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Attenuation of acute and chronic effects of morphine by the imidazoline receptor ligand 2-(2-benzofuranyl)-2-imidazoline in rat locus coeruleus neurons

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1 The aim of this study was to determine if 2-(2-benzofuranyl)-2-imidazoline (2-BFI) interacts with the opioid system in the rat locus coeruleus, using single-unit extracellular recordings.

2 In morphine-dependent rats, acute administration of the selective imidazoline receptor ligands 2-BFI (10 and 40 mg kg^{-1}, i.p. and 100 μg, i.c.v.) or valldemossine (10 mg kg^{-1}, i.p.) did not modify the naloxone-induced hyperactivity of locus coeruleus neurons compared with that observed in the morphine-dependent control group.

3 After chronic administration of 2-BFI (10 mg kg^{-1}, i.p., three times daily, for 5 days) and morphine, naloxone-induced hyperactivity and tolerance to morphine were attenuated. This effect was not observed when a lower dose of 2-BFI (1 mg kg^{-1}, i.p.) or valldemossine (10 mg kg^{-1}, i.p.) were used.

4 Acute administration of 2-BFI (10 and 40 mg kg^{-1}, i.p. and 100 μg, i.c.v.) but not valldemossine (40 mg kg^{-1}, i.p.) diminished the potency of morphine to inhibit locus coeruleus neuron activity *in vivo* (ED_{50} values increased by 2.3, 2.9; and 3.1 fold respectively). Similarly, the potency of Met^5-enkephalin to inhibit locus coeruleus neurons was decreased when 2-BFI (100 μM) was applied to rat brain slices (EC_{50} increased by 5.6; $P<0.05$).

5 The present data demonstrate that there is an interaction between 2-BFI and the opioid system in the locus coeruleus. This interaction leads to an attenuation of both the hyperactivity of locus coeruleus neurons during opiate withdrawal and the development of tolerance to morphine when 2-BFI is chronically administered. These results suggest that imidazoline drugs may prove to be useful agents for the management of opioid dependence and tolerance.

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Abbreviations: 2-BFI, 2-(2-benzofuranyl)-2-imidazoline; ANOVA, analysis of variance; LC, locus coeruleus; ME, Met^5-enkephalin

Introduction

Over the last 20 years it has been well established that a group of substances with an imidazoli(di)ne/guanidine structure binds with high affinity to sites which have been termed imidazoline receptors. These receptors have been classified into three subtypes on the basis of their pharmacological profiles: I_1-imidazoline receptors (labelled by [^{3}H]-clonidine), I_2-imidazoline receptors (labelled by [^{3}H]-idazoxan) and non-I_1/I_2-imidazoline receptors (Eglen *et al*., 1998). I_2-imidazoline receptors have been divided in I_{2A} and I_{2B} subtypes according to their affinity for amiloride (Miralles *et al*., 1993). Imidazoline receptors are present in a wide range of mammalian tissues including the central nervous system and some evidence indicates that these receptors are involved in several physiological and pathological processes (Eglen *et al*., 1998). Consequently, it has been proposed that ligands of these receptors may have a therapeutic interest in hypertension, pancreatic endocrine dysfunction, inflammation, neuroprotection and some psychiatric disorders (Eglen *et al*., 1998; García-Sevilla *et al*., 1999).

A number of studies have suggested that substances with high affinity for imidazoline receptors, such as 2-BFI or valldemossine, may interact with the opioid system. Thus, imidazoline receptor ligands, including agmatine, the putative endogenous agonist, modulate the analgesic effect of morphine in mice (Kolesnikov *et al*., 1996; Fairbanks & Wilcox, 1997; Sánchez-Blázquez *et al*., 2000; Yesilyurt & Uzbay, 2001) and in rats (Horváth *et al*., 1999) and block the development of tolerance to morphine effects (Kolesnikov *et al*., 1996; Boronat *et al*., 1998). Moreover, acute and chronic treatments with imidazoline substances attenuate the characteristic behavioural signs observed during naloxone-precipitated morphine withdrawal (Aricioglu-Kartal & Uzbay, 1997; Hudson *et al*., 1999). In addition, the density of I_2 imidazoline sites is decreased in the brains of heroin addicts (Sastre *et al*., 1996) and of rats chronically treated with morphine (Hudson *et al*., 1999). Overall, these results suggest that imidazoline ligands could be of use in the treatment of chronic pain, since these substances may attenuate the development of tolerance and dependence.

