

RESEARCH PAPER

Neutral antagonist activity of naltrexone and 6 β -naltrexol in naïve and opioid-dependent C6 cells expressing a μ -opioid receptor

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Background and purpose: Adenylyl cyclase sensitization occurs on chronic agonist activation of μ -opioid receptors and is manifested by an increase in cAMP levels (overshoot) on challenge with antagonist. It has been proposed that a long lasting constitutively active receptor is formed on chronic μ -opioid exposure and that antagonists with inverse agonist activity rapidly return the receptor to a basal state causing a cAMP overshoot and a more severe withdrawal response *in vivo*. This hypothesis depends on an accurate characterization of neutral and inverse agonist properties of opioid antagonists.

Experimental approach: C6 glioma and HEK293 cells expressing μ -opioid receptors were used. Opioid antagonists were examined for their ability to induce a cAMP overshoot following chronic treatment with the agonist DAMGO ([D-Ala²,N-Me-Phe⁴,Glyol⁵]-enkephalin). The compounds were also characterized as agonists, inverse agonists or neutral antagonists by using assays for competitive binding, [³⁵S]GTP γ S (guanosine-5'-O-(3-[³⁵S]thio)triphosphate) binding and changes in cell surface receptor expression.

Key results: Naltrexone, 6 β -naltrexol and naloxone were indistinguishable to the μ -opioid receptor in the opioid-naïve or dependent state and acted as neutral antagonists. The δ -opioid receptor inverse agonist RTI-5989-25 [(+)-N-[*trans*-4'-(2-methylphenyl)-2'-butenyl]-(3*R*,4*R*)-dimethyl-4-(3-hydroxyphenyl)piperidine], a 3,4-dimethyl-4-(3-hydroxyphenyl)-piperidine, was an inverse agonist at the μ -opioid receptor, and the peptide antagonist CTAP (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) showed variable, assay-dependent properties. All the antagonists precipitated the same degree of cAMP overshoot in opioid-dependent cells.

Conclusions and implications: Antagonists at the μ -opioid receptor may be neutral or show inverse agonist activity. Formation of a constitutively active μ -opioid receptor is not a requirement for the development or expression of adenylyl cyclase sensitization.

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Abbreviations: [³⁵S]GTP γ S, guanosine-5'-O-(3-[³⁵S]thio)triphosphate; CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO, [D-Ala²,N-MePhe⁴,Glyol⁵]-enkephalin; DTT, dithiothreitol; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; RTI-5989-25, (+)-N-[*trans*-4'-(2-methylphenyl)-2'-butenyl]-(3*R*,4*R*)-dimethyl-4-(3-hydroxyphenyl)piperidine

Introduction

The μ -opioid receptor belongs to the class of G-protein coupled receptors (GPCRs) that activate G $\alpha_{i/o}$ proteins and inhibit the family of adenylyl cyclase (AC) enzymes. Chronic exposure of these receptors, including the μ -opioid receptor,

to agonist results in sensitization of AC as an adaptive cellular response (Watts and Neve, 2005). This occurs to maintain homeostatic control of AC activity and may be a cellular model of dependence (Christie, 2008). Following challenge with antagonist there is an expression of the developed sensitization, resulting in an increased accumulation of cAMP, so-called 'cAMP overshoot'. This cAMP overshoot is seen not only in cultured cells exposed to μ -opioids (Clark *et al.*, 2004; Zhao *et al.*, 2006; Wang *et al.*, 2007b) but also *in vitro* in CNS tissues from μ -opioid-dependent animals (Bohn *et al.*, 2000). AC sensitization has been shown to be isoform-dependent,

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Pertussis toxin sensitive with an important role for G $\beta\gamma$ subunits and may involve an increased interaction between AC and G α_s (Watts and Neve, 2005). The mechanism behind this AC adaptation is not known, but kinase enzymes such as Raf-1 and PKC that could phosphorylate AC have been implicated.

Numerous studies have shown the μ -opioid receptor itself is a target for phosphorylation leading to desensitization (Johnson *et al.*, 2005). Additionally, phosphorylation of the receptor has been implicated in AC sensitization (Wang *et al.*, 1994; 1999; Sadee and Wang, 1995). Consistent with this, AC sensitization is abolished by mutation of Tyr394 in the C-terminal tail of the μ -opioid receptor expressed in CHO cells (Wang *et al.*, 2007b). Phosphorylation has been suggested to convert the receptor to a persistent constitutively active (R*) state that develops on chronic opioid exposure and continuously signals even in the absence of agonist. Rapid reversal of the persistent R* state back to a basal resting state (R) is then postulated to cause the observed cAMP overshoot (Wang *et al.*, 1994; Sadee and Wang, 1995; Liu and Prather, 2001). Ligands that inhibit constitutive activity are known as inverse agonists or are said to have negative intrinsic activity and bind preferentially to R, thus shifting the equilibrium in favour of this state. In contrast, neutral antagonists bind equally to R and R* and do not alter the equilibrium between receptor states (Milligan, 2003; Kenakin, 2004). Consequently, opioid antagonists that cause a cAMP overshoot have been characterized as inverse agonists (Wang *et al.*, 1994; Sadee and Wang, 1995; Liu and Prather, 2001; Szucs *et al.*, 2004). A role for a constitutively active μ -opioid receptor has been extended to dependence and withdrawal *in vivo*, and again antagonists that cause a more severe withdrawal have been identified as inverse agonists (Bilsky *et al.*, 1996; Wang *et al.*, 2001; 2004; Raehal *et al.*, 2005).

Based on their ability to induce a cAMP overshoot and precipitate a severe withdrawal in opioid-dependent systems, the opioid antagonists naloxone and naltrexone have been characterized as inverse agonists, while the naltrexone metabolite 6 β -naltrexol, and the peptide CTAP (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂) were perceived to be neutral antagonists (Wang *et al.*, 1994; 2001; Bilsky *et al.*, 1996; Liu and Prather, 2001; Raehal *et al.*, 2005). In support of this, a continuum of negative efficacies has been reported for μ -opioid antagonists, determined by their relative abilities to precipitate acute withdrawal in the mouse (Walker and Sterious, 2005). In contrast, we have shown in both the mouse (Divin *et al.*, 2008) and the monkey (Ko *et al.*, 2006) that there is not a qualitative difference between naltrexone and 6 β -naltrexol to precipitate morphine withdrawal, but rather a quantitative difference that may have a pharmacokinetic basis.

