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Stimulation of locus coeruleus neurons by non-I_1/I_2-type imidazoline receptors: an *in vivo* and *in vitro* electrophysiological study

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1 Imidazoline binding sites have been reported to be present in the locus coeruleus (LC). To investigate the role of these sites in the control of LC neuron activity, we studied the effect of imidazolines using *in vivo* and *in vitro* single-unit extracellular recording techniques.

2 In anaesthetized rats, local (27 pmoles) and systemic (1 mg kg^{-1}, i.v.) administrations of 2-(2-benzofuranyl)-2-imidazoline (2-BFI), a selective I-imidazoline receptor ligand, increased the firing rate of LC cells (maximal increase: $22\pm5\%$, $P<0.001$ and $16\pm7\%$, $P<0.001$ respectively). Chronic pretreatment with the irreversible monoamine oxidase inhibitor clorgyline (3 mg kg^{-1}, i.p., every 12 h for 14 days) abolished this effect.

3 In rat midpontine brain slices containing the LC, bath application (1 mM) of the imidazolines 2-BFI, 2-(4,5-dihydroimidaz-2-yl)-quinoline (BU224), idazoxan, efaroxan, phentolamine and (2-2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline (RX821002) reversibly stimulated LC cells. The maximal effect was ~90% except for RX821002 and efaroxan which induced smaller maximal effects (~58% and ~35% respectively). Simultaneous application of idazoxan and 2BFI did not lead to additive effects.

4 Bath application of the α_2-adrenoceptor antagonists, yohimbine (1–10 μM) and N-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (10 μM), failed to modify LC activity. The irreversible blockade of α_2-adrenoceptors with EEDQ (10 μM) did not alter the effect of idazoxan or that of efaroxan. Previous application of clorgyline (10 μM) did not modify the excitatory effect of 2-BFI or efaroxan.

5 Changes in the pH of the bathing solution (6.84–7.84) did not influence the effect caused by idazoxan. Bath application of 2-BFI (1 mM) reversed the inhibition induced by diazoxide (300 μM), an ATP-sensitive K^+ channel opener, whereas application of glibenclamide (3 μM), an ATP-sensitive K^+ channel blocker, partially blocked the effect of 2-BFI.

6 This study shows that imidazoline compounds stimulate the firing rate of LC neurons. This effect is not mediated by α_2-adrenoceptors nor by I_1 or I_2-imidazoline receptors but involves a different subtype of imidazoline receptor. Our results indicate that this receptor is located extracellularly and modulates ATP-sensitive K^+ channels.

Keywords: ATP-sensitive K^+channel; imidazoline ligands; locus coeruleus; extracellular; single-unit recording; *in vivo*; *in vitro*

Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, one-way analysis of variance; 2-BFI, 2-(2-benzofuranyl)-2-imidazoline; BU224, 2-(4,5-dihydroimidaz-2-yl)-quinoline; EEDQ, N-ethoxycarbonyl-1,2-dihydroquinoline; LC, locus coeruleus; MAO, monoamine oxidase; RX821002, (2-2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline

Introduction

It is now well established that imidazoli(di)ne/guanidine compounds recognize binding sites named imidazoline receptors (for review, see Bousquet, 1995; Regunathan & Reis, 1996). An endogenous ligand for these imidazoline receptors was identified to be agmatine, a precursor of the polyamine pathway (Li *et al*., 1994). Two principal imidazoline receptor types have been defined: the I_1-imidazoline receptor, labelled by [^{3}H]-clonidine and the I_2-imidazoline receptor preferentially labelled by [^{3}H]-idazoxan (Michel & Ernsberger, 1992). Further pharmacological binding studies have shown that I_1-imidazoline receptors are recognized with high affinity by imidazolidines, with medium affinity by imidazolines and with low affinity by guanidines, whereas I_2-imidazoline receptors are labelled with high affinity by imidazolines and guanidines and with medium affinity by imidazolidines (Ernsberger *et al*., 1992). More recently, I_2-imidazoline receptors have been further subclassified on the basis of their amiloride sensitivity (I_{2A}) or insensitivity (I_{2B}) (Diamant *et al*., 1992; Miralles *et al*., 1993a). Finally a non- I_1/I_2-imidazoline receptor has been described in various peripheral tissues (Chan *et al*., 1994; Molderings & Göthert, 1995; Olmos *et al*., 1994). These receptor types are distinguished not only by their pharmacological profiles but also by their subcellular distribution: I_1-imidazoline receptors are located on the outer cell membrane (Piletz & Sletten, 1993), whereas I_2-imidazoline receptors are found mostly intracellularly on the outer membrane of mitochondria (Tesson & Parini, 1991). The brain distribution of each of these receptors is also different. I_1-imidazoline sites are found predominantly in the brain stem (Ernsberger *et al*., 1995), where they mediate hypotension elicited by imidazolidines or compounds with a similar chemical structure (for review, see Bousquet, 1995). I_2-imidazoline sites are widely distributed in the brain but their physiological function remains largely unknown. Nevertheless, the fact that several studies have revealed that the density of these binding sites is

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altered in human brain from patients with psychiatric disorders such as depression (Meana *et al*., 1993a,b; Sastre & Garcia-Sevilla, 1997) and heroin addiction (Sastre *et al*., 1996) suggests that these receptors might be implicated in the pathophysiology of such disorders.

One of the brain areas where a high density of I_2-imidazoline sites, labelled by [^{3}H]-rilmelidine or [^{3}H]-RS-45041-190, has been found is in the locus coeruleus (LC) (King *et al*., 1995; MacKinnon *et al*., 1995). This is the main noradrenergic nucleus in the CNS and has been implicated in the control of numerous functions such as anxiety, as well as vigilance attention, learning and memory, and affective behaviours (Svensson, 1987). Recently, we have shown that clonidine and a variety of centrally acting imidazolidines/oxazolines, stimulates the firing activity of LC neurons *in vivo* probably through I_1-imidazoline receptors, when the α_2-adrenoceptors are blocked with N-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (Pineda *et al*., 1993). This is an indirect effect mediated by excitatory amino acids (Ruiz-Ortega *et al*., 1995) which does not originate in the LC but rather in the ventrolateral medulla (Ruiz-Ortega & Ugedo, 1997). In fact, no effect on LC activity is found when clonidine and other imidazoline derivatives (after blocking α_2-adrenoceptors with EEDQ) (Szabo *et al*., 1996) or agmatine (Pineda *et al*., 1996) are applied to midpontine slices.

