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μ Opioid receptor-mediated G-protein activation by heroin metabolites: evidence for greater efficacy of 6-monoacetylmorphine compared with morphine

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Abstract

The efficacy of heroin metabolites for the stimulation of μ opioid receptor-mediated G-protein activation was investigated using agonist-stimulated [^{35}S]guanosine-5′-*O*-(γ-thio)-triphosphate binding. In rat thalamic membranes, heroin and its primary metabolite, 6-monoacetylmorphine (6-MAM), were more efficacious than morphine or morphine-6-β-D-glucuronide. This increased efficacy was not due to increased action of heroin and 6-MAM at δ receptors, as determined by competitive antagonism by naloxone, lack of antagonism by naltrindole, and competitive partial antagonism with morphine. In agreement with this interpretation, the same relative efficacy profile of heroin and its metabolites was observed at the cloned human μ opioid receptor expressed in C6 glioma cells. Moreover, these efficacy differences were GDP-dependent in a manner consistent with accepted mechanisms of receptor-mediated G-protein activation. The activity of heroin was attributed to *in vitro* deacetylation to 6-MAM, as confirmed by HPLC analysis. These results indicate that the heroin metabolite 6-MAM possesses higher efficacy than other heroin metabolites at μ opioid receptors, which may contribute to the higher efficacy of heroin compared with morphine in certain behavioral paradigms *in vivo.*

Keywords: Mu opioid receptor; G-protein; Agonist efficacy; Heroin; 6-Monoacetylmorphine; Signal transduction

1. Introduction

3,6-Diacetylmorphine (heroin) and morphine are both effective analgesics, but also produce undesirable effects such as respiratory depression, tolerance, and dependence. Morphine is widely used clinically, but heroin is an illicit opiate that accounts for a major portion of non-medical opiate use in the United States. Although heroin generally is more potent than morphine when administered *in vivo*, previous studies have found no advantages of heroin over morphine in the treatment of chronic or post-surgical pain [1]. However, a recent study of the agonist actions of opioids in a rodent neuropathic pain model has demonstrated that heroin is both a more potent and efficacious anti-allodynic agent than morphine [2]. Thus, although the increased agonist potency of heroin compared with morphine may be due to pharmacokinetic differences between these two drugs [3,4], it is also possible that a greater intrinsic efficacy of heroin resulting in greater receptor reserve may contribute to its increased potency *in vivo.* Because heroin is metabolized rapidly to 6-MAM, and then to morphine [3,5], it is likely that any greater intrinsic efficacy of heroin over morphine is due to the primary metabolite, 6-MAM. Moreover, since most of the *in vivo* effects of morphine are due primarily to its actions at μ-type opioid receptors [6–9] and since most opiate alkaloids bind to μ receptors with high affinity [10], any increased efficacy of heroin versus morphine may be due to a greater efficacy of 6-MAM as a μ

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Abbreviations: 6-MAM, 6-monoacetylmorphine; GTPγS, guanosine-5′-*O*-(γ-thio)-triphosphate; DAMGO, [D-Ala^2,(*N*-Me)Phe^4,Gly^5(OH)]enkephalin; M-6-G, morphine-6-β-D-glucuronide; and hMOR-C6, C6 rat glioma cells expressing human μ opioid receptors.

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receptor agonist. Alternatively, heroin could exert actions at different opioid receptor subtypes, as suggested by recent studies that demonstrated that heroin analgesia could be mediated by μ receptor splice variants [11,12].

Agonist efficacy at μ opioid receptors can be measured *in vitro* using agonist-stimulated [^{35}S]GTPγS binding [13–15], which is a direct measure of receptor-mediated G-protein activation [16–18]. Thus, under conditions where there is no receptor reserve for G-protein activation, maximal stimulation of [^{35}S]GTPγS binding by the agonist-occupied receptor directly correlates with intrinsic efficacy [19]. In the present study, [^{35}S]GTPγS binding was used to examine the efficacy of heroin and its metabolites, 6-MAM, morphine, and M-6-G, compared with opioids that are known to be full agonists for G-protein activation by μ opioid receptors in brain, DAMGO and methadone [15,19, 20]. These experiments were conducted in membranes from rat thalamus, which contains predominantly μ opioid receptors [21,22], and from transfected C6 glioma cells expressing a pure population of human μ opioid receptors.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats (150–200 *g*) were purchased from Zivic Miller. [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]naloxone (57.5 Ci/mmol) were purchased from the New England Nuclear Corp. Guanosine-5′-diphosphate and GTPγS were purchased from Boehringer Mannheim. DAMGO, naloxone, naltrindole, and adenosine deaminase were purchased from the Sigma Chemical Co. All other opioid drugs were obtained from the Research Triangle Institute through the NIDA drug supply program. Geneticin (G-418), fetal bovine serum, and penicillin-streptomycin were purchased from Gibco/BRL. Dulbecco's Modified Eagle's Medium and Scintisafe Econo-1 scintillation fluid were purchased from Fisher Scientific. All other reagent grade chemicals were purchased from the Sigma Chemical Co. or Fisher Scientific. The human μ opioid receptor cDNA construct was provided by Dr. Erik Mansson of the Adolor Corp.

