasmid encoding the MOR, 2 μg/plate of plasmid encoding either one of the AC isozymes or pXMD1-gal (for mock DNA transfection), and, where indicated, with 1 μg/plate of plasmid encoding the TSH receptor. Twenty-four hours later, the cells were trypsinized and re-cultured in 24-well plates, and after an additional 24 h, the cells were assayed for cAMP content (as a measure of AC activity) as described below. Transfection efficiencies were normally in the range of 40–80%, as determined by transfection with pXMD1-gal and staining for β-galactosidase activity [11].

2.4. AC assay

The assay was performed in triplicate as described previously [10,11]. In brief, cells cultured in 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml of [^{3}H]adenine, and then washed three times with 0.5 ml/well of DMEM containing 20 mM HEPES (pH 7.4) and 0.1 mg/ml bovine serum albumin (BSA). This medium was replaced with 0.5 ml/well of DMEM containing 20 mM HEPES (pH 7.4), 0.1 mg/ml BSA and the phosphodiesterase inhibitors IBMX (0.5 mM) and RO-20-1724 (0.5 mM). AC activity was stimulated by the addition of either 1 μM FS (for the activation of AC in CHO cells or of AC-V in transfected COS cells), 1 μM ionomycin (for the stimulation of the Ca^{2+}-dependent AC-I in COS cells transfected with this isozyme) or 0.1 μM TSH (for the stimulation of AC-II in transfected COS cells; in this case, the cells were also cotransfected with the TSH receptor). After 10 min incubation at room temperature, the medium was removed and the reaction terminated by addition of perchloric acid containing 0.1 mM unlabeled cAMP, followed by neutralization, and the amount of [^{3}H]cAMP was determined by a two-step column separation procedure [10]. Chronic treatment was achieved by incubating the cells for 4 h with the indicated concentrations of agonists, followed by agonist withdrawal (by quick removal of medium and addition of fresh medium containing 1 μM of the opiate antagonist naloxone) and the addition of the appropriate AC stimulator (see above) to assay cAMP accumulation. The incubation with [^{3}H]adenine took place during the last 2 h of the chronic exposure. Uptake of [^{3}H]adenine into the cells was not affected by the chronic agonist treatments.

Statistical analysis of the data was performed using the Student's *t*-test. Curve fitting and calculation of EC_{50} and IC_{50} were done by non-linear regression using the GraphPad software package (ISI Software, Philadelphia, PA, USA).

3. Results

3.1. Effects of acute and chronic morphine and morphine glucuronides on AC activity

Dose–response curves for the effects of acute and chronic agonist exposures on AC activity in CHO cells are depicted in Fig. 2. Significant inhibition of FS-stimulated cAMP accumulation was observed in cells acutely treated with morphine or morphine-6-glucuronide. Both materials showed similar effects on AC inhibition with IC_{50} values of 92 ± 9 nM and 71 ± 25 nM, respectively (Fig. 2a). No significant inhibition was observed for morphine-3-glucuronide.

We have previously shown that chronic exposure to morphine followed by removal of the agonist by extensive wash or the addition of the antagonist naloxone leads to AC superactivation [9]. Fig. 2b shows that chronic treatment of CHO-μ cells by either morphine or morphine-6-glucuronide induces AC superactivation. The level of superactivation is dependent on the concentration of these ligands during the chronic exposure prior to the agonist withdrawal. The EC_{50} values for AC superactivation by these opiate agonists appear to be similar to the IC_{50} values for inhibition: 127 ± 27 nM and 136 ± 56 nM, respectively. This indicates that exposure to

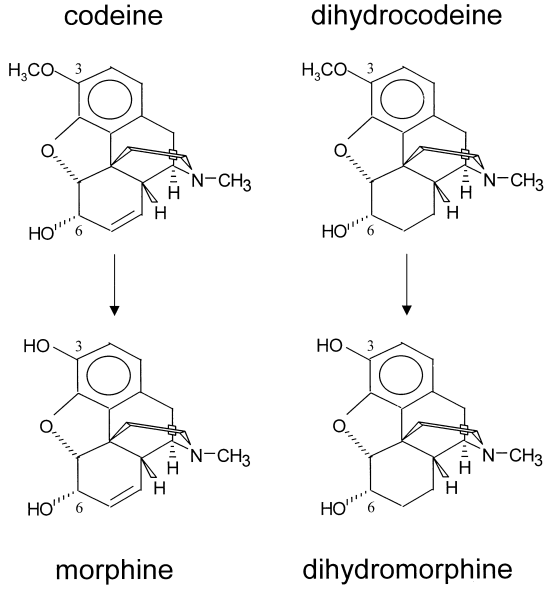

Fig. 1. The *O*-demethylation pathway by CYP2D6 of codeine and dihydrocodeine to morphine and dihydromorphine, respectively.