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The cellular and molecular alterations which underlie opioid addiction are complex and not completely understood. There are many brain areas involved, including the locus coeruleus nucleus (LC) (Maldonado & Koob, 1993). Electrophysiological studies have clearly established that chronic administration of morphine leads to a reduction in the inhibitory effects of morphine (tolerance) in these cells (Aghajanian, 1978) and that morphine withdrawal induces a hyperactivity of LC neurons (Aghajanian, 1978; Rasmussen *et al*., 1990). Thus, some drugs which modulate LC activity attenuate the severity of the opioid withdrawal syndrome (see Melichar *et al*., 2001). In this regard, it is noteworthy that firing of LC neurons have been shown to be modulated by imidazoline drugs (Pineda *et al*., 1993; Ruiz-Ortega & Ugedo, 1997; Ugedo *et al*., 1998).

The aim of this study was to evaluate the interaction between imidazoline compounds and the opioid system, by using single-unit extracellular recordings in LC neurons *in vivo* and *in vitro*. To this end, we examined the modulation of the acute and chronic effects of opioid agonists by the high selective imidazoline ligands, 2-BFI (Lione *et al*., 1996, 1998) and valldemossine (Ozaita *et al*., 1997).

Methods

Animal treatment and preparation

Adult, male, albino Sprague-Dawley rats weighing 250–300 g were housed under controlled environmental conditions (22°C and a 12-h light/dark cycle) with free access to food and water. For chronic treatments with morphine, rats were injected intraperitoneally (i.p.) three times daily (at 0800, 1400 and 2000 h) for 5 days with increasing doses of the opiate as follows: day 1: 10, 10 and 10 mg kg^{-1}; day 2: 10, 10 and 20 mg kg^{-1}; day 3: 20, 20 and 40 mg kg^{-1}; day 4: 40, 40 and 80 mg kg^{-1}; day 5: 80, 80 and 100 mg kg^{-1}. We have previously shown (Pineda *et al*., 1998) that this treatment induces morphine dependence in rats. Consequently, naloxone administration (5 mg kg^{-1}, i.p. on day 6) precipitates a behavioural abstinence syndrome and a hyperactivity of LC neurons; as described by Pineda *et al*. (1998). Control animals were injected with an equivalent volume of saline solution (NaCl 0.9% in water) for 5 days. To evaluate the chronic effect of imidazolines, in some groups, 2-BFI (1 or 10 mg kg^{-1}, i.p), valldemossine (10 mg kg^{-1}, i.p) or saline was injected, for 5 days, 30 min before each morphine or saline administration as described Boronat *et al*. (1998). In another set of experimental groups, 2-BFI (10 or 40 mg kg^{-1}, i.p. or 100 μg, i.c.v.), valldemossine (10 mg kg^{-1}, i.p.) or saline (i.p. and i.c.v.) was injected 30 min before naloxone administration into animals chronically treated with morphine or saline, in order to study the acute effect of imidazolines.

Rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.). For *in vivo* experiments, a tracheal cannula was inserted and the right jugular vein was cannulated for additional administrations of anaesthetic and other drugs. The body temperature was maintained at ~37°C for the entire experiment by means of a heating pad connected to a rectal probe. The head was oriented at 15° to the horizontal plane (nose down), the skull was exposed and a 3-mm burr hole was drilled in the occipital bone over the LC. An electrode was placed 1.1 mm lateral to the midline and 3.7 mm caudal to the lamboid fontanel (Paxinos & Watson, 1986). In some experiments, a 23-gauge steel catheter was inserted into the left lateral ventricle 1.0 mm posterior and 1.3 mm lateral to bregma, at a depth of 4–5 mm from the skull surface, to which it was fixed with dental cement. The intraventricular position was controlled by inspection of the level of an air bubble in a plastic tube connected to the cannula.