2.2. Cell culture and membrane preparation

Cells were cultured at 37° in a humidified atmosphere of 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium containing 100 μg/mL of penicillin and streptomycin, and 5% fetal bovine serum (culture medium). hMOR-C6 cells were grown as above in culture medium containing 1 mg/mL of Geneticin (G-418). Cells were harvested and membranes were prepared as previously described [15,19]. Rat thalamic membranes also were prepared as previously described [15,19].

2.3. Cell transfection

C6 rat glioma cells in exponential growth phase were detached mechanically from culture plates and collected by centrifugation at 345 *g* for 10 min at 4°. Cells were resuspended in culture medium, counted with a hemacytometer, and diluted to a concentration of 3.5×10^6/mL. Human μ opioid receptor cDNA, in *pc*DNA-neo, was linearized with *Pvu*I, and 1- to 10-μg aliquots of linear DNA were transferred to electroporation cuvettes containing 0.4 mL each of cell suspension. Electroporation was performed at 0.26 kV, 1050 μF, with infinite internal resistance, using a Bio-Rad Gene Pulser II electroporation system. Cells were then diluted 20× in culture medium, plated 2 mL/well on a 12-well plate, and incubated for 48 hr under standard culture conditions. The medium then was replaced with culture medium containing 2 mg/mL of Geneticin, and the cells were grown to confluency. Cells were harvested and diluted into 96-well plates at a density of 1 cell/3 wells. Cloned cells were transferred to 100-mm plates and grown to confluency. Membranes from each clone were screened for μ opioid receptor expression by assay of [^{3}H]naloxone binding.

2.4. [^{3}H]Naloxone binding

Cell membranes (100 μg) were incubated with 0.1 to 15 nM [^{3}H]naloxone in assay buffer for 1 hr at 30°. Nonspecific binding was determined with 10 μM levallorphan. The incubation was terminated by rapid vacuum filtration through GF/B glass fiber filters using a 48-well Brandel cell harvester, and washed three times with 3 mL of ice-cold 50 mM Tris–HCl, pH 7.4. The filters were extracted overnight in Scintisafe Econo-1 scintillation fluid, and radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H.

2.5. [^{35}S]GTPγS binding

Agonist-stimulated [^{35}S]GTPγS binding was assayed as previously described [15,19] with minor modifications. Briefly, membranes (10 μg rat thalamus or 30 μg hMOR-C6) were incubated with 0.05 nM [^{35}S]GTPγS, 30 μM GDP, and various concentrations of agonists with and without antagonists in assay buffer [50 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl] at 30° for 2 hr (thalamus) or 90 min (hMOR-C6). In some experiments, 0.05 nM [^{35}S]GTPγS and 30 μM GDP were replaced by 0.1 nM [^{35}S]GTPγS and various concentrations of GDP, respectively. In experiments examining endomorphin-1, membranes (10 μg thalamus, 5 μg striatum, or 10 μg amygdala) were pretreated with protease inhibitors as previously described [20]. Nonspecific binding was determined with 20 μM unlabeled GTPγS. The incubation was terminated by vacuum filtration, the filters were extracted overnight in scintillation fluid, and radioactivity was deter-

mined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S.

2.6. HPLC

Analysis of morphine, heroin, and 6-MAM was conducted by reverse phase HPLC by a modification of a previous procedure [23]. Drug standards were prepared in deionized H_2O at room temperature and stored frozen at −80° until used. Prior to assay, drugs were thawed at room temperature and stored at 4° for not more than 2 hr prior to addition to the incubation mixture. To determine the conditions for chromatographic analysis and to ensure the purity of each drug prior to assay, aliquots of drug standards (100 μM) were applied to the HPLC. Each drug standard yielded a single elution peak, except for heroin which eluted as approximately 92% heroin and 8% 6-MAM. Then aliquots of drug standards (final concentration of 100 μM each) were incubated at 30° with rat thalamic membranes under the same conditions as described above for [^{35}S]GTPγS binding. In one experiment, membranes were immersed in boiling water for 5 min before addition to the incubation mixture. After incubation, membranes were centrifuged at 25,000 *g* for 10 min at 4°, and then aliquots (100 μL) of the supernatant were applied to HPLC for analysis. Separation of drugs was accomplished on a C-18 reverse phase column (3 μm Microsorb C-18, Rainin Instrument Co.), using 0.1 M NaH_2PO_4, pH 7.3, 35% methanol, as the mobile phase (1 mL/min). Drugs were detected by UV absorbance at 225 nm; peak elution times were: morphine, 2.6 min; 6-MAM, 3.7 min; and heroin, 6.8 min. Drug levels were quantified by integration of peak areas, followed by calculation against known quantities of drug standards eluted from the HPLC under identical conditions. At the concentration of drugs used (100 μM), total recovery of opiates from incubated membranes was comparable to that of the opiates incubated under identical conditions with boiled membranes.

