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Mu opioid receptor efficacy and potency of morphine-6-glucuronide in neonatal guinea pig brainstem membranes: Comparison with transfected CHO cells

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ABSTRACT: The major side effect of morphine and its active metabolite, morphine-6-glucuronide (M6G), is respiratory depression, which is mediated by μ opioid receptors in the medulla and pons. Although the effect of morphine on coupling between μ opioid receptors and G proteins has been studied, the effect of M6G on this coupling has not. Therefore, stimulation of guanylyl-5′-O-([γ^{35}S]-thio)-triphosphate ([^{35}S]-GTPγS) binding by these two narcotic analgesic drugs was compared to the μ-specific synthetic opioid peptide [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin in Chinese hamster ovarian cells stably transfected with the murine μ opioid receptor and in brainstem membranes prepared from 3-, 7-, and 14-day-old guinea pigs. All three agonists stimulated [^{35}S]-GTPγS binding in transfected cells and neural tissue, and the stimulation was antagonized by naloxone. In brainstem membranes, but not transfected cells, M6G was less efficacious but more potent than morphine, which may be due to differences between murine and guinea pig μ opioid receptors or in the G proteins in these two tissues. Efficacy of the agonists did not change during development, but overall potency decreased between 3 and 14 days after birth. *In vivo* potency differences for respiratory depression between morphine and M6G are qualitatively similar to *in vitro* potency differences of these drugs to stimulate [^{35}S]-GTPγS binding in neonatal guinea pig brainstem membranes. Tolerance to opioid effects on [^{35}S]-GTPγS binding developed in transfected cells incubated with morphine with the maximum decrease in potency occurring 18 h later than the maximum decline in efficacy. © 2001 Elsevier Science Inc.

KEY WORDS: DAMGO, Development, GTPγS binding, Morphine, Respiration, Cellular tolerance.

INTRODUCTION

In the adult and neonatal guinea pig as well as the human, μ opioid drugs, such as morphine, depress respiration and the major site of action of this effect is in the brainstem. Morphine-6-glucuronide (M6G), an active metabolite of morphine, has many of the attributes of a μ agonist, including depression of breathing. In the opioid naïve neonatal guinea pig, subcutaneously injected M6G is equipotent to morphine in depressing the ventilatory response to carbon dioxide inhalation at 3 days after birth, but it is eightfold more potent by day 7 [18]. This increase in potency persists through day 14 and is not due to changes in M6G pharmacokinetics such as an increased systemic absorption or increased central nervous system distribution compared to morphine [19]. There is, however, a 42% increase in μ receptor density in guinea pig pontomedullary membranes from day 3 to day 7 after birth [20], but this change should affect morphine and M6G potency equally unless these two agonists have different receptor efficacies. μ, along with δ and κ opioid receptors, contains seven transmembrane domains and belongs to the family of G protein coupled receptors [37]. These receptors are activated by endogenous opioid peptides and alkaloids. The μ opioid receptor (MOR) is the molecular target for potent analgesic drugs, such as morphine, as well as the morphine metabolite M6G and the synthetic opioid peptide [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin (DAMGO). The μ opioid receptor selectively activates G proteins of the G_i/G_0 family [5,8] which couple to effectors decreasing adenylyl cyclase activity [5,13,25] and calcium channel conductance [17,27], as well as increasing potassium channel conductance [22]. Upon agonist binding, the receptor interacts with the G protein and decreases the affinity of the GDP-bound α subunit for GDP and increases its affinity for GTP, resulting in a guanine nucleotide exchange [10]. The receptor catalytically activates G proteins, thus a single receptor can activate multiple G proteins [31]. In a random collision-coupling model, the rate of G protein activation is dependent on receptor density, but not G protein levels [35]. A recent report, however, questions this model and suggests that there may be a compartmental organization of opioid receptors and G proteins [26].

The [^{35}S]-GTPγS binding assay examines receptor activation of G proteins in isolated cellular membranes. It measures agonist-stimulated binding of the hydrolysis-resistant GTP analog [^{35}S]-GTPγS in the presence of GDP [11,15]. Established for the μ opioid receptor by Traynor and Nahorski [36], this assay was effective in demonstrating that different μ-selective agonists have different efficacies in stimulating [^{35}S]-GTPγS binding to human neuroblastoma SH-SY5Y cell membranes.