The characterization of a role for a persistent, constitutively active μ -opioid receptor as a contributing factor in AC sensitization and opioid dependence *in vivo* relies on the correct definition of antagonists as neutral antagonists or inverse agonists. To test this in the absence of interference due to distribution and metabolism we have used a heterologous expression system of C6 glioma cells (C6 μ), together with HEK293 cells expressing a FLAG-tagged μ -opioid receptor to study cell surface receptor levels. We have previously used

C6 μ cells in studies of opioid signalling including AC sensitization (Clark *et al.*, 2004; Clark and Traynor, 2006) and have shown similar μ -opioid-mediated sensitization in HEK cells (Clark and Traynor, 2006). We have compared the ability to precipitate expression of AC sensitization and the pharmacological profiles of naltrexone and 6 β -naltrexol, along with the standard opioid antagonist naloxone, the peptidic antagonist CTAP and the known δ -opioid inverse agonist (+)-N-[*trans*-4'-(2-methylphenyl)-2'-butenyl]-(3*R*,4*R*)-dimethyl-4-(3-hydroxyphenyl)piperidine (RTI-5989-25; Zaki *et al.*, 2001). The results show that there is no inherent efficacy difference between 6 β -naltrexol and naltrexone under the conditions studied and furthermore that development and manifestation of AC sensitization is not dependent on the formation of a constitutively active μ -opioid receptor.

Methods

Cell culture and treatments

C6 rat glioma cells stably transfected with the rat μ -opioid receptor (C6 μ) or HEK293 cells stably transfected with the FLAG-tagged mouse μ -opioid receptor were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 0.5 mg·mL⁻¹ or 0.8 mg·mL⁻¹ Geneticin respectively. Cells were grown in the presence of 10% fetal bovine serum at 37°C in 5% CO₂. For chronic opioid treatment, cells were incubated overnight with 10 μ mol·L⁻¹ DAMGO ([D-Ala²,N-Me-Phe⁴,Glyol⁵]-enkephalin). C6 μ cells were used for all experiments except for the determination of cell surface receptor number, which utilized HEK cells expressing a FLAG-tagged μ -opioid receptor. C6 μ cells expressed 3.2 \pm 0.2 pmol·mg⁻¹ protein receptor and HEK cells 9.7 \pm 1.3 pmol·mg⁻¹ protein receptor, determined by [³H]diprenorphine binding.

Membrane preparation

Cells were washed twice with ice cold phosphate-buffered saline (0.9% NaCl, 0.61 mmol·L⁻¹ Na₂HPO₄ and 0.38 mmol·L⁻¹ KH₂PO₄, pH 7.4), detached from the plate by incubation in harvesting buffer (20 mmol·L⁻¹ HEPES, pH 7.4, 150 mmol·L⁻¹ NaCl and 0.68 mmol·L⁻¹ EDTA) and pelleted by centrifugation. The resulting pellet was suspended in cold 50 mmol·L⁻¹ Tris buffer, pH 7.4 and homogenized with a Tissue Tearor (Biospec Products Inc., Bartlesville, OK). The homogenate was centrifuged at 18 000 \times g at 4°C for 20 min, and the pellet resuspended in 50 mmol·L⁻¹ Tris, homogenized with a Tissue Tearor and recentrifuged. The final pellet was resuspended in 50 mmol·L⁻¹ Tris, aliquoted and stored at -80°C until use. Protein concentration was measured by the method of Bradford (1976).

[³H]Diprenorphine binding

For competitive binding, cell membranes were incubated for 75 min at 25°C with varying concentrations (0.1 nmol·L⁻¹–1 μ mol·L⁻¹) of ligand and 0.2 nmol·L⁻¹ [³H]diprenorphine in 50 mmol·L⁻¹ Tris, pH 7.4 with and without the presence of 100 mmol·L⁻¹ NaCl and 10 μ mol·L⁻¹ GTP γ S. Non-specific

binding was determined in the presence of 10 $\mu\text{mol}\cdot\text{L}^{-1}$ naloxone. Assays were stopped by rapid filtration through glass microfiber filtermats, type GF/C (Whatman, Clifton, NJ) by using a Brandell harvester (Gaithersburg, MD) followed by washing with cold 50 $\text{mmol}\cdot\text{L}^{-1}$ Tris buffer. Filtermats were dried, and 0.1 mL Ecolume was added to each sample. Filtermats were heat sealed in polyethylene bags, and radioactivity retained on the filters was measured by liquid scintillation counting in a Wallac 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Perkin Elmer, Boston, MA).

$[^3\text{S}]\text{GTP}\gamma\text{S}$ [*Guanosine-5'-O-(3- $[^3\text{S}$]thio)triphosphate*] binding

C6 glioma cell membranes were incubated for 60 min at 25°C with 0.1 $\text{nmol}\cdot\text{L}^{-1}$ $[^3\text{S}]\text{GTP}\gamma\text{S}$ and with ligand (DAMGO, morphine, 6 β -naltrexol, CTAP, naltrexone, naloxone or RTI-5989-25; 10 $\mu\text{mol}\cdot\text{L}^{-1}$) or vehicle (H_2O) in GTP γS Buffer [50 $\text{mmol}\cdot\text{L}^{-1}$ Tris, pH 7.4, 1 $\text{mmol}\cdot\text{L}^{-1}$ EDTA, 5 $\text{mmol}\cdot\text{L}^{-1}$ MgCl_2 , 100 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, 2.4 $\text{mmol}\cdot\text{L}^{-1}$ dithiothreitol (DTT), 30 $\mu\text{mol}\cdot\text{L}^{-1}$ GDP, 1 mU adenosine deaminase] or GTP γS buffer in which NaCl was replaced with KCl. In certain experiments with CTAP, the DTT was omitted. Alternatively, membranes were incubated with varying concentrations of morphine (1 $\text{nmol}\cdot\text{L}^{-1}$ –0.1 $\text{mmol}\cdot\text{L}^{-1}$) with and without the presence of antagonist (10, 30 or 100 $\text{nmol}\cdot\text{L}^{-1}$) in GTP γS Buffer. Reactions were terminated by rapidly filtering samples through glass microfiber filtermats mounted in a Brandell harvester and rinsing three times with wash buffer (50 $\text{mmol}\cdot\text{L}^{-1}$ Tris, pH 7.4, 5 $\text{mmol}\cdot\text{L}^{-1}$ MgCl_2 and 100 $\text{mmol}\cdot\text{L}^{-1}$ NaCl or KCl as appropriate). Bound $[^3\text{S}]\text{GTP}\gamma\text{S}$ retained on the filtermats was determined as described for binding assays.