2.7. Data analysis

All data are reported as the means ± SEM of at least three experiments that were each performed in triplicate, except for the HPLC data which was the means ± SEM of single determinations from three experiments. Nonlinear regression analysis was conducted by iterative fitting using JMP (SAS for Macintosh). Statistical significance of the data was determined by analysis of variance followed by the Tukey-Kramer HSD test, Dunnett's test, or the two-tailed unpaired Student's *t*-test, also performed using JMP. K_e values were determined by the following relationship: K_e = [antagonist]/(DR − 1), where DR = (EC_{50} + antagonist)/(EC_{50} − antagonist).

3. Results

3.1. Agonist efficacy

Stimulation of [^{35}S]GTPγS binding by heroin and its metabolites was compared with that obtained with the full μ opioid agonist, DAMGO. In rat thalamic membranes (Fig. 1A), DAMGO stimulated [^{35}S]GTPγS binding by 104 ± 5% over basal levels (264 ± 9.6 fmol/mg membrane protein). Heroin, 6-MAM, morphine, and M-6-G all produced concentration-dependent stimulation of [^{35}S]GTPγS binding, with all metabolites showing over 10-fold greater potency than heroin. Maximal stimulation was similar for both morphine and M-6-G (55–60% of DAMGO), and similar to values previously reported for morphine in thalamic membranes [15,20]. In contrast, both heroin and 6-MAM displayed higher maximal stimulation of [^{35}S]GTPγS binding (75–80% of DAMGO) than morphine or M-6-G. Potency and relative efficacy values obtained from non-linear regression analysis of the data from thalamus are shown in Table 1A, which also shows values of another full μ agonist, methadone. These values confirmed that both heroin and 6-MAM were significantly more efficacious than morphine or M-6-G, but less efficacious than DAMGO and methadone.

Stimulation of [^{35}S]GTPγS binding also was examined in membranes from transfected C6 glioma cells stably expressing the human μ opioid receptor (hMOR-C6 cells). In these cells, [^{3}H]naloxone binding exhibited a B_{max} value of 361 ± 27 fmol/mg membrane protein, and a K_D value of 1.45 ± 0.16 nM. DAMGO stimulated [^{35}S]GTPγS binding by 105 ± 5% above basal levels (30 ± 2.9 fmol/mg membrane protein). Agonists stimulated [^{35}S]GTPγS binding with a similar relative efficacy profile as in rat thalamic membranes: DAMGO = methadone > heroin = 6-MAM > morphine = M-6-G (Fig. 1B, Table 1). As in thalamic membranes, heroin was over 10-fold less potent than 6-MAM, morphine, or M-6-G. Non-linear regression analysis of the data confirmed that both heroin and 6-MAM were significantly more efficacious than morphine or M-6-G, but less efficacious than DAMGO or methadone (Table 1B). Therefore, differences in efficacies of these agonists observed in the brain are maintained in hMOR-C6 cells.

Further evidence that 6-MAM is a higher efficacy partial μ agonist than morphine or M-6-G was provided by varying the concentration of GDP in [^{35}S]GTPγS binding assays in rat thalamic membranes. These data (not shown) revealed that all three agonists, as well as DAMGO, produced the same low level of stimulation at low concentrations (1–3 μM) of GDP. However, at 10–100 μM GDP, the percent stimulation by 6-MAM was significantly greater than that obtained with morphine and M-6-G ($P < 0.05$ by the Tukey-Kramer HSD test), but less than that obtained with DAMGO. This increased difference in efficacies with in-

