British Journal of Pharmacology (2005) 146, 68–76
www.nature.com/bjp

Opioid tolerance in periaqueductal gray neurons isolated from mice chronically treated with morphine

[1]Elena E. Bagley, [1]Billy C.H. Chieng, [1]MacDonald J. Christie & *,[1]Mark Connor

[1]Pain Management Research Institute, E25, Kolling Institute, University of Sydney at Royal North Shore Hospital, St Leonards, NSW 2065, Australia

1 The midbrain periaqueductal gray (PAG) is a major site of opioid analgesic action, and a significant site of cellular adaptations to chronic morphine treatment (CMT). We examined μ-opioid receptor (MOP) regulation of voltage-gated calcium channel currents (I_{Ca}) and G-protein-activated K channel currents (GIRK) in PAG neurons from CMT mice.

2 Mice were injected s.c. with 300 mg kg^{-1} of morphine base in a slow release emulsion three times over 5 days, or with emulsion alone (vehicles). This protocol produced significant tolerance to the antinociceptive effects of morphine in a test of thermal nociception.

3 Voltage clamp recordings were made of I_{Ca} in acutely isolated PAG neurons and GIRK in PAG slices. The MOP agonist DAMGO (Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol enkephalin) inhibited I_{Ca} in neurons from CMT mice (230 nM) with a similar potency to vehicle (150 nM), but with a reduced maximal effectiveness (37% inhibition in vehicle neurons, 27% in CMT neurons). Inhibition of I_{Ca} by the $GABA_B$ agonist baclofen was not altered by CMT.

4 Met-enkephalin-activated GIRK currents recorded in PAG slices were significantly smaller in neurons from CMT mice than vehicles, while GIRK currents activated by baclofen were unaltered.

5 These data demonstrate that CMT-induced antinociceptive tolerance is accompanied by homologous reduction in the effectiveness of MOP agonists to inhibit I_{Ca} and activate GIRK. Thus, a reduction in MOP number and/or functional coupling to G proteins accompanies the characteristic cellular adaptations to CMT previously described in PAG neurons.
British Journal of Pharmacology (2005) **146,** 68–76. doi:10.1038/sj.bjp.0706315;
published online 27 June 2005

Keywords: Analgesic tolerance; opioid dependence; adaptation; cellular tolerance; GAT-1

Abbreviations: CMT, chronic morphine treatment; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAMGO, Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol enkephalin; GIRK, G-protein-activated inwardly rectifying potassium channels; I_{Ca}, voltage-gated calcium current; ME, methionine enkephalin; MOP, μ-opioid receptor; PAG, periaqueductal gray; PKA, protein kinase A; Rp-8-CPT-cAMPS, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphothioate, Rp-isomer; RVM, rostral ventromedial medulla

Introduction

The midbrain periaqueductal gray (PAG) is an important site of opioid analgesia (Tsou & Jang, 1964; Akil *et al*., 1976; Yaksh *et al*., 1976), and tolerance to the antinociceptive effects of both systemic and locally applied morphine has been demonstrated following local application of morphine in the PAG (Siuciak & Advokat; 1987; Lane *et al*., 2004). During opioid administration, projection neurons from the PAG to the rostral ventromedial medulla (RVM) are activated, and this in turn activates RVM neurons projecting to the dorsal horn of the spinal cord. It is thought that opioids increase the activity of PAG projection neurons by a process of disinhibition, that is, by inhibiting GABA release from nerve terminals adjacent to projection neurons and also directly hyperpolarizing GABAergic interneurons in the PAG. In mouse PAG, μ-opioid receptor (MOP) activation hyperpolarizes most neurons *via* activation of G-protein-gated inwardly rectifying potassium channels (GIRK; Vaughan *et al*., 2003), and also inhibits GABA release from nerve terminals in mouse PAG (Hack *et al*., 2003), consistent with the disinhibitory mechanisms proposed to be responsible for PAG-mediated opioid analgesia. μ-Opioids inhibit voltage-dependent calcium channels (I_{Ca}) in most mouse PAG neurons (Connor *et al*., 1999b), and this could also be reflected in an inhibition of GABA release or change in neuronal excitability, although this has not been directly tested in mouse.

Tolerance is a phenomenon commonly associated with the administration or prolonged misuse of opioid drugs, and repeated administration of morphine or other μ-opioid receptor agonists leads to decreased analgesic effectiveness in humans and animals (McQuay, 1999). The reduced analgesic effectiveness of opioids over time reflects both adaptive changes in neural circuits that modulate nociception and changes in the effects of μ-agonists on the opioid-sensitive cells that form parts of these pathways (Ossipov *et al*., 2004). Several different cellular adaptations to prolonged opioid treatment have been described involving a functional upregulation of cAMP-dependent signaling in neurons that is often reflected by changes in the efficacy or regulation of synaptic transmission (reviewed in Williams *et al*., 2001). Protein kinase A (PKA)-dependent adaptations to prolonged morphine

*Author for correspondence; E-mail: markc@med.usyd.edu.au

treatment in the PAG are unmasked during opioid withdrawal and probably play a major role in producing the behavioral signs of opioid withdrawal (Chieng & Christie, 1996; Christie *et al*., 1997; Ingram *et al*., 1998; Hack *et al*., 2003; Bagley *et al*., 2005). These adaptations may also play a role in the development of analgesic tolerance, but the changes in neuronal excitability produced by these adaptations are most prominently expressed during withdrawal rather than during the continued administration of drug.

During continuous opioid treatment, a reduction in the analgesic effectiveness of opioids could be produced, in part, by a reduction in the capacity of the μ-opioid receptor to couple to the cellular effectors normally activated to produce analgesia (Williams *et al*., 2001). We have previously demonstrated that μ-opioid inhibition of GABA release is reduced in PAG slices from chronically morphine-treated (CMT) animals after the PKA-dependent adaptations to CMT are blocked (Hack *et al*., 2003). However, at present, it is not known whether morphine-induced adaptations in the PAG are accompanied by changes in the effectiveness of signaling *via* the μ-receptor. We hypothesize that CMT is accompanied by a decrease in the effectiveness of μ-opioid receptor coupling to cellular effectors that may contribute to analgesia in the PAG. To test this hypothesis, we have examined the capacity of μ-agonists to modulate two ion channels in PAG cell bodies, I_{Ca} and GIRK. Both the inhibition of I_{Ca} and activation of GIRK are mediated by G-protein $\beta\gamma$ subunit interaction with the channels (Dascal, 2001), and thus provide a relatively direct measurement of the state of μ-opioid receptor/effector coupling. Opioid activation of GIRK is likely to be directly involved in the opioid-induced analgesia arising from the PAG, and a reduction in the effectiveness of this coupling may be directly involved in analgesic tolerance. Opioid inhibition of I_{Ca} in PAG neurons provides a quantitative assay whereby changes in the potency of opioids can be readily detected. Using these two *in vitro* assays, we show that there is a reduction in the effectiveness of μ-opioid receptor signaling in neurons from mice subject to a morphine treatment paradigm that produces analgesic tolerance to systemically delivered morphine.