The aims of the present study were to investigate the functional significance of I-imidazoline binding sites in the LC and to characterize the pharmacological profile of these receptors. To this end, we performed single-unit extracellular recordings in anaesthetized rats and in rat brain slices containing the LC.

Methods

Animals and treatment

Adult male albino Sprague-Dawley rats weighing 200–300 g were used. The animals received a standard diet with water freely available and were housed at 22 ± 2°C with a 12 h light/dark cycle. All rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.). In some experiments, animals were treated with saline or the irreversible inhibitor of the monoamine oxidase (MAO) enzyme, clorgyline (3 mg kg^{-1} i.p., every 12 h for 14 days) and then LC cell firing rate was studied with *in vivo* recording. This protocol for clorgyline treatment down-regulates non-adrenoceptor [^{3}H]-idazoxan binding sites in the rat brain (Olmos *et al*., 1993). Since several authors have suggested that I_2-imidazoline sites are MAO regulatory sites (Tesson *et al*., 1995; Tesson & Parini, 1991) we also evaluated *in vitro* the effect of clorgyline in addition to an imidazoline, on the LC.

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

For *in vivo* experiments, the rat was placed in a stereotaxic frame after a tracheal cannula had been inserted and the right jugular vein had been cannulated for additional administrations of anaesthetic and other drugs. The body temperature of animals was maintained at 37°C throughout the 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 and 3.7 mm caudal to the lambda and 5.5–6.5 mm ventral to the cortical surface.

Brainstem slices were prepared as described (Pineda *et al*., 1996). Coronal slices of 600 μm thickness including the LC were incubated at 33°C in a brain slice chamber continuously perfused 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 aCSF consisted of (in mM): NaCl 126, 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 with a final pH of ~7.34. The electrode was positioned in the LC which was visually identified as a dark oval area in the upper pons on the lateral borders of the central gray and the fourth ventricle, just anterior to the genu of the facial nerve. Drugs were dissolved in the aCSF and applied to the tissue by changing the superfusion medium by means of a three-way stopcock valve; 45 s were required until a drug reached the slice.

Single-unit, extracellular recordings of LC cells were made as previously described (Pineda *et al*., 1993). The recording electrode was an Omegadot glass micropipette filled with a 2% solution of Pontamine sky blue in 0.5% sodium acetate and broken back to a tip diameter of 1–2 μM. The impedance of the electrode, measured in 0.9% saline at 135 Hz, was 2–5 MΩ. The signal from the recording electrode was passed through a high-input impedance amplifier and continuously monitored with an audiomonitor and also with an oscilloscope. Individual neuronal spikes were discriminated in a custom-made unit, fed into a computer, which generated interspike time interval histograms, into an electronic rate counter and finally into a pen chart recorder (creating 10 s consecutive samples). Only one cell was used in each animal when drugs were applied intravenously. In anaesthetized rats LC cells were identified by criteria described by Cedarbaum & Aghajanian (1976), which include a steady spontaneous firing rate between 0.5–5 Hz, a long-lasting positive-negative spike and a typical biphasic excitation-inhibition response to noxious stimuli applied to the contralateral hindpaw (paw pinch). Additional clues to the location of the LC were the relative electrical silence just dorsal to the LC (corresponding to the IVth ventricle) and the presence, just lateral to the LC, of the mesencephalic nucleus of the Vth nerve, whose cells were activated by proprioceptive stimulation of the face (Aston-Jones *et al*., 1982). In assays carried out *in vitro*, the noradrenergic neurons were identified by a steady spontaneous firing rate at ~1 Hz and a long-lasting positive-negative spike (Andrade & Aghajanian, 1984).

Pressure microinjection into the LC

A thick-walled pipette with a calibrated narrow inner diameter was broken at 2 μm from the tip and was glued adjacent to a recording micropipette (Akaoka *et al*., 1992). The calibrated pipettes were filled with 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 of 7.40), with or without 10 mM of 2-BFI. Drug ejection was performed by applying pressure pulses (50–150 ms) using a solenoid-controlled pneumatic pressure device (Picospritzer^{TM}II, General Valve Corp.) driven by synthetic air. The injected volume was measured by monitoring the meniscus movement in the calibrated pipette.

Analysis of data

Data are given as means ± s.e.mean. All effects on firing rates were normalized to the initial basal firing rate of each cell (the firing rate at the beginning of the experiment was taken as

100%). In some cases, cumulative concentration-response curves were constructed and analysed using the Microsoft Excel programme (version 95) as described by Bowen & Jerman (1995). Experimental data were analysed for the best non-linear fit to the logistic three-parameter equation described by Parker & Waud (1971): $E = E_{max}[D]^n/(EC_{50}{}^n + [D]^n)$, where [D] is the concentration of the drug, E is the effect on the firing rate induced by D, E_{max} is the maximal change at 'infinite' concentration, EC_{50} is the effective concentration for eliciting 50% of E_{max}, and n is the slope factor of the function.

Statistical significance was assessed by means of one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons, the Student's *t*-test for comparisons of two independent groups or the paired Student's *t*-test for comparisons of the firing rate before and after drug application. The level of significance was chosen as $P = 0.05$.

Drugs

2-(2-Benzofuranyl)-2-imidazoline HCl (2-BFI), and idazoxan HCl (Lasa Laboratories, Spain); 2-(4,5-dihydroimidaz-2-yl)-quinoline HCl (BU224), diazoxide, efaroxan HCl, (2-2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline HCl (RX821002) and yohimbine HCl (RBI, U.S.A.); phentolamine HCl (Ciba-Geigy, Spain); clonidine HCl, clorgyline HCl, N-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), glibenclamide and noradrenaline bitartrate (Sigma Chemical Co., U.S.A.). EEDQ, glibenclamide and diazoxide were dissolved in 0.1% dimethyl sulfoxide in CSF.