Biochemical mechanisms of cellular tolerance have been studied in many systems, including SH-SY5Y cells [7], C_6 glial cells

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[39], and Chinese hamster ovarian (CHO) cells transfected with the rat μ opioid receptor [12]. It has been shown that chronic exposure of opioid receptors to agonists leads to a cellular tolerance caused most likely by an initial desensitization of the receptor due to uncoupling, followed by down-regulation as the number of cell surface receptors decreases [21]. Studies using 7315c pituitary tumor cells [24] and SH-SY5Y cells [3] examined altered properties of μ opioid receptor/effector components during cellular tolerance. Although the exact mechanisms of cellular tolerance are unknown, it probably involves phosphorylation of the receptor and binding of β-arrestin to the phosphorylated receptor [2,14]. Stimulation of [^{35}S]-GTPγS binding by DAMGO was higher in membranes from cells pre-incubated with partial agonists, including morphine, compared to pre-incubations with full agonists, such as DAMGO [39], once again emphasizing the importance and differing roles of agonist efficacy and potency in the desensitization and down regulation processes [3,23,38]. In SH-SY5Y cells, incubation with morphine (10 μM) for 48 h induced cellular tolerance indicated by a 2.5-fold increase in EC_{50} and reduced E_{max} 31%. It was also shown that this development of cellular tolerance was complete within 24 h and was blocked by the μ opioid receptor antagonist naloxone [7].

M6G has been shown to bind reversibly to the μ opioid receptor [16], however, data concerning the relative potency of M6G to morphine have been inconclusive. Upon measuring cyclic adenosine monophosphate (cAMP) levels, both M6G and morphine showed a dose-dependent inhibition of cAMP accumulation and both were blocked by the antagonist naloxone [1]. Lambert et al. [13] showed that both morphine and M6G were equipotent when measuring cAMP activity, whereas morphine had a higher affinity than M6G for binding to the opioid receptor. In nociceptive stimuli experiments, however, M6G exerts its antinociceptive effect at low fractional occupancy of [^{3}H]-diprenorphine binding sites compared to morphine [9].

In [^{3}H]-DAMGO binding studies in the guinea pig pup brainstem, the K_i for M6G was threefold greater than the K_i for morphine. However, in rat brain homogenates there was a twofold greater binding affinity for M6G compared to morphine [4]. In SK-N-SH cells and rat thalamus membranes, it has been shown that DAMGO is more efficacious than morphine [29], however, in CHO cells expressing the murine μ opioid receptor, no difference in efficacy between DAMGO and morphine was reported [29].

The purpose of this study is to compare μ opioid receptor agonist M6G with its parent molecule morphine and the μ selective synthetic peptide selective for the μ opioid receptor, DAMGO, in both *in vivo* and *in vitro* systems. Development of cellular tolerance to morphine was examined in murine MOR-CHO cells because it is a less complex system than the brainstem tissue in that the transfected cells have one predominant opioid receptor as well as lower basal [^{35}S]-GTPγS binding in contrast to the brainstem. The questions that were being examined were: first, is there cross tolerance to M6G?; second, is the time course for development of cellular tolerance similar for the three agonists?; and third, is the time course similar for changes in EC_{50} and E_{max}? Brainstem tissue was studied because it is the site of opioid induced respiratory depression. The guinea pig was selected as the animal model for the brainstem studies because there is much relevant information in the scientific literature on opioid pharmacology in this species, guinea pig placental permeability is similar to the human, and the guinea pig pup is more neurologically mature at birth compared to the rat and mouse newborn. Efficacy (E_{max}) and potency (EC_{50}) of GTPγS binding was measured with agonist concentration-effect curves.

MATERIALS AND METHODS

Chemicals

[^{35}S]-GTPγS (1250 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). Morphine sulfate, DAMGO, and M6G were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Naloxone and all other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and Cell Lines

Topeka and Dunkin-Hartley guinea pigs (Simonsen Laboratories, Gilroy, CA, USA) were housed in the institutional animal care facilities under strict veterinarian control. The guinea pigs were kept under a 12-h light/dark cycle and were given food and water *ad libitum.*

Stably transfected CHO cells expressing either the murine μ or κ opioid receptor were cultivated in 75 cm^2 sterile tissue culture flasks (Sarstedt Inc., Newton, NC, USA) containing F-12 Nutrient Mixture, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml geneticin (all from GIBCO BRL, Gaithersburg, MD, USA).

Cell Incubations

To study the development of tolerance, cells were first transferred to 100 $\times$ 20 mm sterile tissue culture dishes (Sarstedt Inc.) via 2 ml Trypsin EDTA (GIBCO BRL) and were grown in monolayers to confluency in an incubator (Forma Scientific, Marietta, OH, USA) at a temperature of 37°C in a 5% CO_2 atmosphere. Either sterile morphine (10 μM) or an equal volume of sterile water (for control cells) was added to cells upon reaching confluency and incubated for 6 to 72 h. Morphine solutions were filtered in a sterile environment with 0.2-μm sterile nylon filters (Gelman Sciences, Ann Arbor, MI, USA). Control cells were exposed to morphine (10 μM) for 5 min prior to harvesting to control for any residual opioid effects.

