British Journal of Pharmacology (1999) 126, 1553–1558
http://www.stockton-press.co.uk/bjp

μ-opioid receptor modulation of calcium channel current in periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1

*,[1]Mark Connor, [2]Alwin Schuller, [2]John E. Pintar & [1]MacDonald J. Christie

[1]Department of Pharmacology, University of Sydney, NSW, 2006, Australia and [2]Department of Neuroscience and Cell Biology, UMNDJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854, U.S.A.

1 The actions of opioid receptor agonists on the calcium channel currents (I_{Ba}) of acutely dissociated periaqueductal grey (PAG) neurons from C57B16/J mice and mutant mice lacking the first exon of the μ-opioid receptor (MOR-1) were examined using whole cell patch clamp techniques. These effects were compared with the $GABA_B$-receptor agonist baclofen.

2 The endogenous opioid agonist methionine-enkephalin (met-enkephalin, pEC_{50} 6.8, maximum inhibition 40%), the putative endogenous μ-opioid agonist endomorphin-1 (pEC_{50} 6.2, maximum inhibition 35%) and the μ-opioid selective agonist DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin, pEC_{50} 6.9, maximum inhibition 40%) inhibited I_{Ba} in 70% of mouse PAG neurons. The inhibition of I_{Ba} by each agonist was completely prevented by the μ-receptor antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2). The δ-opioid receptor agonists DPDPE ([D-$Pen^{2,5}$]-enkephalin, 1 μM) and deltorphin II (1 μM), and the κ-opioid receptor agonist U-69593 (1–10 μM), did not affect I_{Ba} in any cell tested.

3 The $GABA_B$ agonist baclofen inhibited I_{Ba} in all neurons (pEC_{50} 5.9, maximum inhibition 42%).

4 In neurons from the MOR-1 deficient mice, the μ-opioid agonists met-enkephalin, DAMGO and endomorphin-1 did not inhibit I_{Ba}, whilst baclofen inhibited I_{Ba} in a manner indistinguishable from wild type mice.

5 A maximally effective concentration of endomorphin-1 (30 μM) partially (19%), but significantly ($P<0.005$), occluded the inhibition of I_{Ba} normally elicited by a maximally effective concentration of met-enkephalin (10 μM).

6 This study indicates that μ-opioid receptors, but not δ- or κ-opioid receptors, modulate somatic calcium channel currents in mouse PAG neurons. The putative endogenous μ-agonist, endomorphin-1, was a partial agonist in mouse PAG neurons.

Keywords: Endomorphin-1; μ-opioid receptor; periaqueductal grey; calcium channels; partial agonist; gene knockout; baclofen

Abbreviations: BSA, bovine serum albumin; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; DAMGO, Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin; DPDPE, [D-$Pen^{2,5}$]-enkephalin; G-protein, heterotrimeric guanine nucleotide-binding protein; I_{Ba}, calcium channel current; met-enkephalin, methionine enkephalin; MOR-1, μ-opioid receptor clone; ORL1, opioid receptor-like protein; PAG, periaqueductal grey; U-69593, (+)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide

Introduction

The midbrain periaqueductal grey (PAG) plays a pivotal role in the integration of an animal's response to threat, stress and pain (reviewed by Bandler & Shipley, 1994). The PAG is an important central site of action for the analgesic actions of opioid drugs (summarized by Yaksh & Rudy, 1978), and there is also considerable evidence that the PAG is critically involved in an animal's responses to withdrawal of opioid drugs (reviewed in Christie *et al*., 1997). In rat PAG, μ-opioid receptor activation modulates the activity of single neurons in many ways. μ-Opioids inhibit both glutamatergic (Vaughan & Christie, 1997) and GABAergic (Vaughan *et al*., 1997) synaptic inputs to all PAG cells. In a sub-population of PAG cells, μ-opioids directly increase an inwardly rectifying potassium conductance (Chieng & Christie, 1994) and inhibit voltage dependent calcium channels (Kim *et al*., 1997; Connor & Christie, 1998). Nothing is known about the cellular actions of opioids in mouse PAG.

All of the potential endogenous opioid agonists identified to date, with the exception of the opioid receptor-like protein (ORL1) agonist nociceptin, interact with native μ-receptors (Lord *et al*., 1977; Chavkin *et al*., 1982). Similarly, both methionine enkephalin (met-enkephalin) and leucine enkephalin, β-endorphin and dynorphin stimulate heterotrimeric guanine nucleotide-binding (G) proteins *via* the cloned human μ-receptor transfected into C6 glioma cells (Alt *et al*., 1998). An additional two putative endogenous μ-receptor agonists, endomorphin-1 and endomorphin-2, have been identified in bovine brain (Zadina *et al*., 1997). Endomorphin-1 and -2 were notable in displaying affinities for rat μ-receptors at least 1000 fold higher than their affinities for guinea-pig κ- and rat δ-receptors, a selectivity which has subsequently been confirmed for murine opioid receptors (Goldberg *et al*., 1998). The selectivity for the μ-receptor led to the suggestion that the endomorphins may be the 'natural ligands for this receptor' (Zadina *et al*., 1997). Subsequently, evidence from biochemical studies measuring μ-receptor mediated G-protein activation has suggested that both endomorphin-1 and -2 are partial