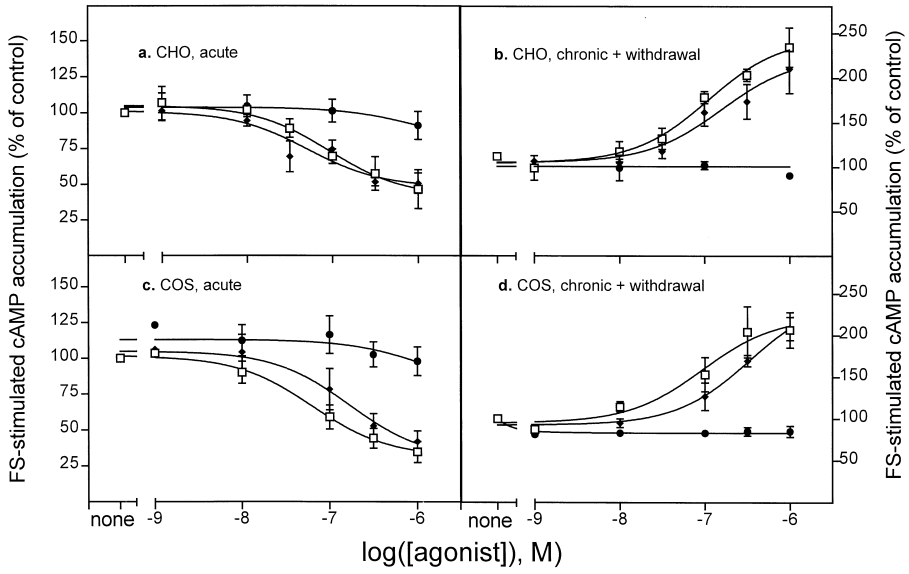

Fig. 2. Dose–response of acute and chronic treatment with morphine and morphine glucuronides on AC activity in CHO cells and on AC-V activity in transfected COS-7 cells. FS-stimulated AC activity was determined (a,b) in CHO-μ cells and (c,d) in COS cells transfected with the cDNAs of AC-V and of the MOR. These CHO-μ cells (a) or COS cells (c) were treated acutely (during the 10 min AC assay) with various concentrations of morphine-3-glucuronide (●), morphine-6-glucuronide (♦) or morphine (□). These CHO-μ cells (b) or COS cells (d) were treated chronically for 4 h with the opiate agonists followed by withdrawal (by a rapid wash and addition of 1 μM naloxone) just prior to the assay of FS-stimulated AC activity. 100% represents the control FS-stimulated cAMP accumulation observed in the absence of opiate agonists (a, b: 1182 ± 96 cpm; c, d: 7175 ± 632 cpm of [^{3}H]cAMP). Data represent the means ± S.E.M. of three experiments.

morphine, and to a lesser extent to morphine-6-glucuronide, did not lead to marked desensitization of the MOR (see also Fig. 2c,d). This is consistent with results obtained previously, which showed that morphine does not cause significant desensitization of the μ-receptor [9,26]. Chronic exposure to morphine-3-glucuronide did not induce AC superactivation, in agreement with its negligible effect on acute AC inhibition.

Taken together, these results indicate that like morphine, morphine-6-glucuronide activates the MOR, leading to inhibition of the endogenous AC in CHO cells upon acute activation, and to AC superactivation upon chronic treatment, while morphine-3-glucuronide has no effect on the μ-receptor.

CHO cells contain a mixture of several AC isozymes which may well be differentially affected by the treatment with the various opiate ligands. In order to examine the effect of morphine and its glucuronides on the various AC isozymes, we employed the COS-7 cell system, which readily allows transient transfection of the MOR together with the desired AC isozymes. Moreover, the endogenous AC in COS is not strongly affected by either acute or chronic agonist exposure [10], allowing the analysis of the effect of exogenous AC isozymes.

