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Continued morphine modulation of calcium channel currents in acutely isolated locus coeruleus neurons from morphine-dependent rats

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1 The actions of the opioid agonists morphine and methionine-enkephalin (met-enkephalin) on the calcium channel currents (I_{Ba}) of acutely isolated locus coeruleus (LC) neurons from morphine-dependent and vehicle-treated rats were examined using whole cell patch clamp techniques.

2 In LC neurons maintained in 5 μM morphine, co-superfusion of naloxone (1 μM) or the μ-opioid receptor antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 1 μM) with morphine resulted in a significant increase in the amplitude of I_{Ba}. The increases in I_{Ba} were not different in neurons from morphine-dependent or vehicle rats. The increase in I_{Ba} was mimicked by washing off morphine, but not by co-superfusion of the κ-receptor antagonist norbinaltorphimine (300 nM) or the δ-receptor antagonist ICI-174864 (1 μM).

3 In spontaneously withdrawn LC neurons from morphine-dependent rats, met-enkephalin (pD_2 7.1, maximum inhibition 49%) and morphine (pD_2 6.5, maximum inhibition 33%), inhibited I_{Ba} in all cells. In cells from vehicle rats the pD_2 for met-enkephalin was 7.3, maximum inhibition 52%, while the pD_2 for morphine was 6.6 and the maximum inhibition 43% ($P<0.05$ versus cells from morphine-dependent rats).

4 I_{Ba} in LC neurons was mostly comprised of ω-conotoxin GVIA- (N-type) and ω-agatoxin IVA- (P/Q-type) sensitive components, with lesser amounts of nimodipine-sensitive current and current resistant to all three blockers. Neither the density of I_{Ba} nor the proportion of any of the components of I_{Ba} differed between neurons from morphine-dependent or vehicle-treated rats.

5 This study demonstrates that in morphine-dependent rats, morphine and met-enkephalin modulation of somatic I_{Ba} in LC neurons displays modest tolerance compared with untreated rats. Further, chronic morphine treatment does not alter the type or density of I_{Ba} in LC neurons. These results provide more evidence that functional μ-opioid receptor coupling is not dramatically altered in the LC in morphine-dependent rats.

Keywords: Morphine; met-enkephalin; opioid dependence; opioid tolerance; locus coeruleus; calcium channels; withdrawal

Abbreviations: BSA, bovine serum albumin; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; G-protein, heterotrimeric guanine nucleotide-binding protein; I_{Ba}, calcium channel current; ICI-174864, N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; I_K, potassium current; LC, locus coeruleus; met-enkephalin, methionine enkephalin; nBNI, norbinaltorphimine; PAG, periaqueductal grey

Introduction

Chronic morphine treatment results in cellular changes in neurons that produce tolerance to morphine, as well as engendering a distinctive withdrawal syndrome upon cessation of morphine treatment. Cellular adaptations to chronic morphine have been proposed to include uncoupling of μ-opioid receptors, changes in adenylyl cyclase activity and G-protein function, as well as alterations in ion channel expression, including calcium channels (Nestler & Aghajanian, 1997; Ramkumar & el-Fakahany, 1984; 1988). Among a number of different brain regions implicated in the expression of the morphine abstinence syndrome, much research has focused on the locus coeruleus (LC) and periaqueductal grey (PAG) (Christie *et al*., 1997; Nestler & Aghajanian, 1997). In the PAG, morphine withdrawal produces a direct excitation of μ-opioid-sensitive cells *via* modulation of a novel conductance (Chieng & Christie 1996), as well as an increase in GABA release from μ-opioid-sensitive presynaptic terminals (Ingram *et al*., 1998). Intriguingly, in the PAG, the efficacy of opioid modulation of GABAergic synaptic transmission is increased by chronic morphine treatment (Ingram *et al*., 1998).

In contrast, previous studies in the LC have shown that chronic morphine treatment produces tolerance to the effects of morphine at the cellular level (Andrade *et al*., 1983, Christie *et al*., 1987). Withdrawal-induced activation of the LC seems to be largely driven by increased afferent drive to the nucleus (Rasmussen & Aghajanian, 1989; Akaoka & Aston-Jones, 1991), but there is still a widespread belief that mechanisms intrinsic to the LC contribute significantly to the excitation of LC neurons (e.g. Nestler & Aghajanian, 1997). Previous studies have demonstrated tolerance to opioids at the level of the inwardly rectifying K channel (I_K) in LC neurons (Christie *et al*., 1987), but nothing is known of the effects of chronic morphine treatment on opioid receptor coupling to calcium channels in the LC. In this study we have examined the effects of chronic morphine treatment on μ-opioid receptor coupling to calcium channels in acutely isolated LC neurons from young adult rats, as well as the effects of chronic morphine treatment on the expression of types of calcium channels in these neurons. Parts of this work have been presented in abstract

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form to the Society for Neuroscience (Connor & Christie, 1998).

Methods

Male Sprague-Dawley rats (28–43 days old at sacrifice) were used for this study. Physical dependence on morphine was induced by a series of three subcutaneous injections of morphine base (100 mg kg^{-1}) in a sustained release emulsion on alternate days over a 5 day period. We (Bellchambers *et al*., 1998; Chieng & Christie 1996) 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 anaesthesia. Vehicle rats were injected with suspension lacking morphine. Vehicle and morphine treatments were performed at the same time, on littermates.

One or two days following the final injection of morphine or vehicle the rats were anaesthetized with halothane and then killed by cervical dislocation. Horizontal slices (between 290–310 μm thick) containing the LC 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 then equilibrated for 30 min at 35°C. The dissociation procedures were based on those outlined in Ingram *et al*. (1997). The 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. The locus coeruleus region was subdissected from each slice with a fine tungsten wire and the cells dissociated from the slices by gentle trituration through silanized pasteur pipettes with firepolished tips. The cells were plated onto plastic culture dishes and kept at room temperature in dissociation buffer. Unless otherwise noted, 5 μM morphine was present in all solutions except the ice cold saline used in the vibratome. The 30 min equilibration period for slices was carried out in morphine. Cells remained viable for up to 6 h after dissociation.