Methods

Experiments were carried out on male C57B16/J mice. All experiments were carried out under protocols approved by the Animal Ethics Committee of the University of Sydney and Royal North Shore Hospital. Mice were kept in 12 h day–night cycle in a low background noise room ventilated at a constant temperature of 21–22°C. Animals were housed in groups of up to six.

Morphine treatment

CMT consisted of a series of three subcutaneous injections of morphine base (300 mg kg^{-1}) in a sustained release emulsion on alternate days over a 5-day period. We (Chieng & Christie 1996; Bellchambers *et al*., 1998) and others (Collier *et al*., 1972) have previously shown that this regime induces profound physical dependence on morphine. The sustained release preparation consisted of 50 mg of morphine base suspended in 0.1 ml of Arlacel A (mannide monooleate), 0.4 ml of light liquid paraffin and 0.5 ml of 0.9% w v^{-1} NaCl. Injections of warmed suspension were made under light halothane anesthesia. Vehicle mice were injected with suspension lacking morphine. Vehicle and morphine treatments were performed in parallel.

Nociceptive testing

At 2 days after the final treatment with morphine base or vehicle, each mouse was placed on the floor inside a cylindrical metal container (dimensions 23 cm height × 16 cm diameter) with the floor heated to 52°C. The time until the mouse either licked its hind paw or jumped in an attempt to escape the hotplate was measured (latency to respond), and then the mouse was removed and returned to its home cage. If mice did not respond on the hotplate within 90 s, they were removed and their latency recorded as 90 s. For each mouse, three hotplate readings, 20 min apart, were collected to establish a stable baseline latency. Mice were then injected with the test compound, either 0.9% saline or morphine hydrochloride (10 mg kg^{-1} i.p.). In preliminary experiments, we found that this dose of morphine produced a substantial but submaximal antinociceptive effect in these conditions. After 20 min in their home cage, mice were again tested in the hotplate. The differences between treatments were assessed by a factorial ANOVA followed by a Scheffe F-test (Statview, SAS Institute, Cary, NC, U.S.A.).

Tissue preparation

Mice (4–8 weeks old for dissociated cells, 4–6 weeks old for slice recordings) were anesthetized with halothane and killed by cervical dislocation. Coronal midbrain slices (220–250 μm thick for slice recording, 350 μm thick for dissociation) containing the PAG were cut with a vibratome in ice-cold physiological saline (ACSF) of composition (mM) NaCl 126, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $NaHCO_3$ 24 and glucose 11, gassed with 95% O_2/5% CO_2 and stored for 30 min at 35°C. Cells were dissociated as described previously (Connor *et al*., 1999b). Briefly, slices were transferred to a dissociation buffer of composition (mM) Na_2SO_4 82, K_2SO_4 30, HEPES 10, $MgCl_2$ 5, glucose 10, containing 20 U ml^{-1} papain, pH 7.3, and incubated for 2 min at 35°C. The slices were then placed in fresh dissociation buffer containing 1 mg ml^{-1} bovine serum albumin (BSA) and 1 mg ml^{-1} trypsin inhibitor and the PAG region was subdissected from each slice with a fine tungsten wire. Cells were dissociated from the slices by gentle trituration, plated onto plastic culture dishes and kept at room temperature in dissociation buffer.

Dissociated neuron recordings

Recordings of currents through Ca^{2+} channels (I_{Ca}) were made using standard whole-cell patch clamp techniques (Hamill *et al*., 1981) at room temperature (20–24°C), as previously described (Connor *et al*., 1999b). Immediately prior to recording, dishes of cells were superfused with a buffer of composition (mM) NaCl 140, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, HEPES 10 and glucose 10, pH 7.3, in order to wash off the dissociation buffer. For I_{Ca} recordings, cells were perfused in solution containing (mM) tetraethylammonium chloride 140, $BaCl_2$ 4, CsCl 2.5, HEPES 10, glucose 10 and BSA 0.05%, pH 7.3. Recordings were made with fire polished borosilicate

pipettes of resistance between 2 and 4 MΩ when filled with intracellular solution of the following composition (mM): CsCl 120, MgATP 5, NaCl 5, Na_2GTP 0.2, EGTA 10, $CaCl_2$ 2 and HEPES 10, pH 7.3. The peak I_{Ca} in each cell was determined by stepping the membrane potential from a holding potential of −90 mV to potentials between −60 and +60 mV, in 10 mV increments. The cells were then repetitively stepped to 0 mV and drugs applied to the neurons *via* an array of sewer pipes positioned above the cell. Cells in which I_{Ca} declined in the absence of drug treatment were discarded. The inhibition by drugs was quantified by measuring the current amplitude isochronically with the peak of the control I_{Ca}. Whole-cell capacitance and series resistance were compensated manually by nulling the capacitive transient evoked by a 20 mV pulse from −90 mV. The series resistance had an average value of 5 MΩ; series resistance compensation of at least 80% was used in all experiments. An approximate value of whole-cell capacitance was read from the amplifier capacitance compensation circuit (Axopatch 1D, Axon Instruments, Union City, CA, U.S.A.). Leak current was subtracted online using a P/8 protocol unless otherwise noted. Cells with an initial holding current of >20 pA at −90 mV were discarded; most cells had holding currents at –90 mV of <5 pA. Evoked I_{Ca} were filtered at 2 kHz, sampled at 5–10 kHz and recorded on hard disk for later analysis. Data were collected and analyzed offline with the PCLAMP (version 5) and Axograph (version 4) suite of programs (Axon Instruments). All data are expressed as mean ± s.e.m., unless otherwise indicated.

Successful recordings were made up to 7 h after cell dissociation. There was no difference in the amount of I_{Ca} between cells recorded at the beginning and end of the day ($P>0.5$), nor did the responses of neurons from CMT animals to maximally effective concentrations of DAMGO (Tyr-D-Ala-Gly-*N*-Me-Phe-Gly-ol enkephalin) differ from the beginning to end of a day ($P>0.3$).

Slice electrophysiology

After cutting, PAG slices were maintained at 34°C in a submerged chamber containing ACSF and were later transferred to a chamber superfused at 2 ml min^{-1} with ACSF (34°C) for recording. Brain slices from both morphine-dependent and vehicle-treated mice were maintained *in vitro* in ACSF containing 5 μM morphine. Slices were spontaneously withdrawn by incubation in morphine-free ACSF for at least 1 h before an experiment. PAG neurons were visualized using infrared Nomarski optics and recordings made from neurons in the ventrolateral region of the PAG (Bagley *et al*., 2005). Perforated patch recordings were made using patch electrodes (4–5 MΩ) filled with (mM) K acetate 120, HEPES 40, EGTA 10, $MgCl_2$ 5, with Pluronic F-127 0.25 mg ml^{-1} and amphotericin B 0.12 mg ml^{-1} (pH 7.2, 290 mosmol l^{-1}). A liquid junction potential for K acetate internal solution of −8 mV was corrected. Series resistance (<25 MΩ) was compensated by 80% and continuously monitored. During perforated patch recordings, currents were recorded using an Axopatch 200A amplifier (Axon Instruments), digitized, filtered (at 2 kHz) and then acquired (sampling at 10 kHz) in pClamp (Axon Instruments) or using Axograph Acquisition software (Axon Instruments). Wortmannin and CGP55845 were dissolved in DMSO (final concentration <0.1%) and all other drugs were dissolved in water. Drugs were added directly to the ACSF and applied by switching the bath superfusion to the ACSF containing the drugs.