Results

2-BFI increases the firing rate of LC neurons in vivo

In an initial series of experiments, the putative modulation by 2-BFI of the firing rate of LC neurons was studied in anaesthetized rats. Local administration of 2-BFI (27 pmoles) into the LC increased the firing rate in 14 out of 20 LC cells (maximal increase in firing rate of responsive cells: $22 \pm 5\%$, $n = 14$, $P < 0.001$) (Figure 1a). The firing rate of the other six neurons was not changed by 2-BFI. Systemic administration of 2-BFI (1 mg kg^{-1} i.v.) also caused a slight but statistically significant increase in the firing rate of all LC neurons recorded (maximal increase: $16 \pm 7\%$, $n = 5$, $P < 0.001$) (Figure 1b). After the administration of 2-BFI the usual inhibitory effect of clonidine on the LC was observed ($n = 2$, data not shown), ruling out an α_2-adrenoceptor antagonistic mechanism for 2-BFI.

Chronic treatment with clorgyline (3 mg kg^{-1} i.p., every 12 h for 14 days) caused a non-significant decrease in the mean basal firing rate of LC cells with respect to the control group (1.8 ± 0.2 Hz, $n = 25$ and 2.3 ± 0.2 Hz, $n = 21$ respectively) (Figure 1a). However, under these circumstances the stimulatory effect of 2-BFI was completely abolished (Figure 1b).

Imidazoline compounds increase the firing rate of LC neurons in vitro

Although imidazoline receptors and α_2-adrenoceptors are distinct, many imidazolines recognize both sites with high affinities. In order to study the modulation mediated by imidazoline receptors in the LC, the effect of bath application of imidazolines with relative selectivity for I_1- or I_2-imidazoline receptors or α_2-adrenoceptors was studied *in vitro*. For the purpose of comparison, a single concentration (1 mM) of the following compounds was applied: 2-BFI and BU224, both with high affinities for I_1- and I_2-imidazoline receptors and negligible affinities for α_2-adrenoceptors; idazoxan, with preferential affinity for I_2-imidazoline receptors and α_2-adrenoceptors over I_1-imidazoline receptors; RX821002 with selectivity for α_2-adrenoceptors over I_1- and I_2-imidazoline receptors and phentolamine and efaroxan, both with preferential affinities for I_1-imidazoline receptors and α_2-adrenoceptors over I_2-imidazoline receptors. Bath application of all these imidazolines increased the firing rate of LC neurons (Figure 2). For all drugs, the maximal effect (Table 1) was reached at 7–10 min after application (Figure 2) and reversed within 30–60 min of washout. Compounds elicited maximal effects in the following order: 2-BFI $\geqslant$ idazoxan $\geqslant$ phentolamine $\geqslant$ BU224 > RX821002 > efaroxan (Table 1). Cumulative concentration-effect curves were plotted for 2-BFI, BU224 and idazoxan (1 μM–1 mM) (Figure 3). The estimated parameters of those curves were: $E_{max} = 141 \pm 41\%$ and $EC_{50} = 80 \pm 24$ μM for 2-BFI, $E_{max} = 79 \pm 15\%$ and $EC_{50} = 67 \pm 18$ μM for BU224 and $E_{max} = 163 \pm 60\%$ and $EC_{50} = 84 \pm 19$ μM for idazoxan. Therefore, these three imidazolines stimulated the firing activity of LC neurons with a similar potency.

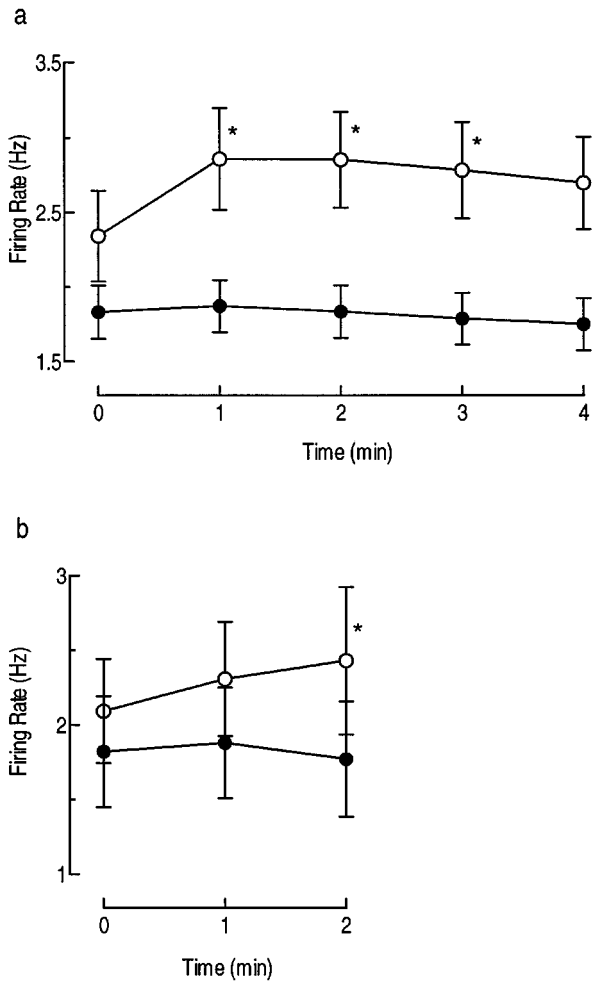

Figure 1 Time course of the effect of 2-BFI on the firing rate of LC neurons in anaesthetized rats in control (open dot) and clorgyline-pretreated rats (3 mg kg^{-1}, i.p., every 12 h for 14 days) (closed dot). The cell firing rate was monitored before ($t = 0$) and after drug application. (a) Local 2-BFI application (27 pmoles) increased the firing rate slightly but significantly over 4 min in control (14 cells) (○) but not in clorgyline-treated rats (18 cells) (●) (b) Intravenous 2-BFI (1 mg kg^{-1}) administration also increased the firing rate in control, ($n = 5$) but not in clorgyline-treated rats ($n = 5$). Bars represent s.e.mean.