For *in vitro* experiments, brains were extracted and coronal brainstem slices of 600 μm thickness including the LC were incubated at 33°C in a brain slice chamber continuously superfused with artificial cerebrospinal fluid (aCSF) at a rate of 1.5 ml min^{-1}. The chamber was a modified design of a gas-liquid interface type, such that an excellent perfusion to the slice could be maintained (Alreja & Aghajanian, 1995). The LC was visually identified as a dark oval area in the upper pons on the lateral border of the central gray and the IVth ventricle, just anterior to the genu of the facial nucleus. Drugs were dissolved in the aCSF and applied to the tissue by changing the superfusion medium by means of a tree-way stopcock valve; 45 s were required until a drug reached the slice.

Extracellular recordings of LC neurons in vivo *and* in vitro

Single-unit extracellular recordings of LC neurons *in vivo* and *in vitro* were performed as described previously (Ugedo *et al*., 1998). The recording electrode was an Omegadot single-barrel glass micropipette filled with a 2% solution of Pontamine Sky Blue in 0.5% sodium acetate (*in vivo*) or 0.25 M NaCl (*in vitro*) and broken back to a tip diameter of 1–2.5 μm. The impedance of the electrode, measured in 0.9% saline at 135 Hz was $2-5 \times M\Omega$. The extracellular signal from the electrode was amplified with a high-input impedance amplifier and then monitored on an oscilloscope and also with an audio monitor. The firing rate was obtained by a window discriminator and represented by the PC computer programme HFCP (Cibertec S.A., Madrid, Spain) as 10 s time histograms. LC neurons were identified by standard criteria that included: spontaneous firing at a regular rhythm, a slow firing rate between 0.5 and 5 Hz and characteristic spikes with a long-lasting > 2 ms, positive–negative waveform.

To study the modulation by imidazoline drugs of chronic morphine effects, three to six consecutive LC cells were randomly recorded (3 min each cell) and the mean firing rate was obtained for each experimental condition: 30 min before imidazoline drug administration, 10–30 min after imidazoline administration and 3–30 min after naloxone injection. To study the inhibitory effect of opioid agonists on LC neurons, dose-effect curves for morphine (0.3–19.8 mg kg^{-1}, i.v.) were performed *in vivo* in each experimental situation, and concentration-effect curves for Met^5-enkephalin (ME, 0.05–10 μM; applied for 1 min) were carried out *in vitro*. These opioid agonists were chosen because they mediate effects on LC neurons through μ-opiod receptor and are very well characterized, *in vivo* and *in vitro*, by other authors (Aghajanian, 1978; Williams & North, 1984; Alreja & Aghajanian, 1993).

Analysis of data and statistics

The inhibition of LC cells by opioid agonists was quantified as the percentage reduction of the basal firing rate. Dose/concentration-effect curves were analysed for the best simple non-linear fit to the three-parameter logistic equation $E = E_{max}/\{1 + (ED_{50}$ or $EC_{50}/A)^n\}$, where E is the effect induced by a certain dose of morphine or concentration of ME (A), E_{max} is the maximal effect, ED_{50} is the dose of morphine and EC_{50} is the concentration of ME needed to elicit 50% of E_{max}, and n is the slope factor of the dose-effect curve (Parker & Waud, 1971). ED_{50}, E_{max} and n were estimated by this analysis (for practical reasons, E_{max} was constrained to be 100% since maximal inhibition was always complete). Experimental data were analysed by using the computer program GraphPad Prism (v. 3.0, GraphPad Software, Inc.). Statistical significance was assessed by means of two-way repeated measures analysis of variance (ANOVA; imidazoline/naloxone effect, treatment effect and imidazoline/naloxone x treatment interaction), one-way ANOVA followed by the Newman–Keuls test for comparisons between more than two groups, or the paired Student's t-test for comparisons of the firing rate before and after drug application. Group differences in ED_{50}, *in vivo*, were evaluated by an analysis of covariance (ANCOVA; taking basal discharge rate into consideration as a covariate). The level of significance was considered as $P < 0.05$. Data are reported as mean ± s.e.mean.