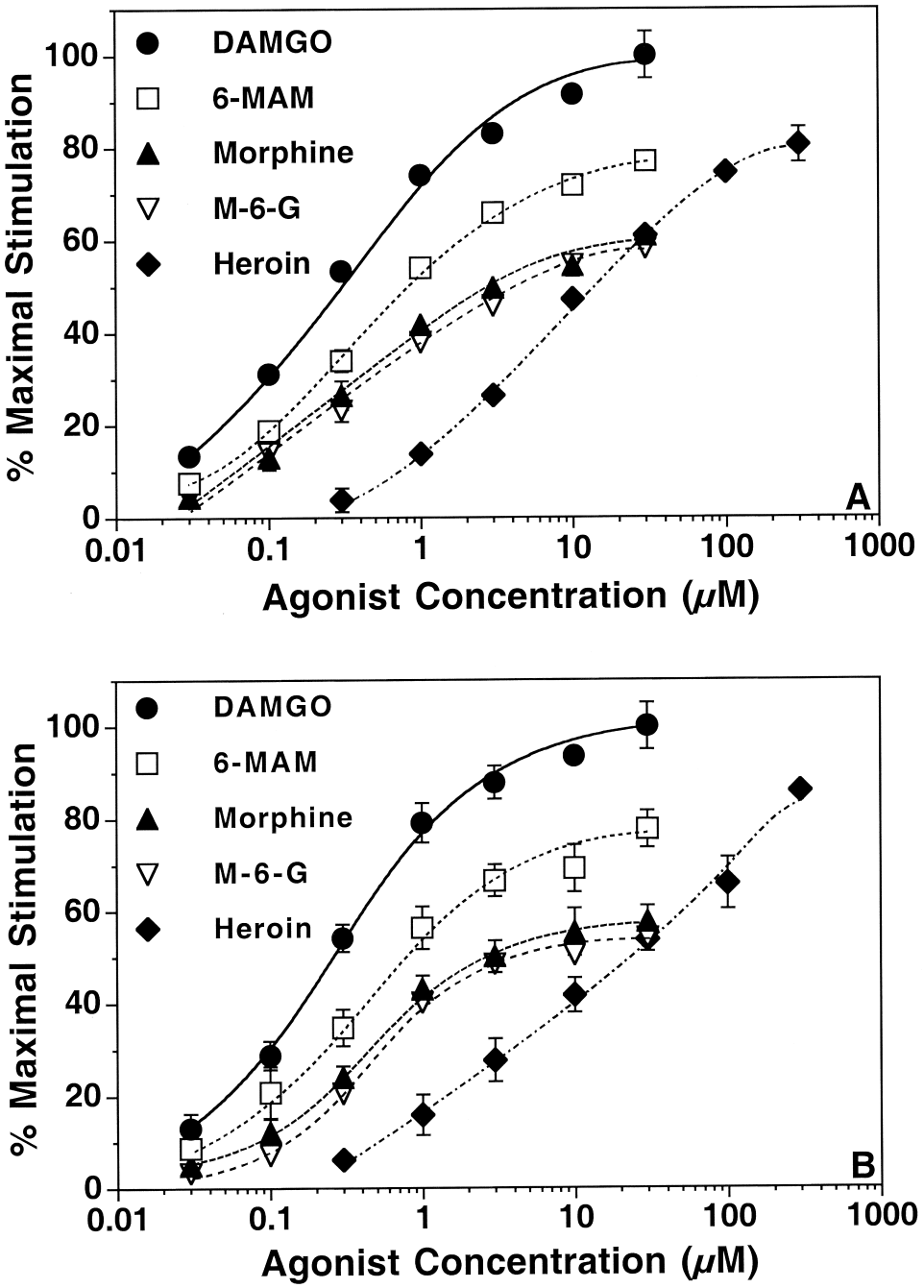

Fig. 1. Stimulation of [^{35}S]GTPγS binding by opioid agonists. Membranes from (A) rat thalamus or (B) hMOR-C6 cells were incubated with 0.05 nM [^{35}S]GTPγS, 30 μM GDP, and various concentrations of opioid agonist as described in "Materials and methods." Data are the means ± SEM (N ≥ 4) of percent maximal stimulation by 30 μM DAMGO.

creasing GDP concentrations is consistent with previous findings with full and partial μ agonists [15,20].

Partial agonists can partially block the activities of higher-efficacy agonists. To demonstrate that morphine can competitively antagonize 6-MAM-stimulated [^{35}S]GTPγS binding, morphine concentration–effect curves were conducted in rat thalamic membranes with 10 μM 6-MAM (Fig. 2). These results showed that morphine produced a significant, concentration-dependent partial blockade of the stimulation by 6-MAM to the level of stimulation produced by morphine alone.

3.2. Receptor specificity

Opioid antagonist experiments confirmed that the stimulation of [^{35}S]GTPγS binding by 6-MAM was mediated by μ receptors in rat thalamus (Fig. 3). Addition of 25 nM naloxone produced a parallel rightward shift in the stimu-

Table 1
EC_{50} and E_{max} values of opioid agonist-stimulated [^{35}S]GTPγS binding

Agonist	EC_{50} (nM)	E_{max} (% Max)
(A) Rat thalamic membranes		
DAMGO	229 ± 18	100 ± 5[a]
Methadone	467 ± 58	99 ± 3[a]
6-MAM	356 ± 32	75 ± 0.4[b]
Heroin	7030 ± 1050*	80 ± 2[b]
Morphine	410 ± 80	59 ± 2[c]
M-6-G	450 ± 89	57 ± 2[c]
(B) hMOR-C6 cell membranes		
DAMGO	312 ± 29	100 ± 5[a]
Methadone	535 ± 65	90 ± 5[a]
6-MAM	357 ± 98	75 ± 4[b]
Heroin	5660 ± 1950*	73 ± 5[b]
Morphine	398 ± 53	57 ± 3[c]
M-6-G	462 ± 24	55 ± 2[c]

Data are means ± SEM (N = 4–7) obtained from non-linear regression analysis of agonist concentration–effect curves. E_{max} values with similar letter designations were not significantly different from each other ($P > 0.05$), whereas those that do not contain any similar letter designations were significantly different from each other ($P < 0.01$ in thalamic membranes and $P < 0.05$ in hMOR-C6 membranes by the Tukey–Kramer HSD test).