Tissue Preparation

Guinea pig neonates were sacrificed by decapitation on 3, 7, or 14 days after birth. After decapitation, the brainstems rostral to the spinal cord and caudal to the inferior colliculi were removed immediately and homogenized in 20 ml/g of ice-cold 0.32 M sucrose and then centrifuged at 2000 $\times$ g for 5 min at 4°C. The supernatant was centrifuged at 18,000 rpm for 25 minutes at 4°C. The resulting pellet was then resuspended in 10 ml/g original weight of 50 mM Tris, pH 7.4 and stored at -80°C. For use, the tissue was brought up to 30 ml/g original weight with 50 mM Tris, pH 7.4 and centrifuged at 30,000 $\times$ g for 20 min. The pellet was then resuspended at 30 ml/g original weight with 50 mM Tris, pH 7.4.

Twelve litters of neonates were used; 8 were of the Topeka strain and 4 were Dunkin-Hartley strain. Each data point ($n = 6$) within an age group represents a different litter, but the data point consisted of pooled membranes from 1 to 3 neonates from the same litter. The data points for days 3 and 14 include three from Topeka litters and three from Dunkin-Hartley litters while day 7 represents two from Topeka litters and four from Dunkin-Hartley litters.

CHO cells were studied fresh without homogenization. Petrie dishes containing confluent CHO cells were drained of nutrient broth. Dulbecco's phosphate buffered saline (PBS), pH 7.2, (Gibco BRL, Grand Island, NY, USA) was added to the dishes and the CHO cells were scraped off, transferred to a centrifuge tube, and
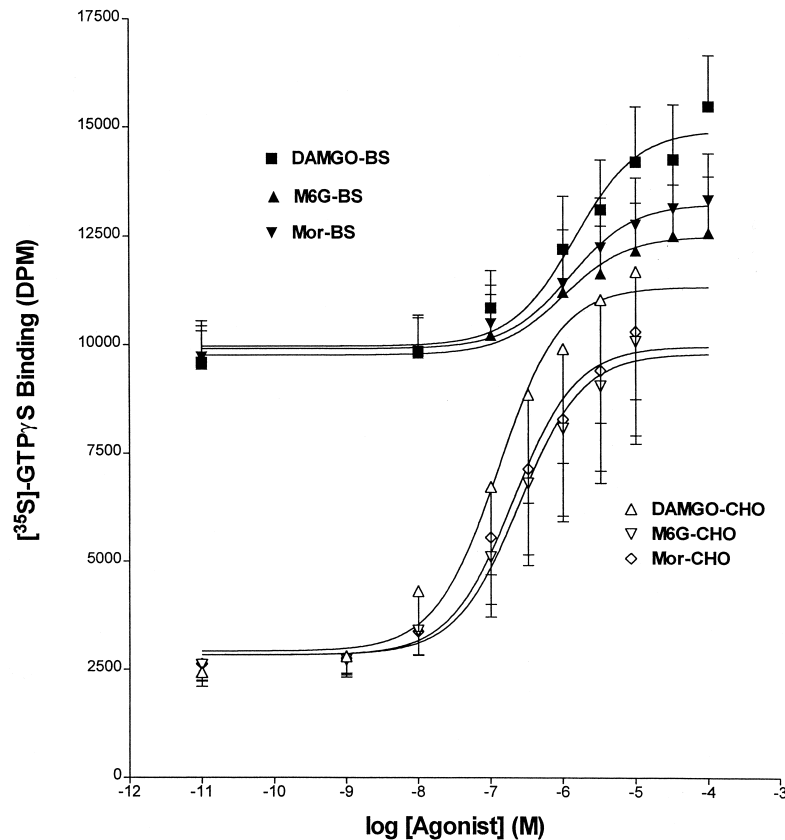
FIG. 1. Opioid stimulation of guanylyl-5′-O-([γ^{35}S]-thio)-triphosphate ([^{35}S]-GTPγS) binding in Chinese hamster ovarian (CHO) Cells and 7-day-old neonatal brainstem (BS) membranes. Data points are the mean ± SEM for 10 CHO cell or 6 neonatal BS experiments. The data points plotted at 10^{-11} M are basal binding data. Basal binding of [^{35}S]-GTPγS was higher for BS membranes than for CHO cells. Lines represent nonlinear regression analysis for a sigmoidal dose-response curve. Abbreviations: DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin; M6G, morphine-6-glucuronide; Mor, morphine.

