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# Effects of $mGlu_1$ and $mGlu_5$ metabotropic glutamate antagonists to reverse morphine tolerance in mice

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**Abstract**

Intracerebroventricular (i.c.v.) injection of phospholipase C inhibitors and structurally dissimilar PKC inhibitors were shown to completely reverse morphine antinociceptive tolerance in mice. Since Group I metabotropic glutamate receptors ($mGlu_1$ and $mGlu_5$) activate phospholipase C through $G\alpha_q$ $G\alpha_{11}$ proteins, we hypothesized that morphine tolerance could occur through an increase in $mGlu_1$ and $mGlu_5$ receptor stimulation. Seventy-two hours after implantation of placebo or 75 mg morphine pellets, mice were tested in the 56 °C warm-water tail-withdrawal test following i.c.v. injection of vehicle or test drug. The $mGlu_1$ receptor antagonist CPCCOEt (7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester) partly but significantly reversed morphine tolerance. The $mGlu_5$ receptor antagonist MPEP (2-Methyl-6-(phenylethynyl)pyridine hydrochloride) also partly reversed the antinociceptive tolerance. Co-administering CPCCOEt with MPEP completely reversed the tolerance. Furthermore, the mixed $mGlu_1$/$mGlu_5$ antagonist AIDA ((*RS*)-1-Aminoindan-1,5-dicarboxylic acid) also completely reversed the tolerance. Thus, greater $mGlu_1$ and $mGlu_5$ receptor stimulation during morphine tolerance may lead to persistent activation of the phosphatidylinositol cascade.


*Keywords:* Metabotrobic glutamate receptors; Opioid tolerance; Phosphatidylinositol cascade; Analgesia; (Mouse)

## 1. Introduction

The chronic administration of opioids appears to activate the phosphatidylinositol cascade (PI). In mice, intracerebroventricular (i.c.v.) injection of the protein kinase C (PKC) inhibitors chelerythrine chloride (intrathecal, i.t.), calphostin C (i.t.) or H7 (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine, 2HCl) were able to prevent or reverse the acute antinociceptive tolerance that developed to administration of a single dose of mu- or delta-opioid receptor agonist (Bilsky et al., 1996; Narita et al., 1995, 1996). In addition, structurally dissimilar PKC inhibitors were demonstrated to reverse antinociceptive tolerance in mice 3 days after morphine pellet implantation (Smith et al., 1999b, 2002, 2003). In rats, concomitant infusion of H7 i.c.v. prevented the development of morphine and butorphanol tolerance, as measured in the tail-flick test (Narita et al., 1994). Chronic co-infusion of i.t. morphine with either bisindolylmaleimide I or chelerythrine prevented the development of morphine tolerance, as measured in the paw-withdrawal test to noxious heat (Granados-Soto et al., 2000). Furthermore, PKC inhibitors were able to reverse tolerance after 5 days of morphine infusion. Recent studies demonstrated that i.t. infusion of anti-sense oligodeoxynucleuotide to PKCalpha mRNA blocked the tolerance to i.t. infused morphine in rats (Hua et al., 2002).

The fact that PKC activity is higher during opioid tolerance suggests that the metabolism of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into diacylglycerol and inositol 1,4,5-trisphosphate by phospholipase C (PLC) may also be higher. Indeed, i.c.v. administration of the PLC inhibitors ET-18-OCH3 (Edelfosine, 1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine) or D609 (Tricyclodecan-9-yl-xanthogenate, K) completely reversed tolerance (Smith et al., 1999b), indicating that chronic morphine administration may increase receptor-mediated PLCβ and/or PLCγ activity. Group I metabotropic glutamate (mGlu) receptor activation ($mGlu_1$ and $mGlu_5$ subtypes) leads to $G\alpha_q$ or $G\alpha_{11}$ subunit binding to the C2 domain of the PLC β1, β3 and β4 isoforms and subse-