* The EC_{50} value of heroin was significantly different from that of all other agonists ($P < 0.01$ by Dunnett's test), but the EC_{50} values of all other agonists were not significantly different from each other ($P > 0.05$ by ANOVA).

lation of [^{35}S]GTPγS binding by 6-MAM, with a calculated K_e value of 0.75 ± 0.12 nM for naloxone. Similarly, the naloxone K_e value for M-6-G-stimulated [^{35}S]GTPγS binding was 1.31 ± 0.17 nM (not shown). In contrast, addition of a 1 nM concentration of the δ opioid-selective antagonist naltrindole (which occupies 93% of δ receptors and 9% of μ receptors, assuming K_D values of 0.15 and 20 nM, respectively [10,24]) had no effect on 6-MAM-stimulated [^{35}S]GTPγS binding (Fig. 3).

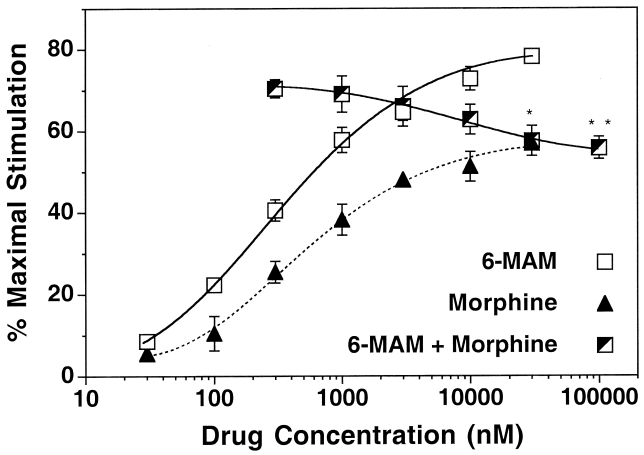

Fig. 2. Competitive partial antagonism of 6-MAM-stimulated [^{35}S]GTPγS binding by morphine. Membranes from rat thalamus were incubated with 0.05 nM [^{35}S]GTPγS, 30 μM GDP, and various concentrations of 6-MAM, or of morphine in the presence and absence of 10 μM 6-MAM. Data are the means ± SEM (N ≥ 3) of percent maximal stimulation by DAMGO. Key: (*) $P < 0.05$, and (**) $P < 0.01$ significantly different from 10 μM 6-MAM alone (by Dunnett's test).

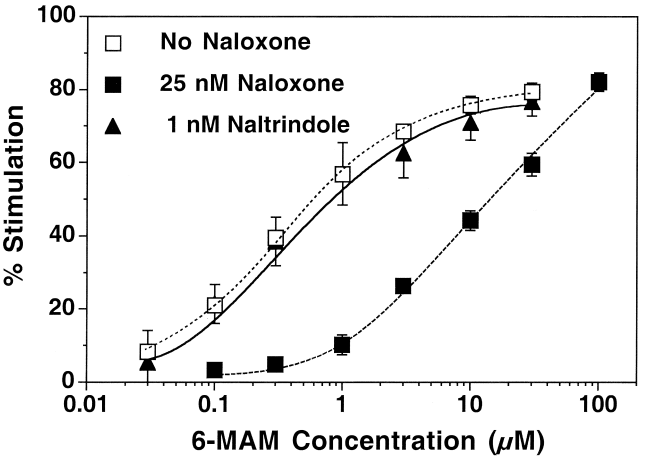

Fig. 3. Effect of naloxone and naltrindole on the stimulation of [^{35}S]GTPγS binding by 6-MAM. Thalamic membranes were incubated with 0.05 nM [^{35}S]GTPγS, 30 μM GDP, and various concentrations of 6-MAM in the presence and absence of 25 nM naloxone or 1 nM naltrindole. Data are the means ± SEM (N ≥ 3) of percent stimulation by 6-MAM.