In order to assess whether these compounds acted at the same receptor, we studied the additivity of the effects when these imidazolines were applied together. As is shown in Figure 4, the effects of idazoxan and 2-BFI were mutually occlusive; that is the maximal effect reached when both compounds were applied together, was smaller than the addition of the maximal effects observed when each drug was applied separately.

The possibility that the *in vitro* effect of imidazoline drugs could be related to an interaction with the MAO enzyme was studied by applying the irreversible MAO inhibitor clorgyline. Bath application of clorgyline (10 μM, for 22 min) did not affect the spontaneous firing rate of LC neurons ($n=4$) or the stimulatory effect of 2-BFI (Table 1) and efaroxan (data not shown).

Taken together, these results indicate that imidazolines stimulate LC cell activity by specific receptors located in the LC nucleus.

α_2-Adrenoceptors do not mediate the effect of imidazolines on LC neurons in vitro

The effect of non-imidazoline compounds with high affinities for α_2-adrenoceptors but negligible affinities for imidazoline receptors was studied in brain slices. Application of yohimbine, a reversible α_2-adrenoceptor antagonist, at concentrations (1–10 μM, for 15 min) that blocked the inhibitory effect of noradrenaline (30–200 μM) did not modify the firing rate of LC neurons (Table 1). Likewise, application of EEDQ (10 μM, for 30 min), an irreversible antagonist of various receptors including the α_2-adrenoceptor (Szabo *et al*., 1996), did not change the firing rate of LC neurons (Table 1).

To provide further evidence that the action observed with imidazolines was independent of an α_2-adrenoceptor mechanism, the effects of idazoxan and efaroxan were studied in each cell before and after the blockade of α_2-adrenoceptors with

Table 1 Effect of imidazoline and non-imidazoline drugs on the firing rate of locus coeruleus neurons in rat brain slices

	Basal firing rate (spikes/10 s)	*% Change in firing rate (above baseline)*	n
Imidazoline compounds with low affinity for α_2-adrenoceptors			
2-BFI (1 mM)	7.8±0.8	+99±13***	20
2-BFI after clorgyline (10 μM)	6.6±0.7	+88±12*	3
BU224 (1 mM)	9.6±1.5	+80±13***	7
Imidazoline compounds with high affinity for α_2-adrenoceptors			
Idazoxan (1 mM)	7.1±0.7	+94±18***	22
Efaroxan (1 mM)	7.3±1.0	+35±9**	12
Phentolamine (1 mM)	6.1±1.1	+90±30*	6
RX821002 (1 mM)	4.2±0.6	+58±9***	5
Non-imidazoline compounds with high affinity for α_2-adrenoceptors			
Yohimbine (1–10 μM)	7.5±1.7	+2±6	3
EEDQ (10 μM)	6.9±1.2	−9±10	9

Brain slices were exposed to the various agents which were dissolved in the superfusion medium. Each value is the mean±s.e.mean of n experiments per group. Basal firing rate is the average of the value obtained for 2 min before drug application. Changes in firing rate were measured as percentages of the baseline. The maximal change in the firing rate was reached at 7–10 min. According to the magnitude of the effect the rank order of potency of the imidazolines was 2-BFI ⩾ idazoxan ⩾ phentolamine ⩾ BU224 > RX821002 > efaroxan. *$P<0.05$, **$P<0.005$, ***$P<0.0005$ when firing rates before versus after drug application were compared by two-tailed, paired Student's t-test.

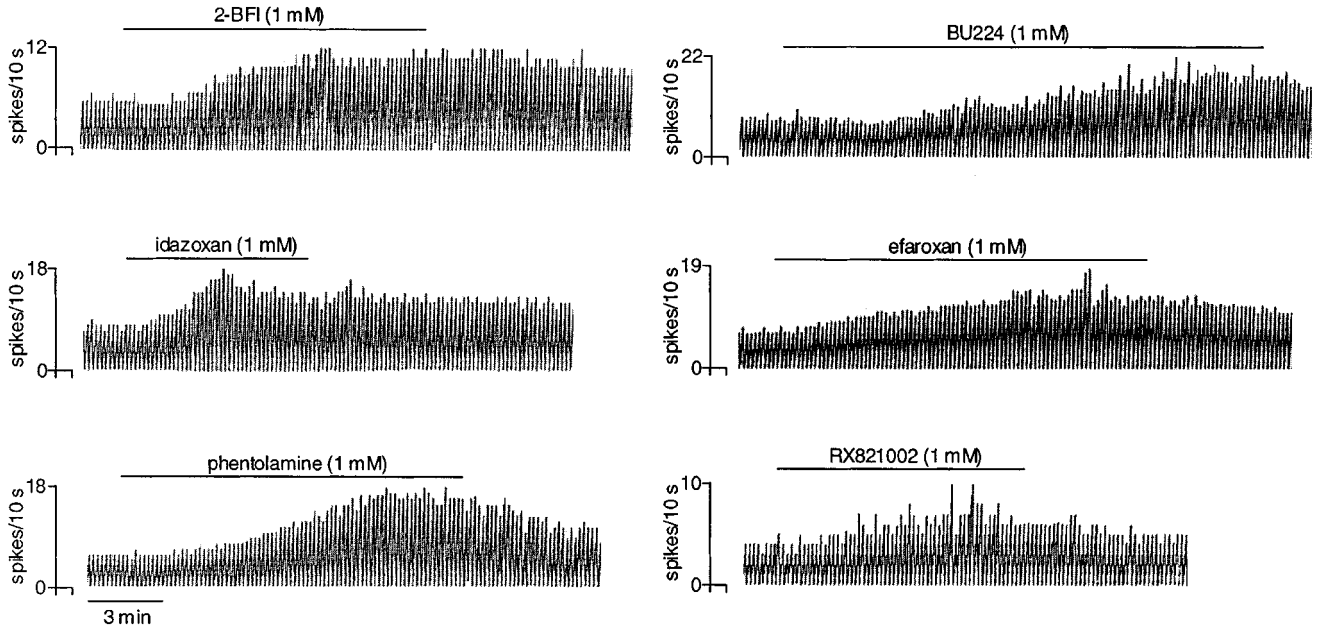

Figure 2 Firing rate recordings of LC neurons in rat brain slices showing the effect of imidazoline compounds. Vertical lines represent the extracellularly recorded firing rates which were displayed on a chart recorder as integrated time histograms (spikes per 10 s). The time scale refers to all traces. Drugs were bath administered at a single concentration (1 mM) for the time indicated by the horizontal bars. Note the increase in the firing rate induced by all imidazolines tested.