centrifuged at 2600 × g for 5 min. The supernatant was discarded and the pellet was resuspended in PBS by rapid pipetting (10 times), and centrifuged again. The supernatant was discarded again and the pellet resuspended, this time in assay buffer (see below) by rapid pipetting (10 times). After protein concentration determination the cell suspension was diluted to the proper concentration with assay buffer containing 5 μM GDP and mixed by rapid pipetting (10 times). Permeability of the cells was increased from a control value of less than 1% to a final value of 80% by the above procedures, as measured by trypan blue exclusion assay.

[^{35}S]-GTPγS Binding Assay

Whole CHO cells (25 mg of protein) or brainstem membranes (15 mg of protein) were incubated for 1 or 2 h [29], respectively, in the presence of excess GDP (5 or 30 μM, respectively) in a final volume of 300 μl at 30°C before harvesting. The incubation vials contained 100 pM of [^{35}S]-GTPγS and agonist or agonist plus the competitive opioid receptor antagonist naloxone (20 nM). Agonist concentrations ranged from 10^{-5} to 10^{-9} M for CHO cells and 10^{-4} to 10^{-8} M for brainstem membranes in assay buffer (50 mM Tris-HCl buffer, 100 mM NaCl, 3 mM MgCl$_2$, 0.2 mM EGTA, pH 7.4). Basal stimulation of binding was determined in the absence of agonist, and nonspecific binding was measured in the presence

of excess unlabeled GTPγS (10 μM). The incubation mixture was filtered with #32 glass filters and washed three times with 3 ml of cold 50 mM Tris-HCl buffer, pH 7.0.

Statistics

The maximum stimulation of [^{35}S]-GTPγS binding (E$_{max}$) was calculated as the percent increase in binding at the greatest agonist concentration divided by basal binding and multiplied by 100. Potency, the effective drug concentration that results in a stimulation that is 50% of the maximum stimulation (EC$_{50}$), was calculated using nonlinear least squares sigmoidal dose-response curve fit with GraphPAD Prism 3.0 software (Fig. 1). Stimulation data are expressed as mean ± SEM The number of experiments for each condition was 10 for CHO cells and 6 for brainstem membranes. Because within each experiment the three agonists were compared on the same tissue source (cell culture batch or pooled neonatal membranes from one litter), the opioid-stimulated [^{35}S]-GTPγS binding was a repeated measure and analyzed as such. Statistical analyses of EC$_{50}$ and E$_{max}$ were done using a one-way repeated measures analysis of variance (ANOVA) for CHO cells for drug effect and two-way ANOVA for the brainstem membranes with one-way repeated measures using SigmaStat 2.03 software for drug and age effect. For the two-way ANOVA, Factor

TABLE 1

GTPγS BINDING IN STABLY TRANSFECTED CHINESE HAMSTER OVARIAN (CHO) CELLS EXPRESSING THE MURINE μ-OPIOID RECEPTOR AND IN GUINEA PIG NEONATAL BRAINSTEM (BS) MEMBRANES

Tissue	Age (Days)	Drug	E_{max} (%)	Log EC_{50}(M)	K_e (nM)
CHO	NA	DAMGO	344 ± 61*	−7.187 ± 0.073*	2.6 ± 0.3
CHO	NA	Morphine	300 ± 57	−6.802 ± 0.077	3.5 ± 0.7
CHO	NA	M6G	296 ± 56	−6.716 ± 0.106	4.1 ± 0.7
BS	3	DAMGO	62.3 ± 4.0*	−6.199 ± 0.044	4.2 ± 0.7
BS	3	Morphine	39.4 ± 2.2†	−6.315 ± 0.078	2.6 ± 0.6
BS	3	M6G	25.1 ± 2.5	−6.648 ± 0.063‡	3.1 ± 0.6
BS	7	DAMGO	61.7 ± 4.6*	−6.197 ± 0.073	4.9 ± 1.1
BS	7	Morphine	38.1 ± 3.5†	−6.243 ± 0.100	6.2 ± 1.8
BS	7	M6G	27.6 ± 3.8	−6.323 ± 0.150‡	6.3 ± 1.8
BS	14§	DAMGO	59.4 ± 6.0*	−6.024 ± 0.124	6.8 ± 1.6
BS	14§	Morphine	36.4 ± 4.1†	−5.980 ± 0.076	7.2 ± 2.5
BS	14§	M6G	23.2 ± 3.9	−6.407 ± 0.187‡	4.6 ± 1.0

Data are expressed as mean ± SEM, $n = 10$ for CHO cells and $n = 6$ for BS membranes except for BS 14 morphine K_e where $n = 5$. DAMGO, [D-Ala[2], N-MePhe[4], Gly-ol[5]] enkephalin; M6G, morphine-6- glucuronide; NA, not applicable.