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

agonists at cloned μ-opioid receptors (MOR-1) (Alt *et al.*, 1998; Hosohata *et al.*, 1998), as well as μ-receptors in brain and spinal cord membranes (Harrison *et al.*, 1998; Narita *et al.*, 1998; Sim *et al.*, 1998). In this study we have examined the regulation of voltage dependent calcium channel currents in acutely dissociated mouse PAG neurons by a variety of opioid agonists, including met-enkephalin, endomorphin-1 and Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin (DAMGO). The actions of these agonists on neurons from both wild type C57B16/J mice and mutant mice lacking the first exon of MOR-1 were compared.

Methods

Wild type and knockout mice homozygous for a deletion of exon 1 of MOR-1 were used as sources of PAG neurons. Production and detailed initial phenotypic analysis of the μ-opioid receptor knockout mice Schuller *et al.* (1999). Briefly, the targeting vector used to produce the gene-targeted mice lacking exon 1 was constructed in two steps. First, a 6.5 kb *Hind*III/*Xho*I fragment corresponding to part of intron 1 of MOR-1 was cloned into the *Hind*III and *Xho*I restriction sites, located between the pMC1neo sequence and the HSV-TK gene of the plasmid knockout vector. Then a 2.5 kb *Not*I/*Bam*HI fragment, located 5′ of exon 1 of MOR-1, was cloned into the *Not*I and *Bam*HI sites 3′ of the Neo gene. Successful targeting events were detected by Southern blot, which was also used to identify embryonic stem cells in which one allele of MOR-1 had been replaced by the mutant allele during homologous recombination. Heterozygous mice were produced following germ-line transmission of microinjected embryonic stem cells, and homozygous MOR-1 knockout mice derived from heterozygote/heterozygote mating were viable and fertile. Knockout mice used in the present study were littermates derived from matings of homozygous MOR-1 knockout mice. Wild type mice were of the parental C57B16/J strain.

Tissue dissociation

Mice of either sex (post natal days 35–103) were anaesthetized with halothane and then killed by cervical dislocation. Horizontal or coronal midbrain slices (between 270–300 μm thick) containing the periaqueductal grey were cut with a vibratome in ice cold physiological saline 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 previously described (Connor & Christie, 1998), using procedures based on those outlined by Ingram *et al.* (1997). 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 units 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 periaqueductal grey 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.

Electrophysiology

Recordings of currents through Ca^{2+} channels were made using standard whole cell patch clamp techniques (Hamill *et al.*, 1981) at room temperature (22–24°C), as previously described (Connor & Christie, 1998). Immediately prior to