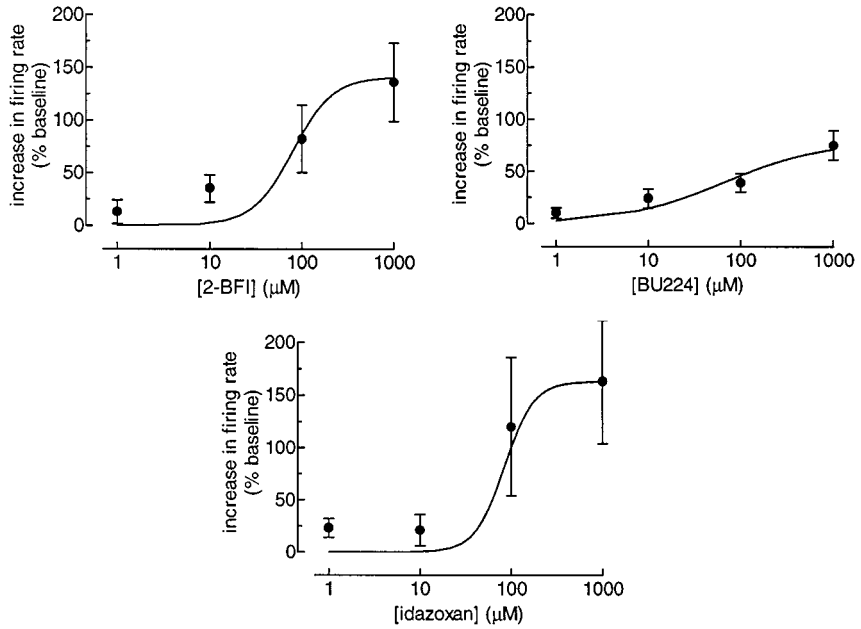

Figure 3 Cumulative concentration-response curves for the effect of 2-BFI, BU224 and idazoxan on the extracellularly recorded firing rate of LC neurons in brain slices. The horizontal axis represents, in semilogarithm scale, the bath concentration of the drug. The vertical axis represents the increase of the firing rate above the baseline when the maximal effect of each concentration was reached. The curves through the data are the theoretical curves calculated by nonlinear regression of the experimental data to a logistic equation (see Methods). Note the concentration dependency and the saturability of the effects. The EC_{50} and the E_{max} of the regressions were: 80 ± 24 μM and 141 ± 41% for 2-BFI, 67 ± 18 μM and 79 ± 15% for BU224 and 84 ± 19 μM and 163 ± 60% for idazoxan, respectively.

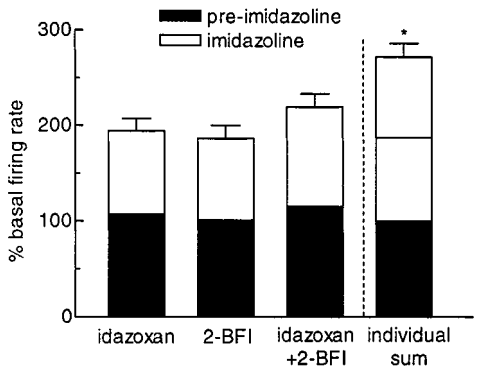

Figure 4 Bar histograms showing the mutual occlusion of idazoxan and 2-BFI effects on the LC. Closed bars represent the basal firing rate and open bars, the maximal firing rate increase reached after bath application of a single concentration (1 mM) of idazoxan, 2-BFI or idazoxan plus 2-BFI. Firing rates are normalized with respect to the initial basal firing rate. (The firing rate at the beginning of the experiment was taken as 100%.) The bar on the right represents the theoretical value of the firing rate that would be reached if the effects of idazoxan and 2-BFI were additive. Bars are the means $\pm$ s.e.mean of five cells. Note the lack of additivity of LC activation by concurrent application of idazoxan plus 2-BFI. $P < 0.001$ (ANOVA followed by Newman-Keuls test) when experimental increase induced by idazoxan plus 2-BFI was compared with the theoretical sum of individual increases.

EEDQ (10 μM, for 30 min). As is shown in Figure 5a and b, both drugs produced the same maximal increase in the firing rate of LC neurons before and after application of EEDQ ($n = 5$).

We compared the recovery from the increase in the firing rate induced by efaroxan (putative non-adrenergic imidazoline-receptor related effect) with the recovery from the blockade induced by this imidazoline of the inhibitory effect of noradrenaline (α_2-adrenoceptor related effect) ($n = 5$). As is shown in Figure 5c, the recovery of the α_2-adrenoceptor antagonism by efaroxan was within 90–130 min, whereas the recovery of the stimulatory effect induced by efaroxan was within 30–60 min (Figure 2).

Therefore, these results indicate that the stimulation of the LC induced by imidazolines is an α_2-adrenoceptor independent effect.

Changes in pH do not alter the effect of imidazolines on LC neurons

In order to examine if the receptor which mediated the observed effects of imidazolines, was located intracellularly, as is the case for I_2-imidazoline receptors (see introduction), we studied the effects of idazoxan under different conditions of pH. Application of an acidic solution (pH = 6.84) for 12 min itself increased the firing rate by 34 ± 7% ($n = 5$, $P < 0.01$) whereas application of a basic solution (pH = 7.84) decreased the firing rate by 24 ± 25% ($n = 5$, N.S.) (Figure 6). However, the effect of idazoxan (1 mM, for 10 min) on the firing rate was similar in both pH conditions (maximal increase in firing rate, at pH 6.84: 88 ± 19% and at pH 7.84: 83 ± 21%; $n = 5$) (Figure 6).

These results suggest that the primary site at which these imidazolines act in the LC may be located at the extracellular level, since augmenting the non-charged fraction of idazoxan, did not lead to greater effects.

