

Long-term antagonism and allosteric regulation of mu opioid receptors by the novel ligand, methocinnamox

Joshua C. Zamora¹ | Hudson R. Smith¹ | Elaine M. Jennings¹ | Teresa S. Chavera¹ |
Varun Kotipalli¹ | Aleasha Jay¹ | Stephen M. Husbands³ | Alex Disney³ |
Kelly A. Berg^{1,2}  | William P. Clarke^{1,2}

¹Department of Pharmacology, UT Health San Antonio, San Antonio, Texas, USA

²Addiction Research, Treatment & Training Center of Excellence, UT Health San Antonio, San Antonio, Texas, USA

³Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom

Correspondence

Kelly A. Berg, Department of Pharmacology-MS7764, UT Health San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA.
Email: berg@uthscsa.edu

Funding information

Addiction Research, Treatment & Training (ARTT) Center of Excellence at UT Health San Antonio; National Institute on Drug Abuse of the National Institutes of Health

Abstract

Opioid overdose is a leading cause of death in the United States. The only treatment available currently is the competitive antagonist, naloxone (Narcan[®]). Although naloxone is very effective and has saved many lives, as a competitive antagonist it has limitations. Due to the short half-life of naloxone, re-narcotization can occur if the ingested opioid agonist remains in the body longer. Moreover, because antagonism by naloxone is surmountable, re-narcotization can also occur in the presence of naloxone if a relatively larger dose of opioid agonist is taken. In such circumstances, a long-lasting, non-surmountable antagonist would offer an improvement in overdose treatment. Methocinnamox (MCAM) has been reported to have a long duration of antagonist action at mu opioid receptors *in vivo*. In HEK cells expressing the human mu opioid receptor, MCAM antagonism of mu agonist-inhibition of cAMP production was time-dependent, non-surmountable and non-reversible, consistent with (pseudo)-irreversible binding. *In vivo*, MCAM injected locally into the rat hindpaw antagonized mu agonist-mediated inhibition of thermal allodynia for up to 96 h. By contrast, antagonism by MCAM of delta or kappa agonists in HEK cells and *in vivo* was consistent with simple competitive antagonism. Surprisingly, MCAM also shifted the concentration-response curves of mu agonists in HEK cells in the absence of receptor reserve in a ligand-dependent manner. The shift in the [D-Ala²,N-MePhe⁴,Gly-ol⁵]-enkephalin (DAMGO) concentration-response curve by MCAM was insensitive to naloxone, suggesting that in addition to (pseudo)-irreversible orthosteric antagonism, MCAM acts allosterically to alter the affinity and/or intrinsic efficacy of mu agonists.

KEYWORDS

allosteric regulation, GPCR, opioid, unsurmountable antagonism

Abbreviations: AUC, area under the curve; BK, bradykinin; DAMGO, [D-Ala²,N-MePhe⁴,Gly-ol⁵]-enkephalin; DPDPE, [D-Pen^{2,5}]-Enkephalin; i.pl., intraplantar; MCAM, methocinnamox; NLX, naloxone; PBS, phosphate buffered saline; PGE2, prostaglandin E2; PWL, paw withdrawal latency; β -FNA, β -funaltrexamine.

Joshua C. Zamora and Hudson Smith have contributed equally to this study.

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1 | INTRODUCTION

In 2018, over 67 000 deaths occurred in the US due to opioid overdose, in which synthetic opioids (e.g., fentanyl) were the main driving force.^{1,2} The increasing availability of very potent opioids that can be lethal when taken in even small doses, and the frequent use of opioids in combination with sedative drugs such as benzodiazepines, has contributed to a dramatic rise in opioid overdose deaths.³⁻⁵

The most effective treatment for opioid overdose is administration of an opioid receptor antagonist, which can quickly reverse the potentially lethal respiratory depression produced by large doses of opioid agonists.⁶ Current pharmacological treatment of opioid overdose relies exclusively on the competitive antagonist **naloxone** (NLX, Narcan).¹ Given either by injection or intranasally, naloxone can reverse completely the respiratory depression and profound sedation produced by opioid agonists. However, naloxone has several limitations. It has a relatively short duration of action, which can result in re-narcotization should a patient overdose on a relatively longer-acting opioid (e.g. **fentanyl**).⁷⁻⁹ Importantly, since NLX is now frequently administered out of a medical setting, re-narcotization may not be recognized leading to increased risk of re-overdose and death when antagonism by administered NLX wanes.¹⁰ To counter re-narcotization, larger, more frequent, or continuous (intravenous drip) administration of naloxone is required. An additional drawback to the use of NLX as treatment for overdose is that its antagonism is surmountable by ingesting a relatively larger dose of an opioid agonist, which can have life-threatening consequences.

In an overdose situation, a long-acting, non-surmountable antagonist would have an advantage over a short acting, competitive antagonist (such as NLX), because a single administration would suffice to protect an individual against re-narcotization, even if a long acting or high dose of a potent opioid agonist had been taken. Methocinnamox (14 β -(4'-methylcinnamoylamido)-7,8-dihydro-N-cyclopropylmethyl-normorphinone, MCAM) has been reported to have long-lasting antagonism at the **mu opioid receptor** in mice¹¹ and rhesus monkeys.¹²⁻¹⁴ Here we compared some pharmacological properties of MCAM with those of the competitive antagonist, NLX, and the irreversible antagonist, **β -funaltrexamine** (β -FNA) in vitro at human mu, **delta** and **kappa** opioid receptors and in vivo in behavioral assays of nociception in the rat.

2 | MATERIALS AND METHODS

2.1 | Drugs and chemicals

Forskolin, **DAMGO**, **(-)-U50488**, β -FNA and NLX were purchased from Sigma-Aldrich. [D-Pen2,D-Pen5]Enkephalin (**DPDPE**), and bradykinin (BK) were purchased from Bachem Americas, Inc.. Prostaglandin E₂ (PGE₂) was purchased from Cayman Chemicals. Hank's balanced salt solution, horse serum, Dulbecco's modified Eagles Medium (DMEM) were purchased from Invitrogen Corp.. All other drugs and chemicals (reagent grade) were purchased from

Sigma-Aldrich. Mammalian expression vectors encoding human mu, delta and kappa opioid receptor cDNAs were obtained from the cDNA Resource Center (cdna@bloom.edu). GloSensor cAMP sensor cDNA, Fugene transfection reagent and CO₂ independent media (formulated for use with cells without a CO₂ incubator), were purchased from Promega. D-Luciferin was purchased from GoldBio. MCAM was synthesized by us as described previously.¹¹

2.2 | Cell line preparation and culture

HEK293 cells, purchased from ATCC (cat # CRC-1573), were used for these studies. The cAMP sensor, GloSensor 22F (Promega, Madison, WI cat # E2301) was transfected into HEK293 cells using the transfection reagent, lipofectamine 3000 (ThermoFisher Scientific) following the manufacturers recommendations. A stable population of HEK cells expressing the biosensor was established in the presence of hygromycin B (300 μ g/ml, Invitrogen) 72 h after transfection. Thereafter, cells were maintained in alphaMEM containing 10% heat inactivated horse serum and 50 μ g/ml hygromycin.

2.2.1 | Opioid receptor transfection

HEK cells expressing the GloSensor cAMP biosensor were stably transfected with human mu, kappa or delta cDNA (purchased from cDNA.org) using the procedure described above. A stable population of cells expressing the cAMP sensor along with a given opioid receptor was established using alphaMEM containing 10% heat inactivated horse serum, 300 μ g/ml Hygromycin B, and 300 μ g/ml G418.

2.2.2 | Transient overexpression of hMOR

HEK293 cells expressing the cAMP GloSensor were transfected transiently with human mu receptor cDNA using FuGENE transfection reagent (Promega #E2311) according to manufacturer's directions. Cells were used in GloSensor cAMP assays 48 h after transfection.