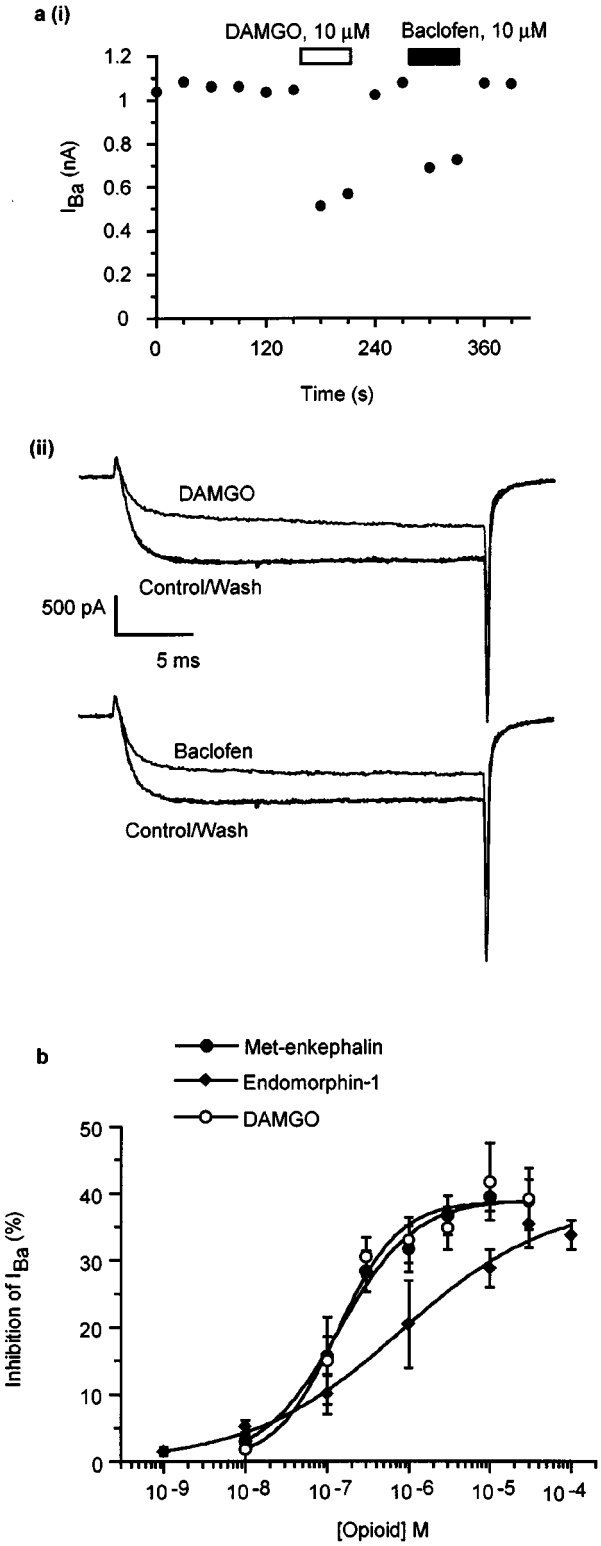

Figure 1 Modulation of PAG calcium channel currents by μ-opioids and baclofen. I_{Ba} was elicited by repetitively stepping the membrane potential from −90 mV to 0 mV. (a) (i) A time plot of the peak amplitude of I_{Ba} illustrating the effects of application of the μ agonist DAMGO and $GABA_B$ agonist baclofen. (ii) Selected traces from the same experiment, showing the inhibition of I_{Ba} by each agonist. (b), Concentration-response relationships for met-enkephalin (EC_{50} 140 nM), DAMGO (EC_{50} 130 nM) and endomorphin-1 (EC_{50} 800 nM) in PAG neurons. Each point represents at least five cells tested.

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, glucose 10, pH 7.3 in order to wash off the dissociation buffer. For calcium channel current (I_{Ba}) recordings, cells were perfused in solution containing (mM) tetraethylammonium chloride 140, $BaCl_2$ 4, CsCl 2.5, HEPES 10, glucose 10, pH 7.3. Recordings were made with fire polished borosilicate pipettes of resistance between 2–4 MΩ when filled with intracellular solution of the following composition (mM): CsCl 110, MgATP 5, Na_2GTP 0.2, EGTA 10, $CaCl_2$ 2 and HEPES 10, pH 7.3. The peak I_{Ba} in each cell was determined by stepping the membrane potential from a holding potential of −90 mV to potentials between −60 and +60 mV, usually for 30 ms, in 10 mV increments. Following this procedure the peak current was evoked every 30 s, and monitored for at least a further 2 min before drugs were applied. The inhibition by drugs was quantified by measuring the current amplitude isochronically with the peak of the control I_{Ba}. Cells in which the I_{Ba} declined in the absence of drug treatment were discarded. 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, Foster City CA, U.S.A.). Leak current was subtracted on line using a P/8 protocol, typically the leak conductance was of the order of 100 pS. Evoked I_{Ba} were filtered at 2 kHz, sampled at 5–10 kHz and recorded on hard disk for later analysis. Data was collected and analysed off line with the PCLAMP suite of programs (Axon Instruments). Cells were exposed to drugs *via* a series of flow pipes positioned above the cells. Drug reservoirs were made from silanized glass syringes and 0.05% bovine serum albumin was included in I_{Ba} recording buffers to prevent adherence of peptides to the tubing. All data are expressed as mean ± s.e.mean, unless otherwise indicated.

Drugs and chemicals Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) was synthesized and purified by Chiron Mimotopes (Clayton, Victoria, Australia). Buffer salts were from BDH Australia or Sigma Australia. Papain was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). DAMGO