Previous studies have indicated that C6 substituted morphine derivatives, such as 6-MAM and M-6-G, may act at a subtype or isoform of the μ opioid receptor that does not have significant affinity for morphine [11,12,25]. If this receptor subtype were contributing to the higher efficacy of 6-MAM for G-protein activation compared with morphine, then the relative efficacy of 6-MAM for stimulation of [^{35}S]GTPγS binding should correlate with levels of this receptor. Because this subtype is found in low levels in the thalamus, experiments were conducted in other brain regions reported to express significant levels of this receptor subtype, including the striatum and amygdala [25], in the presence of 1 nM naltrindole to block δ opioid receptors. Furthermore, because the highly μ-selective opioid peptide endomorphin-1 exhibits a similar relative efficacy [20] to that shown by 6-MAM in the present study, this agonist was included as a control. The results of these experiments (Fig. 4) showed that the relative efficacy of 6-MAM, morphine, and endomorphin-1 did not vary significantly among the thalamus, striatum, and amygdala. In each region, 6-MAM and endomorphin-1 were equally efficacious, and each of these agonists produced greater stimulation than morphine. The relative efficacy of 6-MAM and morphine were confirmed in concentration–effect curves in the striatum (data not shown). The relative E_{max} value of 6-MAM was 71 ± 0.8%, whereas that of morphine was 52 ± 2.8% ($P < 0.005$ different by Student's t-test, df = 4, N = 3), with no significant difference in potency (270 ± 31 vs 156 ± 69 nM, respectively).

3.3. Heroin metabolism

To determine whether heroin was metabolized to 6-MAM and/or morphine during the [^{35}S]GTPγS binding

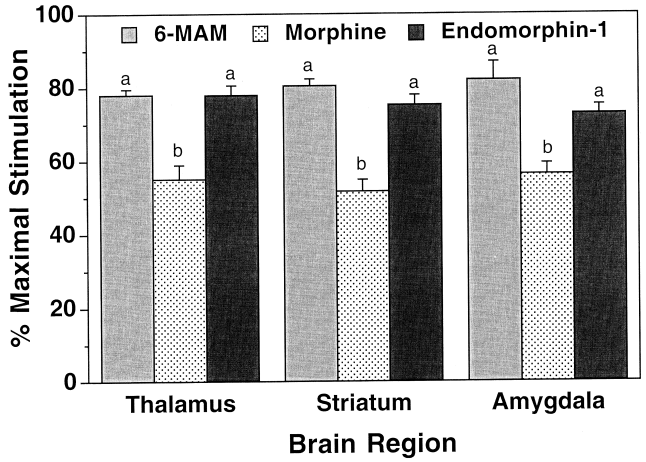

Fig. 4. Relative maximal stimulation of [^{35}S]GTPγS binding by 6-MAM, morphine, and endomorphin-1 in various brain regions. Membranes were incubated with 0.05 nM [^{35}S]GTPγS, 30 μM GDP, and 30 μM 6-MAM, morphine, or endomorphin-1 in the presence of 1 nM naltrindole. Data are the means $\pm$ SEM (N $\geq$ 3) of percent maximal stimulation by 30 μM DAMGO. Values with similar letter designations were not significantly different from each other ($P > 0.05$), whereas those that do not contain any similar letter designations were significantly different from each other ($P < 0.01$ by the Tukey-Kramer HSD test).

assay, these opiates were each incubated for 2 hr with thalamic membranes under [^{35}S]GTPγS binding assay conditions. HPLC analyses of the supernatants were then conducted, and the data were expressed as a percent of the total amount of opiates detected. Results (Fig. 5) showed that only morphine was detected with the addition of morphine to the membranes. When 6-MAM was incubated with the membranes, approximately 95% was recovered as 6-MAM, with 5% recovered as morphine. In contrast, when heroin was incubated with the membranes, only 38% was recovered as heroin, with approximately 60% recovered as 6-MAM and 2% recovered as morphine. This conversion was not enzymatic in nature: when the membranes were boiled for 2 min prior to incubation with the opiates, similar percentages of each compound were detected as when incubated with native thalamic membranes.

This non-enzymatic conversion of heroin to 6-MAM affects the potency, but not the efficacy, of the drug. In Fig. 6, heroin was preincubated in assay buffer for 2 hr prior to the assay of [^{35}S]GTPγS binding. Results showed that this preincubation increased the potency of heroin in stimulating [^{35}S]GTPγS binding by approximately 4-fold (EC_{50} value 1.24 $\pm$ 0.42 μM after preincubation compared with 5.0 $\pm$ 0.36 μM in the control; $P < 0.01$ different by Student's *t*-test, df = 4, N = 3), with no effect on E_{max} values (73 $\pm$ 3% after preincubation compared with 75 $\pm$ 6% in the control).

4. Discussion

The present study demonstrated that heroin and its primary metabolite, 6-MAM, displayed greater efficacy for μ opioid receptor-mediated G-protein activation than morphine. The relative efficacies of heroin and 6-MAM were similar to that previously reported for the highly μ-selective putative endogenous opioid peptide, endomorphin-1 [20]. The finding that the relative efficacy of 6-MAM, compared