Recordings of currents through Ca^{2+} channels (I_{Ba}) were made using standard whole cell patch clamp techniques (Hamill *et al*., 1981) at room temperature (22–24°C). Immediately prior to recording, dishes of cells were perfused 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 out the dissociation buffer. This buffer did not contain morphine unless experiments investigating the acute withdrawal of morphine were being conducted. For I_{Ba} recordings, cells were perfused in solution containing (mM) tetraethylammonium chloride 140, $BaCl_2$ 2, $MgCl_2$ 1, CsCl 2.5, HEPES 10, glucose 10, BSA 0.05%, pH 7.3. Recordings were made with fire polished borosilicate pipettes of resistance approximately 2 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 test current was evoked every 30 s, and monitored for at least a further 2 min before drugs or toxins were applied. The inhibition by drugs or toxins was quantified by measuring the current amplitude isochronically with the peak of the control I_{Ba}. Cells in which I_{Ba} declined in the absence of drug treatment were discarded and were not used in analysis of the population characteristics of LC cells. 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 was between 2 and 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, unless otherwise noted. Typically the leak conductance was less than 1 nS. Evoked I_{Ba} was 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 and toxins *via* a series of flow pipes positioned above the cells.

All data are expressed as mean ± s.e.mean, unless otherwise indicated. Concentration response data for each condition was obtained from a number of cells and the pooled data fitted to a modified version of the Hill equation using the program KaleidaGraph 3.0.5 (Abelbeck Software). The equation fitted to the data was of the form: $E = E_{max}(x^n/(x^n + EC_{50}{}^n))$ where E is the per cent inhibition of I_{Ba}; E_{max} is the maximum inhibition of I_{Ba}; x is the concentration of drug; EC_{50} is the concentration of drug resulting in 50% of the maximal inhibition and n is the slope factor (see Jenkinson *et al*., 1995). Ninety-five per cent confidence intervals were determined using the standard deviation of the potency and maximal inhibition values derived by Kaleidagraph multiplied by the percentiles of the t distribution appropriate for the number of points used to generate each curve. Statistical significance was assessed using a paired or unpaired, two tailed Students t-test, as noted.

Drugs and chemicals

Morphine base and morphine hydrochloride were from Glaxo U.K. Buffer salts were from BDH Australia or Sigma Australia. Papain was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). Arlacel A, BSA and trypsin inhibitor (Type II-O) were from Sigma Australia. ω-Conotoxin GVIA was from Auspep (Melbourne, Australia). Met-enkephalin was from either Sigma or Auspep. CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2), ICI-174864 trifluoracetate (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, where Aib is α-aminoisobutyric acid), naloxone hydrochloride, nimodipine, nor-binaltorphimine hydrochloride (nBNI) and UK14304 were from Research Biochemicals International (Natick, MA, U.S.A.). ω-Agatoxin IVA was from the Peptide Institute (Osaka, Japan).

Results

Dissociated LC neurons were identified as large (membrane capacitance 32 ± 1 pF, $n = 215$), usually multipolar neurons with somata shapes characteristic of LC neurons described in fixed tissue preparations (e.g. Swanson, 1976). The I_{Ba} of all the neurons tested in this study was inhibited by application of met-enkephalin or morphine. When LC neurons were stepped from a holding potential of −90 mV to potentials between −60 and +60 mV inward currents in most cells began to activate at about −40 mV and were invariably greatest at membrane potentials between −10 and 0 mV.

When current/voltage (I/V) relationships for I_{Ba} were determined in LC neurons maintained in 5 μM morphine, the current density and activation kinetics of I_{Ba} did not differ between neurons dissociated from morphine-dependent or vehicle-treated rats (Table 1). Similarly, when I/V relationships were determined in LC cells after 5 μM morphine had been washed out of the recording dish (i.e. cells had been 'withdrawn'), there was also no difference in the current density or activation kinetics of I_{Ba} between cells from morphine-dependent and vehicle-treated rats (Table 1). However, in neurons from both morphine-dependent and vehicle-treated rats the peak I_{Ba} density was significantly less in neurons maintained in 5 μM morphine than in the withdrawn cells ($P<0.01$ for each). The activation kinetics of the peak I_{Ba}, determined by measuring the time taken to reach 95% of the maximum current (0–95% risetime), also differed between cells maintained in 5 μM morphine and withdrawn cells. In neurons from both morphine-dependent and vehicle-treated rats, the 0–95% risetime was significantly longer in cells maintained in 5 μM morphine than in withdrawn cells ($P<0.01$ for each, Table 1).

Table 1 Basic properties of I_{Ba} in LC neurons from vehicle-treated and morphine-dependent rats

	Vehicle-treated		*Morphine-dependent*	
	In morphine	*Withdrawn*	*In morphine*	*Withdrawn*
Peak current density (pA pF^{-1})	-89 ± 5* ($n=37$)	-118 ± 5 ($n=69$)	-80 ± 5* ($n=40$)	-121 ± 5 ($n=80$)
Peak current risetime (0–95%, ms)	3.9 ± 0.4*	2.4 ± 0.1	3.7 ± 0.3*	2.5 ± 0.1

Basic properties were derived from I/V relationships determined either in the continuous presence of morphine (5 μM) or following spontaneous withdrawal from morphine. Peak current was always observed at steps to -10 or 0 mV. *Denotes significantly different from withdrawn cells of same treatment, $P<0.01$, unpaired t-test. There was no difference ($P>0.15$) in the current density between cells from morphine-dependent animals and cells from vehicle treated rats, either in the presence of absence of morphine (5 μM).