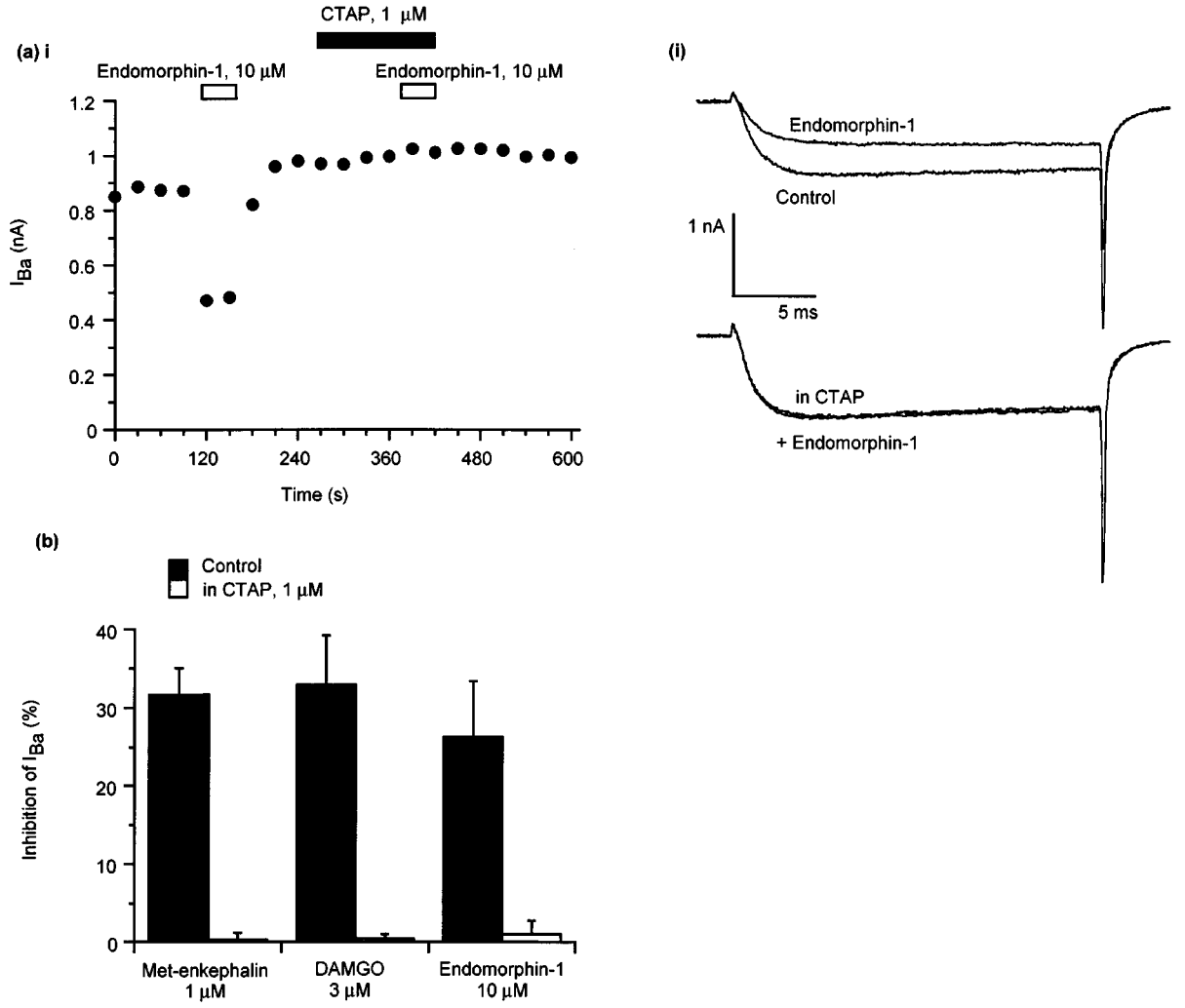

Figure 2 The μ-opioid receptor antagonist CTAP blocks the effects of met-enkephalin, DAMGO and endomorphin-1. The example traces were elicited by repetitively stepping the membrane potential of a PAG neuron from −90 mV to 0 mV. (a) The effects of endomorphin-1 in μ-opioid responsive cell are blocked by the selective μ-opioid receptor antagonist CTAP. (i) A time plot of the peak amplitude of I_{Ba} illustrating the effects of application of endomorphin-1 and subsequent co-application of CTAP and endomorphin-1. (ii) Selected traces from the same experiment. (b) In the presence of CTAP (1 μM) met-enkephalin (1 μM, $P<0.0001$ vs control, unpaired *t*-test, $n=5$ for each), DAMGO (3 μM, $n=5$, $P<0.001$ vs control, $n=7$) and endomorphin-1 (10 μM, $P<0.01$ vs control, $n=5$ for each) do not inhibit I_{Ba} to any significant extent.

(Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin), DPDPE ([D-Pen^{2,5}]-enkephalin), BSA and trypsin inhibitor (Type II-O) were from Sigma Australia. Met-enkephalin was from Auspep (Melbourne, Australia). Baclofen, deltorphin II and U-69593 ((+)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro [4.5]dec-8-yl]benzeneacetamide) were from Research Biochemicals International (Natick, MA, U.S.A.). Morphine hydrochloride was from Glaxo U.K. CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) was kindly donated by the National Institutes on Drug Abuse, U.S.A. In parallel experiments, the U-69593 used in this study inhibited the electrically stimulated contractions of the guinea-pig longitudinal muscle/myenteric plexus; the DPDPE and deltorphin II inhibited the electrically stimulated twitch of the rat *vas deferens* (data not shown).

Results

When PAG neurons were stepped from a holding potential of −90 mV to potentials between −60 mV and +60 mV the inward currents in most cells began to activate at about −40 mV and were invariably greatest at membrane potentials between −10 and +10 mV. The inward currents in PAG neurons were sensitive to Cd^{2+}, a non-selective blocker of high voltage-activated calcium channels. At a concentration of 30 μM, Cd^{2+} blocked 95±0.5% ($n=7$) of the inward current.

The opioid receptor agonists met-enkephalin, DAMGO, endomorphin-1 and morphine inhibited I_{Ba} in 107 of 149 (72%) of PAG neurons tested. The inhibition of I_{Ba} by these agonists was rapid and completely reversible (Figure 1). The inhibition of I_{Ba} by submaximally effective concentrations of met-enkephalin (1 μM), DAMGO (3 μM) and endomorphin-1 (10 μM) was completely prevented by treatment of opioid-sensitive neurons with the μ-opioid receptor selective antagonist CTAP (1 μM, 2 min, Figure 2). Neither the selective κ-opioid receptor agonist U-69593 (1–10 μM, $n=15$), nor the selective δ-opioid receptor agonists DPDPE (1 μM, $n=8$) and deltorphin II (1 μM, $n=7$), inhibited I_{Ba} in any PAG neuron tested.