Drugs

Drugs were obtained from the following sources: Met^5-enkephalin (ME) from Sigma (Madrid, Spain); morphine hydrochloride from Alcaliber, S.A. (Madrid, Spain); naloxone hydrochloride from Research Biochemical Inc (Natick, MA, U.S.A.); 2-(2-benzofuranyl)-2-imidazoline HCl (2-BFI) and valldemossine (LSL 61122) from Lasa laboratories (Spain). For systemic administrations, drugs were dissolved in 0.9% NaCl. For i.c.v. applications, 2-BFI was dissolved in Dulbecco's buffered saline solution containing (in mM): NaCl 136.9, KCl 2.7, NaH_2PO_4 8.1, KH_2PO_4 1.5, $MgCl_2$ 0.5 and $CaCl_2$ 0.9 (pH ~ 7.4). For *in vitro* applications, drugs were dissolved in standard artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 128, KCl 3, NaH_2PO_4 1.25, glucose 10, $NaHCO_3$ 25, $CaCl_2$ 2 and $MgSO_4$ 2 saturated with 95% O_2/5% CO_2 (pH ~ 7.34).

Results

Acute administration of 2-BFI or valldemossine does not modify the naloxone-induced hyperactivity of locus coeruleus neurons in morphine-dependent rats

In an initial series of experiments, we studied the effect of acute administration of 2-BFI and valldemossine on LC neurons in rats chronically treated with morphine. As expected (Pineda *et al*., 1998), the basal firing rate of LC neurons, before imidazoline administration, was slightly reduced in morphine-dependent rats (1.30 ± 0.12 Hz) compared with saline-treated animals (1.53 ± 0.14 Hz) (Figure 1A,B). Administration of 2-BFI (10 and 40 mg kg^{-1}, i.p. and 100 μg, i.c.v.) increased the firing rate of LC neurons in all experimental groups. Two-way repeated measures ANOVA (imidazoline effect, treatment effect and imidazoline x treatment interaction) revealed no significant differences in the effect of the imidazolines on the firing rate of LC neurons between saline and morphine-treated rats, ($F_{[3,34]} = 1.268$, $P = 0.301$; compare Figure 1A and B). On the other hand, valdemossine did not alter the firing rate of LC neurons in either group.

Neurons of morphine-dependent rat were hyperactive after naloxone administration (5 mg kg^{-1}, i.p.), with firing rate values being increased by 3.3 fold (Figure 1B). Two-way repeated measures ANOVA (naloxone effect, treatment effect and naloxone x treatment interaction) revealed that neither 2-BFI (10 and 40 mg kg^{-1}, i.p. and 100 μg, i.c.v.) nor valldemossine (10 mg kg^{-1}, i.p.) administered 30 min before naloxone, significantly modified the naloxone-induced hyperactivity of LC neurons in morphine-dependent rats ($F_{[3,36]} = 0.591$, $P = 0.625$; Figure 1B). Naloxone did not