* DAMGO different than morphine or M6G ($p < 0.05$) for corresponding tissue.

† Morphine different than M6G ($p < 0.05$) for corresponding tissue.

‡ M6G diferent than DAMGO or morphine ($p < 0.05$) for corresponding tissue.

§ Day 14 different than day 3 ($p < 0.05$) for Log EC_{50}.

A was drug with three levels, DAMGO, morphine, and M6G, which was a repeated measurement, and Factor B was age which also had three levels, 3 days, 7 days and 14 days after birth. Multiple comparisons were done on the significant effects of the one- or two-way ANOVA using the Tukey Test. A two-way ANOVA was used for both E_{max} and EC_{50} analyses for the cellular tolerance data. $p < 0.05$ was considered significant.

The estimated affinity (K_e) of the antagonist naloxone (20 nM) for the opioid receptor was calculated using the formula $K_e =$ [naloxone]/(DR − 1), where *DR* is the ratio of agonist EC_{50} in the presence and absence of naloxone. The DR was calculated using the ratio of the antilog of the average log EC_{50} under both conditions. The K_e for the CHO cells was analyzed by one-way ANOVA for agonist effect and for the brainstem membranes by two-way ANOVA for agonist and age effects.

RESULTS

GTPγS Binding in CHO Cells

In CHO cells stably transfected with the murine μ opioid receptor (mMOR-CHO) there was a significant difference among the three drugs in stimulating GTPγS binding for efficacy [$F(2,18) = 12.8, p < 0.001$] and potency (chi-square on ranks with 2 degrees of freedom = 13.4, $p < 0.001$). DAMGO stimulation was greater than either morphine or M6G ($p < 0.05$) (Fig. 1, Table 1). The efficacy for morphine and M6G stimulation, relative to DAMGO (100%), was 87% and 86%, respectively. DAMGO was more potent than either morphine or M6G ($p < 0.05$) (Fig. 1, Table 1). There was, however, no difference between morphine and M6G in either efficacy or potency. The non-specific binding was about 20% of basal binding (data not shown). DAMGO did not stimulate GTPγS binding in CHO cells stably transfected with the kappa opioid receptor (data not shown).

GTPγS Binding in Brainstem

The efficacy of agonist-stimulated GTPγS binding in neonatal guinea pig brainstem membranes depended on the specific opioid

agonist studied [$F(2,30) = 136.8, p < 0.001$], but not on neonatal age and there was no significant interaction between age and drug. DAMGO stimulation was greater than either morphine or M6G stimulation at all ages and morphine stimulation was greater than that for M6G at all ages ($p < 0.001$) (Fig. 1, Table 1). The non-specific binding was about 10% of basal binding. (Data not shown.)

The EC_{50} in brainstem membranes was dependent on neonatal age [$F(2,30) = 3.88, p = 0.044$], and on the agonist studied [$F(2,30) = 7.88, p = 0.002$]. There was no interaction between age and drug effects. The overall potency of the three agonists on day 14 was less than on day 3 ($p < 0.05$), suggesting developmental regulation of the μ opioid receptor, and the potency of M6G was greater than morphine and DAMGO ($p < 0.05$) in the brainstem membranes (Table 1).

Naloxone Antagonism

Naloxone antagonized the stimulation of GTPγS binding for all three agonists in CHO cells and brainstem membranes causing a parallel rightward shift in the dose response curve. The calculated K_e values for all experiments are presented in Table 1. There were no effects of agonist on K_e for CHO cells or neonatal membranes and no effects of neonatal age upon K_e for the brainstem membranes.