Drugs and chemicals Buffer salts were from BDH Australia or Sigma Australia. Papain was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). All other chemicals were from Sigma Australia except the following: Met-enkephalin and DAMGO were from Auspep (Melbourne, Australia). Baclofen was from Research Biochemicals International (Natick, MA, U.S.A.). Rp-8-CPT-cAMPS (8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphothioate, Rp-isomer) was from Biolog Life Sciences (CA, U.S.A.). CNQX was from Tocris Cookson (U.K.). Tetrodotoxin was from Alomone (Israel). Morphine base and morphine hydrochloride were from Glaxo U.K. CGP55845 was a gift from Ciba Ltd (Basel, Switzerland).

Results

The CMT schedule our laboratory has used over several years has been shown to produce significant physical dependence on morphine and profound adaptations in the PAG region. We wanted to establish if this morphine treatment regimen rendered mice tolerant to the antinociceptive effects of morphine. Animals were treated with morphine or vehicle for 5 days, as outlined in Methods, and on day 7 tested on a hotplate assay of thermal nociception (52°C). Each animal was tested three times on the hotplate to establish a stable baseline response, and then animals were injected with either morphine, 10 mg kg^{-1}, or with 0.9% saline. The baseline latency to a response did not differ between vehicle-treated mice (20 ± 1 s, $n=18$) and CMT animals (17 ± 2 s, $n=18$), and was similar in a smaller group of naïve mice (25 ± 4 s, $n=9$) that had not been subject to either vehicle or CMT. Injection of 0.9% saline did not produce a change in the response latency of either vehicle or CMT animals when measured 20 min after the injection. Injection of morphine produced a significant increase in the response latency in vehicle-treated ($P<0.001$) but not morphine-treated animals (Figure 1).

In dissociated PAG neurons from CMT mice isolated and maintained in the absence of morphine, superfusion of the μ-opioid agonist DAMGO continued to inhibit I_{Ca} (Figure 2). Concentration–response curves for DAMGO were constructed by applying one or more concentrations of agonist to neurons repetitively stepped from −90 to 0 mV. Superfusion of high concentrations of DAMGO (up to 30 μM) inhibited I_{Ca} in a similar proportion of PAG neurons taken from vehicle (80%, 56/70 neurons) and CMT animals (81%, 58/72 neurons). The potency of DAMGO to inhibit I_{Ca} was derived by fitting the pooled data to a logistic function in the program Graphpad Prism 4, and was similar in neurons from CMT mice (pD_2 of 6.6, 95% confidence interval 6.9–6.4, $EC_{50}=150$ nM) and in those from vehicle-treated animals (pD_2 of 6.8, 95% confidence interval 7.0–6.65, $EC_{50}=230$ nM). A two-way ANOVA comparing the DAMGO concentration–response curves in vehicle and CMT animals showed a significant main effect of concentration ($P<0.0001$) and treatment ($P<0.0001$), but no significant interaction between concentration and treatment ($P=0.31$). The inhibition of I_{Ca} by maximally effective concentrations of DAMGO (30 μM) was significantly reduced in PAG neurons taken from CMT animals (27 ± 3 *versus*

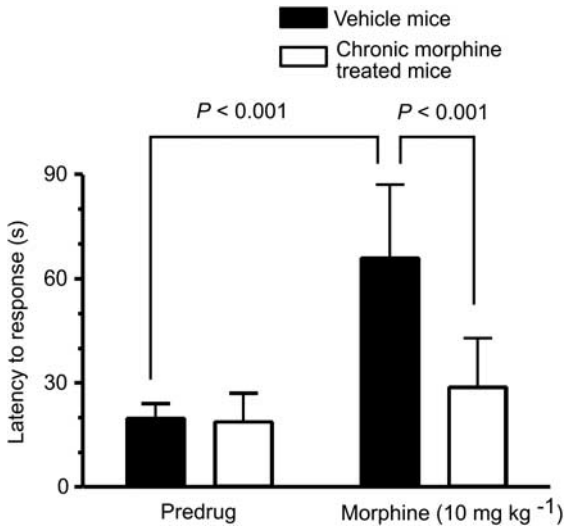


Figure 1 Morphine antinociception is reduced in CMT mice. Mice were treated with morphine base or vehicle for 5 days, as described in Methods, and on day 7 were tested for morphine-induced antinociception. Mice were placed on a hotplate (52°C) and the time taken for the animals to lick their paws or jump was measured. The animals were tested three times to establish a stable baseline, then injected with 10 mg kg^{-1} of morphine hydrochloride, and their response latency tested 20 min later. The response latencies (s) ($\pm$s.d.) are plotted for each condition; $n=10$ for vehicle/morphine, $n=12$ for chronic morphine/morphine. Statistical significance was tested by factorial ANOVA followed by a Scheffe F-test.