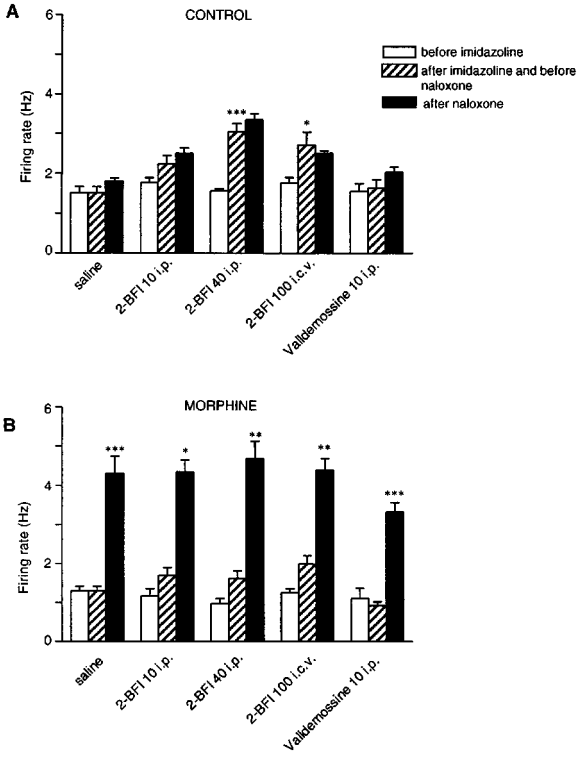

Figure 1 Bar histograms showing the mean firing rate of LC neurons before and after acute administration of the imidazoline receptor ligands 2-BFI (10 and 40 mg kg^{-1}, i.p. and 100 μg, i.c.v.) and valldemossine (10 mg kg^{-1}, i.p.) and after naloxone administration (5 mg kg^{-1}, i.p.) in control (A) and in morphine-dependent (B) rats. Note that 2-BFI was able to increase the firing rate of LC neurons in control and in morphine-dependent rats while valldemossine was without effect. Naloxone administration induced a hyperactivity of LC cells (increase of firing rate with respect to previous value in the same group) in morphine-dependent rats, to the same magnitude in rats acutely pretreated with the imidazoline ligands or saline. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, paired Student's t-test; the firing rate before imidazoline or naloxone was compared with that after the correspondent drug administration. Bars represent mean ± s.e.mean of $n = 5–7$ rats.

produce a significant effect in any of the groups of rats chronically treated with saline (Figure 1A).

Chronic treatment with 2-BFI attenuates the naloxone-induced hyperactivity and morphine tolerance of locus coeruleus neurons in morphine-dependent rats

In morphine dependent animals we studied the effect of chronic treatment (three times daily for 5 days, administered 30 min before each morphine injection) with 2-BFI (1 and 10 mg kg^{-1}, i.p.) or valldemossine (10 mg kg^{-1}, i.p.) on the naloxone-induced hyperactivity of LC neurons. The highest (10 mg kg^{-1}), but not the lowest (1 mg kg^{-1}, i.p.) dose of 2-BFI or valldemossine (10 mg kg^{-1}, i.p.), significantly attenuated; hyperactivity with respect to the group treated only with morphine (firing rate increment after naloxone 123 vs 231%; $P<0.01$; two way ANOVA) (Figures 2C,D and 3).

In consonance with other studies (Pineda *et al*., 1998), the dose-effect curves for morphine (0.3–19.2 mg kg^{-1}, i.v.) were shifted to the right after chronic treatment with morphine, leading to a 4 fold increase in the ED_{50} value (saline-morphine, $ED_{50}=3327\pm216$ μg kg^{-1}, $n=6$, $P<0.01$; one-way ANOVA followed by the Newman–Keuls test) with respect to the values obtained in the saline–treated group (saline–saline, $ED_{50}=784\pm106$ μg kg^{-1}; $n=5$) (Figure 4). However, when animals were treated chronically with morphine and 2-BFI (2-BFI-morphine), there were no significant differences in the dose-response curves ($ED_{50}=1822\pm243$ μg kg^{-1}; $n=6$) with respect to the group chronically treated only with 2-BFI (2-BFI-saline) ($ED_{50}=1072\pm180$ μg kg^{-1}; $n=5$, ANOVA followed by the Newman–Keuls test, $q=1.748$) (Figure 4B). The dose-effect curves for morphine after 2-BFI chronic treatments were not statistically different from those obtained in the saline treated group (ANOVA followed by the Newman–Keuls test, $q=0.643$).

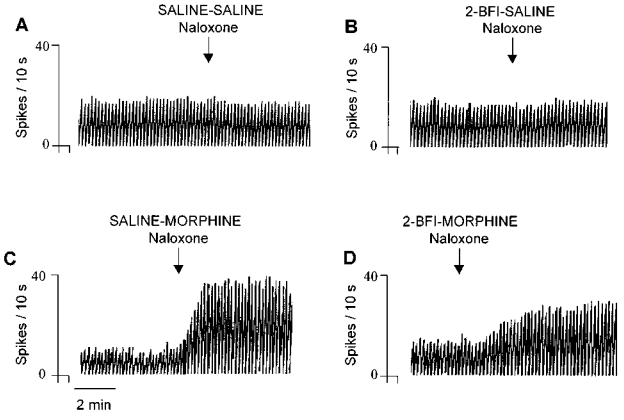

Figure 2 Representative examples of firing rate recordings from LC neurons showing the effect of naloxone (5 mg kg^{-1}, i.p.) in control (A and B) and in morphine-dependent rats (C and D). Vertical lines representing the extracellularly recorded firing rates were displayed as integrated time histograms (spikes per 10 s). The time scale refers to all traces. Note the increase in firing rate due to naloxone administration only in morphine-dependent rats and the attenuation of the naloxone effect in morphine-dependent rats chronically treated with 2-BFI.