cAMP accumulation

Cells were grown in 24-well plates to reach confluence on the day of the assay. To measure AC inhibition cells were treated with varying concentrations of DAMGO (1 $\text{nmol}\cdot\text{L}^{-1}$ –10 $\mu\text{mol}\cdot\text{L}^{-1}$) in DMEM for 15 min in the presence of 10 $\mu\text{mol}\cdot\text{L}^{-1}$ forskolin and 1 $\text{mmol}\cdot\text{L}^{-1}$ phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine), without or with the presence of 6 β -naltrexol or naltrexone (100 $\text{nmol}\cdot\text{L}^{-1}$). To measure AC sensitization, cells were treated overnight with the opioid agonist DAMGO (10 $\mu\text{mol}\cdot\text{L}^{-1}$). To begin the assay, media containing the opioid agonist was removed, and replaced with media containing 10 $\mu\text{mol}\cdot\text{L}^{-1}$ forskolin representing an approximately EC_{30} concentration (Clark *et al.*, 2004), 1 $\text{mmol}\cdot\text{L}^{-1}$ IBMX unless otherwise stated and opioid antagonist (6 β -naltrexol, CTAP, naltrexone, naloxone or RTI-5989-25). Alternatively, cells were washed by quickly removing and replacing media three times to remove the opioid agonist. Cells were incubated at 37°C for 5 min, and the assay was stopped with ice cold 0.1 $\text{mol}\cdot\text{L}^{-1}$ HCl. After 30 min at 4°C, cAMP accumulation was measured by using a cAMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) following the manufacturer's instructions.

Cell surface receptor levels

HEK293-FLAG- μ cells were seeded onto poly-D-lysine coated plates (BD Biosciences, San Jose, CA) and incubated with or

without 10 $\mu\text{mol}\cdot\text{L}^{-1}$ antagonist (6 β -naltrexol, naltrexone, CTAP or RTI-5989-25) for 24 h. Cells were fixed with 3.7% formaldehyde in Tris-buffered saline, washed and blocked with 1% non-fat dry milk. The cells were then washed and incubated with monoclonal anti-FLAG-M2 alkaline phosphatase antibody (Sigma) followed by incubation with p-nitrophenyl-phosphate. At the end of the incubation each sample was added to 3 N NaOH in a 96-well plate, and absorbance at 405 nm was measured. Background absorbance was obtained from similarly treated untransfected HEK293 cells and subtracted from the absorbance of stable HEK293-FLAG- μ cells.

Data analysis and statistics

Data were analysed by using GraphPad Prism 4.0 (San Diego, CA). Antagonist binding affinities derived from competition curves were calculated as K_i ($\text{nmol}\cdot\text{L}^{-1}$) values and as their negative logarithm ($\text{p}K_i$). Antagonist binding affinities from pharmacological experiments were also determined from antagonist-induced shifts in μ -opioid agonist concentration-effect curves as $\text{p}K_B$ or $\text{p}A_2$ values. These values are the negative logarithm of the dissociation constant of an antagonist determined under equilibrium conditions and are a measure of an antagonist's affinity for its receptors. $\text{p}K_B$ values were calculated from shifts in μ -opioid agonist concentration-effect curves caused by a single (100 $\text{nmol}\cdot\text{L}^{-1}$) concentration of antagonist in the cAMP accumulation assays according to the equation $\text{p}K_B = -\log[B/(\text{dose-ratio} - 1)]$, where B equals the concentration of opioid receptor antagonist and dose-ratio represents the EC_{50} concentration in the presence of antagonist divided by the EC_{50} concentration in the absence of antagonist (Divin *et al.*, 2008). $\text{p}A_2$ values were determined from shifts in the DAMGO concentration-effect curves in the $[^3\text{S}]\text{GTP}\gamma\text{S}$ assay experiments in response to three different concentrations of the antagonists according to the Schild method (Arunlakshana and Schild, 1959). The data presented are from at least three experiments performed in duplicate, with results presented as mean \pm SEM. Data were compared by using a two-tailed *t*-test, or two-way ANOVA to compare concentration-response curves. Differences were considered significant if $P < 0.05$.

Drugs and reagents

Tissue culture media, Geneticin, fetal bovine serum and trypsin were from Invitrogen (Carlsbad, CA). $[^3\text{S}]\text{GTP}\gamma\text{S}$ (1250 Ci·mmol $^{-1}$) and $[^3\text{H}]\text{diprenorphine}$ (50 Ci·mmol $^{-1}$) were obtained from Perkin-Elmer Life Sciences (Boston, MA). Adenosine deaminase was obtained from CalBiochem (San Diego, CA). Ecolume scintillation fluid was from ICN (Aurora, OH). Morphine sulphate, 6 β -naltrexol, naltrexone and naloxone were obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). DAMGO, CTAP, GDP, GTP γS , forskolin, IBMX and all other biochemicals were from Sigma (St. Louis, MO) and were of analytical grade. RTI-5989-25 was prepared as previously described (Zaki *et al.*, 2001). FLAG-tagged mouse μ -opioid receptor was a kind gift from Dr Lakshmi Devi, Mt. Sinai School of Medicine, New York, NY.

Results

Adenylyl cyclase sensitization

On chronic treatment and subsequent rapid removal of opioid agonist, cells expressing μ-opioid receptors exhibit an enhanced cAMP accumulation (overshoot) above untreated forskolin-stimulated controls (Watts and Neve, 2005). To assess cAMP overshoot in C6μ cells an approximately EC₃₀ concentration of 10 μmol·L⁻¹ forskolin was employed (Clark et al., 2004). At maximal concentration (10 μmol·L⁻¹) the antagonists, 6β-naltrexol, CTAP, naltrexone, naloxone or RTI-5989-25, were all able to induce a cAMP overshoot following overnight treatment of C6μ cells with the high-efficacy μ-opioid agonist DAMGO (10 μmol·L⁻¹; Figure 1A). All antagonists induced the same degree of cAMP overshoot that was the same as that obtained by washing cells by removing and replacing media to dissociate bound opioid agonist from the receptor ($P > 0.05$). Using morphine (10 μmol·L⁻¹) to induce AC sensitization gave a lower percentage of cAMP overshoot compared with DAMGO across the antagonists, as previously reported (Liu and Prather, 2001), but the antagonists all gave a similar results with the putative inverse agonist naltrexone giving the same degree of overshoot (225 ± 20%) as 6β-naltrexol (248 ± 16%), CTAP (277 ± 14%) or RTI-5989-25 (202 ± 13%). In addition, the phosphodiesterase inhibitor IBMX present in our assays to prevent cAMP breakdown has been reported to block the inverse agonist effect of naltrexone (Wang et al., 1999). In the absence of IBMX the percentage of cAMP overshoot in cells treated overnight with 10 μmol·L⁻¹ DAMGO was reduced, but removing IBMX from the assay did not reveal any significant difference between the degree of overshoot seen with naltrexone (275 ± 13%) compared with 6β-naltrexol (274 ± 16%), RTI (266 ± 68%) or CTAP (313 ± 34%).