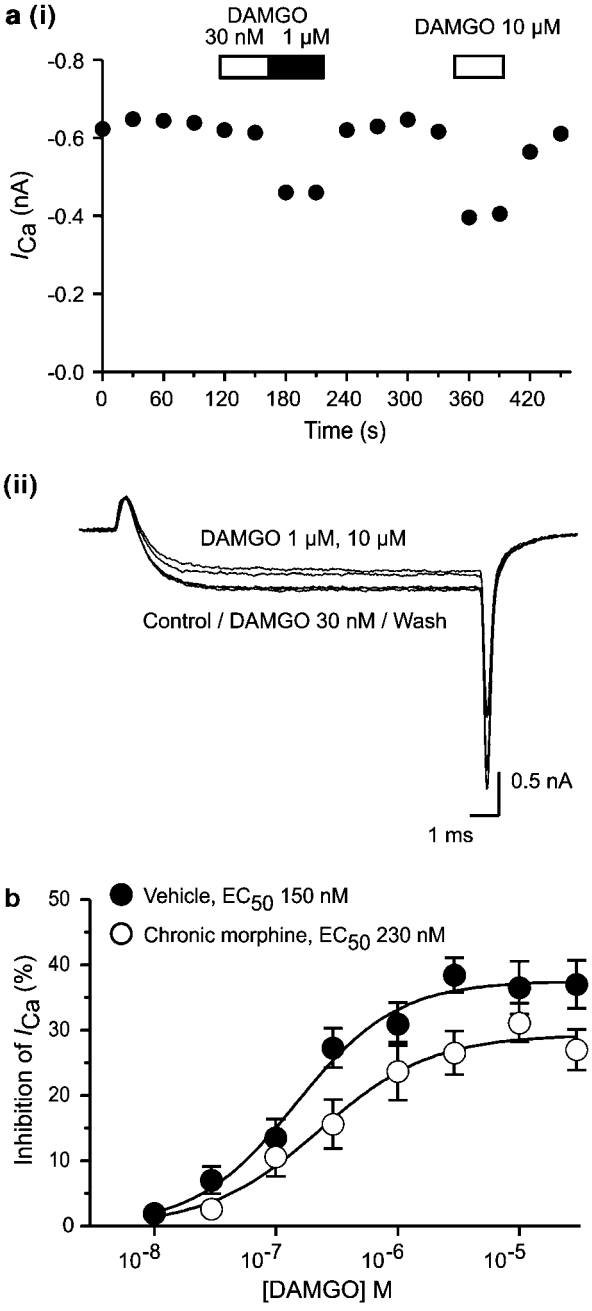


Figure 2 DAMGO continues to inhibit I_{Ca} in PAG neurons from CMT mice. I_{Ca} was evoked by repetitively stepping PAG neurons from −90 to 0 mV. (a) (i) Time plot of the I_{Ca} amplitude of a PAG neuron taken from a morphine-treated mouse, illustrating the effect of a range of DAMGO concentrations. Drugs were applied for the duration indicated by the bars. (ii) Example traces from the experiment illustrated in (i). (b) Concentration–response relationship for DAMGO inhibition of I_{Ca} in PAG neurons from vehicle- and morphine-treated mice. Each point represents the mean $\pm$ s.e.m. of at least nine cells; curves were fit to the pooled data. The maximum effect of DAMGO was reduced in neurons from CMT animals ($P<0.05$, two-way ANOVA followed by Bonferroni *post hoc* test).

37$\pm$4% in vehicle, $P<0.05$, Bonferroni *post hoc* test corrected for multiple comparisons; Figure 2). In neurons from CMT animals, the size of the inhibition of I_{Ca} by 30 μM DAMGO was not dependent on the concentration of agonist that had been previously applied to the cell. In cells probed first with a low concentration of DAMGO (10–100 nM), superfusion of 30 μM DAMGO inhibited I_{Ca} by 25$\pm$4% ($n=9$). In cells initially probed with higher concentrations of DAMGO (300 nM–10 μM), the inhibition of I_{Ca} by 30 μM DAMGO was 29$\pm$4% ($n=11$, $P=0.52$ *versus* low concentration probe, Student's *t*-test).

The basic characteristics of I_{Ca} were not affected by CMT. Peak I_{Ca} density was 261$\pm$15 pA pF^{-1} ($n=56$) in DAMGO-sensitive cells from vehicle animals, and 254$\pm$12 pA pF^{-1} ($n=58$) in DAMGO-sensitive cells from CMT animals ($P=0.74$, Student's *t*-test). In DAMGO-sensitive cells, the 0–95% rise time of I_{Ca} elicited by a step from −90 to 0 mV was 2.80$\pm$0.1 ms in neurons from vehicle animals and 2.85$\pm$0.15 ms in neurons from CMT mice ($P=0.55$, Student's *t*-test). Peak I_{Ca} density in μ-agonist-insensitive cells was 214$\pm$19 pA pF^{-1} ($n=14$) and 230$\pm$23 pA pF^{-1} ($n=14$) in cells from vehicle and CMT mice, respectively. The membrane area of acutely dissociated PAG neurons did not differ between neurons from CMT animals (4.7$\pm$0.2 pF, $n=72$) and vehicles (4.6$\pm$0.2 pF, $n=70$).

The $GABA_B$ receptor agonist baclofen inhibits I_{Ca} in virtually all mouse PAG neurons (Connor *et al*., 1999b), and so to assess any changes in the capacity of I_{Ca} to be modulated in CMT animals, we constructed concentration–response curves for baclofen in DAMGO-sensitive PAG neurons (Figure 3). In cells from vehicle-treated animals, baclofen inhibited I_{Ca} with a pD_2 of 5.7 (95% confidence interval 6.0–5.5, $EC_{50}=1.8$ μM), and in cells from CMT mice with a pD_2 of 5.8 (95% confidence interval 6.2–5.5, $EC_{50}=1.5$ μM). The inhibition of I_{Ca} by maximally effective concentrations of baclofen (100 μM) was similar in PAG neurons taken

from CMT animals (49±2%, $n=10$) and vehicles (49±3%, $n=10$).

Opioid receptor inhibition of I_{Ca} usually proceeds *via* a relatively direct mechanism involving the interaction of G-protein $\beta\gamma$ subunits with the channel α subunit. G-protein $\beta\gamma$ subunit-mediated inhibition of I_{Ca} is characterized by kinetic slowing of the activation of the inhibited current, and can be reversed by strong depolarizing pulses. We examined the kinetic characteristics of opioid inhibition of I_{Ca} using a protocol of two test pulses to 0 mV separated by a 50 ms depolarizing step to +80 mV (Figure 4). In neurons from either CMT or vehicle-treated mice, the amplitude of the first and second test steps did not differ in control conditions, but in the presence of DAMGO (10 μM), there was a significant increase in the amplitude of the second test pulse compared to the first (Figure 4 and Table 1). The amount of relief of DAMGO-induced inhibition of I_{Ca} provided by the conditioning pulse did not differ between neurons from vehicle-treated and CMT mice (58±2% in vehicle and 57±5% in CMT neurons, $n=7$). In neurons from both vehicle and CMT mice, the DAMGO-induced inhibition of the first test step was associated with a significant slowing of the activation of the I_{Ca} (Table 1) and this kinetic slowing was significantly reversed by the depolarizing conditioning step (Table 1). There was no difference in the effect of the depolarizing conditioning step on the kinetic properties of the test steps in the absence of drug between neurons from vehicle and CMT animals (Table 1).

μ-Opioids also increase the conductance of a GIRK in most mouse PAG neurons recorded in brain slices (Figure 5a; see also Vaughan *et al*., 2003). We reanalyzed the experiments presented by Bagley *et al*. (2005) to examine any changes

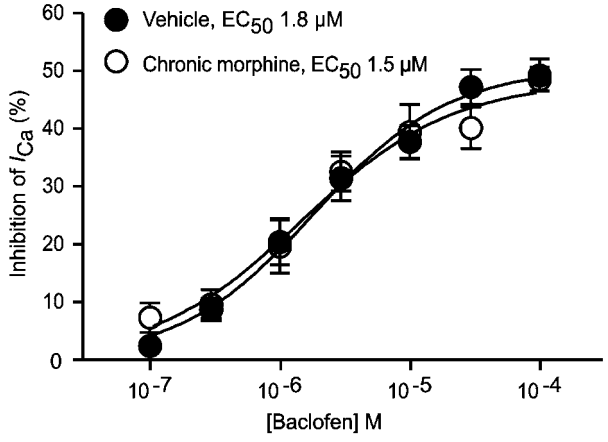


Figure 3 Baclofen inhibition of I_{Ca} in PAG neurons is unaffected by CMT. I_{Ca} was evoked by repetitively stepping PAG neurons from −90 to 0 mV. Concentration–response relationship for baclofen inhibition of I_{Ca} in PAG neurons from vehicle- and morphine-treated mice is shown. Each neuron was tested for DAMGO sensitivity before baclofen application and only opioid-sensitive cells were included in the analysis. Each point represents the mean± s.e.m. of at least six cells; curves were fit to the pooled data.