When LC neurons maintained in 5 μM morphine since sacrifice were repetitively stepped from a holding potential of -90 mV to a test potential of -10 mV, co-application of the opioid antagonist naloxone caused a rapid, significant, increase in the amplitude of I_{Ba} in cells from both morphine-dependent and vehicle treated rats ($P<0.002$ for each, paired t-test, Figures 1 and 2). The increase in I_{Ba} amplitude in

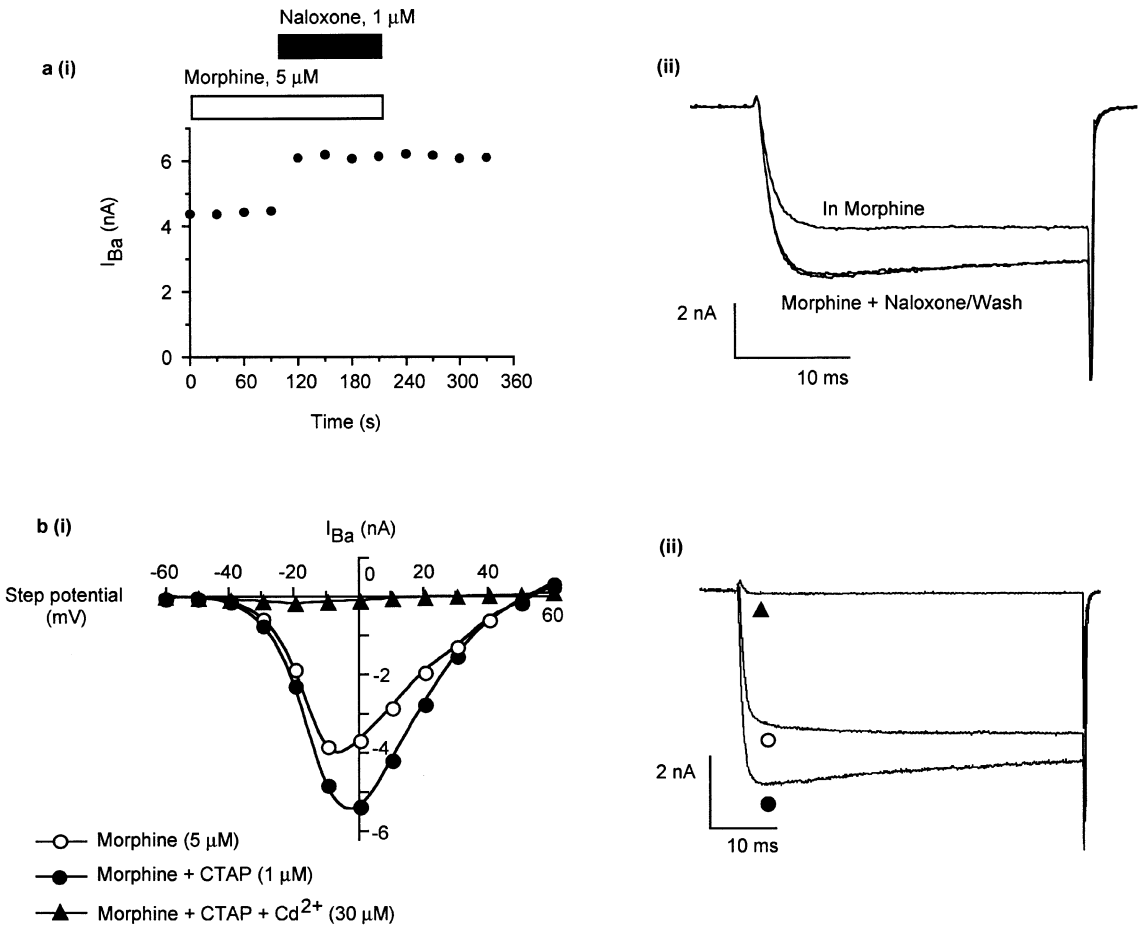

Figure 1 Morphine continues to modulate I_{Ba} in LC neurons from morphine-dependent rats. I_{Ba} was elicited by stepping the membrane potential from -90 to -10 mV (a) or from -90 mV to a range of test potentials from -60 to $+60$ mV (b). (a) (i) A time plot of the amplitude of I_{Ba} of a cell from a morphine-dependent rat maintained in 5 μM morphine since sacrifice, illustrating the effects on I_{Ba} when the opioid antagonist naloxone is co-applied with morphine. (ii) Selected traces from the same experiment, showing the increase in I_{Ba} in the presence of naloxone. (b) (i) A series of current/voltage relationships obtained in the presence of 5 μM morphine, in the presence of morphine and the μ-opioid antagonist CTAP, and in the presence of morphine, CTAP and the I_{Ba} blocker Cd^{2+}, illustrating that the opioid antagonist induced increase in I_{Ba} occurs at a range of membrane potentials, and further that there is no substantial outward current in these recording conditions. (b) (ii) Example traces from the same experiment, showing the I_{Ba} elicited by a step from -90 to 0 mV in each condition.

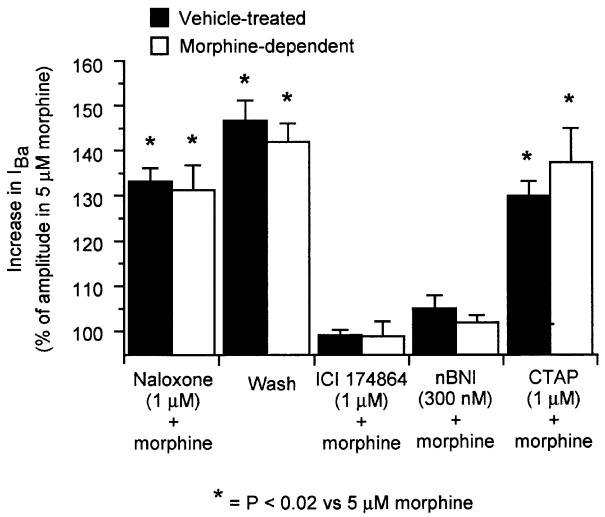

Figure 2 μ-Opioid receptors are responsible for continued morphine modulation of I_{Ba} in LC neurons from morphine-dependent rats. LC neurons, maintained in 5 μM morphine since sacrifice, were repetitively stepped from -90 to -10 mV. They were then superfused with a variety of opioid receptor antagonists in the continued presence of morphine, or the morphine was simply washed off the cells ('spontaneous withdrawal'). Each bar represents the mean$\pm$s.e.mean of between 5 and 15 experiments. Superfusion of naloxone, the μ-opioid selective antagonist CTAP, or spontaneous withdrawal from morphine resulted in significant increases in the amplitude of I_{Ba}.