Three types of AC isozymes, representing the three major classes of AC isozymes according to their properties and sequence homologies, were studied. All these AC isozymes can be stimulated by $G_{\alpha s}$, although they differ in their response to Ca^{2+} and $G_{\beta\gamma}$. The three classes of AC isozymes were represented here by: AC-I, which, like AC-VIII, is stimulated by Ca^{2+}/calmodulin; AC-V, which, like AC-VI, is inhibited by Ca^{2+} ions, stimulated by FS and inhibited by $G_{\beta\gamma}$; and AC-II, which is similar to AC-IV and AC-VII, and is stimulated by $G_{\beta\gamma}$ subunits in the presence of activated $G_{\alpha s}$ [20,21,27,28]. The choice of these particular isozymes was also based on the fact that these are the only three AC isozymes whose mRNAs are expressed at high levels in the rat brain [20]. Due to the differences between them, the three AC isozymes were stimulated using three types of stimulants. The Ca^{2+} ionophore ionomycin was used to activate AC-I, FS to activate AC-V and α_s activation (via activation of the TSH receptor cotransfected into the COS cells) was used to activate AC-II.

The experiment presented in Fig. 2c shows that AC-V in transfected COS cells is dose-dependently inhibited by acute activation with morphine or morphine-6-glucuronide, with IC_{50} values of 69 ± 22 nM and 129 ± 33 nM, respectively, values similar to those obtained with CHO cells. On the other hand, morphine-3-glucuronide had no significant effect on AC-V. Similarly, chronic activation of the μ-receptor by morphine or morphine-6-glucuronide led to a dose-dependent AC superactivation with EC_{50} of 122 ± 30 nM and 450 ± 100 nM, respectively, while no superactivation was observed with morphine-3-glucuronide.

Fig. 3 compares the effect of acute and chronic opiate exposures on AC-V vs. AC-I and AC-II. Acute activation of the MOR by either morphine or morphine-6-glucuronide led to inhibition of the stimulated activity of both AC-V and AC-I, albeit much less strongly for the latter, while chronic activation of the MOR with either agonist followed by agonist withdrawal led to superactivation of both of these isozymes (Fig. 3a,b). Again, the superactivation was much stronger with AC-V. Neither acute nor chronic application of morphine-3-glucuronide had significant effects on the activities of these two AC isozymes.

Stimulation of AC-II by 0.1 μM TSH led to a large increase in cAMP accumulation as compared to the basal level (see legend to Fig. 3). However, in contrast to AC-I or V, acute exposure of cells transfected with AC-II to either morphine or morphine-6-glucuronide induced a significant increase in AC-II activity (Fig. 3c), a result similar to the observations made earlier with several other $G_{i/o}$-coupled receptor agonists