A concentration-response relationship for μ-opioid agonist inhibition of PAG I_{Ba} was determined by application of one or more concentrations of agonist to cells stepped repetitively from −90 mV to the membrane potential that evoked the largest I_{Ba} in each neuron (either −10 or 0 mV) (Figure 1b). Desensitization of the inhibition of I_{Ba} by any of the agonists was not observed during brief applications of all but the highest concentrations tested. A logistic function was fitted to the concentration response relationship for each agonist inhibition of I_{Ba}, and a pEC_{50} and slope factor determined. Met-enkephalin inhibited I_{Ba} with a pEC_{50} of 6.8±0.1 and slope factor of 0.9±0.1, the maximum inhibition of I_{Ba} was about 40%. DAMGO inhibited I_{Ba} with a pEC_{50} of 6.9±0.1, a slope factor of 1.1±0.3, to a maximum of about 40%. Endomorphin-1 inhibited I_{Ba} with a pEC_{50} of 6.2±0.2, a slope factor of 0.5±0.1, to a maximum of about 35%. Morphine (10 μM) inhibited I_{Ba} by 25±4% ($n=5$). The $GABA_B$ agonist baclofen inhibited I_{Ba} in all PAG neurons tested (45/45), with a pEC_{50} of 5.9±0.2, a slope factor of 0.9±0.3, to a maximum of about 42%.

The maximal inhibition of I_{Ba} by endomorphin-1 appeared to be less than that by met-enkephalin or DAMGO. To determine if endomorphin-1 was acting as a partial agonist at μ-receptors the occlusion of the effects of a high concentration of met-enkephalin by a maximally effective concentration of endomorphin-1 was examined (Figure 3). In these experiments cells were exposed to endomorphin-1 (30 μM) and then switched to buffer containing endomorphin-1 (30 μM) and met-enkephalin (10 μM). The same cells were also exposed to met-enkephalin (10 μM) alone. The two treatments were separated by 5 min and the order of treatment was alternated between cells. A concentration of 30 μM endomorphin-1 was chosen because the inhibition of I_{Ba} by this concentration of agonist did not appear to desensitize significantly in the first 60 s of agonist exposure, while the inhibition of I_{Ba} by 100 μM endomorphin-1 did (data not shown). Met-enkephalin (10 μM) applied alone inhibited I_{Ba} by 38±2%; when met-enkephalin (10 μM) was applied to the same cells in the

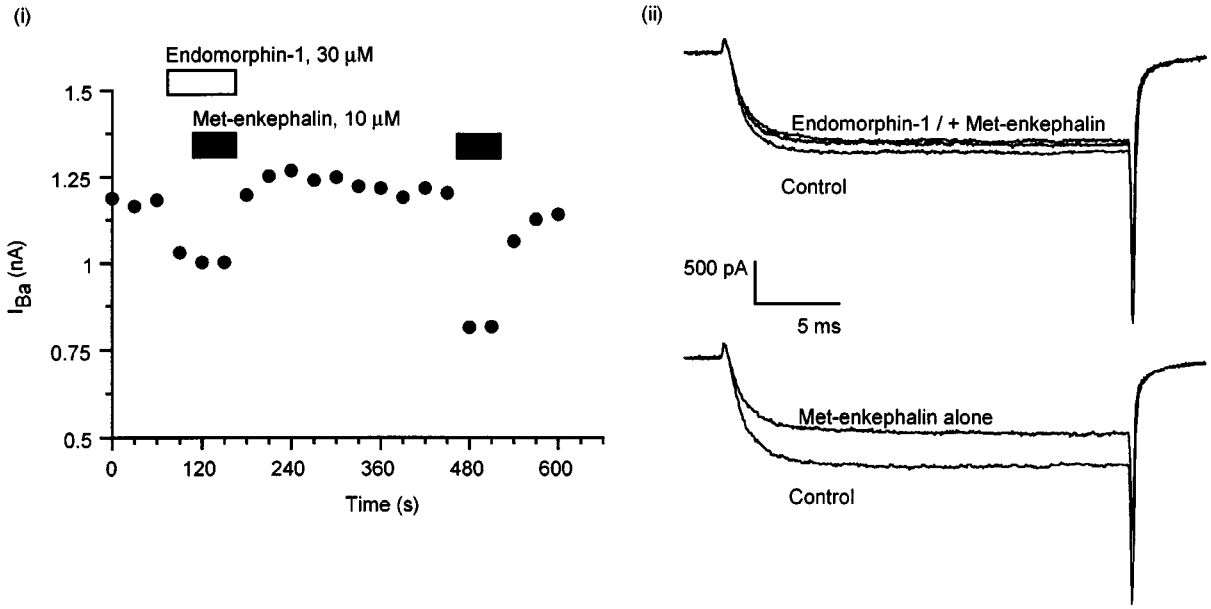

Figure 3 Endomorphin-1 is a partial agonist in PAG neurons. (i) A time plot of the peak amplitude of I_{Ba} illustrating the effects of application of endomorphin-1, endomorphin-1 with met-enkephalin and subsequently met-enkephalin alone. I_{Ba} was elicited by repetitively stepping the membrane potential of the neuron from −90 mV to 0 mV. Note that the abscissa has been truncated so the effects of the agonists can be more easily compared. Endomorphin-1 occluded the effects of met-enkephalin by about 20% in nine cells tested. (ii) Selected traces from the same experiment, showing the I_{Ba} in each condition.