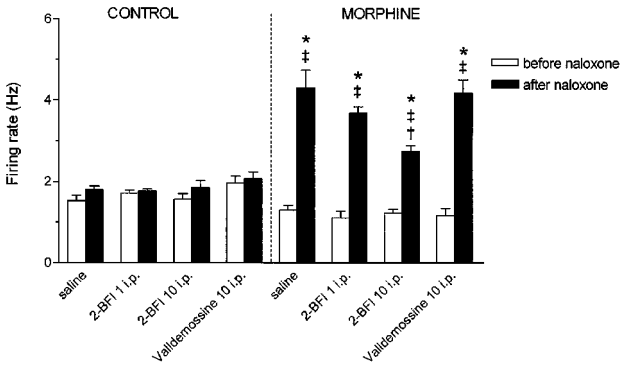

Figure 3 Bar histograms showing the mean firing rate before and after naloxone (5 mg kg^{-1}, i.p.) in control and morphine-dependent animals simultaneously treated (three times daily for 5 days), with 2-BFI (1 or 10 mg kg^{-1}, i.p.) or valldemossine (10 mg kg^{-1}, i.p.). *$P<0.001$; the firing rate after naloxone was compared with that before naloxone; paired Student's t-test. ‡$P<0.005$; the effect of naloxone in each morphine group was compared with the corresponding effect in the control group; two-way ANOVA. †$P<0.05$; the effect of naloxone in the 2-BFI-morphine group was compared with that of the saline-morphine group; two-way ANOVA. Bars represent mean±s.e.mean of $n=5–7$ rats.

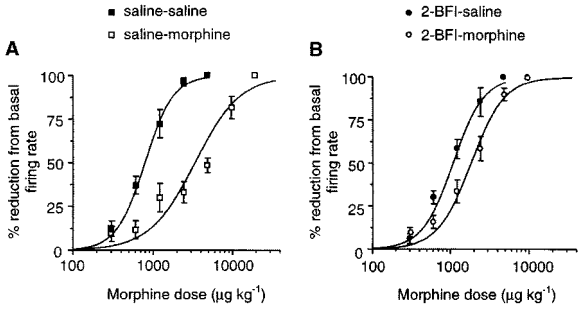

Figure 4 Dose-response curves illustrating the inhibitory effect of morphine on LC neurons in control and in morphine-dependent rats after chronic treatment with saline solution (A) or 2-BFI (B). Symbols represent means±s.e.mean of the percentage reduction from the basal firing rate of $n=5–7$ rats. The horizontal axis represents the cumulative doses of morphine administered i.v. Note that in morphine-dependent rats (saline-morphine), the ED_{50} value was shifted to the right 4 fold with respect to the control group ($P<0.01$; one-way ANOVA followed by Newman–Keuls test) (A). After chronic treatment with 2-BFI, no significant differences were observed between morphine-dependent and control rats (B).

Acute administration of 2-BFI but not valldemossine reduces the potency of morphine and ME to inhibit LC neuron activity in naïve rats in vivo *and* in vitro

We next studied the effect of 2-BFI administration on the dose-effect curves for morphine (0.3–19.2 mg kg^{-1}, i.v.) in LC neurons from naïve rats. After 2-BFI administration (10 and 40 mg kg^{-1}, i.p. or 100 μg, i.c.v. 30 min before morphine), the dose-response curves for morphine were shifted to the right (Figure 5). ANCOVA revealed that there were significant differences of ED_{50} values between 2-BFI pretreated and the control groups ($F_{[4,18]}=4.357$, $P=0.012$) with the ED_{50} values being increased by 2.3 ($n=5$, $P<0.05$), 2.9 ($n=5$, $P<0.01$) and 3.1 ($n=4$, $P<0.05$) fold respectively