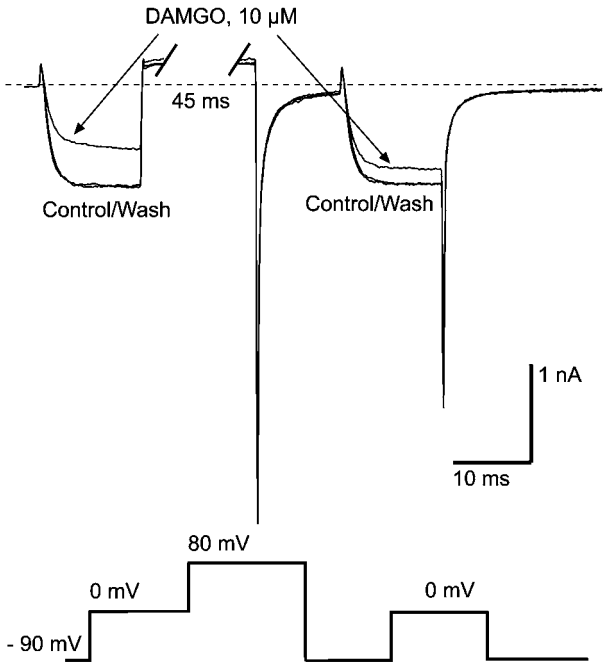


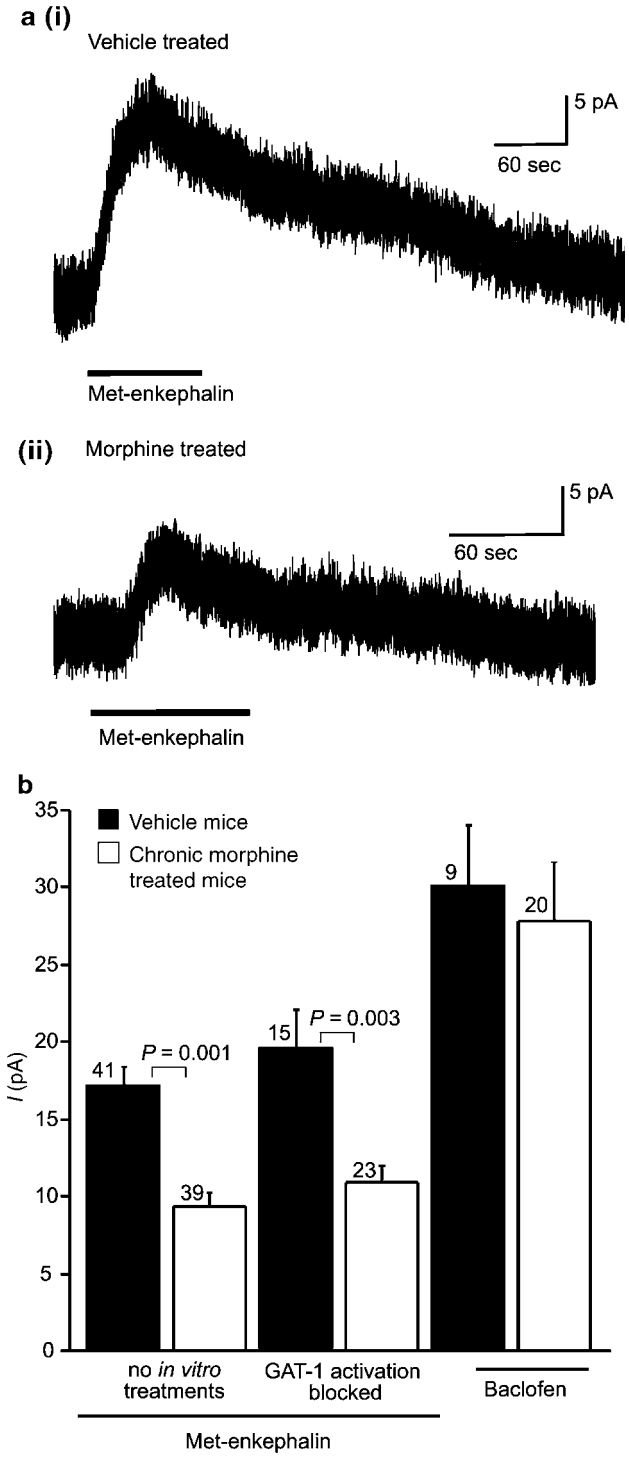
Figure 4 μ-Opioid receptor inhibition of I_{Ca} is still mediated by G-protein $\beta\gamma$ subunits in morphine-treated mice. Example traces of a PAG neuron from a morphine-treated mouse subjected to a conditioning pulse paradigm, which is illustrated beneath the traces. The depolarizing step to +80 mV partially relieved the DAMGO-mediated inhibition of the test step. Note that 45 ms of the conditioning step has been removed from the traces for clarity. These experiments were performed without online leak subtraction, and zero holding current is represented by the dotted line.

Table 1 Effects of a depolarizing conditioning step on DAMGO modulation of I_{Ca} in PAG neurons

	Vehicle (n=7)		*Morphine treated* (n=7)	
	Control	*In DAMGO*	*Control*	*In DAMGO*
0–95% rise time of step 1 (ms)	3.1±0.4	5.3±0.3[a]	2.7±0.2	4.4±0.6[b]
0–95% rise time of step 2 (ms)	3.0±0.3	3.3±0.3[c]	2.7±0.2	2.9±0.3[d]
Peak I_{Ca} ratio, step 2 : step 1	1.01±0.02	1.38±0.05[e]	0.99±0.03	1.28±0.08[f]

PAG neurons were stepped twice from −90 to 0 mV with an 50 ms conditioning step to +60 mV preceding the second step (see Figure 4). Each cell was exposed to 10 μM DAMGO.
[a]$P=0.0002$ *versus* predrug, paired *t*-test.
[b]$P=0.003$ *versus* predrug, paired *t*-test.
[c]$P=0.01$ *versus* predrug, paired *t*-test.
[d]$P=0.04$ *versus* predrug, paired *t*-test.
[e]$P=0.0002$ *versus* predrug, paired *t*-test.
[f]$P=0.004$ *versus* predrug, paired *t*-test.