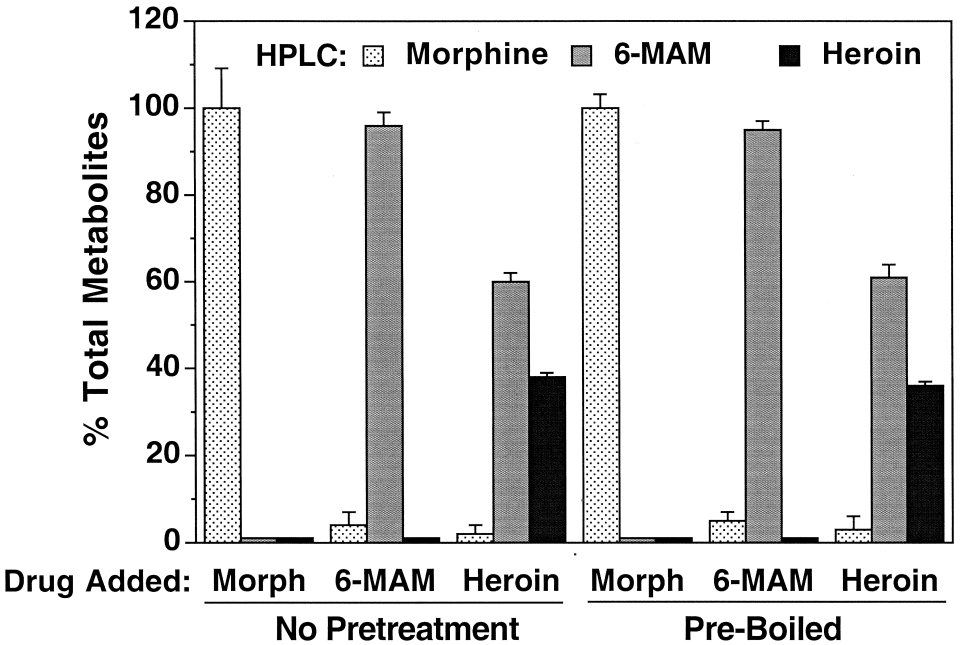

Fig. 5. *In vitro* metabolism of opiates determined by HPLC analysis. Thalamic membranes were incubated with 0.05 nM GTPγS, 30 μM GDP, and 100 μM morphine, 6-MAM, or heroin. After centrifugation of membranes, aliquots of the resulting supernatants were subjected to isocratic reverse phase HPLC as described in "Materials and methods." Data are the means $\pm$ SEM (N = 3) of the percent of total opiates recovered under each condition.

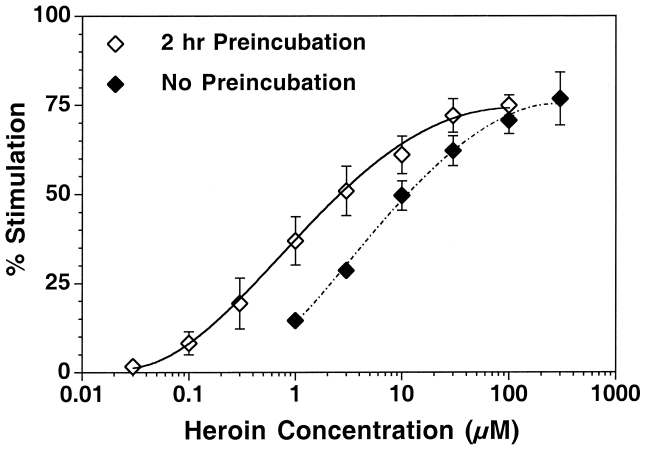

Fig. 6. Effect of heroin preincubation on the stimulation of [^{35}S]GTPγS binding. Thalamic membranes were incubated with 0.05 nM [^{35}S]GTPγS, 30 μM GDP, and various concentrations of heroin that had or had not been preincubated in assay buffer for 2 hr at 30° prior to the assay. Data are the means ± SEM (N = 3) of percent stimulation by heroin.

with that of other opioids, was dependent upon the concentration of GDP agrees with previous [^{35}S]GTPγS binding studies with μ opioid [15,20] and several other $G_{i/o}$-coupled receptors [26–28]. Thus, 6-MAM displayed typical characteristics of a high efficacy partial agonist.