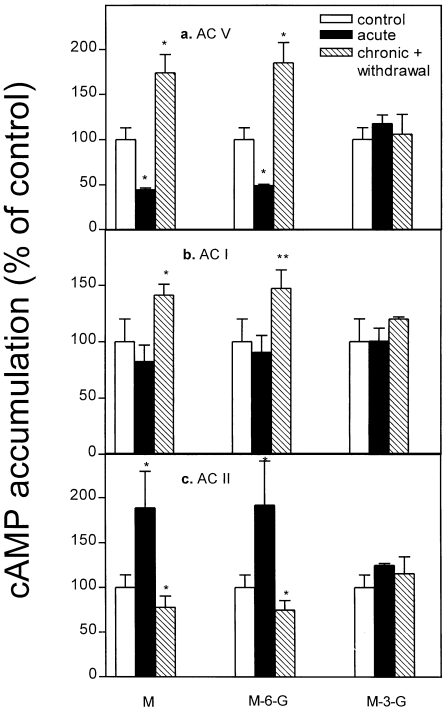

Fig. 3. Acute and chronic MOR activation by morphine and morphine glucuronides differentially regulates the activity of various AC isozymes. COS-7 cells were transfected with the cDNAs of the indicated AC isozymes together with the cDNAs of the MOR and, in the case of AC-II, also of the TSH receptor. Acute, the cells were treated with 1 μM opiate for the 10 min of the AC assay; Chronic+withdrawal, the cells were treated with 1 μM opiate for 4 h followed by its withdrawal and immediate AC assay. (a) AC-V was stimulated with 1 μM FS, (b) AC-I was stimulated with 1 μM ionomycin and (c) AC-II was stimulated with 0.1 μM TSH. These stimulations increased AC activity by 5.4-, 2.0- and 6.8-fold, respectively, compared to unstimulated AC activity (demonstrating expression of the transfected AC molecule). M, morphine; M-3-G, morphine-3-glucuronide; M-6-G, morphine-6-glucuronide. cAMP accumulation observed under these stimulating conditions (in the absence of morphine or morphine glucuronides) was defined as 100% (a, 6004 ± 384 cpm; b, 1853 ± 188 cpm; c, 9929 ± 696 cpm). Data represent the means ± S.E.M. of triplicate determinations of a representative experiment. $*P < 0.05$, $**P < 0.01$ vs. control.

[7,11,29,30]. Moreover, following withdrawal from chronic agonist treatment, not only was no superactivation of this isozyme apparent, but a reduction in the activity of AC-II was actually observed. As observed with AC isozymes I and V, morphine-3-glucuronide had no significant effect on AC-II activity when applied either acutely or chronically.

3.2. *Differential effects of 3-substituted and 3-unsubstituted opiates on the regulation of AC activity*

We investigated the effects on AC activity of acute and chronic treatment with several 3-substituted opiates (all at 1 μM) and their corresponding unsubstituted derivatives in CHO cells stably transfected with the μ-receptor. Codeine and dihydrocodeine (which differ from morphine and dihydromorphine by being methylated at the 3 position) showed only minute inhibition after acute treatment and minute activation of AC after chronic treatment in comparison with the unmethylated compounds morphine and dihydromorphine (Fig. 4). A similar result was obtained with 3-ethylmorphine (where an ethyl group replaced the methyl group of codeine).

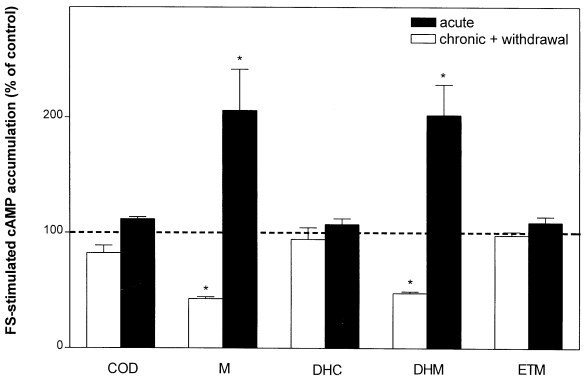

Fig. 4. Effects of acute and chronic treatment with different 3-substituted and 3-unsubstituted opiates on AC activity in CHO-μ cells. FS-stimulated AC activity of CHO-μ cells was determined following acute treatment with 1 μM of the indicated opiates, or after 4 h chronic treatment followed by agonist withdrawal. COD, codeine; M, morphine; DHC, dihydrocodeine; DHM, dihydromorphine; ETM, ethylmorphine. FS-stimulated cAMP accumulation in the absence of opiates is represented as 100% (1125 ± 82 cpm of [^{3}H]cAMP). Data represent the means ± S.E.M. of triplicate determinations of a representative experiment out of three experiments which gave similar results. $*P < 0.02$ vs. control.

These results demonstrate that like in morphine-3-glucuronide, substitution at the 3′ OH position of morphine or dihydromorphine markedly reduces the activity of the opiate ligand.

4. Discussion

The results presented here indicate that morphine-6-glucuronide parallels the effects of morphine upon both acute and chronic administration for different AC isozymes. Morphine-6-glucuronide is one of the major metabolites of morphine, and has demonstrated analgesic effects in both animal and human studies. While in animal studies morphine-6-glucuronide presented superior analgesic effects compared to morphine, the analgesic effects in humans are still contradictory [31]. Osborne et al. [32] observed analgesic effects of morphine-6-glucuronide in cancer patients suffering severe pain, while in a single-dose study in healthy volunteers, morphine-6-glucuronide did not show any analgesic effect [33]. As morphine-6-glucuronide is rather hydrophilic compared to morphine, its penetration via the blood–brain barrier might be impaired and therefore, an effective concentration of the drug at the receptor within the central nervous system might not have been achieved [34]. Concerning binding to the μ-receptor, morphine-6-glucuronide has shown an affinity comparable to that of morphine [13,15,16]. Our results are in agreement with these binding studies, as morphine-6-glucuronide presents comparable results to morphine in both the inhibition and superactivation of transfected AC-V, as well as of the endogenous AC of CHO cells. These results, however, are not able to explain the increased physical dependence after chronic treatment of mice with morphine-6-glucuronide compared to morphine, as observed by Frances et al. [15].