in amplitude of the met-enkephalin-induced GIRK current in CMT mice. The amplitudes of the ME currents in neurons from CMT animals were significantly smaller than those in neurons from vehicle-treated mice (Figure 5b). However, following withdrawal of morphine from brain slices taken from CMT mice, a PKA-dependent current through the GABA transporter GAT-1 is apparent in opioid-sensitive PAG neurons (Bagley *et al*., 2005). The GAT-1 current can be blocked completely by inhibitors of PKA (Rp-8-CPT-cAMPS, 100 μM), by the selective GAT-1 inhibitors NO-711 (10 μM) or SKF 88976A (10 μM) or by depleting GABA vesicular stores in PAG slices by preincubating slices in a combination of PI3Kinase inhibitor wortmannin (5 μM) and hypertonic sucrose (50 mM) (Bagley *et al*., 2005). In neurons from vehicle-treated animals, met-enkephalin-induced GIRK currents reversed at -103 ± 2 mV ($n=41$), and in each of the conditions designed to block the GAT-1 current, the GIRK currents in neurons from CMT animals also reversed close to the calculated equilibrium potential for K (in GAT-1 blockers NO-711 and SKF 88976A, ME currents reversed at -112 ± 4 mV, $n=13$; in Rp-8-CPT-cAMPS, ME currents reversed at -103 ± 7 mV, $n=4$; in wortmannin, ME currents reversed at -107 ± 10 mV, $n=6$). In each of these conditions, the amplitudes of the ME currents in neurons from CMT animals were significantly smaller than those in neurons from vehicle-treated mice recorded under the same conditions (Figure 5a and b). The amplitudes of the GIRK currents produced by the $GABA_B$ receptor agonist baclofen in PAG neurons from CMT and vehicle-treated animals were not different (Figure 5b).



Discussion

Neurons in the PAG play a pivotal role in the analgesic actions of μ-opioids. This study shows that there is significant tolerance to the effects of μ-opioid receptor agonists in PAG neurons taken from mice treated with a morphine regimen that produces antinociceptive tolerance. The PAG is unlikely to be the only region affected by the morphine administered in our experiments, but it has been repeatedly shown to be of central importance in mediating the systemic antinociceptive effects of μ-opioid agonists. The PAG has also been demonstrated to be more liable to develop tolerance to repeated morphine treatments than other regions crucial for opioid-activated antinociception (Morgan *et al*., 2005).

The reduced activation of GIRK channels observed in this study could directly contribute to weaker disinhibition of PAG output neurons, which may lead directly to less PAG-mediated antinociception. Another likely mechanism of tolerance to the analgesic effects of morphine in the PAG is a weaker opioid

Figure 5 Homologous tolerance of opioid-activated potassium conductance after CMT. (a) Example traces of currents from neurons voltage clamped at -56 mV in spontaneously withdrawn slices. Met-enkephalin (30 μM, superfusion shown by bars) induced an outward current in (i) a vehicle-treated neuron and a smaller outward current in (ii) a neuron from a morphine-treated mouse in a slice pretreated with wortmannin (5 μM)/sucrose (50 mM). (b) Average outward current induced by met-enkephalin (30 μM) or baclofen (10 μM) for neurons voltage clamped to -56 mV. The met-enkephalin-induced current was smaller in neurons from dependent mice than in neurons from vehicle mice when GAT-1 activation was prevented or not (no *in vitro* treatment). GAT-1 activation was blocked by GAT-1 inhibitors (NO-711 10 μM or SKF88976A 10 μM), after wortmannin (5 μM)/sucrose (50 mM) pretreatment and in the presence of Rp-8-CPT-cAMPS (100 μM). All experiments using GAT-1 inhibitors, wortmannin and Rp-8-CPT-cAMPS were performed in the presence of tetrodotoxin (1 μM), CNQX (10 μM), picrotoxin (100 μM) and CGP55845 (1 μM). The number of neurons is shown above the bars and each current amplitude is presented as mean $\pm$ s.e.m. Statistical significance was tested using a Student's *t*-test.

inhibition of GABA release, which would be expected to produce a similar effect to weaker activation of GIRK. We have previously shown that when morphine withdrawal-induced adaptations in GABA neurotransmission are blocked using PKA inhibitors, DAMGO is significantly less effective at inhibiting GABA release in CMT animals than normal (Hack *et al*., 2003).

The role of inhibition of I_{Ca} in μ-opioid receptor-mediated disinhibition in the PAG is less clear, because opioid inhibition of GABA release is not prominently mediated by inhibition of I_{Ca} in either normal or CMT rat PAG *in vitro* (Vaughan *et al*., 1997; Ingram *et al*., 1998). However, because opioid inhibition of I_{Ca} in PAG neurons is robust and rapid, it is well suited as a quantitative reporter of receptor/effector coupling. A similar tolerance to μ-opioids at the level of I_{Ca} was previously demonstrated in locus coeruleus neurons taken from morphine-treated rats (Connor *et al*., 1999a). In the locus coeruleus, a system with more μ-opioid receptor reserve than PAG, CMT reduced the potency of ME to inhibit I_{Ca} without an accompanying decrease in its maximal effectiveness, but the maximal effectiveness of the partial agonist morphine was significantly reduced (Connor *et al*., 1999a). In the present study, the potency of DAMGO was unaltered but its maximal effectiveness was reduced, consistent with a reduction in the number of functionally coupled receptors for an agonist that has no 'spare receptors' in the normal state. There is unlikely to be a substantial excess of μ-receptors available to couple to inhibition of I_{Ca} in PAG neurons because the partial μ-opioid agonist endomorphin 1 does not inhibit I_{Ca} to the same extent as DAMGO or ME (Connor *et al*., 1999b), and the maximal inhibition of I_{Ca} by DAMGO is significantly less than that produced by baclofen, even though $GABA_B$ and μ-opioid receptors couple to the same type of I_{Ca} (Rhim *et al*., 1996; Chieng & Bekkers, 1999).

Opioid-activated GIRK currents are small in mouse PAG neurons, particularly in neurons from CMT animals, and it was not feasible to construct a full concentration–response curve for met-enkephalin. However, the response to high concentrations of met-enkephalin was significantly reduced in neurons from morphine-treated mice, which is consistent with the data obtained from measuring opioid inhibition of I_{Ca} in the same cells. Tolerance has also been demonstrated at the level of μ-opioid receptor/GIRK coupling in LC neurons from morphine-treated rats, with a reduction in the potency of DAMGO to activate currents, and decrease in the potency and maximal response of the less efficacious μ-opioid agonist normorphine reported (Christie *et al*., 1987). As in the present study, the coupling of other receptors to GIRK channels in opioid-sensitive neurons of the LC was unchanged by CMT (Christie *et al*., 1987; Dang & Williams, 2004).