Tolerance in CHO Cells

Following chronic incubation of mMOR-CHO cells with the opioid agonist morphine, cellular tolerance was observed. There was a significant effect of the duration of incubation with morphine [$F(5,108) = 12.8, p < 0.001$] and agonist [$F(2,108) = 81.4, p < 0.001$] on potency. Efficacy was also affected by duration of the incubation [$F(5,108) = 12.6, p < 0.001$] and agonist [$F(2,108) = 33.1, p < 0.001$]. There was no significant interaction between duration of morphine exposure and agonist for either efficacy or potency. The E_{max} data showed that DAMGO was significantly more efficacious overall than either morphine or M6G
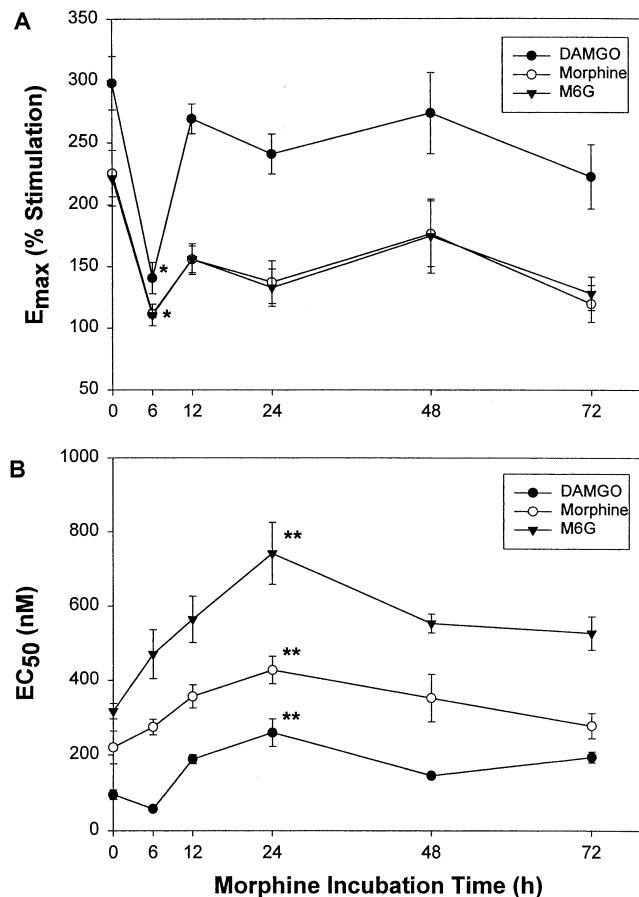
FIG. 2. Tolerance development in transfected Chinese hamster ovarian (CHO) cells. After 6 h of chronic morphine exposure, E_{max} values (A) of all agonists decreased. After 24 h of morphine incubation, peak increases in EC_{50} (B) were attained with all three agonists. Data are mean ± SEM, n = 4–11. Abbreviations: DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin; M6G, morphine-6-glucuronide; Mor, morphine. *Peak in maximum cellular tolerance significantly different than zero time point ($p < 0.05$). **Peak in potency change significantly different than zero time point ($p < 0.05$).

($p < 0.05$) with no significant difference between morphine and M6G while the EC_{50} data showed an overall significant difference in potency among the three agonists ($p < 0.05$).

As early as 6 h after incubation, the maximal effects of the agonists DAMGO, morphine, and M6G fell to 47.2%, 49.5%, and 49.9% of their respective original levels ($p < 0.001$) (Fig. 2). There was a trend, which was not significant, for a simultaneous increase in the amount of agonist required to produce an EC50 response in the case of morphine and M6G [124.8% and 148.6%, respectively, $p > 0.05$)], but not in the case of DAMGO ($p > 0.05$]. After 12 h of morphine exposure, maximal effect levels for DAMGO, morphine, and M6G decreased to 90.3%, 69.2%, and 70.4% of their respective control levels ($p = 0.006$). At this time point, EC_{50} values for DAMGO, morphine, and M6G increased to 198.0%, 162.3%, and 178.3% of their respective control levels ($p = 0.002$).

After 24 h of incubation, peak changes in the EC_{50} were attained with all three agonists. DAMGO, morphine, and M6G maximal effect levels dropped to 80.8%, 60.9%, and 60.0% of their respective control levels ($p < 0.001$). Corresponding EC_{50}

values for DAMGO, morphine, and M6G rose to 272.7%, 194.8%, and 234.3% of their original levels ($p < 0.001$). The changes from control for EC_{50} values were diminished in incubations exceeding 24 h, however, the values never returned to control levels by 72 h for either E_{max} ($p < 0.001$) or EC_{50} ($p = 0.004$).