2.3 | Opioid agonist-mediated inhibition of cellular cAMP levels

Cellular levels of cAMP were measured using the GloSensor cAMP assay according to the instructions of the manufacturer (Promega). Briefly, cells (40 000 cells/well) seeded into white-walled, clear bottom 96-well plates coated with poly-L-ornithine were incubated in 100 μ l CO₂ independent media containing 450 μ g/ml of the substrate, D-Luciferin (GoldBio) and 10% heat-inactivated horse serum. Cells were equilibrated with substrate for 2 h in the dark at 30°C prior to data collection. Bioluminescence was measured using a FluoStar Omega microplate reader (BMG Labtech) with internal temperature

set to 30°C. Baseline measurements were taken at 1 Hz for 5 min to establish baseline luminescence counts for each well. After collection of baseline measurements, opioid agonists were added and additional readings taken for 5 min at 1 Hz. Next forskolin (FSK) was added to all wells (final concentration of 10 μ M) and luminescence was recorded for 30 min at 1 Hz. For experiments with antagonists, ligands were incubated with the cells at final concentrations as follows: naloxone, 100 nM¹⁵; β -FNA, 10 nM¹⁵; MCAM, 10 nM for μ , 20 nM for δ , and 50 nM for κ ¹¹ for either 15 min, 2 h (during substrate loading period) or 24 h (final 2 h were during substrate loading) as indicated before addition of an opioid agonist. For wash-out experiments, media was removed from the wells by flicking and replaced with 200 μ l of fresh media containing D-luciferin substrate, repeated twice. After the wash interval, media was replaced with 100 μ l of fresh media containing D-luciferin substrate and baseline, opioid agonist- and forskolin-mediated bioluminescence measurements were recorded as described above.

2.4 | Opioid agonist-mediated inhibition of cellular cAMP accumulation

For experiments where we incorporated a more rigorous wash paradigm to remove high concentrations of antagonist (e.g., Figure 8), we measured μ opioid agonist-mediated inhibition of forskolin-stimulated cAMP accumulation as we have done before.¹⁶⁻²¹ Briefly, cells were incubated with antagonists as indicated and then washed 5 times at 37°C to remove free ligand. Cells were then incubated with forskolin (1 μ M) in the presence or absence of opioid agonists along with the phosphodiesterase inhibitor, rolipram (100 μ M) for 15 min at 37°C. Cellular cAMP was extracted and measured with radioimmunoassay.

2.5 | Animals

Adult male Sprague-Dawley rats (Charles River Laboratories) weighing 250–300 g were used in this study. The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to International Association for the Study of Pain and federal guidelines. Animals were housed for 1 week with food and water available ad libitum before behavioral testing.

2.6 | Behavioral experiments

Opioid agonist-mediated antinociception was measured with a thermal (heat) plantar test apparatus²² as described previously.^{17,19,20,21,23,24} The radiant heat stimulus intensity was set to produce baseline paw withdrawal latency (PWL) of 10 ± 2 s, with a cutoff time of 25 s to prevent tissue damage. Because peripheral opioid receptor-mediated antinociception requires an inflammatory

stimulus,^{17,19,20,21,23,24,25} bradykinin (BK) was administered to enhance opioid receptor-mediated antinociception. After baseline PWL was measured, animals were pre-treated (15 min) with BK (25 μ g) via intraplantar (i.pl.) injection (50 μ l) with or without MCAM. BK injection produces a transient (<10 min) allodynia such that PWL returns to baseline before opioid administration. 15 min after BK injection, rats received a co-injection (i.pl.) of PGE₂ (0.3 μ g) with either DAMGO (μ agonist), DPDPE (δ agonist) or U50488 (κ agonist) or vehicle. Measurements of PWL were taken in duplicate at least 30 s apart at 5 min intervals for 20 min after injections with PGE₂ \pm opioid agonist. Time-course data are expressed as the change (sec) from individual PWL baseline values and represent mean \pm SEM with 6 animals per group. As shown in Figure S3, i.pl. administration of MCAM locally into the rat hindpaw had no effect on baseline PWL, PGE₂-evoked thermal hypersensitivity or BK-mediated hypersensitivity. Figure S4 shows the time-line for paw withdrawal testing and drug administration.

Drugs were solubilized as follows: BK was solubilized in PBS; DAMGO, DPDPE and U50488 were solubilized in ddH₂O; PGE₂ was solubilized in ethanol with a final dilution of 0.1% ETOH in PBS. All drugs were administered via i.pl. injection at a final volume of 50 μ l. At doses tested, none of the drugs altered PWL in the contralateral paw, indicating that changes in PWL observed in the ipsilateral paw were due to local, not systemic, drug action. Experimenters were blinded to the treatment allocation.

2.7 | Data analysis

All statistical analysis was done using Prism software (GraphPad Software, Inc., version 8.0).

For in vitro data, individual concentration-response curves with peak cAMP data were fitted to a logistic equation (Equation 1) using non-linear regression analysis to provide estimates of maximal response (R_{max}) and potency (EC₅₀).

$$R = R_o - \left((R_o - R_i) / \left(1 + \left(10^{[A] - \text{Log}(EC_{50})} \right) \right) \right) \quad (1)$$

Where R is the measured response at a given agonist concentration (A), R_o is the response in the absence of agonist, R_i is the response after maximal inhibition by the agonist, and EC_{50} is the concentration of agonist that produces half-maximal response. Experiments were repeated at least four times with at least triplicate replicates within each experiment. All data analysis and statistical evaluations between treatment groups were done using the individual curve fit parameters and these statistics (including the geometric mean \pm SEM) are reported in the text and/or figure legends. Statistical differences between treatment groups with the same agonist were analyzed with either a paired t test or one-way ANOVA followed by Sidak's multiple comparison test. $p < .05$ was considered statistically significant.

For behavior experiments, full time-course data were analyzed with two-way, repeated measures ANOVA (time and

treatment as factors), followed by Bonferroni's post-test. Area under the curve data were analyzed by one-way ANOVA followed by Dunnett's post-test. Data are presented as mean \pm SEM of at least 6 animals per group, and $p < .05$ was considered statistically significant.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to Pharmacology,²⁶ and are permanently archived in the Concise Guide to Pharmacology 2021/22.²⁷

3 | RESULTS

3.1 | Comparison of antagonist properties of MCAM versus the competitive antagonist, naloxone, and the noncompetitive antagonist, β -FNA at mu opioid receptors

3.1.1 | MCAM

As shown in Figure 1, incubation with the mu agonist, DAMGO, inhibited forskolin stimulated cAMP levels with a maximal inhibition of $43\% \pm 2\%$ and pEC_{50} of 7.93 ± 0.15 (12 nM). Pretreatment with MCAM (10 nM, $10 \times K_i$ as reported by¹¹) for either 15 min or 2 h shifted the DAMGO concentration response curve significantly to the right ($F(2, 26) = 9.89, p = .001$; one-way ANOVA). The pEC_{50} of DAMGO did not differ between different pretreatment times and was 6.18 ± 0.51 (661 nM) and 5.97 ± 0.77 (1 μ M) for 15 min and 2 h MCAM pretreatment, respectively ($p = .98$). Pretreatment with MCAM also reduced the maximal response to DAMGO indicating that the antagonist effects of MCAM were not surmounted by higher agonist concentrations ($F(3, 30) = 57.5, p < .0001$; one-way ANOVA). However, the reduction in the DAMGO maximal response was time dependent (Figure 1A, $p = .01$, 15 min vs. 2 h MCAM pretreatment and 2 vs. 24 h MCAM pretreatment; Sidak's multiple comparisons test). By 24 h of treatment the response to DAMGO was essentially abolished. Treatment with MCAM did not alter forskolin-stimulated cAMP levels in the absence of DAMGO with either 15 min, 2 or 24 h pretreatment (see Figure S1B).