naloxone was accompanied by a significant decrease in the activation time of the current for cells from morphine-dependent (from 3.1 ± 0.2 to 2.5 ± 0.2 ms, $n=8$, $P<0.006$, paired t-test) and vehicle treated rats (from $3.3.\pm0.2$ to 2.4 ± 0.4 ms, $n=7$, $P<0.001$ paired t-test). There was no difference in the amount of the naloxone-induced increase of I_{Ba} between neurons from morphine-dependent and vehicle-treated rats. The increase in I_{Ba} amplitude and decrease in activation time of the current was also observed when morphine was simply washed off cells (Figure 2). After washout of morphine, the activation time of the current decreased from 3.7 ± 0.4 to 2.9 ± 0.2 ms ($P<0.005$, $n=14$) in cells from morphine-dependent rats, and from 3.7 ± 0.3 to 3.1 ± 0.1 ms ($P<0.002$, $n=15$) in rats from vehicle-treated animals. The effects of naloxone could also be mimicked by co-application of 5 μM morphine with the selective μ-opioid antagonist CTAP (1 μM), but not by co-application of 5 μM morphine with the δ-opioid selective antagonist ICI 174864 (1 μM) or the κ-opioid selective antagonist nBNI (300 nM) (Figure 2). The withdrawal increase in I_{Ba} following co-application of morphine (5 μM) and CTAP (1 μM) or simple wash of morphine was evident over a range of membrane potentials (Figure 1, $n=6$).

In LC cells maintained in morphine (5 μM) since sacrifice, the amplitude of I_{Ba} could be increased by a strong positive depolarizing step shortly before the test step. In the experiments illustrated in Figure 3, LC cells were stepped twice to -10 mV, with an 80 ms depolarizing step to $+80$ mV between the test steps. These experiments were performed without leak subtraction. In cells from morphine-dependent animals maintained in morphine, the amplitude of the I_{Ba} evoked by the first test step (T1) was always less than the amplitude of the I_{Ba} evoked by the test step (T2) that followed the conditioning step to $+80$ mV ($n=6$). When morphine was washed off the cells, the amplitude of both T1 and T2 was increased significantly ($P<0.01$, $n=6$, Table 2, Figure 3a). The amplitude of T1 was increased significantly more than the amplitude of T2 after morphine wash ($61\pm7\%$ for T1, $22\pm4\%$ for T2, $P<0.01$, unpaired t-test), and the amplitude of the first step was always greater than the amplitude of the second step after morphine washout ($n=6$). Similar results were obtained from cells isolated from vehicle-treated rats ($n=9$, Table 2), the amplitude of T1 increased by $55\pm5\%$, the amplitude of T2 increased by $18\pm2\%$ ($P<0.01$, unpaired t-test). The conditioning step to $+80$ mV also partly reversed the kinetic slowing of I_{Ba} observed in the presence of morphine (Table 2). In cells from morphine-dependent rats maintained in morphine since sacrifice, the risetime of the I_{Ba} evoked by the first test step (T1) to -10 mV was significantly slower than that of the I_{Ba} evoked by the test step following the conditioning step (T2, $P<0.05$, $n=6$). When morphine was

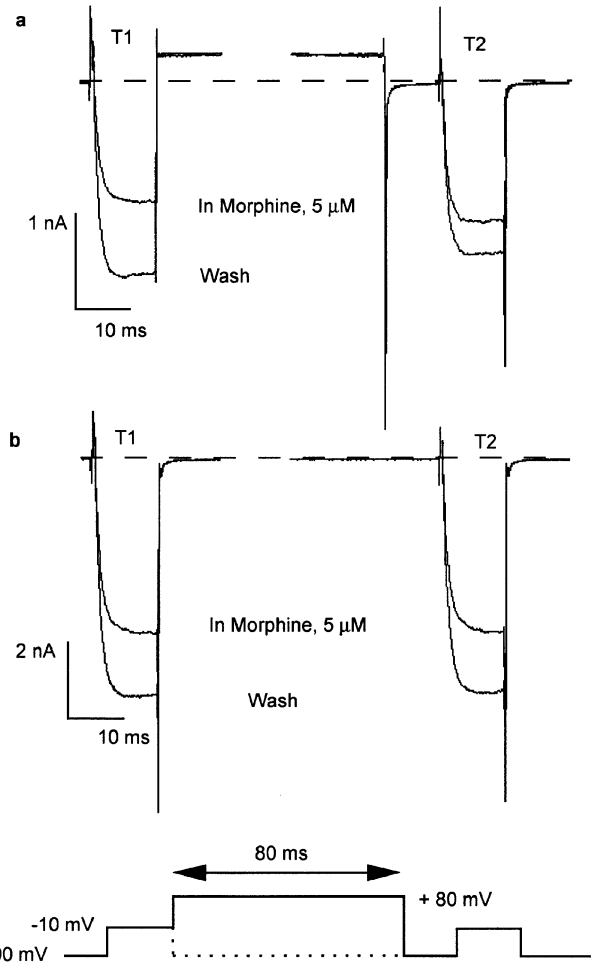

Figure 3 Continued morphine modulation of I_{Ba} is relieved by a positive prepulse. LC neurons from morphine-dependent rats were maintained in morphine (5 μM) after sacrifice. Cells were voltage clamped at -90 mV and stepped twice to a test potential of -10 mV. In (a), an 80 ms positive step to $+80$ mV was applied to the cell immediately after the first test pulse, in (b), the cell was held at -90 mV for the 90 ms between test pulses. The resulting raw current traces for steps in the presence of morphine and following the wash of morphine are shown. The dashed line represents the zero current line, because of the complex step paradigm leak subtraction was not used. The break in the current traces represent a section of about 50 ms that has been omitted for clarity. Note that in (a), the amplitude T2 in morphine is facilitated compared with the T1, while in (b) there is no facilitation of T2 in the presence of morphine. The facilitation of T2 is abolished following morphine wash. The experiment in (a) is typical of six cells, the experiment in (b) of four cells. Similar results were obtained from cells isolated from vehicle-treated rats.