It is probable in the present study that both 6-MAM and M-6-G were acting through a μ receptor-mediated mechanism. Naloxone K_e values for the reversal of 6-MAM- and M-6-G-stimulated [^{35}S]GTPγS binding in rat thalamus (0.75 to 1.3 nM) were similar to those previously reported for reversal of the stimulation of [^{35}S]GTPγS binding by the highly μ-selective agonist DAMGO in both rat thalamus and CHO cells expressing cloned μ receptors [19,22], and to measurements of the affinity of naloxone for binding to cloned μ opioid receptors in both the present and previous studies [19,29]. In certain strains of mice, heroin and 6-MAM have been reported to show antinociceptive activity that was due to activation of δ receptors [30–32]. However, the possibility that a small number of δ opioid receptors present in rat thalamus [21,22] may have contributed to the increased efficacy of 6-MAM was ruled out by the lack of effect of naltrindole. It is also possible that the increased efficacy of 6-MAM compared with morphine was due to its actions at an alternative splice variant of the μ opioid receptor which binds 6-MAM and M-6-G with high affinity, but to which morphine binds poorly [11,12,25]. The present study cannot address this question conclusively; however, two lines of evidence argue against this possibility. First, the thalamus is a brain region that has been reported to contain very low levels of this receptor subtype, yet the relative efficacies of 6-MAM and morphine in striatum and amygdala, which contain this receptor [25], were similar to that in the thalamus. Moreover, the relative efficacy of the highly μ-selective opioid peptide endomorphin-1 was not significantly different from that of 6-MAM in all three regions, suggesting that both were acting through a μ receptor-mediated mechanism. Second, the same relative efficacy profile for heroin and 6-MAM versus M-6-G and morphine was obtained in brain as in hMOR-C6 cells expressing a pure population of cloned human μ receptors. Although C6 cells have been reported to endogenously express opioid receptors under certain conditions [25,33], the untransfected C6 cells used in the present study did not bind [^{3}H]naloxone (data not shown). Thus, regardless of the efficacy of heroin metabolites at δ receptors or at variants of opioid receptors present in other tissues, the present study shows that 6-MAM is more efficacious than morphine or M-6-G at both the rat and human μ receptor. These findings do not, however, disprove the existence of a 6-MAM-selective μ receptor isoform, as this receptor may be present at low abundance or coupled to a non-$G_{i/o}$ type of G-protein and therefore remains undetected in the [^{35}S]GTPγS binding assay. Nonetheless, these results suggest that in interpreting any study comparing the effects of heroin or 6-MAM with those of morphine, the relative efficacy of these drugs at the μ receptor should be taken into consideration.

The low potency of heroin for G-protein activation suggests that *in vitro* degradation to 6-MAM is required for receptor activation to be detected. This interpretation agrees with previous observations that rapid degradation of heroin to 6-MAM in brain membrane homogenates accounts for its binding affinity [34], and is supported by HPLC results in the present study. It is known that heroin is deacetylated to 6-MAM by non-enzymatic mechanisms [35–37], and this appears to be the case in the present study, since similar levels of 6-MAM were detected after incubation with boiled membranes. Moreover, the finding of increased apparent potency of heroin after preincubation in assay buffer suggests that the *in vitro* deacetylation of heroin to 6-MAM was due, at least in part, to non-enzymatic degradation.

The structural basis of the increased efficacy profile of 6-MAM compared with that of morphine is due to acetylation of the sixth carbon position; however, it is uncertain whether increased bulk and/or electronegativity contribute to this difference. It is unlikely to be due to bulk alone because M-6-G was no more efficacious than morphine in stimulating G-protein activation. Furthermore, the 6-methyl ether derivative of morphine, heterocodeine, did not show increased efficacy compared with morphine in the μ receptor-mediated stimulation of [^{35}S]GTPγS binding (Selley D, Wojno H, and May E, unpublished observations). Thus, although acetylation of the sixth carbon of morphine appears to be optimal for increasing efficacy at the μ receptor, further studies will be required to determine whether other substitutions can produce similar results.

These efficacy differences between heroin, 6-MAM, and morphine may be relevant to opiate actions *in vivo.* However, it is difficult to gauge *in vivo* efficacy differences from the literature because most experimental paradigms show full agonist effects with morphine. Although heroin has been reported to exhibit higher efficacy than morphine in suppressing allodynia after lumbar nerve ligation in the rat

[2], few other studies have compared heroin or 6-MAM with morphine in behavioral tests of intrinsic efficacy, such as with the use of irreversible antagonists to measure receptor reserve. Inferring intrinsic efficacy directly from potency measurements can be misleading. For example, 6-MAM was found previously to have a slightly lower antinociceptive potency than morphine after i.c.v. administration in mice [38], suggesting the possibility of lower receptor reserve. However, heroin has been shown to modify reward and aversion thresholds of electrical brain stimulation in rats with 6- to 40-fold greater potency than morphine [39], suggesting higher receptor reserve for 6-MAM. Although heroin is known to show receptor reserve for maintaining self-administration in rats [40], it has not been directly compared with morphine. Thus, *in vivo* potencies depend upon a variety of factors in addition to intrinsic efficacy, including differences in receptor affinity and possible pharmacokinetic differences related to species and route of administration, and differences in the sensitivities of different behavioral endpoints to opiates. Nonetheless, the present results indicate that heroin and 6-MAM display greater efficacy than morphine at the level of μ opioid receptor-mediated G-protein activation. Considering that 6-MAM is rapidly concentrated in the brain and cerebrospinal fluid following heroin administration in humans [41], the findings of increased receptor signaling efficacy of 6-MAM suggest that heroin may possess higher intrinsic efficacy than morphine with regard to analgesia and/or behavioral reinforcement in humans. Furthermore, it is also possible that 6-MAM possesses a different intrinsic efficacy than morphine at δ or κ opioid receptors, which could also contribute to any differences in the behavioral effects of these two opiates. This possibility will be addressed in future studies.

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