We also examined the reversibility of MCAM antagonism for DAMGO-mediated inhibition of forskolin stimulated cAMP levels. As shown in Figure 1B, after a 2 h pretreatment with MCAM, a washout procedure did not alter the reduction in potency ($F(2, 20) = 16.72; p < .0001$, one-way ANOVA) nor the reduction in maximal response ($p = .32$, Sidak's multiple comparisons test, $n = 4$) to DAMGO. The pEC_{50} values for DAMGO were 7.99 ± 0.12 (10 nM) and 6.56 ± 0.52 (275 nM) for vehicle and MCAM treatment, respectively, following washout. The E_{max} values were $35\% \pm 2\%$ and

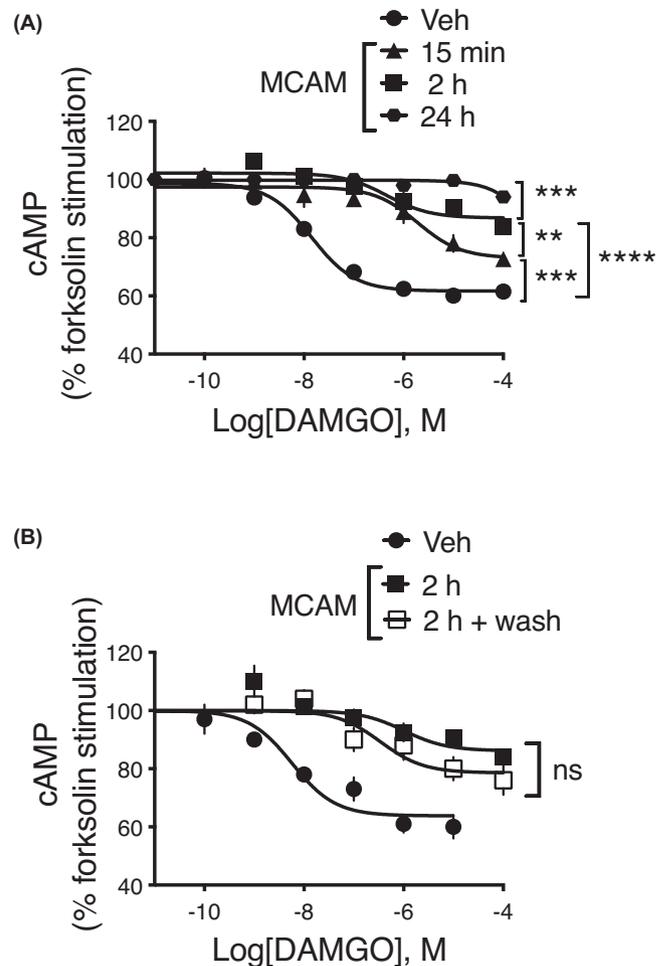


FIGURE 1 MCAM antagonism of DAMGO is time-dependent, non-surmountable and resistant to washout. (A) HEK cells expressing human mu opioid receptors were pretreated with either vehicle or MCAM (10 nM) for 15 min, 2 or 24 h before measurement of forskolin-stimulated cAMP levels with or without DAMGO. (B) Cells were treated with either vehicle or MCAM (10 nM) for 2 h, followed by a wash as indicated. As there was no difference in response to DAMGO in vehicle-treated cells with or without a wash ($p = .32$, Sidak's multiple comparisons test, $n = 4$), data were pooled for vehicle-treated conditions (also done in Figures 2 and 3). Each data point on the graph represents the mean \pm SEM of at least 4 experiments, each done in triplicate, as a percent of forskolin stimulation. For some data points, error bars are contained within the symbol. Curve fit lines represent the non-linear regression of the mean data. Individual concentration response curve data were fit to a logistical equation (see Methods) to estimate pEC_{50} and E_{max} values which are provided in the Results section and summarized in table 1 of the Data S1 file. ** $p < .01$; *** $p < .001$, **** $p < .0001$; ns = not significant, one-way ANOVA with Sidak's post-test

$17\% \pm 5\%$ for inhibition of forskolin-stimulated activity following vehicle or MCAM treatment, respectively. A summary of pEC_{50} and E_{max} parameters derived from nonlinear regression analysis of individual curves (Methods) for DAMGO with or without MCAM pretreatment are shown in table 1 in Data S1.

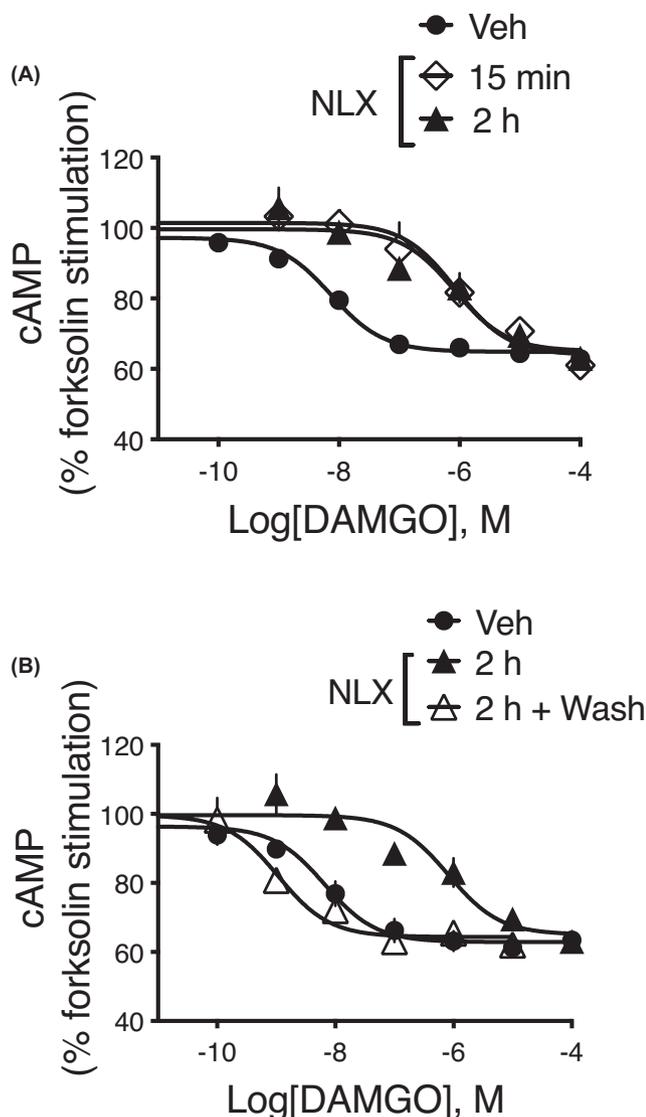


FIGURE 2 Antagonism of DAMGO by naloxone is time-independent, surmountable, and reversible. HEK cells expressing human mu opioid receptors were pretreated with either vehicle or naloxone (NLX, 100 nM) for 15 min or 2 h (A) or with vehicle or NLX for 2 h followed by no wash or a wash step (B) before measurement of DAMGO-mediated inhibition of forskolin stimulated cAMP levels. Data are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of at least 4 experiments in triplicate. Curve fit lines represent the non-linear regression of the mean data. Individual concentration response curve data were fit to a logistical equation (see Methods) to estimate pEC₅₀ and E_{max} values which are provided in the Results section

3.1.2 | Naloxone

As shown in Figure 2, the DAMGO curve was shifted significantly to the right (\approx 100-fold) in a parallel and surmountable manner following either 15 min or 2 h pretreatment with naloxone (NLX, 100 nM, $F(5, 23) = 8.84$, $p < .0001$, one-way ANOVA). The pEC₅₀ for DAMGO was 8.24 ± 0.13 (6 nM), 6.44 ± 0.35 (363 nM), and 6.25 ± 0.35 (562 nM),

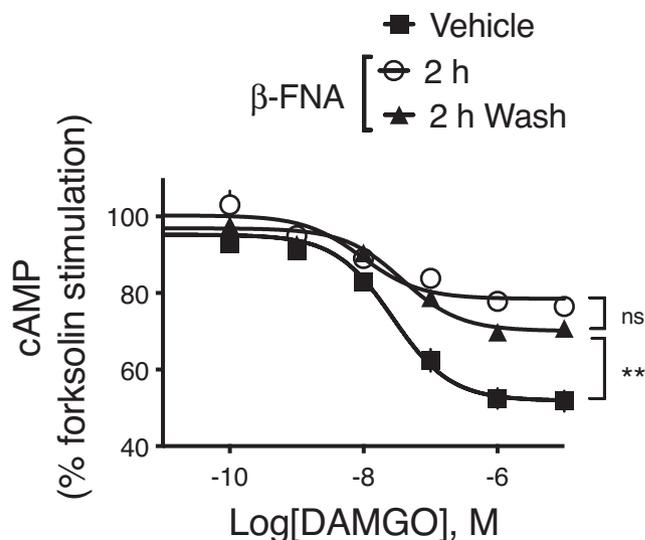


FIGURE 3 Antagonism of DAMGO by β -FNA is non-surmountable and resistant to washout. HEK cells expressing human mu opioid receptors were pretreated with vehicle or β -FNA (10 nM) for 2 h, followed by a washout procedure or not, before measurement of DAMGO-mediated inhibition of forskolin-stimulated cAMP levels. Data are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of 4 (with wash step) or 5 experiments in triplicate. As done for Figures 1 and 2, data for DAMGO with or without a wash step were pooled for vehicle-treated conditions. The pEC₅₀ and E_{max} values, calculated from individual concentration response curve data fit to a logistical equation (see Methods), are provided in the Results section. ** $p < .01$, versus vehicle; ns = not significant, one-way ANOVA with Sidak's post-test

for vehicle, 15 min NLX and 2 h NLX pretreatment, respectively. The effect of NLX was independent of pretreatment time as there was no difference in DAMGO potency between the 15 min and 2 h pretreatments with NLX ($p = .99$, Sidak's multiple comparisons test). As expected for a competitive antagonist, the antagonism by NLX was fully surmounted by DAMGO. The maximal inhibition by DAMGO was $46\% \pm 3\%$, $44\% \pm 2\%$ and $45\% \pm 2\%$ for vehicle, 15 min NLX and 2 h NLX pretreatment, respectively ($F(2,14) = 0.069$, $p = .93$, one-way ANOVA). Furthermore, the effect of NLX was completely reversed following a wash step (Figure 2B). As shown in Figure S1B, treatment with NLX alone did not alter forskolin-stimulated cAMP levels.