Table 2 Effects of a depolarizing conditioning step on morphine modulation of I_{Ba} in LC neurons

	Morphine-dependent		*Vehicle-treated*	
	In morphine	*Withdrawn*	*In morphine*	*Withdrawn*
With conditioning step	$n=6$		$n=9$	
Ratio T2:T1 amplitude	1.19 ± 0.05	0.90 ± 0.02*	1.2 ± 0.03	0.91 ± 0.01*
0–95% Risetime of T1 (ms)	3.8 ± 0.2	3.0 ± 0.1*	3.3 ± 0.2	2.6 ± 0.1*
0–95% Risetime of T2 (ms)	3.3 ± 0.2#	3.3 ± 0.1	2.9 ± 0.1#	2.8 ± 0.1
Without conditioning step	$n=4$		$n=6$	
Ratio T2:T1 amplitude	1.00 ± 0.04	0.98 ± 0.03	0.99 ± 0.01	0.97 ± 0.01
0–95% Risetime of T1 (ms)	4.0 ± 0.2	3.2 ± 0.2*	3.1 ± 0.2	2.5 ± 0.1*
0–95% Risetime of T2 (ms)	4.0 ± 0.1	3.2 ± 0.2*	3.2 ± 0.2	2.6 ± 0.2*

LC neurons were stepped twice from −90 to −10 mV, with or without an 80 ms conditioning step to +80 mV preceding the second step (see Figure 3). T1 is the first test step, T2 the second test step. Cells were withdrawn by washing off morphine. #Denotes significantly different between T1 and T2 in the same condition ($P<0.05$, paired t-test); *Denotes significantly different between cells in morphine and following morphine wash ($P<0.05$, paired t-test).

washed off the cells, the risetime of T1 became significantly faster ($P<0.01$, $n=6$), but the risetime of T2 did not change. Similar results were obtained from cells isolated from vehicle treated rats (Table 2).

When there was no depolarizing step between the two test pulses, there was no difference between the amplitude or risetime of the I_{Ba} evoked by the test pulses in the presence of morphine (Table 2, Figure 3b). Withdrawal from morphine produced a similar increase in the amplitude of each test step in cells from morphine-dependent (T1 increased by $46\pm7\%$, T2 by $43\pm7\%$) and vehicle-treated (T1 increased by $57\pm10\%$, T2 by $54\pm9\%$) animals. The 0–95% risetime of the I_{Ba} evoked by both T1 and T2 was significantly shortened by washing off morphine (Table 2).

Concentration-response relationships for morphine and met-enkephalin inhibition of I_{Ba} were determined in neurons washed free of morphine (Figure 4). One or more concentrations of agonist were applied to cells stepped repetitively from −90 to −10 mV. Estimates of potency (pD_2) were made by fitting a logistic function to the pooled concentration-response data (Figure 4 and Table 3). Met-enkephalin and morphine both effectively inhibited I_{Ba} in neurons from morphine-dependent and vehicle-treated rats. The potency of met-enkephalin to inhibit I_{Ba} was slightly reduced in cells from morphine-dependent rats, with no apparent difference in the response elicited by maximally effective concentrations of agonist (3–10 μM) between cells from either treatment group. Morphine was less potent than met-enkephalin in inhibiting I_{Ba} in LC neurons, and maximally effective concentrations of morphine produced less inhibition of I_{Ba} than met-enkephalin (Table 3). There was no difference in the potency of morphine between morphine-dependent and vehicle-treated rats, however, the inhibition of I_{Ba} by maximally effective concentrations of morphine (3–10 μM) was less in neurons from morphine-dependent rats than in vehicle-treated rats ($P<0.025$, unpaired t-test). The inhibition of I_{Ba} in spontaneously withdrawn LC neurons by the α2-adrenoceptor agonist UK14304 (3μM) was the same in cells from morphine-dependent ($41\pm2\%$, $n=17$) and vehicle-treated ($41\pm2\%$ $n=13$) rats.

The contribution of various types of calcium channel to the I_{Ba} in LC neurons was determined by application of maximally effective concentrations of one or more calcium channel inhibitors to spontaneously withdrawn cells stepped repetitively from −90 to −10 mV (Figure 5). The order of application of inhibitors was varied between experiments. Application of ω-conotoxin GVIA (1 μM) or ω-agatoxin IVA (500 nM) inhibited the largest components of I_{Ba}, while lesser amounts were sensitive to nimodipine (3 μM), or resistant to application of all three blockers (Figure 5). There was no difference in the amount of I_{Ba} inhibited by any blocker between neurons from morphine-dependent or vehicle-treated rats, and there was no difference in the amount of resistant I_{Ba}. In these experiments no attempt was made to assess the reversibility of the toxins employed and a more detailed description of the actions of these agents on LC neurons will be presented elsewhere.

Discussion

In this study we have demonstrated that the opioid agonists morphine and met-enkephalin effectively modulate the I_{Ba} in acutely isolated LC neurons from young adult rats, and that this modulation is only modestly attenuated in neurons from morphine-dependent rats. Neither the total density of calcium channels nor the proportions of the various types of pharmacologically identified types of calcium channel differed between neurons taken from vehicle-treated or morphine-dependent rats. These results are consistent with previous studies demonstrating a modest tolerance to the *in vitro* μ-opioid activation of potassium channels in LC neurons from morphine-dependent rats (Christie *et al*., 1987), as well as tolerance to morphine inhibition of firing rates in LC neurons *in vitro* (Andrade *et al*., 1983; Bell & Grant, 1998) and *in vivo* (Aston-Jones *et al*., 1997).