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quent increases in $PIP_2$ turnover (Conn and Pin, 1997). Furthermore, the βγ subunits from $G_q$, $G_{11}$, and other G-proteins (e.g., $G_{i/o}$ from mu-opioid receptor stimulation) can bind the pleckstrin homology domain of PLC to stimulate enzyme activity. Thus, chronic administration of morphine could lead to increases in the activity of the PI cascade through greater group I mGlu receptor stimulation. This led to the hypothesis that group I mGlu receptor antagonists would reverse antinociceptive tolerance in mice implanted with morphine pellets, while having no effect in non-tolerant placebo-pelleted mice. Our results demonstrate that the $mGlu_1$ and $mGlu_5$ antagonists CPCCOEt (7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester) and MPEP (2-Methyl-6-(phenylethynyl)pyridine hydrochloride), respectively, were partly effective in reversing morphine tolerance, while the mixed $mGlu_1/5$ antagonist AIDA ((*RS*)-1-Aminoindan-1,5-dicarboxylic acid) completely reversed morphine tolerance.

## 2. Methods

### 2.1. Methods of handling mice

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30 g were housed 6 to a cage in animal care quarters maintained at 22 ± 2 °C on a 12-h light–dark cycle. Food and water were available ad libitum. The mice were brought to a test room (22 ± 2 °C, 12-h light–dark cycle), marked for identification and allowed 24-h to recover from transport and handling. The Institutional Animal Care and Use Committee (IACUC) at the Virginia Commonwealth University School of Medicine approved all procedures. IACUC procedures comply with the European Communities Council Directive of 24 November 1986 (86/609/EEC) to minimize pain or discomfort.

### 2.2. Surgical implantation of pellets

Mice were anesthetized with 8% vaporized diethyl ether before shaving the hair around the base of the neck. Adequate anesthesia was noted by the absence of the righting-reflex and lack of response to toe-pinch, according to IACUC guidelines. The skin was cleansed with 10% providone iodine (General Medical, Prichard, WV) and rinsed with alcohol before making a 1-cm horizontal incision at the base of the neck. The underlying subcutaneous space toward the dorsal flanks was opened using a sterile glass rod. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision and subcutaneous space. A placebo pellet or 75 mg morphine pellet was inserted in the space before closing the site with Clay Adams Brand, MikRon® AutoClip® 9mm Wound Clips (Becton Dickinson, Sparks, MD) and again applying iodine to the surface. The animals were allowed to recover in their home cages where they remained throughout the experiment.

### 2.3. Intracerebroventricular injections

Intracerebroventricular (i.c.v.) injections were performed as described by Pedigo et al. (1975). Mice were anesthetized with ether and a transverse incision was made in the scalp. A free-hand 5 μl injection of the drug or the vehicle was made into the lateral ventricle. The extensive experience of this laboratory has made it possible to inject drugs with greater than 95% accuracy. Immediately upon testing the animals were euthanized to minimize any type of distress, according to IACUC guidelines.

### 2.4. The tail-withdrawal test

The warm-water tail-withdrawal apparatus used to assess for antinociception in the mice was maintained at 56 ± 0.1 °C. Before injecting the mice, the base-line (control) latency was determined. Only mice with a control reaction time from 2 to 4 s were used. The average baseline tail-withdrawal latency for these experiments was 3.1 s. The test latency after drug treatment was assessed at the appropriate time, and a 10-s maximum cut-off time was imposed to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (1964) as the percentage of maximum possible effect (% MPE) which was calculated as: $\%MPE=[(\text{test}-\text{control})\ (10-\text{control})^{-1}]\times 100$. Percent MPE was calculated for each mouse using at least 6 mice per dose.