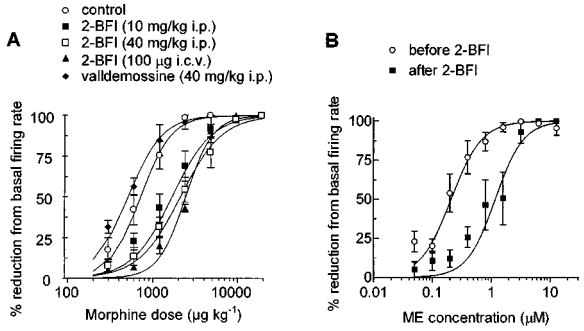

Figure 5 (A) Effects of acute treatments with imidazoline drugs on the morphine-induced inhibition of the firing rate of LC neurons in anaesthetized rats. Dose-effect curves for the inhibitory effect of morphine were elaborated from data obtained 30 min after imidazoline drug administration. (B) Concentration-effect curves for the inhibitory effect of ME on the firing rate of LC neurons *in vitro* were constructed before and after the application of 2-BFI. Symbols represent means ± s.e.mean of the percentage reduction from the basal firing rate of $n = 5-6$ rats. The horizontal axis represents the cumulative doses of morphine administered i.v. and cumulative concentrations of ME.

with respect to the values in the control group ($ED_{50} = 720 \pm 127$ μg kg^{-1}; $n = 5$). However, administration of valldemossine (40 mg kg^{-1}, i.p. 30 min before morphine), which did not alter the basal firing rate (1.74 ± 0.20 and 1.76 ± 0.18 Hz; $n = 5$, before and after drug administration, respectively), did not significantly modify the dose-response curves for the inhibitory effect of morphine on LC neurons ($ED_{50} = 514 \pm 44$ μg kg^{-1}; $n = 5$) (Figure 5A).

For *in vitro* experiments, ME was used, as μ opioid receptor agonist since it causes a rapidly reversible effect that can be tested in the same cell before and after 2-BFI perfusion. The concentration of 2-BFI applied (100 μM for 10 min) did not significantly change the firing rate of LC neurons as previously shown (Ugedo *et al*., 1998). Bath perfusion with ME (0.05 – 10 μM, 45 s) caused a concentration-dependent inhibition of the spontaneous firing rate of LC cells (Figure 5B). Perfusion with 2-BFI (100 μM for 10 min) shifted to the right the concentration-effect curve for ME (EC_{50} 0.21 ± 0.06 μM vs 1.18 ± 0.31 μM, $n = 6$, $P < 0.05$; paired Student's *t*-test) (Figure 5).

Discussion

The main finding of this work is that the imidazoline ligand 2-BFI reduces some effects of morphine on LC neuron firing rate. Thus 2-BFI, when administered chronically, induces an attenuation of morphine tolerance and of the electrophysiological hyperactivity induced by morphine withdrawal. In addition, when administered acutely, it decreases the potency of opioid agonists to inhibit LC neurons.

Agmatine, the putative endogenous ligand for imidazoline receptors, dose dependently and significantly attenuates all of the behavioural signs of the morphine abstinence syndrome, which is precipitated in morphine-dependent rats by naloxone (Aricioglu-Kartal & Uzbay, 1997). In addition, the imidazoline selective ligand, BU224, alleviates parts of the morphine-abstinence syndrome (Hudson *et al*., 1999). Accordingly, in the present study chronically administered 2-BFI reduced in a dose-dependent manner the LC cell hyperactivity induced by naloxone in morphine dependent rats. It has repeatedly been shown by *in vivo* and *in vitro* studies that morphine inhibits the firing of LC neurons through μ-opioid receptors located in this nucleus (Aghajanian, 1978; Williams & North, 1984). Moreover, repeated morphine administration induces a state of morphine dependence, which is responsible for the hyperactivity of LC neurons during the morphine abstinence syndrome and also a state of tolerance to morphine (Aghajanian, 1978; Rasmussen *et al*., 1990; Pineda *et al*., 1998). Thus, a good correlation between LC hyperactivity and the degree of the behavioural syndrome associated with morphine withdrawal has been established (Rasmussen *et al*., 1990). Therefore the present results provide some clues about the neurobiological substrate for the alleviation of the morphine withdrawal syndrome by imidazoline ligands. Moreover, we also show that 2-BFI attenuated morphine tolerance in agreement with previous studies that have shown that concurrent, chronic administration of agmatine or 2-BFI also prevents tolerance to morphine-induced spinal antinociception in mice and rats (Kolesnikov *et al*., 1996; Boronat *et al*., 1998).