This study also demonstrates that LC neurons express a range of pharmacologically distinguishable calcium channel types. Most of the I_{Ba} was inhibited by either ω-conotoxin GVIA or by ω-agatoxin IVA, with a lesser component sensitive to nimodipine and another insensitive to all three blockers used. The types and relative proportions of the calcium channels in LC neurons from young adult rats are similar to those recently described in very young animals (Chieng & Bekkers, 1999). Irreversible inhibition by ω-conotoxin GVIA is generally accepted to define N-type calcium channels, which are formed by the Class 1B α-subunit (Dubel *et al*., 1992), while ω-Agatoxin IVA-sensitivity is generally accepted to define P/Q-type calcium channels (Mintz *et al*., 1992; Randall & Tsien 1995), which are formed by alternative splicing of the α_{1A} subunit (Mori *et al*., 1991; Bourinet *et al*., 1999). In this study we made no attempt to distinguish P-type channels from Q-type channels. However, preliminary studies in LC neurons from untreated rats indicate a low concentration of ω-Agatoxin IVA (50 nM), which is maximally effective for inhibiting for P-type channels (Mintz *et al*., 1992), only inhibits a small fraction of the whole cell I_{Ba} (Connor & Christie, unpublished observations). The pharmacological identification of N-type, P/Q-type and L-type

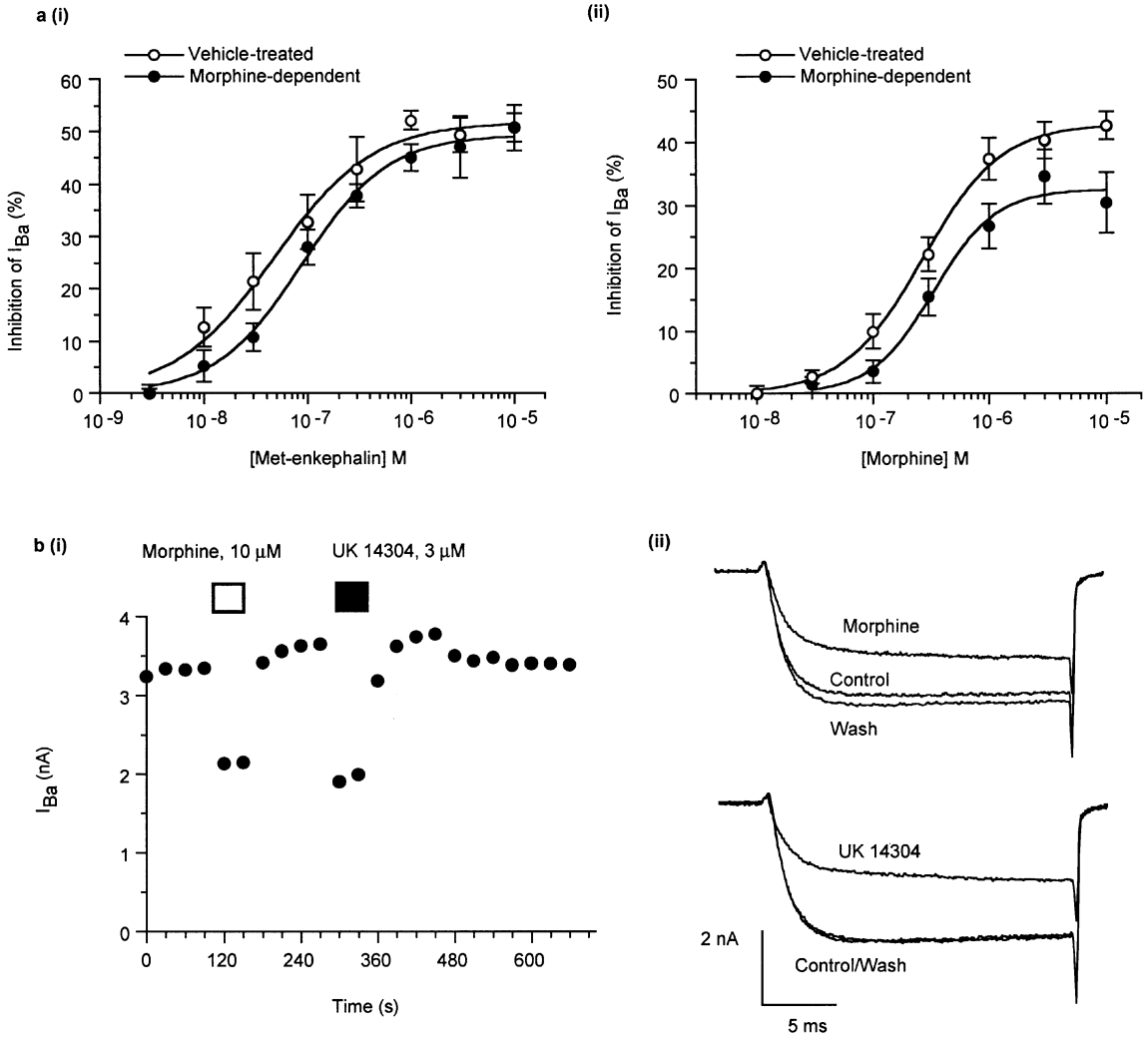

Figure 4 Concentration-dependent modulation of I_{Ba} in LC neurons from morphine-dependent rats by met-enkephalin and morphine. Neurons were spontaneously withdrawn from morphine, I_{Ba} was evoked by stepping the cells repetitively from −90 to −10 mV. Each point on the concentration-response curves represents between 5 and 9 cells tested. a (i) Concentration-response relationships for met-enkephalin in neurons from morphine-dependent (EC_{50} 90 nM) and vehicle-treated (EC_{50} 45 nM) rats. The EC_{50} and maximum effect of met-enkephalin did not differ between treatments. (ii) Concentration-response relationships for morphine in neurons from morphine-dependent (EC_{50} 330 nM) and vehicle-treated (EC_{50} 270 nM) rats. The maximum effect of morphine was significantly less in morphine-dependent rats ($P<0.05$). b (i) A time plot of the amplitude of I_{Ba} at −10 mV, illustrating the effects of morphine and the α2-adrenoceptor agonist UK14304 on I_{Ba}. (ii) Selected traces from the same experiment.