presence of endomorphin-1 (30 μM) the inhibition of I_{Ba} was only 31±4% ($P<0.004$, paired t-test, $n=9$) (Figure 4). Met-enkephalin (10 μM) applied in the presence of endomorphin-1 (30 μM) did not inhibit I_{Ba} more than endomorphin-1 alone (29±4%; $P>0.1$, paired t-test).

In neurons from mice containing a deletion of the first exon of the μ-opioid receptor (MOR-1), neither DAMGO (3 μM, $n=9$), met-enkephalin (3 μM, $n=10$) nor endomorphin (10 μM, $n=9$) inhibited I_{Ba} in any cell. There was also no inhibition of I_{Ba} by either U-69593 (1–10 μM, $n=12$), DPDPE (1–10 μM, $n=3$) or deltorphin II (1 μM, $n=7$). The inhibition of I_{Ba} by baclofen (10 μM) was not different between neurons from wild type and μ-receptor knock out mice. The inhibition of I_{Ba} by baclofen was 41±2% ($n=10$) in wild type mice and 36±4%, ($n=12$) in exon 1 deletion mice). There was no apparent difference in the amount of I_{Ba} between wild type or μ-receptor knock out mice. The peak I_{Ba} density was 253±21 pA pF^{-1} in μ-receptor knock out mice ($n=29$) and 271±17 pA pF^{-1} in parallel experiments in wild type animals ($n=31$).

Discussion

In mouse PAG neurons, μ-opioid receptor activation inhibited I_{Ba} in most neurons, while the $GABA_B$ receptor agonist, baclofen, inhibited I_{Ba} in all neurons. Neither δ- nor κ-opioid agonists affected I_{Ba} in any mouse PAG neuron examined. The results of the present study are broadly consistent with previous studies showing that μ-opioids inhibit I_{Ba} in a sub-population of acutely dissociated rat PAG neurons (Kim *et al*., 1997; Connor & Christie, 1998), whilst δ- and κ-opioid agonists are ineffective (Connor & Christie, 1998). Notably, μ-opioids inhibited I_{Ba} in a far greater proportion of mouse PAG neurons than rat PAG neurons (72% vs 38%). Autoradiographic studies have identified both κ- and δ-opioid receptor messenger RNA localized to the mouse PAG (DePaoli *et al*., 1994; Jenab *et al*., 1995; Kitchen *et al*., 1997) and a moderate to high density of [^{3}H]-U-69593 binding has been reported in the PAG of C57BL/6 mice (Jamensky & Gianoulakis, 1997). The biochemical consequences of κ-opioid receptor activation in the PAG are poorly characterized but a number of studies have demonstrated δ-opioid receptor stimulation of G-protein activity in mouse PAG (see Garzon *et al*., 1997). The lack of effect of either δ- or κ-receptor agonists on I_{Ba} in mouse PAG is intriguing. A similar conundrum exists in the rat PAG where several studies have also failed to find any effects of δ- and κ-opioids on the membrane currents of PAG neurons or on synaptic transmission within PAG slices (Chieng & Christie, 1994; Vaughan & Christie, 1997), despite the presence of δ- and κ-opioid receptor messenger RNA and radioligand binding (Kalyuzhny *et al*., 1996; Mansour *et al*., 1996). It should be noted that the present study utilized Ba^{2+} as a charge carrier and strong intracellular Ca^{2+} buffering in order to minimize current rundown mediated by Ca^{2+}-dependent processes. It is likely that any Ca^{2+}-dependent mechanisms of I_{Ba} modulation were suppressed under these conditions and thus the possibility that δ- and κ-opioid receptors may act *via* such pathways in PAG neurones cannot be excluded.

The type(s) of calcium channel present in mouse PAG neurons is not known at present, however rat PAG neurons possess predominantly N-type and P/Q-type currents (approximately 40% of each), with lesser amounts of L-type and resistant current (Connor & Christie, 1998). We did not attempt to determine which types of calcium channel current were being modulated by μ-opioids in mouse PAG, however previous studies have shown that μ-opioids predominantly inhibit the N-type and to a lesser extent P/Q-type calcium channels in central neurons (e.g. Rhim & Miller, 1994; Kim *et al*., 1997; Soldo & Moises, 1997). In our previous study in rat PAG neurons, nociceptin, the endogenous ligand for the opioid-receptor like protein (ORL1), strongly inhibited both N-type and P/Q-type currents, while having little effect on the residual L- and R-type currents (Connor & Christie, 1998).