Imidazolines reverse the effect of the ATP-sensitive K^+ channel opener diazoxide

Some studies have established that the permeability of ATP-sensitive K^+ channels in pancreatic cells is modulated by imidazolines (Chan *et al*., 1991; Olmos *et al*., 1994). To test if ATP-sensitive K^+ channels were involved in the effect of imidazolines on the LC, the ability of imidazolines to reverse the

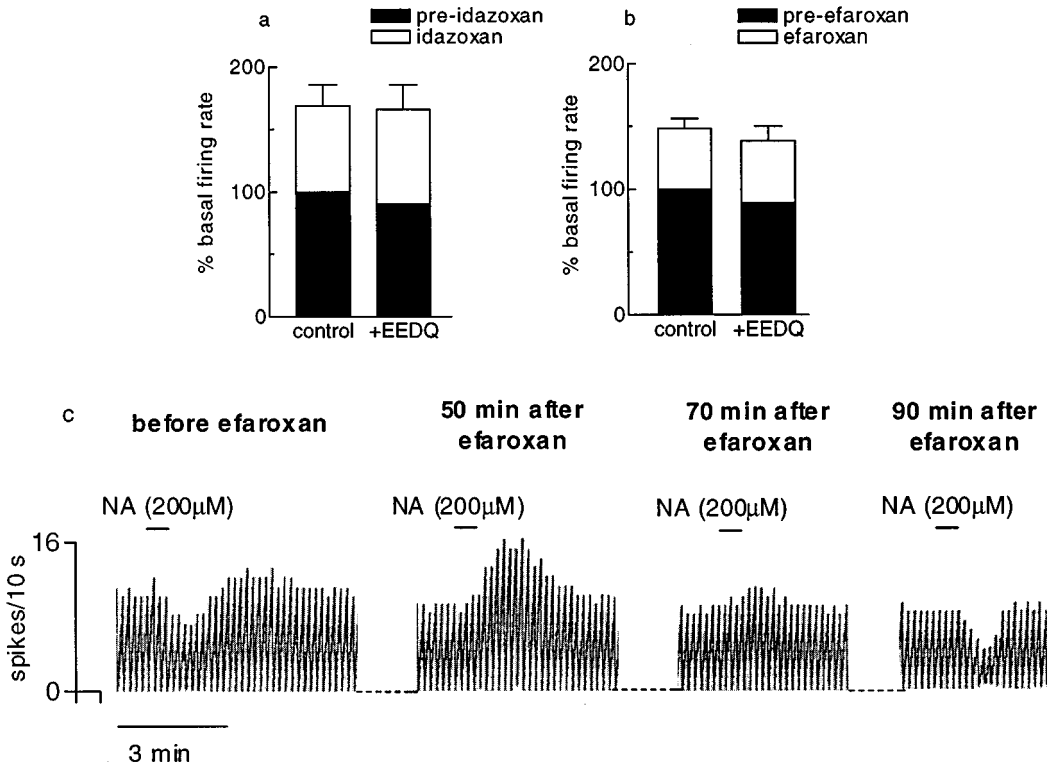

Figure 5 Bar histograms showing the lack of effect of bath application of the irreversible α_2-adrenoceptor antagonist EEDQ on the stimulatory effects induced by idazoxan (1 mM) (a) or efaroxan (1 mM) (b) on LC neurons. The vertical axis shows the percentage of the basal firing rate (closed bars) and the maximal firing rate increase (open bars) reached after drug application. Bars are the means ± s.e.mean of five cells. Note that the effects of bath applications of idazoxan or efaroxan were not modified by previous application of EEDQ which did not change the basal firing rate either. (c) Firing rate recording of LC neurons in brain slices showing the recovery of the inhibitory effect of noradrenaline after the α_2-adrenoceptor antagonism induced by efaroxan (1 mM). Note that NA causes excitation of LC cells while recovering from its inhibitory effect; this effect could be mediated by α_2-adrenoceptors which are unmasked when α_2-adrenoceptors are blocked (Williams & Marshall, 1987). Note that the α_2-adrenoceptor antagonist effect of efaroxan lasted 90 min (see Figure 2).

Figure 6 Bar histograms showing the lack of effect of changing the pH of the slice bathing medium on the effect induced by idazoxan on the LC neuron firing rate. Closed bars represent the basal firing rate and open bars, the maximal firing rate increase reached after bath application of a single concentration (1 mM) of idazoxan. Firing rate values were normalized with respect to initial baseline values in each cell (the firing rate at the beginning of the experiment, at a pH of 7.34, was taken as 100%). Bars are the means ± s.e.mean of five cells. Note that the magnitude of the effect of idazoxan was the same at pH 6.84 and 7.84. The increase in basal firing rate after application of acidic aCSF was significantly different ($P<0.01$).

inhibition of the firing rate induced by diazoxide, an ATP-sensitive K^+ channel opener, was studied. In each cell, 2-BFI was applied before and after perfusion with diazoxide and the corresponding effects were compared. Application of diazoxide (300 μM) decreased the firing rate by 57 ± 12% ($n=6$, $P<0.001$) (Figure 7a and b), whereas 2-BFI (1 mM, for 7 min) reversed the inhibition of LC cells induced by diazoxide. The magnitude of the increase in the firing rate elicited by 2-BFI after dizoxide was even greater than in the absence of the opener (Figure 7a and b), which suggests that this effect of 2-BFI could not be due only to a physiological antagonism but rather to a modulation of ATP-sensitive K^+ channels. To further investigate this hypothesis, the effect of 2-BFI was studied in the presence of glibenclamide, an ATP-sensitive K^+ channel blocker in the LC (Finta *et al.*, 1993). Application of glibenclamide (3 μM for 15 min) did not modify significantly the firing rate of LC cells ($n=5$), in agreement with previous results (Finta *et al.*, 1993). However, the effect of 2-BFI (1 mM, for 7 min) was blocked by 44% ($n=5$, $P<0.01$) in the presence of glibenclamide (Figure 7c), whereas the stimulatory effect of glutamate (300 μM for 30 s) was not changed ($n=5$) (Figure 7d).