As these results contrasted with previous reports, we further compared the ability of 6β-naltrexol and naltrexone to induce a cAMP overshoot in chronic DAMGO-treated cells (Figure 1B). Both compounds concentration-dependently induced an increase in cAMP in treated cells over control, vehicle-treated cells with similar EC₅₀ values of 28.2 ± 5.2 nmol·L⁻¹ and 22.4 ± 6.0 nmol·L⁻¹ ($P > 0.05$) respectively. This was confirmed when cAMP overshoot was precipitated with a combination of the two antagonists. Thus, following overnight DAMGO, 100 nmol·L⁻¹ 6β-naltrexol, 100 nmol·L⁻¹ naltrexone or 50 nmol·L⁻¹ 6β-naltrexol with 50 nmol·L⁻¹ naltrexone precipitated the same level of cAMP overshoot ($P > 0.05$; Figure 1C).

These results demonstrate that the loss of a constitutively active receptor is not required for cAMP overshoot but that removing the chronic μ-opioid agonist, either by challenge with antagonist or by washing, is sufficient. Consequently in this assay all the antagonists appeared operationally the same, and so we undertook a more comprehensive pharmacological analysis to better define their relative efficacies as neutral antagonists or inverse agonists.

Receptor affinity

The affinity of ligands for GPCRs is greatly influenced by the presence or absence of Na⁺ and guanine nucleotides in the

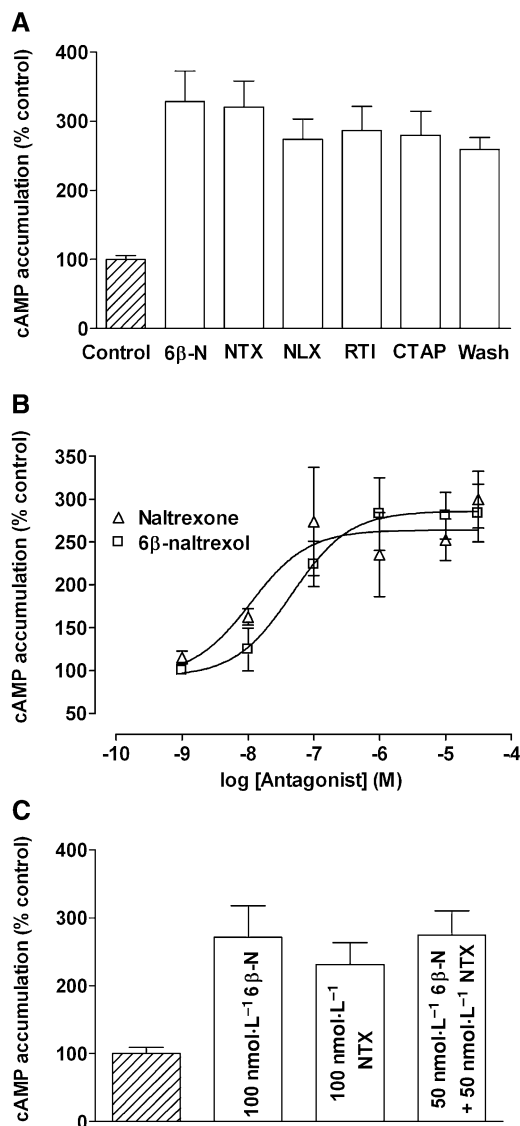


Figure 1 Adenylyl cyclase sensitization in C6 glioma (C6μ) cells. C6μ cells were treated overnight with 10 μmol·L⁻¹ DAMGO, and cAMP overshoot was precipitated in the presence of 10 μmol·L⁻¹ forskolin and 1 nmol·L⁻¹ IBMX to prevent hydrolysis of cAMP. (A) cAMP overshoot was induced with 10 μmol·L⁻¹ 6β-naltrexol (6β-N), naltrexone (NTX), naloxone (NLX), RTI-5989-25 (RTI), CTAP or by washing. (B) cAMP overshoot was precipitated with 1 nmol·L⁻¹–10 μmol·L⁻¹ 6β-naltrexol or naltrexone. (C) cAMP overshoot was precipitated with 100 nmol·L⁻¹ 6β-naltrexol, 100 nmol·L⁻¹ naltrexone or 50 nmol·L⁻¹ 6β-naltrexol with 50 nmol·L⁻¹ naltrexone. cAMP accumulation is expressed as a percentage of 10 μmol·L⁻¹ forskolin-stimulated cAMP levels in vehicle-treated control cells (4.7 ± 0.5 pmol·μg⁻¹ protein). Values represent mean ± SEM of three to five experiments performed in duplicate. CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO, [D-Ala²,N-MePhe⁴,Glyol⁵]-enkephalin; IBMX, 3-isobutyl-1-methylxanthine; RTI-5989-25, (+)-N2-[trans-4'-(2-methylphenyl)-2'-butenyl]2-2(3R,4R)-dimethyl-4-(3-hydroxyphenyl)piperidine.

assay buffer that inhibit the formation of activated, R* receptor conformations and inhibit coupling to G-proteins respectively, to promote the basal, R state of the receptor. In contrast, in low ionic strength buffers, high affinity G-protein coupled states (R*G) exist (Strange, 2008). The ability of 6β-

Table 1 Opioid antagonist affinities for the μ -opioid receptor in C6 glioma cells

Opioid	Competitive binding		$[^{35}\text{S}]\text{GTP}\gamma\text{S}$	cAMP
	Tris buffer + Na ⁺ , GTP γ S	K _i (nmol·L ⁻¹) (pK _i)	pA ₂	K _B (nmol·L ⁻¹) (pK _B)
6 β -naltrexol	0.93 \pm 0.04 (9.03)	1.26 \pm 0.13 (8.90)	8.91 \pm 0.32	0.63 \pm 0.19 (9.20)
Naltrexone	0.38 \pm 0.08 (9.42)	0.46 \pm 0.21 (9.33)	9.35 \pm 0.36	0.52 \pm 0.12 (9.28)
RTI-5989-25	0.062 \pm 0.024 (10.21)	0.011 \pm 0.005* (10.96)	11.26 \pm 0.17 ^a	ND
CTAP	1.52 \pm 0.31 (8.82)	7.00 \pm 1.59** (8.15)	7.96 \pm 0.30	ND
Naloxone	2.44 \pm 0.49 (8.61)	1.62 \pm 0.47 (8.79)	ND	ND

K_i values were determined by competitive displacement of [³H]diprenorphine (0.2 nmol·L⁻¹) binding in 50 mmol·L⁻¹ Tris buffer, pH 7.4, in the presence and absence of 100 mmol·L⁻¹ NaCl and 10 μ mol·L⁻¹ GTP γ S. pA₂ values were determined by Schild analysis of antagonism of morphine-stimulated [³⁵S]GTP γ S binding. The 95% confidence interval of all slopes in the Schild analysis contained unity. K_B values were determined by measurement of the ability of 100 nmol·L⁻¹ antagonist to shift the concentration–response curve for DAMGO-induced inhibition of forskolin-stimulated cAMP accumulation. Details of all assays are in the *Methods*. For comparison, pK_i and pK_B values were calculated as $-\log(K)$. Values represent means \pm SEM of three experiments performed in duplicate.