### 2.5. Reversal of morphine tolerance

Group I mGlu receptor antagonists were tested for their ability to reverse morphine tolerance 72-h after the implantation of morphine pellets. Their effects in placebo pellet-implanted mice were also determined. Baseline tail-withdrawal latencies were obtained before i.c.v. injection of vehicle or Group I mGlu receptor antagonist. Morphine was then immediately injected s.c., and test tail-withdrawal latencies were obtained 30-min later. Test times were based on preliminary time-course experiments, and the literature. The highest soluble doses of CPCCOEt and MPEP were tested, since they only partly reversed morphine tolerance. In another experiment, 5-fold lower doses of CPCCOEt and MPEP were co-administered together to determine whether simultaneous $mGlu_1/mGlu_5$ receptor antagonism would completely reverse the tolerance. Statistical determination of tolerance reversal was established by generating morphine dose–response curves for calculation of $ED_{50}$ values using least squares linear regression analysis followed by calculation of 95% confidence limits by Bliss (1967). Tests for parallelism were conducted before calculation of potency ratio values and 95% confidence limits by the method of Colquhoun (1971). A potency ratio value greater than one, with the lower 95% confidence limit greater than one, was considered a significant difference in potency.

Table 1
The reversal of morphine tolerance with group I metabotropic glutamate receptor antagonists

| Group | Treatment i.c.v. | $ED_{50}$ mg/kg (95% c.l.) | Potency ratio (95% c.l.) | |
|---|---|---|---|---|
| Placebo-P | Veh | 3.3 (2.7–4.1) | | – |
| Morphine-P | Veh | 33.4 (27.2–41.0)[a] | vs. Pbo Veh | 9.4 (6.8–12.8)[a] |
| Placebo-P | CPCCOEt (1.0 μmol) | 2.5 (1.4–4.4) | | |
| Morphine-P | CPCCOEt (1.0 μmol) | 11.2 (7.4–16.8)[b,c] | vs. Pbo CPCCOEt | 4.3 (2.0–10.8)[c] |
| Placebo-P | MPEP (1.1 μmol) | 3.1 (2.3–4.3) | | |
| Morphine-P | MPEP (1.1 μmol) | 15.7 (11.2–22.0)[b,c] | vs. Pbo MPEP | 4.5 (2.6–7.6)[c] |
| Placebo-P | CPCCOEt (200 nmol) + MPEP (220 nmol) | 3.1 (2.1–4.7) | | |
| Morphine-P | CPCCOEt (200 nmol) + MPEP (220 nmol) | 2.7 (1.7–4.2) | vs. Pbo CP + MPEP | 1.1 (0.7–1.9) |
| Placebo-P | AIDA | 3.4 (2.3–5.2) | | |
| Morphine-P | AIDA | 3.9 (2.8–5.4)[b] | vs. Pbo AIDA | 1.1 (0.7–1.9) |

Mice were implanted with placebo or 75 mg morphine pellets. At 72-h, CPCCOEt (1.0 μmol), MPEP (1.1 μmol) or AIDA (1.1 μmol) were administered i.c.v. In the co-administration studies, CPCCOEt (200 nmol) and MPEP (220 nmol) were administered together. Immediately afterwards, the mice were challenged with various doses of morphine s.c.

[a] Significantly different compared to Placebo-P/Vehicle control.

[b] Significantly different compared to Morphine-P/Vehicle control.

[c] Significantly different compared to respective Placebo-P/mGlu receptor antagonist.

### 2.6. Drugs and chemicals

The 75 mg morphine pellets were obtained from the National Institute on Drug Abuse, Bethesda, MD. Morphine sulfate (Mallinckrodt, St. Louis, MO) was dissolved in pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, IL). MPEP (2-Methyl-6-(phenylethynyl)pyridine hydrochloride), CPCCOEt (7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester) and AIDA ((*RS*)-1-Aminoindan-1,5-dicarboxylic acid) were dissolved in 10% dimethyl sulfoxide (DMSO), 20% emulphor, 70% distilled water. We have previously published on the use of this vehicle for i.c.v. injections (Smith et al., 1999a,b, 2002, 2003). The corresponding vehicle-injected mice were injected with 10% DMSO, 20% emulphor, 70% distilled water.