3.1.3 | β -FNA

We also assessed the effects of β -FNA, a well characterized irreversible antagonist at mu opioid receptors,²⁸⁻³⁰ on DAMGO-mediated inhibition of forskolin-stimulated cAMP levels. Pretreatment with β -FNA (10 nM) for 2 h had no effect on the pEC₅₀ for DAMGO, which was 7.90 ± 0.23 (13 nM), and 8.22 ± 0.18 (6 nM); vehicle versus β -FNA pretreatment, respectively (mean \pm SEM, $n = 5$, $p = .36$, paired t test). However, as expected, pretreatment with β -FNA significantly

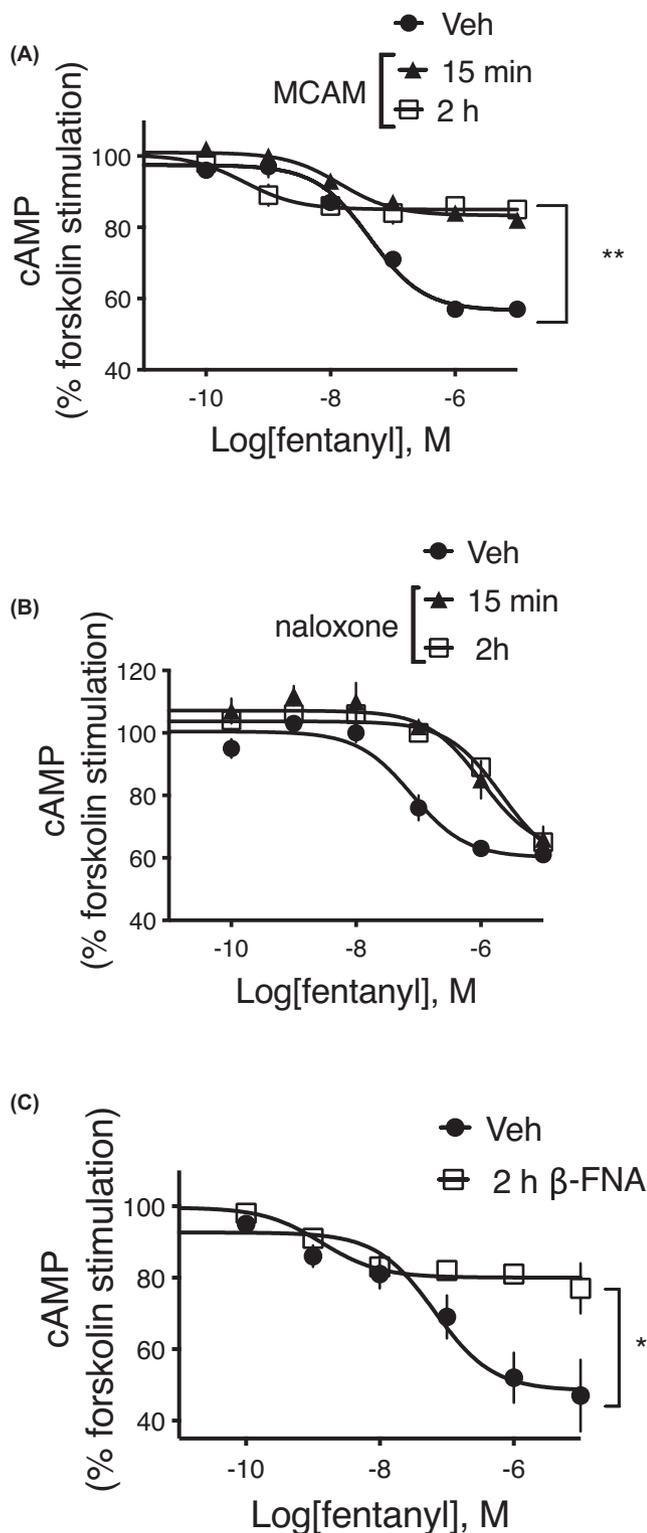


FIGURE 4 Comparison of the effects of MCAM, naloxone (NLX) and β -FNA for antagonism of fentanyl-mediated inhibition of forskolin-stimulated cAMP signaling. HEK cells expressing human mu opioid receptors were pretreated with vehicle or MCAM, 10 nM (A), naloxone, 100 nM (B) or β -FNA, 10 nM (C) for 15 min or 2 h (as indicated) followed by measurement of fentanyl-mediated inhibition of forskolin-stimulated cAMP levels. Data are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of 6 (MCAM), 6 (NLX) or 3 (β -FNA) experiments. The pEC_{50} and E_{max} values, calculated from individual concentration response curve data fit to a logistical equation (see Methods), are provided in the Results section. *** $p < .01$, E_{max} with MCAM treatment versus E_{max} vehicle, one-way ANOVA with Sidak's post-test. * $p < .05$, E_{max} with β -FNA treatment versus E_{max} vehicle, paired t -test. Treatment with β -FNA had no effect on the EC_{50} for fentanyl, $p = .45$, paired t test

antagonist, the reduction in the maximal response to DAMGO by β -FNA was not sensitive to washout ($p = .68$, unpaired t test, Figure 3). Lastly, treatment with β -FNA alone had no effect on forskolin-stimulated cAMP levels (Figure S1B).

The lack of shift in potency by β -FNA suggests a lack of receptor reserve in our mu opioid receptor-expressing HEK cells. To confirm that β -FNA would shift the DAMGO curve in a system with receptor reserve, we tested the effect of β -FNA in HEK cells with a higher density of the human mu opioid receptor. As shown in Figure S2, the EC_{50} for DAMGO in the higher expressing cells was ~ 10 -fold lower than that in the lower expressing cells ($pEC_{50} = 7.53 \pm 0.12$ [29 nM] vs. 8.93 ± 0.18 [1 nM] low vs. overexpression of receptors, $p = .002$, unpaired t test), indicating receptor reserve. Following treatment of overexpressing cells with β -FNA, the DAMGO curve was shifted significantly to the right about 100-fold ($pEC_{50} = 6.95 \pm 0.26$ [113 nM]).

3.2 | Comparison of effects of MCAM, NLX and β -FNA on fentanyl-mediated inhibition of cellular cAMP levels

We next sought to determine effects of MCAM, NLX and β -FNA on a structurally different mu opioid receptor agonist. Because of its role in the current opioid epidemic, we chose to compare the effects of the antagonists on fentanyl-mediated inhibition of cAMP signaling. As shown in Figure 4A, fentanyl inhibited forskolin-stimulated cAMP levels with a maximal inhibition of $43\% \pm 2\%$ and pEC_{50} of 7.36 ± 0.11 (44 nM). Pretreatment with MCAM (10 nM) for 15 min did not alter the EC_{50} of fentanyl ($F(2,19) = 38.16$; $p = .08$ vs. vehicle, one way ANOVA with Dunnett's post-test) however the maximal response was reduced to $17\% \pm 2\%$ of forskolin stimulation ($F(2,19) = 346.75$; $p < .0001$ vs. vehicle, one way ANOVA with Dunnett's post-test) indicating that MCAM antagonism was not surmountable by fentanyl. Interestingly and in contrast to effects on DAMGO, following a 2 h pretreatment, MCAM increased the potency of fentanyl by ~ 10 -fold (pEC_{50} of 9.38 ± 0.30 [0.4 nM], $p < .0001$ 2 h MCAM vs. vehicle) with no further reduction in the maximal response.