[³⁵S]GTP γ S, guanosine-5'-O-(3-[³⁵S]thio)triphosphate; CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO, [D-Ala²,N-MePhe⁴,Glyol⁵]-enkephalin; ND, not determined.

^aAffinity determined by single concentration of antagonist.

P* < 0.05, *P* < 0.01, compared with Tris buffer.

naltrexol, CTAP, naltrexone, naloxone and RTI-5989-25 to concentration-dependently displace the binding of the non-selective opioid antagonist [³H]diprenorphine in membranes from C6 μ cells was measured in Tris-HCl buffer without and with 100 mmol·L⁻¹ NaCl and 10 μ mol·L⁻¹ GTP γ S. All compounds showed high μ -opioid receptor affinity in the order RTI-5989-25 > naltrexone > 6 β -naltrexol > CTAP > naloxone (Table 1). The affinities of 6 β -naltrexol, naltrexone and naloxone in the presence or absence of NaCl and GTP γ S were not significantly different (*P* > 0.05), indicating an inability to distinguish R and R*G states of the μ -opioid receptor. However, CTAP was shifted to a lower affinity in a buffer containing NaCl and GTP γ S (***P* < 0.01), showing preferable binding to R*G states suggesting a compound with agonist activity in this assay. In contrast, RTI-5989-25 had a higher affinity in the NaCl and GTP γ S containing buffer (**P* < 0.05) showing preference for the basal R state as expected for an inverse agonist.

Antagonist affinity was also determined in a functional assay by measuring the ability of the antagonists to inhibit morphine-stimulated binding of [³⁵S]GTP γ S to G-protein (Table 1). All of the antagonists concentration-dependently induced parallel rightward shifts in the morphine concentration–response curve. Analysis of these results showed that the affinity values determined by Schild analysis (pA₂) for naltrexone and 6 β -naltrexol in the [³⁵S]GTP γ S assay were similar to their affinity values (pK_i) determined in competition binding assays in Tris-HCl buffer in the absence or presence of NaCl and GTP γ S, confirming equivalent affinity for basal and active states of the receptor. With CTAP, the pA₂ matched its pK_i in the presence of NaCl and GTP γ S because of the predominance of low affinity (R) states of the receptor in the [³⁵S]GTP γ S assay. In contrast to results obtained for naltrexone and 6 β -naltrexol, the affinity of RTI-5989-25 measured in the [³⁵S]GTP γ S assay matched the competitive binding affinity values in Tris-HCl buffer in the presence of NaCl and GTP γ S (Table 1), but not in Tris-HCl buffer alone, suggesting a higher affinity for the basal R state of the receptor indicating inverse agonism. Additionally, using acute DAMGO-mediated inhibition of forskolin-stimulated cAMP formation as a measure of agonism, 100 nmol·L⁻¹ 6 β -naltrexol or

100 nmol·L⁻¹ naltrexone resulted in approximately the same degree of rightward shift in the DAMGO concentration–effect curve, inducing a 196 \pm 62-fold shift and a 218 \pm 36-fold shift respectively. These data yielded a similar affinity value (K_B or pK_B) for both antagonists (Table 1) again confirming 6 β -naltrexol and naltrexone were indistinguishable to the μ -opioid receptor.

Binding affinities in buffers promoting high or low affinity states of the receptor are not necessarily indicative of agonism or inverse agonism at a receptor. For example, the highly efficacious opioid agonists etorphine and BW373U86 bind no differently in buffers promoting high and low affinity states of their respective receptors (Childers *et al.*, 1993; Lee *et al.*, 1999). Moreover, the antagonists 7-benzylidenenaltrexone and naltriben that show inverse agonism at the δ -opioid receptor do not bind preferentially to low affinity states (Neilan *et al.*, 1999). Therefore, additional measures of ligand efficacy were examined.

Efficacy measures using the [³⁵S]GTP γ S binding assay

DAMGO (10 μ mol·L⁻¹) stimulated [³⁵S]GTP γ S binding in C6 μ cell membranes by approximately sixfold (Table 2), indicating very efficient receptor–G-protein coupling. At a maximal concentration of 10 μ mol·L⁻¹, 6 β -naltrexol, CTAP, naltrexone, naloxone and RTI-5989-25 alone did not significantly alter G-protein activation from basal values. However, there was a small, but non-significant increase in [³⁵S]GTP γ S binding for naloxone, naltrexone and CTAP as previously reported (Wang *et al.*, 2001; 2007a), indicating a very high sensitivity to agonist stimulation in this system.

Sodium ions by decreasing the level of active R* receptor also decrease basal G-protein activation. Consequently, basal signalling can be increased by replacing Na⁺ ions with K⁺ ions (Szekeres and Traynor, 1997; Selley *et al.*, 2000). Under these conditions, basal [³⁵S]GTP γ S stimulation was almost doubled (14.9 fmol·mg⁻¹ in NaCl, 27.2 fmol·mg⁻¹ in KCl). Due to this increased basal activity, DAMGO stimulation, measured as percentage increase over basal, was reduced to approximately one-third of its level in the presence of Na⁺ ions. Even under these conditions of enhanced basal signalling, 6 β -naltrexol,

Table 2 Opioid effects on [³⁵S]GTPγS binding in membranes from C6 glioma cells without or with overnight pretreatment with 10 μmol·L⁻¹ DAMGO

Opioid (10 μmol·L ⁻¹)	% Basal [³⁵ S]GTPγS binding			
	Na ⁺ containing buffer		K ⁺ containing buffer	
	Vehicle-treated	DAMGO-treated	Vehicle-treated	DAMGO-treated
DAMGO	580 ± 59**	330 ± 21**	226 ± 11**	170 ± 11**
6β-Naltrexol	92.4 ± 3.2	114 ± 9.1	104 ± 3.8	103 ± 6.6
Naltrexone	115 ± 6.9	106 ± 5.6	111 ± 6.7	97.7 ± 4.0
RTI-5989-25	96 ± 3.1	107 ± 7.6	73.9 ± 5.8**	90.6 ± 5.2
CTAP	105 ± 6.4	101 ± 6.7	80.3 ± 5.2**	82.9 ± 5.3*
CTAP (-DTT)	132 ± 9.1*	103 ± 13	108 ± 3.4	95.5 ± 3.2
Naloxone	107 ± 4.1	98.4 ± 7.0	102 ± 3.8	99.2 ± 3.7
Basal binding				
+DTT	14.9 ± 1.1	10.8 ± 0.8	27.2 ± 1.7	13.0 ± 1.2
-DTT	56.3 ± 3.9	57.8 ± 13.9	42.6 ± 7.0	31.9 ± 4.7