## 3. Results

Implantation of a 75 mg morphine pellet resulted in approximately a 9.4-fold reduction in the antinociceptive potency of morphine, consistent with the level of tolerance exhibited in previous studies from this laboratory (Table 1) (Smith et al., 1999b, 2002). The mice were tested with vehicle i.c.v. or the $mGlu_1$ receptor antagonist CPCCOEt. The highest soluble dose of 1.0 μmol partly, but significantly, reversed morphine tolerance (Fig. 1A, Table 1). In

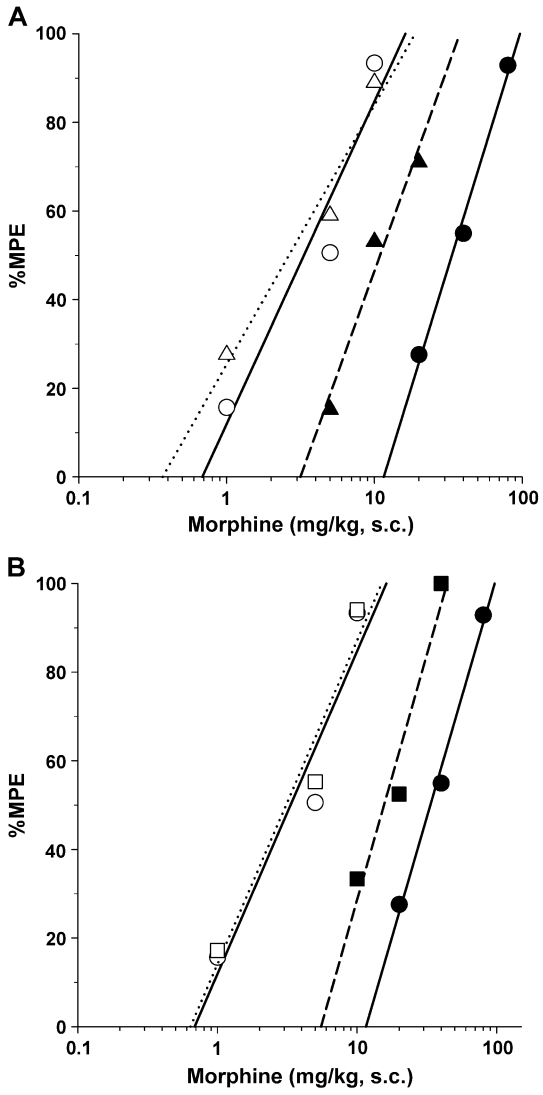


Fig. 1. (A) Partial reversal of morphine tolerance with the mGlu1 receptor antagonist CPCCOEt. Mice were surgically implanted with placebo or 75 mg morphine pellets as described in Materials and methods. Seventy-two hours later, vehicle or CPCCOEt was injected i.c.v. immediately followed by s.c. morphine. Tail-withdrawal latencies were obtained 30-min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/CPCCOEt (1.0 μmol) (△, dotted line); morphine-P/veh (●, solid line); morphine-P/CPCCOEt (1.0 μmol) (▲, dashed line). Each curve represents 18 mice. (B) Partial reversal of morphine tolerance with the mGlu5 receptor antagonist MPEP. Mice were surgically implanted with placebo or 75 mg morphine pellets as described in the Methods. At 72-h, vehicle and MPEP was injected i.c.v. immediately followed by s.c. morphine. Tail-withdrawal latencies were obtained 30-min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/MPEP (1.1 μmol) (□, dotted line); morphine-P/veh (●, solid line); morphine-P/MPEP (1.1 μmol) (■, dashed line). Each curve represents 18 mice.

addition, the $mGlu_5$ receptor antagonist MPEP was tested. Yet like CPCCOEt, the highest soluble dose of MPEP (1.1 μmol, i.c.v.) only partly reversed morphine tolerance (Fig. 1B, Table 1). Since antagonizing both $mGlu_1$ and $mGlu_5$ receptors partly reversed the tolerance to morphine, experiments were conducted to determine whether simultaneously antagonizing $mGlu_1$ and $mGlu_5$ receptors would be more effective. Co-administration of 5-fold lower doses of CPCCOEt (200 nmol) and MPEP (220 nmol) i.c.v. completely reversed morphine tolerance (Fig. 2A, Table 1). Furthermore, the mixed $mGlu_1/mGlu_5$ receptor antagonist AIDA (1.1 μmol) also completely reversed morphine tolerance (Fig. 2B, Table 1). It is notable that none of the selective receptor antagonists or AIDA affected morphine's potency in the placebo pellet-implanted mice.