reduced the maximal inhibition of forskolin-stimulated cAMP levels by DAMGO indicating that the antagonist effects of β -FNA were not surmountable by higher concentrations of DAMGO (see Figure 3). The maximal inhibition by DAMGO was reduced from $48\% \pm 5\%$ forskolin stimulated cAMP levels with vehicle pretreatment to $22\% \pm 1\%$ with 2 h β -FNA pretreatment, respectively (mean \pm SEM, $n = 5$; $p = .003$, paired t test). As expected for an irreversibly bound

As expected for a competitive antagonist, NLX produced similar degree of surmountable antagonism on fentanyl as was observed for DAMGO. As shown in Figure 4B, pretreatment with 100 nM NLX shifted the fentanyl curve to the right in a fully surmountable manner following. In addition, the effect of NLX was independent of pretreatment time as expected. In these experiments, the pEC_{50} for fentanyl was 7.12 ± 0.13 (76 nM), 5.59 ± 0.14 (2.6 μ M), and 5.88 ± 0.19 (1.3 μ M), for vehicle, 15 min NLX and 2 h NLX pretreatment, respectively ($F(2,15) = 23.93$, $p < .0001$ 15 min vs. vehicle and $p = .0002$ for 2 h vs. Veh; one-way ANOVA with Dunnett's post-test). The maximal inhibition by fentanyl was not altered by NLX and was $41\% \pm 2\%$, $46 \pm 3\%$ and $42\% \pm 4\%$ forskolin stimulated activity for vehicle, 15 min NLX and 2 h NLX pretreatment, respectively ($F(2,15) = 0.89$, $p = .43$, one-way ANOVA).

Similar to the effects on DAMGO in HEK cells with low mu receptor expression (i.e., no receptor reserve), pretreatment with β -FNA (10 nM) for 2 h did not alter the fentanyl EC_{50} , but significantly reduced the maximal inhibition of forskolin-stimulated cAMP levels (see Figure 4C). The pEC_{50} for fentanyl was 7.43 ± 0.30 (38 nM), and 8.04 ± 0.96 (9 nM) for vehicle and 2 h β -FNA pretreatment, respectively (mean \pm SEM, $n = 3$; $p = .45$, paired t test). The maximal inhibition of forskolin-stimulated activity was $49\% \pm 9\%$ and $18\% \pm 2\%$ for vehicle pretreatment and 2 h β -FNA pretreatment, respectively (mean \pm SEM, $n = 3$; $p = .03$, paired t test).

3.3 | Effects of MCAM on morphine-mediated inhibition of cAMP levels

We also examined effects of pretreatment with MCAM (10 nM) for 15 min and 2 h on morphine-mediated inhibition of cAMP signaling on HEK-GloSensor cells expressing mu receptors. As shown in Figure 5, pretreatment with MCAM for 15 min shifted the morphine concentration response curve significantly to the right 10-fold. The pEC_{50} of morphine was 6.88 ± 0.13 (131 nM) and 5.81 ± 0.28 (1.5 μ M) for vehicle versus 15 min MCAM treatment, respectively (mean \pm SEM, $n = 5$, $p = .004$, paired t test). Further, the maximal response to morphine was significantly reduced from $42\% \pm 5\%$ to $24\% \pm 3\%$ inhibition of forskolin stimulated activity, vehicle versus 15 min MCAM pretreatment, respectively, (mean \pm SEM, $n = 5$, $p = .04$, paired t test). As shown in Figure 5, there was no significant response to morphine following a 2 h treatment with MCAM.

3.4 | MCAM is a competitive, surmountable and short acting antagonist at delta- and kappa-opioid receptors

In addition to mu, MCAM has been reported to have affinity for both delta- and kappa-opioid receptors,¹¹ thus we sought to assess the pharmacological properties of MCAM at delta and kappa receptors both in vitro and in vivo. We first tested effects of MCAM on concentration-response curves for the inhibition of

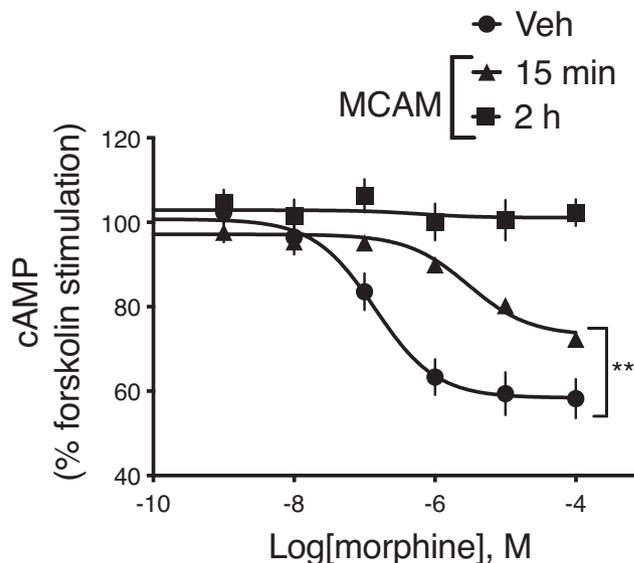


FIGURE 5 MCAM antagonism of morphine is time-dependent and non-surmountable. HEK cells expressing human mu opioid receptors were pretreated with either vehicle or MCAM (10 nM) for 15 min or 2 h before measurement of morphine-mediated inhibition of forskolin-stimulated cAMP levels. Data points are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of at least 4 experiments in triplicate. The Mean \pm SEM of individual curve fit parameters (E_{max} and pEC_{50} values) are provided in the Results section. $**p < .01$, one-way ANOVA with Sidak's post-test

forskolin-stimulated cAMP levels by the delta opioid receptor agonist, DPDPE, and the kappa opioid receptor agonist, U50488. As shown in Figure 6, following pretreatment with MCAM (20 nM, $10 \times K_i$ at delta receptors,¹¹) for either 15 min or 2 h, the curve for DPDPE was shifted to the right (~10-fold) with no change in the maximal response. Similarly, the curve for U50488 was shifted to the right (~10-fold) with no change in the maximal response (i.e., fully surmountable) following pretreatment for either 15 min or 2 h with MCAM (50 nM, $10 \times K_i$ at kappa receptors,¹¹). Further, antagonism produced by MCAM at both delta and kappa receptors was fully reversed following washout (Figure 6).

3.5 | MCAM antagonism in vivo

We next examined the ability of MCAM to antagonize peripheral opioid receptor-mediated antinociception in the rat hindpaw. MCAM or vehicle was administered by intraplantar (i.pl.) injection, 30 min, 24, 48 or 96 h before testing for opioid agonist-mediated antinociception. As shown in Figure 7, with vehicle pretreatment, DAMGO, DPDPE and U50488 each reduced PGE₂-stimulated thermal allodynia. Pretreatment with MCAM, i.pl., for 15 min antagonized the antinociceptive response to all three agonists, without altering baseline thermal sensitivity, or the allodynic effects of BK or PGE₂ (Figure S3). When tested 24 h after administration, MCAM