Table 3 Potency of opioid agonist inhibition of I_{Ba} in LC neurons from vehicle-treated and morphine-dependent rats

	Met-enkephalin		*Morphine*	
	pD2	*Maximum inhibition*	*pD2*	*Maximum inhibition*
	(95% Confidence intervals)			
Vehicle-treated	7.32 (7.12–7.52)	52% (47–56%)	6.57 (6.51–6.63)	43% (41–44%)
Morphine-dependent	7.06 (6.94–7.17)	49% (46–52%)	6.47 (6.30–6.65)	33% (29–36%)*

Opioid potencies were derived from concentration response relationships generated as described in the text. *Denotes significantly different from vehicle treated cells ($P<0.05$) of same treatment.

calcium channels in LC neurons is consistent with the presence of mRNA for A-, B-, C- and D-class α subunits in the region, as detected by *in situ* hybridization (Tanaka *et al*., 1995). Cells in the region of the LC have also been shown to express α_{1A}-subunit-like immunoreactivity (Craig *et al*., 1998). Acutely isolated LC cells did not have a prominent low voltage-activated component of their whole cell I_{Ba}, consistent with very low levels of mRNA for the α_{1G}, α_{1H} and α_{1I} subunits detected by *in situ* hybridization in the LC region (Talley *et al*., 1999). A substantial fraction of the whole cell I_{Ba} was resistant to all three antagonists used, but completely abolished by 30 μM Cd^{2+}, which is consistent with this current being of the R-type (Zhang *et al*., 1993). Some toxin-resistant currents are likely to be encoded by the α_{1E} pore forming subunit (Soong *et al*., 1993), unfortunately there is no information regarding the expression of α_{1E} mRNA in the brainstem.

The molecular basis for tolerance to morphine in the LC has not been established. A change in opioid receptor binding in the LC following chronic morphine treatment has not been demonstrated, nor is there consistent evidence for such changes in other brain regions (reviewed in Cox, 1993). Similarly, no changes in the numbers or types of ion channels modulated by morphine have been observed (this study, Christie *et al*., 1987). Several studies have reported alterations in parameters of G-protein function in the LC region, but it is not clear how these changes would produce homologous

μ-opioid receptor tolerance. In membranes from the LC region of morphine-dependent rats, the absolute amount of basal and μ-opioid agonist-stimulated G-protein activation was reduced compared to control membranes (Selley *et al*., 1997; Sim *et al*., 1996). However, the relative stimulation of G-protein activity by either morphine or DAMGO was unchanged, indicating little change in the functional effectiveness of opioid agonists in this paradigm (Selley *et al*., 1997; Sim *et al*., 1996). Unfortunately, the effects of opioid agonists on G-protein function were not compared with those of agonists at other receptors, so no conclusions can be drawn about the specificity of the observed effects. These studies on G-protein function contrast with an earlier study demonstrating a modest increase in the amount of immunoreactive $G_{\alpha i}$ and $G_{\alpha o}$ in membranes from the LC region of dependent rats (Nestler *et al*., 1989).

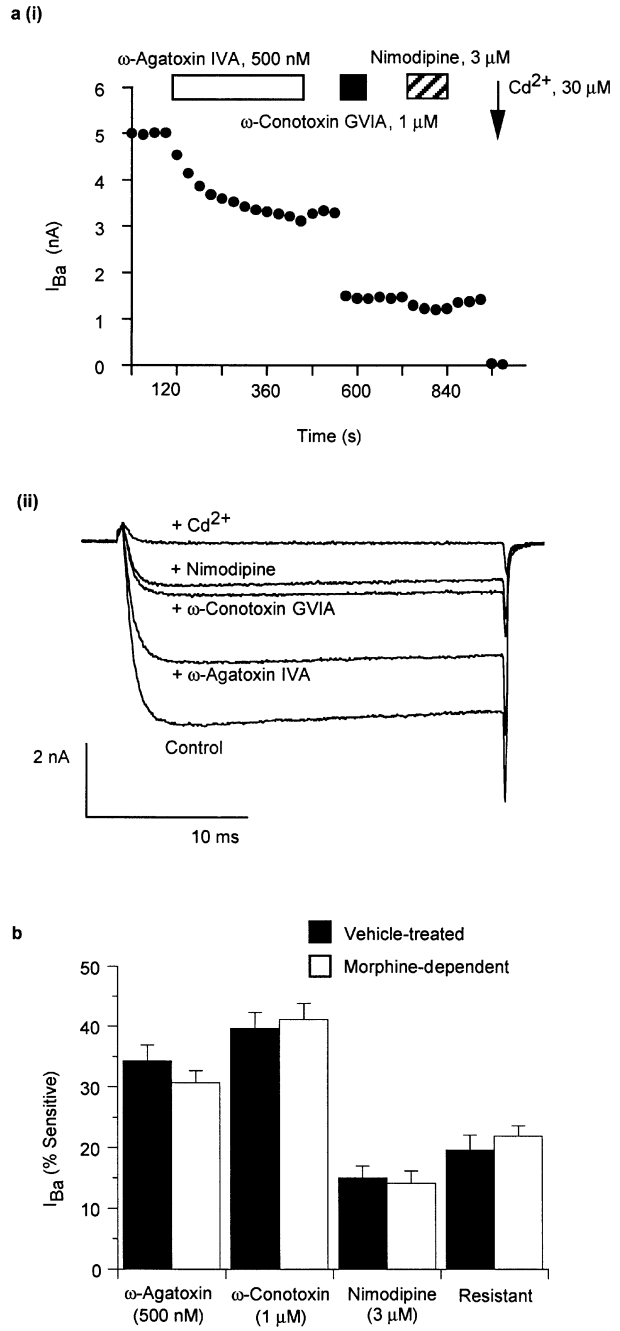

Figure 5 Pharmacological identification of the types of calcium channels in LC neurons from morphine-dependent rats. Neurons were spontaneously withdrawn from morphine, I_{Ba} was evoked by stepping the cells repetitively from -90 to -10 mV. a (i) A time plot of the amplitude of I_{Ba} at -10 mV, illustrating the effects of superfusion of a series of calcium channel antagonists on I_{Ba}. (ii) Selected traces from the same experiment, showing the current remaining after superfusion of the P/Q-type calcium channel antagonist ω-agatoxin IVA, the N-type calcium channel antagonist ω-conotoxin GVIA, the L-type calcium channel antagonist nimodipine and the non-selective calcium channel blocker Cd^{2+}. (b) A summary of the relative contributions of each of the components of I_{Ba} to the whole cell current. For antagonists, each bar represents the mean $\pm$ s.e.mean of between 9 and 14 cells. Resistant I_{Ba} represents the current insensitive to all of the antagonists, and was determined in seven cells from vehicle-treated rats, and nine cells from morphine-dependent rats.