Assays were performed in the presence of 100 mmol·L⁻¹ NaCl or 100 mmol·L⁻¹ KCl as described in the *Methods*. All assays were performed in the presence of 2.4 mmol·L⁻¹ dithiothreitol with the exception of CTAP where noted. Values represent means ± SEM for three to five experiments performed in duplicate. Basal binding values are given as fmol·mg⁻¹ protein.

[³⁵S]GTPγS, guanosine-5'-O-(3-[³⁵S]thio)triphosphate; CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO, [D-Ala²,N-MePhe⁴,Glyol⁵]-enkephalin; DTT, dithiothreitol; RTI-5989-25, (+)-N-[trans-4'-(2-methylphenyl)-2'-butenyl]-(3R,4R)-dimethyl-4-(3-hydroxyphenyl)piperidine.

*P < 0.05, **P < 0.001, significantly different from basal values.

naltrexone and naloxone did not alter G-protein activation from basal values. In contrast, RTI-5989-25 and CTAP significantly decreased basal binding of [³⁵S]GTPγS (***P < 0.001), suggesting inverse agonist activity in this assay.

CTAP is a cyclic peptide constrained by a disulphide bridge, and so the integrity of this structure may be compromised by the presence of the disulphide reducing agent, DTT present in the [³⁵S]GTPγS assay buffer. Indeed, in the absence of DTT, CTAP no longer reduced [³⁵S]GTPγS binding below basal values, but rather showed partial agonist activity that was significant in the presence of Na⁺ ions (Table 2). This reversal of CTAP efficacy in the absence of DTT was not, however, due to breaking of the disulphide bond of CTAP, which was stable to incubation with 2.5 mmol·L⁻¹ DTT for 1 h at 25°C as determined by mass spectrometry (data not shown), in agreement with the stability of this compound *in vivo* (Abbruscato *et al.*, 1997). Additionally, the receptor binding affinity for CTAP was not significantly different in the presence or absence of DTT (K_i: 1.52 ± 0.31 nmol·L⁻¹ in the absence of DTT; 1.75 ± 0.41 nmol·L⁻¹ in the presence of DTT) confirming stability of the peptide.

Chronic agonist treatment has been reported to reveal inverse agonist activity at the level of [³⁵S]GTPγS binding in HEK293 cells stably expressing the μ-opioid receptor (Burford *et al.*, 2000), in GH₃ cells (Liu and Prather, 2001) and in brain membranes from chronically morphine-treated mice (Wang *et al.*, 2004). Although our findings with cAMP overshoot do not support this, we examined [³⁵S]GTPγS binding after chronic agonist treatment. C6μ cells were treated overnight with 10 μmol·L⁻¹ DAMGO, which causes an eightfold shift in the potency of DAMGO and a 50% reduction in maximal effect of DAMGO to stimulate [³⁵S]GTPγS binding in these cells (Yabaluri and Medzihradsky, 1997). [³⁵S]GTPγS binding was then examined in the presence of either 100 mmol·L⁻¹ NaCl or KCl (Table 2). There was no change in the basal level of [³⁵S]GTPγS binding suggesting no increase in active states of

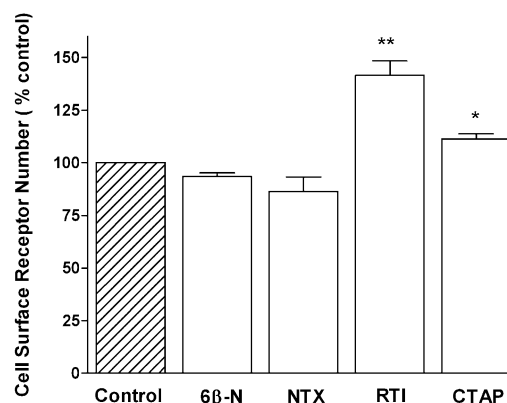


Figure 2 Cell surface receptor levels in HEK293-FLAG-μ cells treated for 24 h with 10 μmol·L⁻¹ 6β-naltrexol, naltrexone, RTI-5989-25 (RTI) or CTAP. Values are expressed as percentage of control, vehicle-treated cells and represent mean ± SEM of three experiments performed in duplicate. *P < 0.05, **P < 0.01, significantly different from vehicle. 6β-N, 6β-naltrexol; CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; NTX, naltrexone RTI-5989-25, (+)-N-[Trans-4'-(2-methylphenyl)-2'-butenyl]-(3R,4R)-dimethyl-4-(3-hydroxyphenyl)piperidine.

the receptor. However, the effect of DAMGO (10 μmol·L⁻¹) to stimulate G-protein activation was markedly reduced in both NaCl (by 43%) and KCl (by 25%) containing buffers, confirming tolerance. Neither 6β-naltrexol, naltrexone nor naloxone significantly altered G-protein activation from basal values. The ability of RTI-5989-25 to reduce basal levels of [³⁵S]GTPγS binding was lost following DAMGO pretreatment, although the effect of CTAP in the presence of DTT to decrease basal signalling activity in Na⁺ free buffer was unchanged.

Cell surface receptor expression

Chronic treatment with inverse agonists increases GPCR cell surface receptor expression, possibly by inhibiting constitutive recycling (Zaki *et al.*, 2001; Miserey-Lenkei *et al.*, 2002). To further compare antagonists, changes in cell surface receptor expression following chronic antagonist exposure were determined in HEK293 cells stably expressing a FLAG-tagged μ -opioid receptor. Cells were treated for 24 h with 10 $\mu\text{mol}\cdot\text{L}^{-1}$ 6 β -naltrexol, naltrexone, CTAP or RTI-5989-25 (Figure 2). Neither 6 β -naltrexol nor naltrexone treatment resulted in a change in the number of cell surface μ -opioid receptors, while treatment with RTI-5989-25 increased cell surface receptor levels by $41.5 \pm 6.9\%$ (** $P < 0.01$) and CTAP increased cell surface receptors by $11.3 \pm 2.5\%$ (* $P < 0.05$).