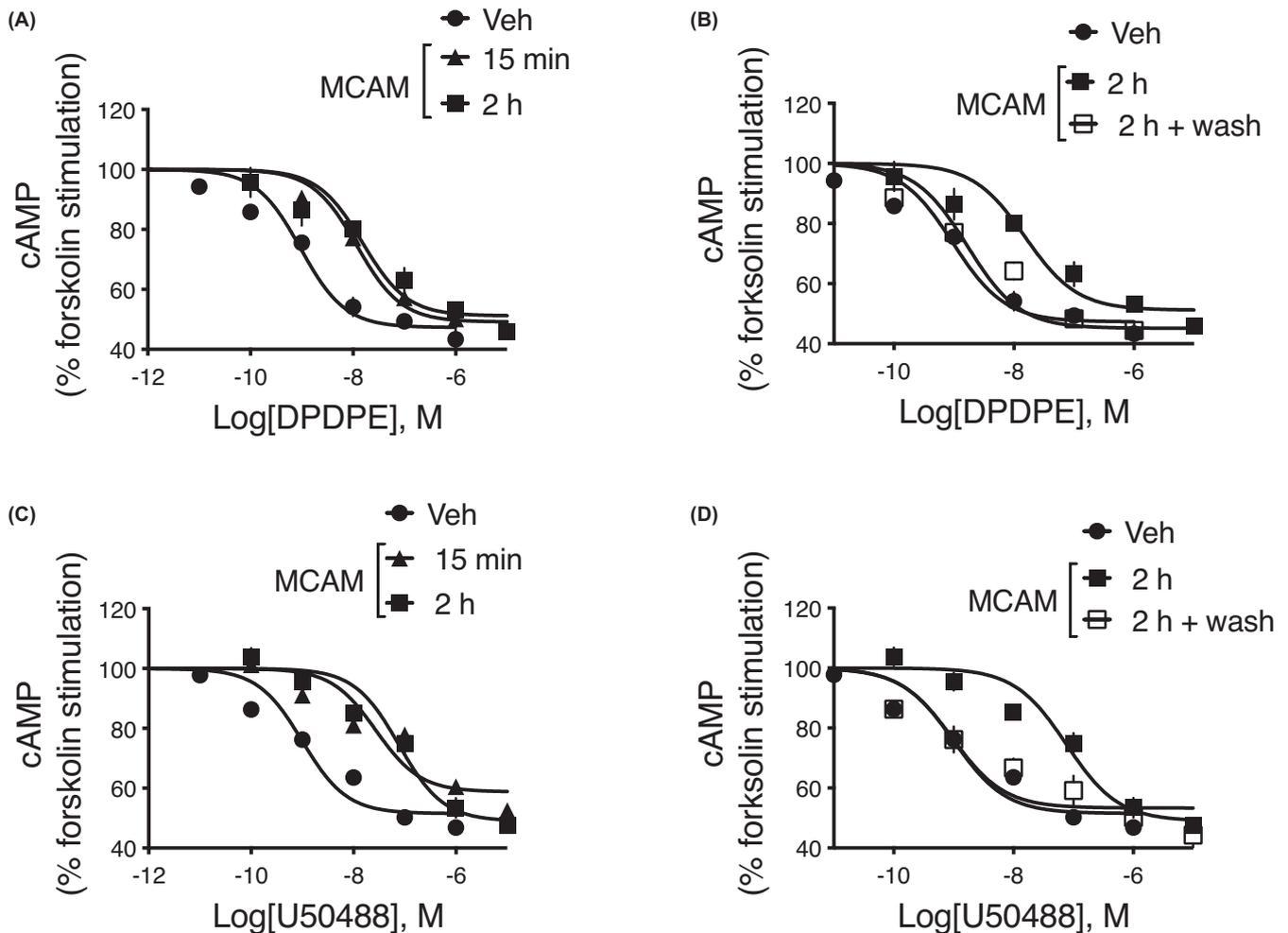


FIGURE 6 MCAM is a competitive (surmountable and reversible) antagonist at delta (A, B) and kappa (C, D) opioid receptors. (A) HEK293 cells expressing human delta opioid receptors were pretreated with vehicle or MCAM (20 nM, $10 \times \text{Ki}$) for 15 min or 2 h before measurement of DPDPE-mediated inhibition of forskolin-stimulated cAMP levels. (B) Cells were pretreated with vehicle or MCAM (20 nM) for 2 h, followed by a wash procedure as indicated, before measurement of DPDPE-mediated inhibition of forskolin-stimulated cAMP levels. (C) HEK293 cells expressing human kappa opioid receptors were pretreated with vehicle or MCAM (50 nM, $10 \times \text{Ki}$) for 15 min or 2 h before measurement of DPDPE-mediated inhibition of forskolin-stimulated cAMP levels. (D) Cells were pretreated with vehicle or MCAM (50 nM) for 2 h, followed by a wash procedure as indicated, before measurement of DPDPE-mediated inhibition of forskolin-stimulated cAMP levels. Data points are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of at least 4 experiments in triplicate

pretreatment did not block either DPDPE- or U50488-mediated antinociceptive responses. However, DAMGO-mediated antinociception remained blocked for up to 96 h after the single intraplantar injection of MCAM.

3.6 | Does MCAM bind to an allosteric site on mu opioid receptors?

The dextral shift in the DAMGO concentration-response curve produced by MCAM in a cell system without receptor reserve was puzzling. In addition, the shifts in the agonist curves produced by MCAM were ligand dependent. Together, this suggested that MCAM may have an allosteric action to modulate orthosteric ligand properties (affinity and/or intrinsic efficacy). We hypothesized that

the non-surmountable reduction in the agonist maximal response was likely due to irreversible (or pseudo-irreversible) orthosteric site binding by MCAM, whereas the shift in the agonist potency was due to the binding of MCAM to an allosteric site on the mu receptor. To test this hypothesis, we assessed the naloxone sensitivity for the shift in the DAMGO EC_{50} versus the naloxone sensitivity for the reduction in E_{max} by MCAM.

Cells were treated with vehicle or with a concentration of NLX to fully occupy the orthosteric site of the mu receptor ($10 \mu\text{M}$; $10\,000 \times \text{Ki}$) for 15 min, followed by the addition of MCAM (10 nM) or vehicle for 15 min and then an extensive wash to remove NLX and unbound MCAM. As shown in Figure 8, the concentration-response curve to DAMGO in cells pretreated with NLX alone (no MCAM) was not different from the response in cells treated with vehicle, indicating that NLX was removed completely from the system by the wash