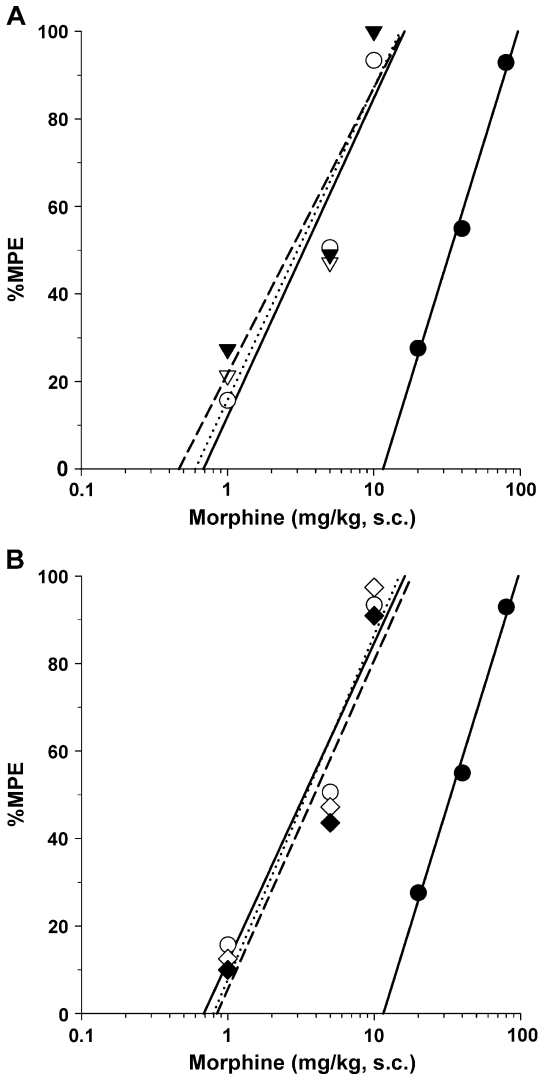


Fig. 2. (A) Co-administration of CPCCOEt and MPEP completely reversed morphine tolerance. Mice surgically implanted with placebo or 75 mg morphine pellets 72-h earlier were injected i.c.v. with vehicle, or were co-administered CPCCOEt and MPEP immediately followed by s.c. morphine. Tail-withdrawal latencies were obtained 30-min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/CPCCOEt (200 nmol) + MPEP (200 nmol) (▽, dotted line); morphine-P/veh (●, solid line); morphine-P/CPCCOEt + MPEP (▼, dashed line). Each curve represents 18 mice. (B) The mixed $mGlu_1/mGlu_5$ receptor antagonist AIDA completely reverses morphine tolerance. Mice were surgically implanted with placebo or 75 mg morphine pellets as described in Materials and methods. Seventy-two hours later, vehicle or AIDA was injected i.c.v. immediately followed by s.c. morphine. Tail-withdrawal latencies were obtained 30-min later. The treatment groups consisted of: placebo-P/veh (○, solid line); placebo-P/AIDA (1.1 μmol) (◇, dotted line); morphine-P/veh (●, solid line); morphine-P/AIDA (1.1 μmol) (◆, dashed line). Each curve represents 18 mice.