Both naloxone and CTAP induced an increase of I_{Ba} in cells maintained in 5 μM morphine, indicating the involvement of μ-opioid receptors in the continued morphine modulation of I_{Ba}. High concentrations of the κ-opioid receptor antagonist nBNI (Birch *et al*., 1987; Takemori *et al*., 1988) or the δ-opioid receptor antagonist ICI-174864 (Cotton *et al*., 1984) had no effect on the morphine modulation of I_{Ba}. There is no evidence for the presence of post-synaptic κ-opioid receptors in rat LC (Williams & North, 1984; Mansour *et al*., 1996), however modest δ-opioid receptor immunoreactivity has been found in the dendrites of LC neurons (van Bockstaele *et al*., 1997). We found no evidence in this study that morphine modulates I_{Ba} *via* δ-receptors, in either morphine-dependent or vehicle-treated rats, which is consistent with previous reports that δ-receptor are also not involved in opioid activation of inward rectifier I_K in LC neurons in slices (Williams & North, 1984).

Several lines of evidence indicate that the increase in I_{Ba} amplitude following washout of morphine or superfusion of μ-antagonists is likely to represent a simple reversal of continued morphine modulation of the current rather than a novel withdrawal effect. Firstly, the activation time of I_{Ba} was decreased by the removal of morphine in cells from dependent animals. Slowing of the activation of I_{Ba} is characteristic of the ubiquitous, G-protein $\beta\gamma$-subunit-mediated pathway of calcium channel inhibition (reviewed in Dolphin, 1998). Secondly, in LC cells maintained in morphine, an 80 ms step to $+80$ mV caused a significant facilitation of the evoked I_{Ba} as well as modest decrease in the risetime of the evoked I_{Ba}. This reflects another characteristic of the G-protein $\beta\gamma$-subunit-mediated pathway, namely relief from $\beta\gamma$ inhibition of the calcium channel which is afforded by a depolarizing conditioning step before the test step (Dolphin, 1998). It has been shown previously that such depolarizing steps relieve the acute inhibition of calcium channels by opioids in LC neurons (Ingram *et al*., 1997). Taken together, the decrease in the activation time of I_{Ba} accompanied by an increase in I_{Ba} amplitude upon agonist removal or following a positive conditioning step strongly suggests that washing off morphine simply reverses an ongoing, G-protein mediated modulation of I_{Ba}. Unfortunately we have been unable to keep LC neurons alive for the 8–10 h necessary to treat them with pertussis toxin in an attempt to block inhibitory G-protein function, and the size and geometry of LC cells does not allow for ready dialysis of acute inhibitors of G-proteins such as GDPβS, so we have been unable to test this hypothesis more directly.

The increase in I_{Ba} when morphine was removed was of a similar magnitude in cells from morphine-dependent and non-dependent animals, which suggests the increase did not represent a novel withdrawal effect. In cells from morphine-dependent rats I_{Ba} in the continued presence of 5 μM morphine was inhibited by 28% when compared to the current measured after removal of morphine, in cells from non-dependent animals 5 μM morphine inhibited I_{Ba} by 26%. These values

compare with a predicted inhibition for 5 μM morphine of about 33% for cells from morphine-dependent rats and 43% for cells from vehicle-treated rats. One might have expected the withdrawal increase to have been greater in the cells from vehicle-treated rats, and we cannot offer a definite explanation why this was not so. However, cells from vehicle-treated rats were exposed to 5 μM morphine for various lengths of time from sacrifice to 'withdrawal' of morphine, and it is possible that acute desensitization of the μ-receptor in vehicle-treated animals may have contributed to a lesser withdrawal-induced increase in I_{Ba}. The cells in which the potency of morphine and met-enkephalin were determined had been spontaneously withdrawn for periods of up 1 h, which has previously been shown to be sufficient time for the complete reversal of acute desensitization of μ-opioid responses in LC neurons *in vitro* (Harris & Williams, 1991).

The findings of the present study are similar to those of the only other study to directly address the effects of chronic morphine treatment on calcium channel function (Kennedy & Henderson, 1991; 1992). In the SH-SY5Y human neuroblastoma cell line, which expresses both μ- and δ-opioid receptors, chronic treatment with 1 μM morphine for 3–7 days induced a 7 fold decrease in the potency of the μ-opioid agonist DAMGO to inhibit I_{Ba}. There was, however, no change in the maximal inhibition by DAMGO. In contrast, the maximal inhibition of I_{Ba} by morphine was strongly reduced (Kennedy & Henderson, 1991). The inhibitory actions of noradrenaline on I_{Ba} were unchanged by chronic morphine treatment. SH-SY5Y cells express predominantly N-type I_{Ba} with a small amount of L-type current; neither the relative amount of each type of current nor their kinetic properties were changed by morphine treatment or acute morphine withdrawal (Kennedy & Henderson, 1992). Thus although chronic opioid treatment has been shown to alter the number of calcium channel antagonist binding sites in mouse and rat brain (Ramkumar & el-Fakahany, 1984; 1988), there is as yet no evidence for alterations in calcium channel expression or function in opioid receptor expressing cells made tolerant to morphine (this study, Kennedy & Henderson, 1991; 1992).

In conclusion, this study demonstrates that in morphine-dependent rats, morphine and met-enkephalin mediated modulation of somatic I_{Ba} in LC neurons displays only very modest tolerance when compared to untreated rats. Further, chronic morphine treatment does not alter the type or amount of I_{Ba} in LC neurons. These results provide more evidence that functional μ-opioid receptor coupling is not dramatically altered in the LC in morphine-dependent rats, as has been previously shown for μ-opioid receptor inhibition of LC firing rates (Andrade *et al*., 1983), activation of inwardly rectifying I_K (Christie *et al*., 1987), and inhibition of adenylyl cyclase (Duman *et al*., 1988). These modest changes in μ-opioid receptor function in the LC are similar to those found in hippocampus (Wimpey *et al*., 1989) and other brainstem nuclei (Sim *et al*., 1996); but are in sharp contrast to the profound changes in the nature of opioid-mediated inhibition of synaptic transmission in the PAG of morphine-dependent rats (Ingram *et al*., 1998).

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