In mouse PAG neurons, the putative endogenous μ-opioid receptor selective agonist endomorphin-1 inhibited I_{Ba} as a partial agonist, with a relatively low potency. The partial agonist characteristics of endomorphin-1 have previously been noted by a number of investigators utilizing assays that measure G-protein interaction with both native (Harrison *et al*., 1998; Narita *et al*., 1998; Sim *et al*., 1998) and cloned (Alt *et al*., 1998; Hosohata *et al*., 1998) μ-opioid receptors. In those experiments endomorphin-1 and -2 consistently stimulate G-protein activity to levels between 40 and 70% of the maximal amounts stimulated by agonists such as DAMGO. In several studies endomorphin-1 was also shown to occlude the maximal stimulatory effects of DAMGO, confirming its partial agonist activity (Alt *et al*., 1998; Sim *et al*., 1998). The efficacy of endomorphin-1 in stimulating G-protein activity appears to be similar to that of morphine (Alt *et al*., 1998; Hosohata *et al*., 1998; Narita *et al*., 1998; Sim *et al*., 1998).

The actions of endomorphin-1 and -2 on single cells are poorly characterized. In NG108-15 neuroblastoma X glioma cells transfected with the rat μ-opioid receptor, both endomorphin-1 and -2 inhibited the voltage-dependent calcium currents with EC_{50}s of about 10 nM (Mima *et al*., 1997; Higashida *et al*., 1998), which is much more potent than observed in mouse PAG cells. The high potency in the latter studies presumably results at least partly from the over-expression of μ-receptors in the NG108-15 cells.

Endomorphin-1 and -2 are potent analgesics when injected i.c.v. in mice (Zadina *et al*., 1997; Goldberg *et al*., 1998). This activity presumably arises at least in part from actions within the PAG, which is thought to be a major site of action for opioid mediated analgesia (Yaksh & Rudy, 1978). It is not known what part the inhibition of calcium channels may have in mediating opioid effects in mouse PAG but clearly μ-opioid agonists, including endomorphins, have the potential to directly modulate the somatic currents of most neurons in the mouse PAG.

This study was supported by The University of Sydney Medical Foundation. M.C. is the recipient of a Rolf Edgar Lake Fellowship from the Faculty of Medicine, University of Sydney. A.S. and J.E.P. supported by NIH Grant DA-09040. The donation of CTAP by the National Institutes on Drug Abuse is gratefully acknowledged.

References

ALT, A., MANSOUR, A., AKIL, H., MEDZIHRASKY, F., TRAYNOR, J.R. & WOODS, J.H. (1998). Stimulation of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding by endogenous opioids acting at a cloned *mu* receptor. *J. Pharmacol. Exp. Ther.*, **286,** 282 – 288.

BANDLER, R. & SHIPLEY, M. (1994). Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *TiNS,* **17,** 379 – 388.

CHAVKIN, C.I., JAMES, I.F. & GOLDSTEIN, A. (1982). Dynorphin is a specific endogenous ligand of the κ opioid receptor. *Science,* **215,** 413 – 415.

CHIENG, B. & CHRISTIE, M.J. (1994). Hyperpolarization by opioids acting on μ-receptors of a subpopulation of rat periaqueductal gray neurones *in vitro*. *Br. J. Pharmacol.,* **113,** 121 – 128.

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

CONNOR, M. & CHRISTIE, M.J. (1998). Modulation of the calcium channel currents of acutely dissociated rat periaqueductal gray neurones. *J. Physiol.,* **509,** 47 – 58.

DE PAOLI, A.M., HURLEY, K.M., YASADA, K., REISINE, T. & BELL, G. (1994). Distribution of κ opioid receptor mRNA in adult mouse brain: an *in situ* hybridization histochemistry study. *Mol. Cell. Neurosci.,* **5,** 327 – 335.

GARZON, J., MARTINEZ-PENA, Y., SANCHEZ-BLAZQUEZ, P. (1997). Gx/z is regulated by μ but not δ opioid receptors in the stimulation of low K_m GTPase activity in mouse periaqueductal grey matter. *Eur. J. Neurosci.,* **9,** 1194 – 1200.

GOLDBERG, I.E., ROSSI, G.E., LETCHWORTH, S.R., MATHIS, J.P., RYAN-MORO, J., LEVENTHAL, L., SU, W., EMMEL, D., BOLAN, E.A. & PASTERNAK, G.W. (1998). Pharmacological characterization of endomorphin-1 and endomorphin-2 in mouse brain. *J. Pharmacol. Exp. Ther.,* **286,** 1007 – 1013.

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.

HARRISON, L.M., KASTIN, A.J. & ZADINA, J.E. (1998). Differential effects of endomorphin-1, endomorphin-2, and Tyr-W-MIF-1 on activation of G-proteins in SH-SY5Y human neuroblastoma membranes. *Peptides,* **19,** 749 – 753.

HIGASHIDA, H., HOSHI, N., KNIJNIK, R., ZADINA, J.E. & KASTIN, A.J. (1998). Endomorphins inhibit high-threshold Ca^{2+} channel currents in rodent NG108-15 cells overexpressing μ-opioid receptors. *J. Physiol.,* **507,** 71 – 75.