## 4. Discussion

Administration of the selective antagonists CPCCOEt or MPEP i.c.v. only partly reversed morphine tolerance in mice. It could be argued that higher doses should have been tested by using other vehicle solvents for these water-insoluble drugs. However, the syringe concentrations of the 1.0 and 1.1 μmol doses injected i.c.v. were 200 and 220 mM, respectively. Assuming complete distribution of 5 μl into ~ 100 μl of cerebrospinal fluid, the final concentrations were still 9.5–10 mM before penetration into the sites of action in neuronal tissue. Of course if the drug was lipophilic, its rapid movement of out of the ventricles after i.c.v. administration could have resulted in much lower estimated cerebrospinal fluid drug concentrations. In microdialysis studies of the periaquiductal grey of rats, 1 mM CPCCOEt was required to block (*S*)-3,5-DHPG $mGlu_1$ receptor agonist-stimulated release of glycine (de Novellis et al., 2002) or glutamate and gamma aminobutyric acid (GABA) (de Novellis et al., 2003). Concentrations of 0.5–2 mM MPEP were tested to block CHPG $mGlu_5$ receptor agonist-stimulated release of neurotransmitter from the periaquiductal grey, as well. Therefore, the concentrations of CPCCOEt and MPEP should have been sufficient to maximally antagonize the receptors following penetration to their sites of action.

Although no studies have determined whether chronic opioid administration alters the levels of $mGlu_1$ and $mGlu_5$ receptors in specific regions of the brain, studies of other glutamate-activated receptor systems indicate that changes in receptor levels are possible. In rats, chronic morphine treatment increased the levels of GluR1 AMPA glutamate receptors in the ventral tegmental area (Carlezon and Nestler, 2002). In addition, chronic morphine administration altered the steady-state mRNA levels of the NMDA receptor NR1 subunit in a brain region specific manner in rats (Zhu et al., 2003). In addition to the possible effects of chronic morphine on brain mGlu receptor levels, $mGlu_1$ receptors are located on postsynaptic sites, whereas $mGlu_5$ receptors are found on presynaptic terminals and stimulate neurotransmitter release when activated (Herrero et al., 1992; Jia et al., 1999; Lorrain et al., 2002). Thus, the inability of the selective antagonists to completely reverse morphine tolerance could have been due to the pre- vs. post-synaptic location of the receptor subtypes, as well as the ability of

glutamate to still stimulate non-antagonized receptors. To address this issue, much lower doses of CPCCOEt and MPEP were co-administered to determine whether simultaneous antagonism of both $mGlu_1$ and $mGlu_5$ receptors would reverse morphine tolerance. The complete reversal of tolerance by this treatment, in addition to the effects of the mixed antagonist AIDA, indicate that both $mGlu_1$ and $mGlu_5$ receptors play an important role in the expression of morphine tolerance.

Most studies to date have utilized animal models in which mGlu receptor drugs are administered simultaneously during chronic opioid treatments to prevent the development of tolerance and dependence. Fundytus and Coderre (1994) demonstrated that daily administration of non-selective group I mGlu receptor antagonists (i.e., mixed $mGlu_1/mGlu_5$) prevented the development of morphine physical dependence. However, it is notable that studies that focus on preventing the development of opioid tolerance appear to require a less vigorous pharmacological approach than those that attempt to reverse tolerance that is already present. For example, i.t. infusion of antisense oligonucleotide to the $mGlu_1$ receptor during 6 days of s.c. morphine administration prevented the development of morphine tolerance (Sharif et al., 2002). Morphine antinociceptive tolerance was prevented in mice by daily s.c. administration of the $mGlu_5$ antagonist MPEP (Kozela et al., 2003). Thus during the development of tolerance, daily pharmacologic antagonism of a single mGlu receptor subtype (either $mGlu_1$ or $mGlu_5$) prevented the development of tolerance. However, in our case, once tolerance was established, the reversal of tolerance required the antagonism of both $mGlu_1$ and $mGlu_5$ receptors.

In summary, the results of this and other studies indicate that chronic administration of opioids leads to the activation of the PI cascade. Higher levels of Group I mGlu receptors, or more efficient receptor coupling to $G_{q/11}$ proteins, could contribute to the activation of PLCβ, resulting in higher PKC activity in animals tolerant to opioids. A future aim of this research would be to identify the brain regions into which these i.c.v. drugs penetrate to reverse tolerance. Another goal would be to determine whether chronic morphine administration significantly affects the levels of $mGlu_1$ and $mGlu_5$ receptors in these regions, and whether these changes can be correlated with alterations in the activity of PLCβ.

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