Discussion

The present study provides evidence that imidazolines increase the firing activity of LC neurons in the rat by an action on non-I_1/I_2-type imidazoline receptors located in this brain nucleus. This is a specific effect mediated by sites which seem to be located extracellularly and to modulate ATP sensitive-K^+ channels.

Most imidazolines used in this study bind not only to imidazoline receptors but also to α_2-adrenoceptors. Indeed

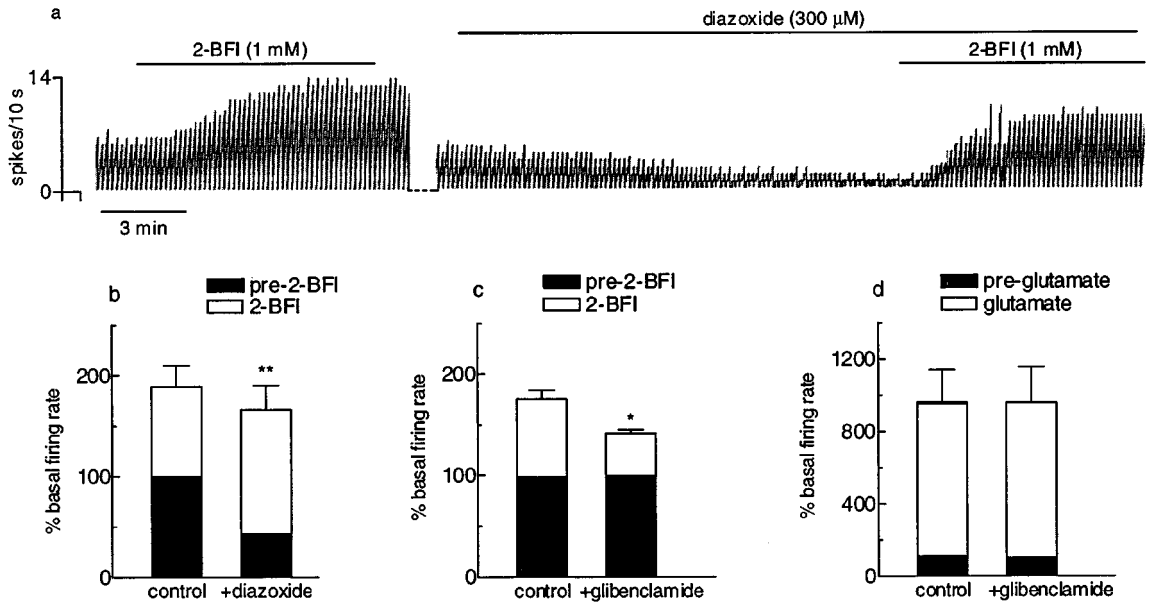

Figure 7 Firing rate recording of one LC neuron from a rat brain slice showing the effect of 2-BFI before and after bath application of diazoxide (a). Vertical lines represent the firing rates extracellularly recorded and displayed on a chart recorder as integrated time histograms (spikes per 10 s). The time scale refers to both traces. Bar histograms showing the effect of 2-BFI (b and c) or glutamate (d) on LC neurons. Closed bars represent the basal firing rates before and after bath application of diazoxide (300 μM) or glibenclamide (3 μM) and open bars represent the maximal firing rate increase reached after bath application of a single concentration of 2-BFI (1 mM) or glutamate (300 μM). The experiments included in c and d were performed in the same neurons. Bars are the means $\pm$ s.e.mean of five cells in each group. Note that 2-BFI application overcame the inhibition induced by diazoxide and glibenclamide application reduced the effect of 2-BFI. $^*P<0.01$, $^{**}P<0.001$ two-tailed, paired Student's t-test, when the increase induced by 2-BFI in control was compared with that after diazoxide inhibition or when the effects of 2-BFI or glutamate were compared with those after glibenclamide. The decrease in the basal firing rate after diazoxide application was also significant ($P<0.001$).

Szabo *et al*. (1996) have reported that there are no functional imidazoline receptors on LC neurons. However, there are several reasons to suggest that the stimulatory effect induced by imidazolines is not mediated by the antagonism of α_2-adrenoceptors. First, in binding studies 2-BFI and BU224, two of the compounds with a stimulatory effect on LC neurons, have very low affinities for any subtype of α_2-adrenoceptors (Hudson *et al*., 1994; Lione *et al*., 1996; Piletz *et al*., 1996; Alemany *et al*., 1997; Hosseini *et al*., 1997). Accordingly, 2-BFI did not block the α_2-adrenoceptor-mediated inhibitory effect of clonidine *in vivo*. Second, yohimbine and EEDQ did not alter the LC cell firing rate in agreement with previous works using these and other α_2-adrenoceptor antagonists in brain slices (Williams *et al*., 1995; Illes & Norenberg, 1990; Pineda & Aghajanian, 1997). Third, the stimulatory effect induced by imidazolines was not affected by preincubation with EEDQ, which *in vitro* markedly decreases the density of α_2-adrenoceptors without affecting the density of I_2-imidazoline sites (Miralles *et al*., 1993b). Finally, there were two different time-courses for the recovery of the effects induced by efaroxan (an imidazoline receptor and α_2-adrenoceptors ligand): the stimulatory effect (a putative imidazoline receptor-mediated effect) and the recovery from the blockade of the noradrenaline effect (an α_2-adrenoceptor antagonistic effect).