Antagonists in combination

Neutral antagonists inhibit the observable effects of inverse agonists (Costa and Herz, 1989; Neilan *et al.*, 1999; Milligan, 2003). If antagonists have different degrees of efficacy then they should compete; alternatively if they have the same efficacy their effects should be additive. The ability of a combination of 6 β -naltrexol and naltrexone to inhibit agonist action in the [^{35}S]GTP γS binding assay was measured (Figure 3A). Morphine concentration-dependently stimulated [^{35}S]GTP γS binding in C6 μ cell membranes. Antagonist treatment resulted in rightward shifts of the morphine concentration–response curve with 10 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol inducing a 13.7 ± 4.9 -fold shift, 10 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone inducing a 14.7 ± 2.0 -fold shift and a combination of 5 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol and 5 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone inducing a similar 11.9 ± 2.8 -fold shift in the morphine concentration–effect curve ($P > 0.05$) (Figure 3A), showing the compounds are indistinguishable to the receptor. In support of this, treatment with 100 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol, 100 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone or a combination of 50 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol and 50 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone antagonized maximal DAMGO-induced inhibition of forskolin-stimulated cAMP accumulation, resulting in $47.3 \pm 4.4\%$, $42.7 \pm 8.5\%$ and $48.0 \pm 7.9\%$ inhibition respectively ($P > 0.05$; Figure 3B).

Discussion

The present results suggest that, at least in C6 μ cells, RTI-5989-25 is an inverse agonist at the μ -opioid receptor; CTAP has variable efficacy that depends on the assay conditions and naltrexone; naloxone and 6 β -naltrexol are all neutral antagonists. Moreover, all of the antagonists examined, including the inverse agonist RTI-5989-25, promoted the same level of cAMP overshoot in cells chronically treated with μ -opioid agonist. This indicates that rapid formation of R from a putatively phosphorylated, constitutively active R* form was not involved in the development or expression of AC sensitization.

The putative inverse agonist naltrexone and the putative neutral antagonist 6 β -naltrexol appeared indistinguishable to the μ -opioid receptor *in vitro* and were operationally the same in precipitation of cAMP overshoot, supporting our findings in the mouse (Divin *et al.*, 2008), reinforced by our data in

the monkey (Ko *et al.*, 2006), that differences between the antagonists may not be pharmacodynamic, but rather due to differential access to μ -opioid receptors in the CNS. Opioid withdrawal is rapidly induced following administration of an opioid antagonist before steady-state concentrations are likely to be established. Thus, a differential rate of access will result in non-equivalent concentrations of antagonists at the receptor, resulting in different degrees of agonist displacement and consequently differences in the severity of the observed withdrawal behaviours. This idea is substantiated by *in vitro* findings from Zhao *et al.* (2006) who reported differences between μ -opioid agonists to induce AC sensitization are not due to agonist-dependent effects in the development of sensitization, but rather due to variation in the expression of AC sensitization caused by the ability of antagonists to displace agonist from the receptor.

Constitutive activity and increased basal signalling of the μ -opioid receptor in naïve cells has been difficult to detect (Neilan *et al.*, 1999), but has been observed in HEK293 cells (Burford *et al.*, 2000), in CHO cells (Szucs *et al.*, 2004) and in dorsal root ganglion neurons from β -arrestin2 knockout

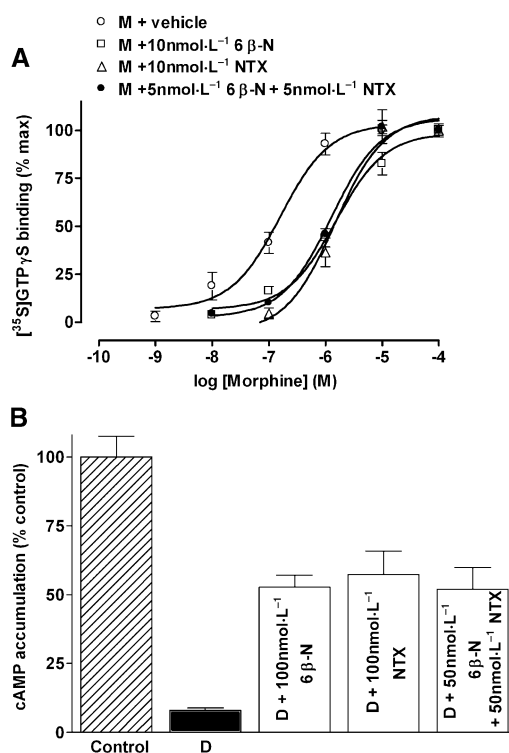


Figure 3 Effects of opioid antagonists in combination. (A) Morphine (M)-induced [^{35}S]GTP γS binding in C6 μ glioma cell membranes in the absence and presence of 10 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol (6 β -N), 10 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone (NTX) or 5 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol and 5 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone in combination. [^{35}S]GTP γS binding is expressed as percentage maximal. (B) Inhibition of forskolin-stimulated cAMP accumulation by 1 $\mu\text{mol}\cdot\text{L}^{-1}$ DAMGO (D) in the absence and presence of 100 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol, 100 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone or 50 $\text{nmol}\cdot\text{L}^{-1}$ 6 β -naltrexol and 50 $\text{nmol}\cdot\text{L}^{-1}$ naltrexone in combination. Accumulation of cAMP is expressed as percentage of vehicle-treated cells. Values represent mean \pm SEM of three experiments performed in duplicate. [^{35}S]GTP γS , guanosine-5'-O-(3- ^{35}S)thio)triphosphate; DAMGO, [D-Ala²,N-MePhe⁴,Gly⁵]enkephalin.

mice (Walwyn *et al.*, 2007). However, constitutive activity of μ -opioid receptors and the inverse agonist activity of naltrexone or naloxone has been reported following chronic pretreatment with the μ -opioid agonists morphine or DAMGO in several systems including GH₃ cells (Liu and Prather, 2001), HEK293 cells (Wang *et al.*, 1999; 2001), SH-SY5Y cells (Wang *et al.*, 1994) and mouse brain homogenates (Wang *et al.*, 2004). Our results suggest this does not occur in C6 cells. Similarly, an inverse agonist effect of naloxone was not seen in morphine-treated CHO cells (Wang *et al.*, 1999), and no development of constitutive μ -opioid signalling has been observed at the level of whole cell calcium currents in locus ceruleus or periaqueductal grey neurons from chronically morphine-treated rodents (Connor *et al.*, 1999; Bagley *et al.*, 2005). Consequently, the ability to observe the development of constitutive activity of the μ -opioid receptor on chronic opioid treatment and an inverse action of naltrexone or naloxone appears to be highly system- and/or assay-dependent.