The basis for the reduction in μ-agonist recruitment of G-protein $\beta\gamma$ subunit-dependent effectors in the PAG is unknown. Our results are consistent with a reduction in surface μ-receptor number, a reduction in the efficiency with which the receptors couple to G proteins, or both. We cannot distinguish between these possibilities, and previous studies have shed little light on the issue. Radioligand binding studies in mice have demonstrated either no change, modest increases or modest decreases in μ-opioid receptor density following CMT (Yoburn *et al*., 1993; Petruzzi *et al*., 1997; Bohn *et al*., 2000; Patel *et al*., 2002) although two studies that utilized Western blots reported a decrease of about 50% in μ-opioid receptor protein in midbrain membranes from morphine-treated mice (Bernstein & Welch, 1998; Cichewicz *et al*., 2001). Similarly, conflicting results have come from studies utilizing agonist-stimulated GTPγS binding to measure μ-opioid receptor coupling to G proteins following CMT. Only one study has examined GTPγS binding in PAG from CMT animals, and it found no change in the efficiency of μ-receptor/G-protein coupling in PAG, although there were changes in other regions (Sim *et al*., 1996). Another study found no changes in receptor/G-protein coupling in any of several rat brain regions examined (Kirschke *et al*., 2002). By contrast, a complete abolition of DAMGO-stimulated GTPγS binding in mouse brain was reported following chronic treatment with a modest dose of morphine (10 mg kg^{-1} per day), despite continued analgesic responses elicited by higher doses of systemic morphine (Bohn *et al*., 2000).

Several mechanisms have been proposed to account for acute μ-opioid receptor desensitization, including receptor phosphorylation and sequestration (reviewed in von Zastrow *et al*., 2003; Connor *et al*., 2004) and sequestration of G-protein subunits coupled to the μ-opioid receptor (Garzon *et al*., 2005). The mechanisms underlying acute μ-opioid receptor desensitization are affected by CMT, in particular the processes that are responsible for recovery from desensitization are slowed (Dang & Williams, 2004). It is possible that the reduced opioid receptor coupling we observed in this study after removal of the morphine at killing reflects the non-reversal of the processes responsible for 'acute desensitization', although consistency of opioid responses for up to 7 h after cell isolation argues against this. Although most of the processes that are proposed to acutely regulate μ-opioid receptor reverse in a few hours, sequestration of G-protein subunits coupled to the μ-opioid receptor has been reported to persist for up to 24 h after a single drug exposure (Garzon *et al*., 2005), and the time course of recovery from acute desensitization in CMT neurons is likely to be of the order of hours rather than minutes (Dang & Williams, 2004). It is unlikely that the decrease in the maximal effect produced by DAMGO was an artifact of our experimental design, in which several concentrations of agonist were applied to each cell. In theory, such a paradigm could produce a reduction of the second response because the first application of agonist had begun to recruit desensitization/sequestration mechanisms. However, the inhibition of I_{Ca} by 30 μM DAMGO was not different between cells previously challenged with low (up to 100 nM) or higher (300 nM–10 μM) concentrations of DAMGO. Further, we have previously shown that the EC_{50} concentrations of DAMGO that produced acute μ-opioid receptor desensitization and receptor internalization were approximately five- and 30-fold greater than that for acute inhibition of I_{Ca}, indicating that these processes proceed less efficiently than acute receptor coupling (Borgland *et al*., 2003).

We did not detect any changes in the basic properties of the whole-cell I_{Ca} in PAG neurons from CMT mice. This is similar to the findings of previous studies in locus coeruleus and SH-SY5Y cells, where neither the type nor the amount of I_{Ca} was changed by CMT (Kennedy & Henderson, 1992; Connor *et al*., 1999a). We did not determine the contributions of different types of I_{Ca} to the whole-cell current in mouse PAG neurons, so it is possible that the proportions of different calcium channels were reciprocally altered without any change in the overall amount of current. However, the unaltered effects of

baclofen in cells from CMT animals argue against any change in the inhibitable current.

Regardless of how μ-opioid receptor signaling is abrogated in PAG neurons, the observation that signaling persists in CMT animals is consistent with the idea that continued μ-opioid receptor signaling is required for the induction of the cellular adaptations that characterize the morphine-tolerant/dependent PAG (Chieng & Christie, 1996; Ingram *et al*., 1998; Hack *et al*., 2003; Bagley *et al*., 2005). Our results do not directly speak to the current controversies regarding the role of μ-opioid receptor trafficking in the neuronal adaptations to chronic agonist treatment (Von Zastrow *et al*., 2003; Connor *et al*., 2004; Waldhoer *et al*., 2004), but they do suggest that efforts directed at maintaining opioid receptor function during chronic agonist treatment may be confounded by the development of more profound adaptations. Thus, pharmacological interventions that disrupt these adaptations to chronic opioid treatment may be the most effective way of maintaining opioid analgesia over extended periods of time.

This study was supported by an NH&MRC of Australia C.J. Martin Fellowship to E.E.B., NH&MRC Project Grant 302087 to B.C., NH&MRC Project Grant 302002 to M.C. and National Institute on Drug Abuse Grant DA12926-01 to M.J.C. M.J.C. is an NH&MRC Senior Principle Research Fellow.

References

AKIL, H., MAYER, D.J. & LIEBESKIND, J.C. (1976). Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science,* **191,** 961–962.

BAGLEY, E.E., GERKE, M.E., VAUGHAN, C.W., HACK, S.P. & CHRISTIE, M.J. (2005). GABA transporter currents activated by protein kinase A excite midbrain neurons during opioid withdrawal. *Neuron,* **45,** 433–445.

BELLCHAMBERS, C.E., CHIENG, B., KEAY, K.A. & CHRISTIE, M.J. (1998). Swim-stress but not opioid withdrawal increases expression of c-fos immunoreactivity in rat periaqueductal gray neurons which project to the rostral ventromedial medulla. *Neuroscience,* **83,** 517–524.

BERNSTEIN, M.S. & WELCH, S.P. (1998). μ-opioid receptor down-regulation and cAMP-dependent protein kinase phosphorylation in a mouse model of chronic morphine tolerance. *Mol. Brain Res.,* **55,** 237–242.

BOHN, L.M., GAINETDINOV, R.R., LIN, F.-T., LEFKOWITZ, R.J. & CARON, M.G. (2000). μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature,* **408,** 720–723.

BORGLAND, S.L., CONNOR, M., OSBORNE, P.B., FURNESS, J.B. & CHRISTIE, M.J. (2003). Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J. Biol. Chem.,* **278,** 18776–18784.

CHIENG, B. & BEKKERS, J.M. (1999). $GABA_B$, opioid and α2 receptor inhibition of calcium channels in acutely dissociated locus coeruleus neurons. *Br. J. Pharmacol.,* **127,** 1533–1538.

CHIENG, B. & CHRISTIE, M.J. (1996). Local opioid withdrawal in rat single periaqueductal grey neurons *in vitro*. *J. Neurosci.,* **16,** 7128–7136.

CHRISTIE, M.J., WILLIAMS, J.T. & NORTH, R.A. (1987). Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Mol. Pharmacol.,* **32,** 633–638.

CHRISTIE, M.J., WILLIAMS, J.T., OSBORNE, P.B. & BELLCHAMBERS, C. (1997). Where is the locus in opioid withdrawal? *Trends Pharmacol. Sci.,* **18,** 134–140.