2-BFI is a highly selective imidazoline ligand which has little affinity for opioid receptors ($K_i > 100{,}000$ nM) (Lione *et al*., 1996; 1998) but, like morphine (see references above), it acts directly on LC neurons (Ugedo *et al*., 1998). This imidazoline reduced the effects of morphine *in vivo* and of ME *in vitro* (Figure 5). The latter finding indicates that the interaction between 2-BFI and the opioid system occurs within the LC, rather than being the consequence of a pharmacokinetic interaction. In a previous study we have shown that 2-BFI increases the firing rate of LC neurons *in vivo* and *in vitro* and that the sites, which mediate this effect, resemble non-I_1/I_2-imidazoline receptors. These sites are coupled to ATP-sensitive K^+ channels both in the LC (Ugedo *et al*., 1998) and in other tissues (Chan *et al*., 1991; Olmos *et al*., 1994). On the other hand, it has been suggested that morphine induces part of its antinociceptive effects by opening ATP-sensitive K^+ channels (Ocaña *et al*., 1995). Consequently, it is possible that 2-BFI may interact with opiate agonists at the level of these ATP-sensitive K^+ channels present in the LC (Finta *et al*., 1993). Alternatively, some analogies have been described between these K^+ channels and G protein-coupled-inward rectifying K^+ channels (Inanobe & Kurachi, 2000), which have been shown to be coupled to μ opioid receptors in the LC (Alreja & Aghajanian, 1993). Thus, it is also reasonable to propose that 2-BFI may modulate opiate action on these G-protein-coupled-inward rectifier K^+ channels.

Several studies have reported that chronic opioid agonist exposure reduces the levels of imidazoline receptor binding (Sastre *et al*., 1996; Hudson *et al*., 1999). However, no significant change in the potency of 2-BFI to stimulate LC neurons was observed in the present study, since the magnitude of the increase of LC neuron firing rates after morphine chronic treatment was similar to that observed in control animals (Figure 1A,B). This discrepancy may be due to the fact that binding studies may not distinguish changes in affinity due to conformational state changes. Additionally, differences in the ligands used in each study and in the chronic treatment protocols cannot be ruled out.

Although the potency of morphine to induce supraspinal analgesia is unaltered (Boronat *et al*., 1998) or increased (Sánchez-Blázquez *et al*., 2000) by acute imidazoline treatment, we observed a decrease in the inhibitory effect of morphine on LC neuron firing rates following application of 2-BFI. This apparent discrepancy may be due to the fact that imidazolines can modulate the synaptic levels of neurotransmitters used in analgesia pathways outside the LC. Thus, changes in the levels of dopamine (Barrot *et al*., 2000; Sastre-Coll *et al*., 2001) and 5-HT (Ugedo *et al*., 1999) in response to 2-BFI have been described in the central nervous system. Another explanation may be that while the effect of the imidazoline on the LC is mediated by non-I_1/I_2-imidazoline receptors (Ugedo *et al*., 1998), the analgesic effect is mediated by I_2-imidazoline receptors (Boronat *et al*., 1998; Sánchez-Blázquez *et al*., 2000). This latter hypothesis may also explain the discrepancy with regard to the lack of effect of valldemossine in our study, since it shows high affinity for I_2-imidazoline receptors (Ozaita *et al*., 1997) and interacts with morphine in the analgesic test (Sánchez-Blázquez *et al*., 2000). On the other hand, the lack of effect of valldemossine, in the present study, could also be due to pharmacokinetic reasons.

In conclusion, our study provides evidence that 2-BFI, an imidazoline drug with high affinity for imidazoline receptors, interacts with the opioid system in the rat LC. This interaction leads to a reduction in both the development of tolerance to morphine and the withdrawal-induced hyperactivity of LC neurons. On the other hand, it has been shown that 2-BFI also prevents tolerance to morphine-induced spinal anti-nociception in rats and may induce neuroprotective effects (Boronat *et al*., 1998). Altogether these results raise the possibility of reducing opiate dependence while preserving the anti-nociceptive effect of morphine, by concomitant administration of 2-BFI with morphine. Given the great importance of tolerance and dependence during the treatment of chronic pain with opioids in clinical practice, our results could be useful for the development of new strategies to treat chronic pain.

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