DISCUSSION

Opioid efficacy and potency can be measured by the [^{35}S]-GTPγS binding assay in cells [36] and tissue [29]. This assay has been utilized to compare M6G with its parent molecule morphine as well as with the synthetic peptide to the μ opioid receptor, DAMGO. Studies of the three agonists were performed on stably transfected mMOR-CHO cells and the neonatal guinea pig brainstem at 3, 7, and 14 days after birth. This is the first report to compare these three agonists in transfected CHO cells and brainstem tissue membranes. [^{35}S]-GTPγS binding assays were performed on murine MOR-CHO cells to compare M6G with morphine and DAMGO. In acute incubations of the three agonists in opioid naïve cells, DAMGO was significantly more efficacious and potent than either morphine or M6G, with no significant difference between morphine and M6G. In a similar experiment, Selley et al. [29] showed no difference in efficacy between DAMGO and morphine (M6G was not studied) in transfected CHO cells, but DAMGO was four times more potent than morphine. Differences in efficacy between the Selley et al. study and the current experiments may be related to a twofold increase in [^{35}S]-GTPγS concentration used in the current study, a difference in the cells used and/or a difference in the preparation of the cells before incubation. In this regard it is important to note that efficacy and potency differences in opioid agonists have been reported in other studies. For example, in SK-N-SH cells, M6G was more efficacious than morphine [1], whereas in SH-5Y5Y cells, M6G and morphine were equally efficacious, but M6G was more potent than morphine [13] for inhibition of cAMP accumulation.

The ability of naloxone to decrease the potency of opioid agonist stimulation of [^{35}S]-GTPγS binding in the transfected CHO cells and brainstem membranes confirms that the effect is mediated by opioid receptors. In addition, because these agonists did not stimulate GTPγS binding in CHO cells expressing the murine κ opioid receptor, the stimulation of GTPγS binding is most likely mediated by the μ opioid receptor. The estimated affinity of the antagonist naloxone for the μ opioid receptor (K_e values) ranged from 2.6 to 7.2 nM in Table 1. This is comparable to the K_e values reported in the literature of 2.5 nM for guinea pig hypothalamus [6] and the K_D values of 4.4 nM reported for mMOR CHO cells and 5.1 nM reported for rat thalamus membranes [28].

There have been a number of published studies that provide evidence of μ opioid receptors in brainstem areas related to cardiovascular control. Two recent reports should be cited in this context because the opioid-stimulated [^{35}S]-GTPγS binding technique was used. The first was a study of the rat brainstem after chronic morphine administration [30]. In that study, many brainstem areas contained significant amounts of μ opioid receptors and DAMGO-stimulated [^{35}S]-GTPγS binding was reduced by chronic morphine exposure including the locus coeruleus, lateral and medical parabrachial nuclei, and the commissural nucleus tractus solitarius. Using the same technique, a second study showed that chronic heroin exposure also reduced DAMGO-stimulated [^{35}S]-GTPγS binding in these same areas of the rat brainstem [32]. A very interesting finding in the latter paper was that the decrease in [^{35}S]-GTPγS binding occurred in some brainstem areas even though there was increased μ opioid receptor binding measured by [^{3}H]-naloxone. That is, there was a decrease in the

efficacy of receptor/G-protein coupling. This seems to be a new finding because previous examination of the effect of chronic morphine [34] or etorphine [33] on μ opioid receptors in rats did not examine brainstem areas.

To explore the development of morphine cellular tolerance in mMOR-CHO cells and to compare M6G to DAMGO and morphine in this context, incubations of the three agonists were performed with cells before and after chronic morphine exposure. The results of these experiments confirm those of previous studies showing that chronic exposure to opioid agonists such as morphine reduces the ability of opioid agonists to stimulate $[^{35}S]$-GTPγS binding. This reduction is demonstrated through a loss of opioid maximum effect and an increase in the EC_{50}. In terms of maximum opioid effect, DAMGO-induced stimulation was greater than either morphine or M6G stimulation at each time interval. In addition, after 6 h of incubation with morphine, the E_{max} for all three agonists dropped to roughly 50% of their original levels. This time point exhibited the largest drop in maximum effect for all three agonists suggesting a similar mechanism for the development of cellular tolerance for each agonist examined. In contrast, the greatest increase in EC_{50} compared to control values occurred after 24 h of incubation for all three agonists. The decreases in the E_{max} for morphine and M6G were significantly lower than the zero time at all time periods except at 48 h, whereas, the EC_{50} values for both these agonists were significantly higher than the zero time at each time point. The EC_{50} value for DAMGO was lower than both morphine and M6G at each time point. The fact that DAMGO produced a greater maximum effect and that the EC_{50} was at a lower concentration for every time interval is consistent with the acute data in the transfected opioid naïve CHO cells that suggests that DAMGO is more efficacious and potent than morphine and M6G.