CICHEWICZ, D.L., HALLER, V.L. & WELCH, S.P. (2001). Changes in opioid and cannabinoids receptor protein following short-term combination treatment with Δ9-tetrahydrocannabinol and morphine. *J. Pharmacol. Exp. Ther.,* **297,** 121–127.

COLLIER, H.O.J., FRANCIS, D.L. & SCHNEIDER, C. (1972). Modification of morphine withdrawal by drugs interacting with humoral mechanisms: some contradictions and their interpretation. *Nature,* **237,** 220–223.

CONNOR, M., BORGLAND, S.L. & CHRISTIE, M.J. (1999a). Continued morphine modulation of calcium channel currents in acutely isolated locus coeruleus neurons from morphine-dependent rats. *Br. J. Pharmacol.,* **128,** 1561–1569.

CONNOR, M., OSBORNE, P.B. & CHRISTIE, M.J. (2004). μ-Opioid receptor desensitization: is morphine different? *Br. J. Pharmacol.,* **143,** 685–696.

CONNOR, M., SCHULLER, A., PINTAR, J.E. & CHRISTIE, M.J. (1999b). μ-Opioid receptor modulation of calcium channel current in periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. *Br. J. Pharmacol.,* **126,** 1553–1558.

DANG, V.C. & WILLIAMS, J.T. (2004). Chronic morphine treatment reduces recovery from opioid desensitization. *J. Neurosci.,* **24,** 7699–7706.

DASCAL, N. (2001). Ion-channel regulation by G proteins. *Trends Endocrinol. Metab.,* **12,** 391–398.

GARZON, J., RODRIGUEZ-MUNOZ, M., LOPEZ-FERNANDO, A. & SANCHEZ-BLAZQUEZ, P. (2005). Activation of μ-opioid receptors transfers control of Gα subunits to the regulator of G-protein signaling RGS9-2. *J. Biol. Chem.,* **270,** 8951–8960.

HACK, S.P., VAUGHAN, C.W. & CHRISTIE, M.J. (2003). Modulation of GABA release during morphine withdrawal in midbrain neurons *in vitro*. *Neuropharmacology,* **45,** 575–584.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell free membrane patches. *Pflugers Archiv.,* **391,** 85–100.

INGRAM, S.L., VAUGHAN, C.W., BAGLEY, E.E., CONNOR, M. & CHRISTIE, M.J. (1998). Enhanced opioid efficacy in opioid dependence is due to an additional signal transduction pathway. *J. Neurosci.,* **18,** 10269–10276.

KENNEDY, C. & HENDERSON, G. (1992). Chronic exposure to morphine does not induce dependence at the level of the calcium channel current in human SH-SY5Y cells. *Neuroscience,* **49,** 937–944.

KIRSCHKE, C., SCHADRACK, J., ZIEGLGANSBERGER, W. & SPANAGEL, R. (2002). Effect of morphine withdrawal on μ-opioid receptor-stimulated guanylyl 5′-[γ-[^{35}S]thio]-triphosphate autoradiography in rat brain. *Eur. J. Pharmacol.,* **446,** 43–51.

LANE, D.A., TORTORICI, V. & MORGAN, M.M. (2004). Behavioral and electrophysiological evidence for tolerance to continuous morphine administration into the ventrolateral periaqueductal gray. *Neuroscience,* **125,** 63–69.

MCQUAY, H. (1999). Opioids in pain management. *Lancet,* **353,** 2229–2232.

MORGAN, M.M., CLAYTON, C.C. & BOYER-QUICK, J.S. (2005). Differential susceptibility of the PAG and RVM to tolerance to the antinociceptive effect of morphine in the rat. *Pain,* **113,** 91–98.

OSSIPOV, M.H., LAI, J., KING, T., VANDERAH, T.W., MALAN JR, T.P., HRUBY, V.J. & PORRECA, F. (2004). Antinociceptive and nociceptive actions of opioids. *J. Neurobiol.,* **61,** 126–148.

PATEL, M.B., PATEL, C.N., RAJASHEKARA, V. & YOBURN, B.C. (2002). Opioid agonists differentially regulate μ-opioid receptors and trafficking proteins *in vivo*. *Mol. Pharmacol.,* **62,** 1464–1470.

PETRUZZI, R., FERRARO, T.N., KURSCHNER, V.C., GOLDEN, G.T. & BERRETTINI, W.H. (1997). The effects of repeated morphine exposure on mu opioid receptor number and affinity in C57BL/6J and DBA/2J mice. *Life Sci.,* **61,** 2057–2064.

RHIM, H., TOTH, P.T. & MILLER, R.J. (1996). Mechanism of inhibition of calcium channels in rat nucleus solitarius by neurotransmitters. *Br. J. Pharmacol.,* **118,** 1341–1350.

SIM, L.J., SELLEY, D.E., DWORKIN, S.I. & CHILDERS, S.R. (1996). Effects of chronic morphine administration on μ opioid receptor-stimulated [^{35}S]-GTPγS autoradiography in rat brain. *J. Neurosci.,* **16,** 2684–2692.

SIUCIAK, J.A. & ADVOKAT, C. (1987). Tolerance to morphine microinjections in the periaqueductal gray (PAG) induces tolerance to systemic, but not intrathecal morphine. *Brain Res.,* **424,** 311–319.

TSOU, K. & JANG, C.S. (1964). Studies on the site of analgesic action of morphine by intracerebral micro-injection. *Sci. Sinica,* **13,** 1099–1109.

VAUGHAN, C.W., BAGLEY, E.E., DREW, G.M., SCHULLER, A., PINTAR, J.E., HACK, S.P. & CHRISTIE, M.J. (2003). Cellular actions of opioids on periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. *Br. J. Pharmacol.,* **139,** 362–367.

VAUGHAN, C.W., INGRAM, S.L., CONNOR, M.A. & CHRISTIE, M.J. (1997). How opioids inhibit GABA-mediated neurotransmission. *Nature,* **390,** 611–614.

VON ZASTROW, M., SVINGOS, A., HABERSTOCK-DEBIC, H. & EVANS, C. (2003). Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr. Opin. Neurobiol.,* **13,** 348–353.

WALDHOER, M., BARTLETT, S.E. & WHISTLER, J.L. (2004). Opioid receptors. *Annu. Rev. Biochem.,* **73,** 953–990.

WILLIAMS, J.T., CHRISTIE, M.J. & MANZONI, O. (2001). Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev.,* **81,** 299–343.

YAKSH, T.L., YEUNG, J.C. & RUDY, T.A. (1976). Systematic examination in the rat of brain sites sensitive to the direct application of morphine: observation of differential effects within the periaqueductal grey. *Brain Res.,* **114,** 83–103.

YOBURN, B.C., BILLINGS, B. & DUTTAROY, A. (1993). Opioid receptor regulation in mice. *J. Pharmacol. Exp. Ther.,* **265,** 314–320.

(Received April 1, 2005
Revised May 18, 2005
Accepted May 23, 2005
Published online 27 June 2005)