HOSOHATA, K., BURKEY, T.H., ALFARO-LOPEZ, J., VARGA, E., HRUBY, V.J., ROESKE, W.R. & YAMAMURA, H.I. (1998). Endomorphin-1 and endomorphin-2 are partial agonists at the human μ-opioid receptor. *Eur. J. Pharmacol.,* **346,** 111 – 114.

INGRAM, S., WILDING, T.J., MCCLESKEY, E.W. & WILLIAMS, J.T. (1997). Efficacy and kinetics of opioid action on acutely dissociated neurons. *Mol. Pharmacol.,* **52,** 136 – 143.

JAMENSKY, N.T. & GIANOULAKIS, C. (1997). Content of dynorphins and κ-opioid receptors in distinct brain regions of C57BL/6 and DBA/2 mice. *Alcohol. Clin. Exp. Res.,* **21,** 1455 – 1464.

JENAB, S., KEST, B., FRANKLIN, S.O. & INTURRISI, C.E. (1995). Quantitative distribution of the delta opioid receptor mRNA in the mouse and rat CNS. *Life Sci.,* **56,** 2343 – 2355.

KALYUZHNY, A.E., ARVIDSSON, U., WU, W. & WESSENDORF, M.W. (1996). μ-Opioid and δ-opioid receptors are expressed in brainstem antinociceptive circuits: studies using immunocytochemistry and retrograde tract tracing. *J. Neurosci.,* **16,** 6490 – 6503.

KIM, C.J., RHEE, J.-S. & AKAIKE, N. (1997). Modulation of high-voltage activated Ca^{2+} channels in the rat periaqueductal gray neurons by μ-type opioid agonist. *J. Neurophysiol.,* **77,** 1418 – 1424.

KITCHEN, I., SLOWE, S.J., MATTHES, H.W.D. & KIEFFER, B. (1997). Quantitative autoradiographic mapping of μ-, δ- and κ-opioid receptors in knockout mice lacking the μ-opioid receptor gene. *Brain Res.,* **778,** 73 – 88.

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1977). Endogenous opioid peptides: multiple agonists and receptors. *Nature,* **267,** 495 – 499.

MANSOUR, A., BURKE, S., PAVLIC, R.J., AKIL, H. & WATSON, S.J. (1996). Immunohistochemical localization of the cloned κ_1 receptor in the rat CNS and pituitary. *Neurosci.,* **71,** 671 – 690.

MIMA, H., MORIKAWA, H., FUKUDA, K., KATO, S., SHODA, T. & MORI, K. (1997). Ca^{2+} channel inhibition by endomorphins via the cloned μ-opioid receptor expressed in NG108-15 cells. *Eur. J. Pharmacol.,* **340,** R1 – R2.

NARITA, M., MIZOGUCHI, H., OJI, G.S., TSENG, E.L., SUGANUMA, C., NAGASE, H. & TSENG, L.F. (1998). Characterization of endomorphin-1 and -2 on [^{35}S]GTPγS binding in the mouse spinal cord. *Eur. J. Pharmacol.,* **351,** 383 – 387.

RHIM, H. & MILLER, R.J. (1994). Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. *J. Neurosci.,* **14,** 7608 – 7615.

SCHULLER, A.G.P., KING, M., ZHANG, J., BOLAN, E., PAN, Y.-X., MORGAN, D.J., CHANG, A., CZICK, M.E., UNTERWALD, E., PASTERNAK, G.W. & PINTAR, J.E. (1999). Retention of heroin and morphine-6-glucuronide analgesia in a new line of MOR-1 knockout mice insensitive to morphine. *Nature Neurosci.,* **2,** 1 – 8.

SIM, L.J., LIU, Q., CHILDERS, S.R. & SELLEY, D.E. (1998). Endomorphin-stimulated [^{35}S]GTPγS binding in rat brain: evidence for partial agonist activity at μ-opioid receptors. *J. Neurochem.,* **70,** 1567 – 1576.

SOLDO, B.L. & MOISES, H.C. (1997). μ-Opioid receptor activation decreases N-type Ca^{2+} current in magnocellular neurons of the rat basal forebrain. *Brain Res.,* **758,** 118 – 126.

VAUGHAN, C.W. & CHRISTIE, M.J. (1997). Presynaptic inhibitory action of opioids on synaptic transmission in the rat periaqueductal grey *in vitro*. *J. Physiol.,* **498,** 463 – 472.

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

YAKSH, T.L. & RUDY, T.A. (1978). Narcotic analgetics: CNS sites and mechanisms of action as revealed by intracerebral injection techniques. *Pain,* **4,** 299 – 359.

ZADINA, J.E., HACKLER, L., GE, L.-J. & KASTIN, A.J. (1997). A potent and selective endogenous agonist for the μ-opioid receptor. *Nature,* **386,** 499 – 502.

(Received October 13, 1998
Revised December 23, 1998
Accepted January 11, 1999)