This time difference between the peak E_{max} and EC_{50} changes suggests that the early and parallel decrease in E_{max} may be due to different mechanisms than the change in EC_{50}, although the mechanisms were not examined in this study. The development of cellular tolerance does imply that there is a decrease in the number of receptors and/or a decrease in efficiency of coupling of the receptor to G proteins. Decreases in receptor number in turn could be due to decreased synthesis of receptor and/or increased receptor degradation. Because the promoter that regulates μ opioid receptor expression in the transfected CHO cells in the current study is viral, cytomegalovirus, transcriptional regulation of the μ opioid receptor should be independent of chronic morphine exposure, however, inactivation of receptors by phosphorylation should be intact and may explained the development of cellular tolerance to morphine. A recent study of cellular adaptation of CHO cells expressing the rat μ opioid receptor suggested that the decreased radio-ligand binding observed following chronic opioid exposure was caused by increased degradation rather than decreased synthesis of the μ opioid receptor [12].

In previous studies involving cellular tolerance to morphine with SH-SY5Y cells, tolerance, which peaked at 48 h of incubation [7], was defined by a 31% decrease in morphine's maximal effect and a 250% increase in the EC_{50} value. The changes for morphine (39.1% decrease in maximal effect and 194.8% increase in EC_{50} value) from control values were similar after 24 h of incubation at the same concentration as those in the current study. Although the effects of cellular tolerance between these two experiments appear to be similar, the incubation times required to achieve this tolerance are not. Similar data has been shown in CHO cells stably transfected with rat μ opioid receptor DNA for $[^3H]$-DAMGO binding studies in which DAMGO dose-dependently down-regulated the μ opioid receptor density with a maximal decrease at 4 h [12].

$[^{35}S]$-GTPγS binding assays were performed to examine the developmental changes of the efficacy and potency of DAMGO, morphine and M6G for the stimulation of neonatal guinea pig brainstem membranes. With regards to efficacy, DAMGO was the most efficacious of the three agonists at all three ages and morphine was significantly more efficacious than M6G. The potency of the three agonists was age dependent with potency decreased by 2 weeks of age compared to 3 days after birth. Potency was also drug dependent with M6G being more potent than DAMGO or morphine.

Previous $[^3H]$-DAMGO binding data in the guinea pig pup brainstem [20] showed a 42% increase in agonist binding (B_{max}) from days 3 to 7, however the affinities of the three agonists for the μ opioid receptor did not change between days 3 and 7. M6G was also shown to have a threefold lower affinity (K_i) than morphine in inhibiting $[^3H]$-DAMGO binding. In spite of the increase in μ opioid receptor binding sites with development, the current studies indicate that this had no effect upon the efficacy of receptor coupling to the G protein with the three agonists studied. A surprising finding was that M6G may be more potent than either DAMGO or morphine in the brainstem membranes. This finding was a modest twofold difference, but statistically significant, and is qualitatively similar to the *in vivo* potency differences for induction of respiratory depression between morphine and M6G [18]. There are quantitative differences, however, in that there was no potency difference between the two drugs at 3 days after birth for the *in vivo* studies [18] and the potency differences at day 7 and 14 were greater in the *in vivo* study compared to the *in vitro* experiments reported here.

There are at least three possible explanations for these differences. First, a large *in vivo* physiological effect may be caused by small differences in GTPγS stimulation that are amplified at some downstream signaling step. Second, it may be a subset of G proteins that facilitate respiratory depression, whereas our assay measures the net stimulation of all G proteins. Third, the *in vivo* effects on respiratory depression may be dependent on a subset of brainstem cells with different pharmacological properties than whole brainstem tissue. Other explanations, or a combination of the above, are possible as well.

The current studies provide new information on M6G stimulation of $[^{35}S]$-GTPγS binding for the μ opioid receptor. In all cases, DAMGO was more efficacious than either morphine or M6G. In both the opioid naïve and morphine tolerant murine MOR-CHO cell studies, morphine and M6G were equally efficacious, however, M6G was shown to be less efficacious than morphine in the brainstem membranes. In the studies using opioid naïve and tolerant murine MOR-CHO cells, M6G was less potent than morphine, but M6G was more potent than morphine in the brainstem.

This study furthers our understanding of the development of the neonatal guinea pig brainstem with respect to the μ opioid receptor and its link to G proteins and indicates that the guinea pig receptor may be functionally different from the murine receptor or that M6G is interacting with other receptor types in the brainstem. It also demonstrates that M6G, along with morphine, differs in its ability compared to DAMGO to interact with the effector system of the μ opioid receptor. This information may eventually help elucidate the biochemical mechanisms underlying opioid cellular tolerance to the respiratory depressant effects of these three agonists.

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