In this regard, recent studies suggest a new and specific binding site for morphine-6-glucuronide, heroin and 6-acetylmorphine [19]. Moreover, it was recently shown using MOR-1 knockout mice that heroin and morphine-6-glucuronide retain analgesic effects in mice lacking exon 1 of the MOR-1 gene,

while the analgesia by morphine was lost. However, morphine, heroin and morphine-6-glucuronide were without effect in the exon 2 MOR-1 deficient mice [18]. According to these results, it was hypothesized that morphine-6-glucuronide, heroin and 6-acetylmorphine bind with high affinity and specificity to a novel μ-receptor subtype [18,19]. Future studies are needed to investigate the role of chronic exposure to morphine-6-glucuronide and heroin on this new μ-receptor subtype, and whether it leads to AC superactivation.

As previously shown with morphine, the 6-glucuronide inhibited AC-V and AC-I, and stimulated AC-II, while reverse modulation of these AC isozymes was observed after chronic opiate exposure. This is important, as different parts of the brain differ in their isozyme distribution pattern, thus leading to a different profile of AC modulation by the opiates in different brain regions. For example, it has been shown that opioid receptor activation can stimulate AC activity in the rat olfactory bulb [35], while opiates are inhibitory in most other parts of the brain.

Using CHO cells transfected with the MOR, we examined the effects of different opiates on AC activity after acute and chronic treatment. We found that opiates substituted in position 3, such as morphine-3-glucuronide, codeine, dihydrocodeine and 3-ethylmorphine, presented neither significant inhibition after acute treatment nor pronounced superactivation after chronic opiate administration. This result is in agreement with binding studies showing that substitution on the free phenolic group (i.e. position 3) of morphine causes a large decrease in binding to the MOR [36]. In contrast to these 3-substituted materials, the corresponding non-substituted opiates, morphine and dihydromorphine, exhibit significant inhibition after acute treatment and superactivation after chronic treatment of the endogenous AC in CHO cells. Morphine also displayed this for individual AC isozymes from the AC-I/VIII and AC-V/VI families [11].

Codeine and dihydrocodeine are among the most widely used and abused prescription drugs (analgesics, cough syrups), and although they have been classified as weak opiates, they have been shown to lead to addiction and withdrawal symptoms. Moreover, these drugs are rising steadily in the drug abuse rankings [22,23]. Codeine and dihydrocodeine, however, can be transformed into morphine and dihydromorphine by CYP2D6 [25,37,38], an enzyme expressed in liver as well as in brain [39]. However, CYP2D6 presents a genetic polymorphism, in that 7–10% of the Caucasian population do not express a functional enzyme [40] and can therefore not form more than trace amounts of morphine or dihydromorphine from codeine or dihydrocodeine, respectively [24,25]. Clinical studies by us and others with healthy volunteers showed that only those with functional CYP2D6 activity obtained analgesic effects from codeine administration. This suggests that the analgesic effects obtained upon codeine administration are mediated by the metabolite morphine, rather than by codeine itself [24,38]. Our current results show that codeine and dihydrocodeine do not significantly affect AC activity. These results suggest that the metabolically formed morphine and dihydromorphine, rather than the weak opiates codeine and dihydrocodeine themselves, may lead to the analgesic effects as well as to the development of addiction and withdrawal symptoms. Furthermore, these results may explain the individual differences in the development of addiction symptoms by codeine and dihydrocodeine as a result of the genetic polymorphism of the metabolizing enzyme, CYP2D6. These considerations are supported by a recently published study, which demonstrates that individuals lacking CYP2D6 activity are protected against oral codeine, oxycodone and hydrocodone dependence [41]. As a consequence, subjects addicted to weak opiates have been successfully treated with fluoxetin, an inhibitor of CYP2D6, resulting in a reduction of the usage of these weak opiates [41]. Taken together, our results help to understand the development of addiction and withdrawal symptoms following the use of weak opioid receptor agonists such as codeine and dihydrocodeine, supporting the hypothesis that the metabolic transformation of weak opiates to stronger opiates (e.g. morphine and dihydromorphine) is an important step in the devolopment of addiction to these drugs.

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