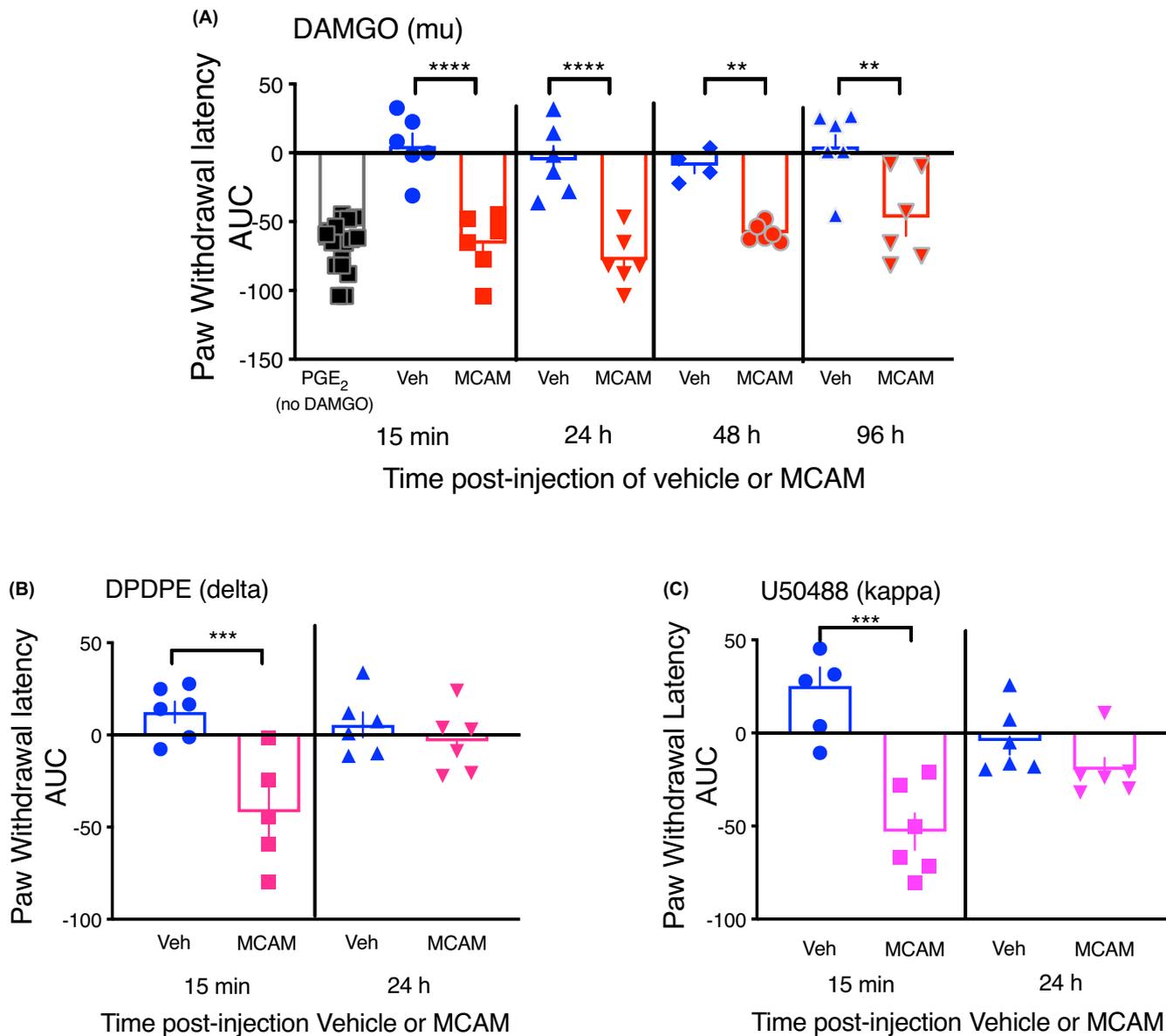


FIGURE 7 Prolonged antagonism by MCAM for peripheral μ -, but not δ - or κ -opioid receptor-mediated reduction of PGE₂-evoked thermal allodynia in the rat hindpaw. Rats received intraplantar (i.pl.) injections of bradykinin (BK, 25 μ g, to induce opioid receptor functional competence, see methods) with or without MCAM (0.25 μ g/50 μ l i.pl, ~10 nM for μ), (0.5 μ g/50 μ l i.pl, ~20 nM for δ) or (1.25 μ g/50 μ l i.pl, ~50 nM κ) followed 15 min later by i.pl. injection with PGE₂ (0.3 μ g/50 μ l i.pl) along with either vehicle, or peripherally restricted doses of DAMGO (μ , 20 μ g), DPDPE (δ , 20 μ g) or U50488 (κ , 0.1 μ g). Paw withdrawal latencies were measured in duplicate before (baseline) BK injection and every 5 min for 20 min after the last injection. MCAM itself had no effect baseline or on PGE₂-induced thermal hypersensitivity (Figure S3). Data are from 6 animals per group and are expressed as area under the 20 min time-course curve (AUC) in arbitrary units. Data were analyzed by one-way ANOVA followed by Sidak's post-test. ** $p < .01$, *** $p < .001$, **** $p < .0001$. Time-course data for each opioid agonist are provided in Figures S3–S6

procedure. The pEC₅₀ values for DAMGO were 7.75 ± 0.10 (18 nM) versus 7.48 ± 0.24 (33 nM), (mean \pm SEM, $n = 4$; ($F(3, 13) = 8.839$, $p = .86$, one-way ANOVA with Sidak's post-test). The DAMGO E_{max} values were $85\% \pm 7\%$ versus $84\% \pm 8\%$ inhibition of forskolin stimulated cAMP levels, for vehicle and NLX pretreatment, respectively, (mean \pm SEM, $n = 4$; $F(3,13) = 13.12$, $p > .99$). Similar to our previous results (see Figure 1), 15 min pretreatment with 10 nM MCAM, in the absence of NLX, shifted the DAMGO curve to the right and significantly reduced the maximal response. The pEC₅₀ value for

DAMGO was 6.55 ± 0.23 (282 nM, $p = .006$ vs. vehicle treatment) and the maximal inhibition was $32\% \pm 6\%$ ($p = .006$ vs. vehicle treatment). In cells pretreated with NLX, the MCAM-induced reduction in the DAMGO maximal response was abolished ($72\% \pm 6\%$ inhibition of forskolin activity, $p = .48$ vs. vehicle treatment), but the shift in DAMGO potency remained (pEC₅₀ of 6.50 ± 0.30 [316 nM], $p < .01$ vs. vehicle treatment). Further, the pEC₅₀ for DAMGO in cells pretreated with NLX and MCAM did not differ from the pEC₅₀ values for DAMGO in cells treated with MCAM alone ($p = .99$).

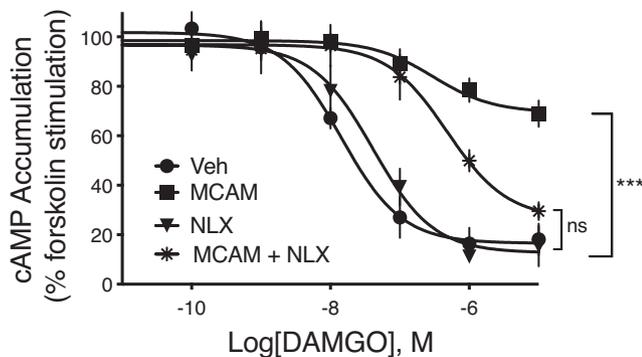


FIGURE 8 MCAM shifts the DAMGO concentration-response curve to the right in a naloxone-insensitive manner. HEK293 cells expressing the human mu opioid receptor were incubated with vehicle or naloxone (NLX, 10 μ M) for 15 min followed by incubation with vehicle or MCAM (10 nM) for an additional 15 min. Cells were washed extensively before measurement of DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation. Data points are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of 4 experiments in triplicate. Individual curve fit parameters (E_{max} and pEC_{50} values) are provided in the Results section. *** p < .001, E_{max} values for MCAM versus vehicle; ns = not significant; one-way ANOVA followed by Sidak's post-test

These data suggest that pretreatment with NLX was able to block MCAM binding to the orthosteric site as MCAM's effect to reduce the DAMGO maximal response was abolished) and suggests that the shift in DAMGO potency was due to an action of MCAM at an NLX-insensitive site.

We also tested effects of a 100-fold lower concentration of MCAM on DAMGO-mediated inhibition of forskolin stimulated cAMP levels. As shown in Figure 9, following a 2 h pretreatment with 0.1 nM MCAM, the maximal response to DAMGO was significantly reduced from 38% \pm 1.1% inhibition of forskolin stimulated activity to 22.9% \pm 2.0% inhibition of forskolin stimulated activity. The DAMGO maximal response was further reduced to 12% \pm 2.0%, following 24 h of incubation with MCAM (p < .0001, one-way ANOVA $F(2,16) = 78.50$). Further, the effect of 2 h pretreatment with 0.1 nM MCAM on the maximal response to DAMGO was insensitive to washout (Figure S7). By contrast to effects of higher concentrations of MCAM, there was no shift in the DAMGO concentration-response curve after treatment with 0.1 nM MCAM ($p = .26$, one-way ANOVA $F(2,16) = 1.466$) suggesting perhaps that MCAM has higher affinity for the orthosteric binding site on the mu receptor. The pEC_{50} values for DAMGO were 8.05 \pm 0.20 (9 nM), 8.97 \pm 0.14 (1 nM) and 8.54 \pm 1.00 (3 nM) for vehicle, 0.1 nM MCAM for 2 h and 0.1 nM MCAM for 24 h, respectively.

4 | DISCUSSION

MCAM has been reported to have selective long-lasting antagonist effects at mu, but not delta or kappa, opioid receptors *in vivo*¹¹ and it was suggested that MCAM acts pseudo-irreversibly at the mu opioid

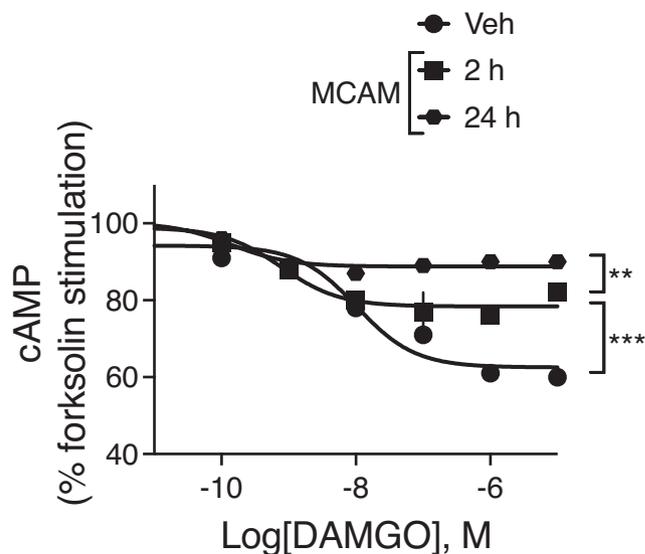


FIGURE 9 A low dose of MCAM reduces the DAMGO maximal response without reducing potency. HEK293 cells expressing the human mu opioid receptor were incubated with vehicle or MCAM (0.1 nM) for 2 or 24 h, before measurement of DAMGO-mediated inhibition of forskolin-stimulated cAMP levels. Data points are expressed as the percent of forskolin stimulation and represent the mean \pm SEM of 10 (Veh) or 5 (2 and 24 h) experiments in triplicate. Individual curve fit parameters (E_{max} and pEC_{50} values) are provided in the Results section and in table 1 of Data S1. ** p < .01 versus 2 h, *** p < .0001 versus vehicle; one-way ANOVA followed by Sidak's post-test. Treatment with 0.1 nM MCAM had no effect on the EC_{50} for DAMGO, $F(2,16) = 1.466$, $p = .26$

receptor, but reversibly at delta and kappa receptors. Here we provide additional evidence that MCAM binds in a pseudo-irreversible manner to the orthosteric site of the human mu opioid receptor, and as a reversible competitive antagonist at human delta and kappa receptors. Moreover, MCAM acts at a naloxone-insensitive allosteric site at the mu receptor to alter the pharmacological properties of mu receptor agonists.