The imidazoline receptor which mediated the stimulatory effect on the LC neurons appears to be different from the I_1- or I_2-imidazoline receptor. First, I_1-imidazoline binding sites have not been found in the LC (Ernsberger *et al*., 1995). The stimulatory effect of i.v. administration of clonidine which is mediated by the I_1-imidazoline receptor (Pineda *et al*., 1993) does not occur when this drug is applied directly into this nucleus in EEDQ-pretreated anaesthetized rats (Ruiz-Ortega & Ugedo, 1997). Moxonidine, rilmenidine and clonidine, three preferential I_1-imidazoline receptor ligands do not have any effect on the LC of EEDQ-pretreated slices (Szabo *et al*., 1996). In a similar manner, the relative potency of idazoxan to stimulate the LC (idazoxan $\sim$ 2BFI $\sim$ BU224) further suggests that I_1-imidazoline receptors do not mediate the stimulatory effect observed in this study. In contrast, various lines of evidence point to the participation of an I_2-imidazoline receptor in mediating the effect observed. Thus, in the rat the LC contains a relatively high density of I_2-imidazoline binding sites as determined by autoradiographic techniques with [^{3}H]-rilmenidine and [^{3}H]-RS-45041-190 (King *et al*., 1995; MacKinnon *et al*., 1995). Moreover, some drugs used in this study (2-BFI, BU224 and idazoxan) have high affinities for I_2-imidazoline receptors. On the contrary, a number of findings do not support the implication of I_2-imidazoline receptors. First, efaroxan and RX821002, which have very low affinities for I_2-imidazoline binding sites (Ernsberger *et al*., 1992; Callado *et al*., 1996), also stimulated LC cell activity. Second, it has been shown that preincubation with clorgyline decreases the density of I_2-imidazoline binding sites as labelled with [^{3}H]-idazoxan (Olmos *et al*., 1993) or [^{3}H]-2-BFI (Alemany *et al*., 1997) in rat brain and liver membranes respectively. However, our results show that preincubation with clorgyline did not modify the response of LC neurons to bath application of 2-BFI or efaroxan. Third, purification studies of subcellular fractions, from human and rabbit liver have demonstrated that [^{3}H]-idazoxan binding sites (I_2-imidazoline receptors) are located intracellularly on the outer membrane of mitochondria (Tesson & Parini, 1991; Piletz & Sletten, 1993). However, raising the pH of the bathing solution for idazoxan from 6.84 to 7.84, which is expected to increase by 10 times the concentration of the uncharged and thus diffusible form of

the compound (assuming a pK_a of 8.59 at 33°C), did not change the magnitude of the idazoxan effect. Since the effect of idazoxan is concentration-dependent, these results suggest that the drug interacts with a site located at the extracellular level of the cell plasma membrane.

Several studies have demonstrated that the I_2-imidazoline site is a MAO regulatory site (Tesson *et al*., 1995; Tesson & Parini, 1991). The results obtained with microdialysis techniques suggest that 2-BFI may act as a MAO inhibitor (Nutt *et al*., 1995). Moreover, some imidazolines such as 2-BFI and idazoxan can inhibit MAO activity (Carpéné *et al*., 1995; Ozaita *et al*., 1997). In electrophysiological *in vivo* experiments the administration of MAO inhibitors decreases the activity of LC neurons (Campbell *et al*., 1985; Oreland & Engberg, 1986); in our study however, the application of clorgyline *in vitro* did not change the LC cell activity or the effect of 2-BFI on the LC neurons. Therefore, it is unlikely that a mechanism at the MAO level could account for the *in vitro* effects observed with imidazoline drugs on the LC. On the other hand, binding studies have shown that chronic treatment with clorgyline decreases the density of non-adrenoceptor [^{3}H]-idazoxan binding sites in the rat brain (Olmos *et al*., 1993). Accordingly, in our study, chronic treatment with clorgyline abolished the effect of 2-BFI *in vivo*. Since in *in vitro* experiments clorgyline did not alter the effect of 2-BFI, these findings might be an adaptative change of the receptor after chronic treatment due to the increase in the levels of cerebral noradrenaline after chronic MAO inhibition (Finberg *et al*., 1993).

Functional studies have revealed the existence of a new type of imidazoline receptor which cannot be ascribed to any of the I_1/I_2-imidazoline receptor types (Chan *et al*., 1994; Molderings & Göthert, 1995; Olmos *et al*., 1994). This non-I_1/I_2-imidazoline receptor mediates the insulinotropic effect of some imidazolines used in this study such as efaroxan, RX821002, phentolamine and idazoxan in pancreatic β-cells and insulinoma cells (Chan *et al*., 1994; Olmos *et al*., 1994). Therefore the site that mediates the effect of imidazolines on the LC resembles that described for non-I_1/I_2-imidazoline receptor. In addition, imidazolines acting through these non-I_1/I_2-imidazoline receptors have been proposed to act as blockers of ATP-sensitive K^+ channels (Chan *et al*., 1991; Olmos *et al*., 1994). Electrophysiological studies have shown that noradrenergic LC neurons are endowed with ATP-sensitive K^+ channels. ATP-sensitive K^+ channel opener drugs decrease spontaneous discharge, whereas some blockers of these channels increase the firing rate of LC cells (Finta *et al*., 1993). The results of the present study show that diazoxide, an opener of ATP-sensitive K^+ channels, decrease the firing rate of LC cells and 2-BFI reverses this effect. Since the effect of 2-BFI, after diazoxide, was greater than under basal conditions, we can expect that the reversal induced by 2-BFI was due to an antagonism at the ATP-sensitive K^+ channel. In addition, glibenclamide, which blocks ATP-sensitive K^+ channels in the LC (Finta *et al*., 1993), partially blocked the effect of 2-BFI. This antagonism seems to be a specific effect since the stimulatory effect of glutamate was not modified by glibenclamide. Therefore, imidazolines in our *in vitro* model may act as ATP-sensitive K^+ channel blockers in a manner similar to that reported for the imidazoline receptors in β pancreatic and insulinoma cells. Indeed, high doses of the imidazolines that have been tested in our study should be needed to modulate a cation channel, as has been suggested in other *in vitro* studies (see Molderings 1997). Nevertheless further intracellular electrophysiological studies are required to confirm this hypothesis.

In summary the results of the present study demonstrate that imidazoline compounds modulate the firing activity of the LC. This is an effect mediated by imidazoline receptors with a pharmacological profile which is distinct from that of I_1- and I_2-imidazoline receptors. These receptors seem to regulate an ATP-sensitive K^+ channel in a manner similar to the imidazoline receptor-type described in pancreatic cells. Our finding extends the range of imidazoline receptor types found in the CNS. Since the LC plays an important role in the pathophysiology of psychiatric disorders, this receptor type may also be involved in the etiology and progression of these disorders.

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