It is possible that, in systems where an inverse agonist effect of naloxone or naltrexone is not seen, the level of μ -opioid receptor constitutive activity is low (Neilan *et al.*, 1999), even in the opioid-dependent state and consequently ligands that differentiate only weakly between R and R* appear as neutral antagonists, except under particular conditions. For example, our assays use 5 mmol·L⁻¹ Mg²⁺, but inhibition of basal μ -opioid signalling, as measured by inhibition of basal [³⁵S]GTP γ S binding by β -chlornaltrexamine is seen in naïve CHO cells only at low levels of Mg²⁺, although the level of Mg²⁺ is not important to observe this response in naïve GH₃ cells (Wang *et al.*, 2001). Thus, specific environments, interacting proteins and receptor conformations, perhaps including distinctive receptor phosphorylation, may be needed to show the inverse agonist properties of naltrexone and naloxone. Indeed, Li *et al.* (2001) using a mutation in the DRY (Asp-Arg-Tyr) region of the second intracellular loop to give a constitutively active μ -opioid receptor, suggested naloxone and naltrexone to have inverse agonist activity. However, at another constitutively active μ -opioid receptor mutant formed by alanine replacement of two cysteine residues in the C-terminal tail, naloxone and naltrexone were neutral antagonists (Brillet *et al.*, 2003). In the current study using wild-type μ -opioid receptors, naloxone, naltrexone and 6 β -naltrexol behaved as neutral antagonists but RTI-5989-25 and CTAP did show inverse agonist properties confirming the cells can distinguish between antagonists on the basis of the presence or absence of negative efficacy and therefore the effects of antagonists on the expression of AC sensitization.

The variable properties of CTAP support the highly situation-sensitive nature of inverse agonism. CTAP acted as an inverse agonist in the [³⁵S]GTP γ S assay when performed in the presence of the reducing agent DTT, and CTAP increased μ -opioid receptor cell surface expression. On the other hand, CTAP stimulated [³⁵S]GTP γ S binding in the absence of DTT indicating partial agonist activity, and bound preferentially to the μ -opioid receptor in Tris-HCl buffer that promotes high agonist-affinity (R*) states. Condition-dependent properties of CTAP can also be inferred from other reports on this compound. CTAP did not precipitate withdrawal in mice following a single injection of a high dose of morphine (Bilsky *et al.*,

1996) yet, precipitated withdrawal symptoms in chronically morphine-pelleted rats (Maldonado *et al.*, 1992) and evoked contractions in guinea-pig ilea treated overnight with morphine (Munday *et al.*, 2000). The differential ability of CTAP to induce withdrawal in these situations may be a consequence of the severity of dependence. On the other hand, CTAP did not precipitate a cAMP overshoot in SH-SY5Y cells (Wang *et al.*, 1994) or GH₃ cells (Liu and Prather, 2001), treated for long periods with high concentrations of morphine and/or DAMGO but showed inverse agonist properties in both naïve and chronic morphine-treated CHO cells expressing a μ -opioid receptor, possibly through a mechanism involving G α_s (Szucs *et al.*, 2004). CTAP has been shown to antagonize DAMGO *in vivo* much more efficiently than other peptides and non-peptides and may non-competitively interact with the alkaloids etorphine and morphine and the antagonist naltrexone (Sterious and Walker, 2003; Walker, 2006), so it is possible that these varied reports are due to an unusual mode of binding to the μ -opioid receptor. Overall, CTAP appears to be a protean ligand, and it can behave as a positive and inverse agonist on the same receptor (Kenakin, 2004; Neubig, 2007), with properties highly dependent on the assay conditions.

Our assay-dependent results with CTAP are not due to instability of the peptide so may be caused by the presence of alternative conformational states of the receptor under the different assay conditions. The μ -opioid receptor is not very sensitive to the reducing action of DTT (Shahrestanifar *et al.*, 1996). Nonetheless, the increased basal [³⁵S]GTP γ S binding and the loss of effect of Na⁺ suggests that the receptor itself might be involved. Like other GPCRs, the μ -opioid receptor contains two conserved cysteine residues in the first and second extracellular loops that form a disulphide bond. The integrity of this disulphide bond controls receptor conformation of GPCRs (Pedersen and Ross, 1985; Lin *et al.*, 1996) and so could alter the properties of CTAP, in particular if this compound does have an atypical interaction with the μ -opioid receptor (Sterious and Walker, 2003; Walker, 2006). Studies with purified receptors may be needed to explain these observations.

RTI-5989-25 has been previously identified as an inverse agonist at the δ -opioid receptor (Zaki *et al.*, 2001), and this study has characterized RTI-5989-25 as an inverse agonist at the μ -opioid receptor. This definition is based on a greater affinity for the μ -opioid receptor in a buffer system that promotes low affinity (R) states of the receptor and a decrease in [³⁵S]GTP γ S binding below basal levels when constitutive signalling is enhanced in Na⁺-free buffer by formation of R* and R*G. RTI-5989-25 treatment also resulted in an increase in cell surface μ -opioid receptor expression in HEK293-FLAG- μ cells. A surprising finding of the present study was the loss of negative intrinsic activity of RTI-5989-25 in cells chronically treated with the μ -opioid agonist DAMGO. This suggests that rather than being more active in the dependent state compounds with negative intrinsic activity lose inverse agonist activity. This could be due to a reduction in the level of μ -opioid receptors (Yabaluri and Medzihradsky, 1997) and/or desensitization of the receptor (Johnson *et al.*, 2005), thus reducing the chance of receptor-G-protein collisions. It is unclear why this is opposite to effects seen in other systems,

but this situation may predominate in the absence of factors that provide for constitutive activity. However, this observation does not support the need for formation of a constitutively active receptor in AC sensitization.

In summary, the results show that in systems that are capable of identifying compounds with inverse agonist activity, naltrexone and 6 β -naltrexol are neutral antagonists that are indistinguishable to the μ -opioid receptor. The degree of cAMP overshoot following chronic opioid sensitization of AC precipitated by opioid antagonists, whether characterized as neutral, inverse or protean, was the same as that seen by washing cells with buffer to dissociate receptor-bound agonist. AC sensitization is a highly complex process that is likely to depend on a variety of cell-specific factors including the G-protein and AC isoform profile (Watts and Neve, 2005), which may determine whether constitutive activity and inverse agonism are involved. However, the findings from the present study, together with our previous *in vivo* studies (Ko *et al.*, 2006; Divin *et al.*, 2008) indicate that formation of a stable constitutively active (R*) state of the μ -opioid receptor is not a necessary prerequisite for the development of AC sensitization or μ -opioid dependence and withdrawal.

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Conflict of interest

The authors state no conflict of interest.

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