In cells expressing the human mu opioid receptor, pre-treatment with MCAM reduced the maximal response to the mu opioid receptor agonist, DAMGO. This effect was resistant to washout and was time dependent. By contrast, the competitive antagonist, naloxone, was fully surmountable by DAMGO and the antagonism by naloxone (parallel shift to the right of the DAMGO concentration-response curve) was independent of time and fully reversible upon washout. The effect of MCAM on the DAMGO response was similar as that of the irreversible antagonist, β -FNA, which reduced the DAMGO maximal response in a non-washable manner. These results indicate that MCAM binding to the mu receptor was non-competitive and irreversible over the duration of the experiment. However, unlike β -FNA, which binds covalently to the mu receptor via a Michael acceptor,²⁹ MCAM shares the same potential weak Michael acceptor group as its close analogue clocinnamox. However, a variety of studies³¹⁻³³ indicate that there is no covalent bond formation to the mu receptor (and therefore no true irreversible binding) and therefore

the binding of MCAM is likely to be very slowly reversible or pseudo-irreversible.³⁴ This is consistent with the long-lasting mu receptor antagonism observed in behavioral antinociception experiments when MCAM was administered locally to the rat hindpaw (up to 96 h) and that has been reported in mice¹¹ and rhesus monkeys.¹²⁻¹⁴

MCAM also has affinity for delta and kappa opioid receptors,¹¹ however and by contrast to its action at mu, MCAM behaves as a reversible, competitive antagonist at delta and kappa receptors in vitro and in vivo. In cells expressing the human delta or kappa receptors, pretreatment with MCAM shifted the concentration-response curves to the delta agonist, DPDPE, and the kappa agonist, U50488, to the right in a surmountable, time independent and fully washable manner. In the rat hindpaw, MCAM blocked the antinociceptive effects of DPDPE and U50488 when tested 15 min after MCAM injection, but not when tested 24 h later, suggesting that MCAM binding to delta and kappa is reversible, unlike binding to mu. Broadbear et al.,¹¹ reported that, in brain tissue taken from mice treated with MCAM, ³H-DAMGO binding was reduced, but not binding of ³H-DPDPE (delta) or ³H-bremazocine (kappa), which is consistent with long-lasting occupancy by MCAM of mu, but not of delta and kappa receptors.

An (pseudo-)irreversible antagonist for the mu opioid receptor could provide an improved treatment for opioid overdose. The current treatment for overdose is administration of the competitive antagonist, naloxone.¹ Since opioid agonists are competitive (bind reversibly) at the mu opioid receptor, administration of a relatively higher dose of a competitive antagonist would reduce receptor occupancy by the agonist and thus reverse the respiratory depression associated with an overdose of agonist. In this regard, naloxone works quite well. It has been estimated that tens of thousands of lives have been saved in the United States by treatment of overdose victims with naloxone.³⁵ In fact, naloxone is now available over the counter in pharmacies in several states.^{36,37} However, because naloxone is a competitive antagonist with a relatively short half-life in vivo, its antagonism can be surmounted if a revived overdose victim re-ingests a higher dose of agonist or if blood levels of the administered naloxone fall before that of the ingested agonist. Thus, re-narcotization and respiratory depression can re-occur, with an increased risk of death.⁷⁻⁹ Even if a long-acting formulation of naloxone were to be developed, there is still a risk for re-overdose if a higher dose of agonist were to be taken. An antagonist, like MCAM, that binds essentially irreversibly would not have these limitations of naloxone.

We were surprised to find that in addition to suppressing the maximal response of DAMGO, MCAM also shifted the DAMGO concentration-response curve to the right. A dextral shift in an agonist concentration-response curve by an (pseudo-)irreversible antagonist can occur if there is "receptor reserve" in the system. Receptor reserve is a term used to describe a system where there is a high efficiency of receptor-effector coupling (high receptor or signaling molecule density) such that the response saturates before receptor occupancy saturates.³⁸ The irreversible antagonist, β -FNA, while reducing the maximal response to DAMGO, did not shift the

DAMGO concentration-response curve indicating that there is no receptor reserve for DAMGO in our mu receptor expressing cell system. The effect of MCAM to shift agonist concentration-response curves was also agonist-dependent. MCAM decreased the potency of morphine to an extent less than the decrease in DAMGO potency and increased the potency of fentanyl. Ligand-dependent changes in agonist potency are characteristic of allosterism.³⁹⁻⁴¹

The opposite effect of MCAM on the potency of fentanyl, compared with that on DAMGO or morphine, highlights the unusual pharmacology of fentanyl (see,⁴² for an excellent review). In addition to differences in fentanyl relative potency between in vitro and in vivo studies, signaling bias and reduced sensitivity to naloxone, molecular modeling studies predict that fentanyl may bind in the orthosteric binding pocket in multiple orientations.^{43,44} In a recent report in bioRxiv,⁴⁵ coarse-grained molecular dynamics simulations and free energy calculations revealed two distinct poses of fentanyl in the orthosteric binding pocket that were 180° reversed. It is conceivable that MCAM differentially interferes with one of the two possible binding poses of fentanyl in the orthosteric binding pocket of MOR.

To test the hypothesis that MCAM may have an allosteric action to shift mu agonist potency in addition to a (pseudo-)irreversible orthosteric action to reduce agonist maximal response, we pre-treated cells with naloxone at a concentration to fully occupy the orthosteric site of the mu receptor prior to administration of MCAM. Following the treatments, cells were subjected to a rigorous wash procedure to remove naloxone and unbound MCAM. The wash procedure was effective in removing bound and unbound naloxone as shown by the return of the DAMGO concentration-response curve to the control (vehicle-pretreated) position. Pretreatment with naloxone prevented the MCAM-induced reduction in the maximal response to DAMGO but did not block the dextral shift in the DAMGO concentration-response curve. This indicates that the decrease in DAMGO potency by MCAM is mediated by MCAM binding to a naloxone-insensitive (allosteric) site. It appears that the affinity of MCAM for this allosteric site may be less than that for the orthosteric site because a 100-fold lower concentration of MCAM was effective at reducing the DAMGO maximal response without shifting the concentration-response curve to the right. Currently, the location of this allosteric binding site for MCAM is not known.

In summary, the time-dependent nature of the antagonism, the non-surmountability by agonists, and the lack of reversibility are consistent with MCAM having irreversible (or pseudo-irreversible) binding properties at mu receptors. Importantly, the fact that MCAM acted as a simple competitive antagonist and did not elicit long-term antagonism at either delta or kappa opioid receptors, suggests that MCAM's long duration of action in vivo is selective for mu opioid receptors. Perhaps the most intriguing finding from this study is that MCAM binds to a naloxone-insensitive, allosteric site that leads to differential modulation of opioid agonist responses and could be responsible for the slow off-rate of MCAM from the orthosteric site.

ACKNOWLEDGEMENTS

We thank Drs. Jim Woods and Gail Winger for many enjoyable discussions and valuable feedback. We also thank Dr. Michael Wedemeyer for helpful discussions. This work was supported by NIH/NIDA RO1 DA038645 to WPC, and a pilot project grant to KAB from the Addiction Research, Treatment & Training (ARTT) Center of Excellence at UT Health San Antonio.

DISCLOSURE

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Participated in research design: Berg and Clarke. Conducted experiments: Zamora, Smith, Jennings, Chavera, Kotipalli, and Jay. Contributed new reagents or analytic tools: Disney and Husbands. Performed data analysis: Berg, Clarke, Zamora, Smith, and Jennings. Wrote or contributed to the writing of the manuscript: Berg and Clarke.

DATA AVAILABILITY STATEMENT

Datasets that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Kelly A. Berg  <https://orcid.org/0000-0003-2977-6411>

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Zamora JC, Smith HR, Jennings EM, et al. Long-term antagonism and allosteric regulation of mu opioid receptors by the novel ligand, methocinnamox. *Pharmacol Res Perspect*. 2021;9:e00887. <https://doi.org/10.1002/prp2.887>