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Protection by imidazol(ine) drugs and agmatine of glutamate-induced neurotoxicity in cultured cerebellar granule cells through blockade of NMDA receptor

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1 This study was designed to assess the potential neuroprotective effect of several imidazol(ine) drugs and agmatine on glutamate-induced necrosis and on apoptosis induced by low extracellular K^+ in cultured cerebellar granule cells.

2 Exposure (30 min) of energy deprived cells to L-glutamate (1–100 μM) caused a concentration-dependent neurotoxicity, as determined 24 h later by a decrease in the ability of the cells to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) into a reduced formazan product. L-glutamate-induced neurotoxicity ($EC_{50} = 5$ μM) was blocked by the specific NMDA receptor antagonist MK-801 (dizocilpine).

3 Imidazol(ine) drugs and agmatine fully prevented neurotoxicity induced by 20 μM (EC_{100}) L-glutamate with the rank order (EC_{50} in μM): antazoline (13) > cirazoline (44) > LSL 61122 [2-styryl-2-imidazoline] (54) > LSL 60101 [2-(2-benzofuranyl) imidazole] (75) > idazoxan (90) > LSL 60129 [2-(1,4-benzodioxan-6-yl)-4,5-dihydroimidazole] (101) > RX821002 (2-methoxy idazoxan) (106) > agmatine (196). No neuroprotective effect of these drugs was observed in a model of apoptotic neuronal cell death (reduction of extracellular K^+) which does not involve stimulation of NMDA receptors.

4 Imidazol(ine) drugs and agmatine fully inhibited [^{3}H]-(+)-MK-801 binding to the phencyclidine site of NMDA receptors in rat brain. The profile of drug potency protecting against L-glutamate neurotoxicity correlated well ($r = 0.90$) with the potency of the same compounds competing against [^{3}H]-(+)-MK-801 binding.

5 In HEK-293 cells transfected to express the NR1-1a and NR2C subunits of the NMDA receptor, antazoline and agmatine produced a voltage- and concentration-dependent block of glutamate-induced currents. Analysis of the voltage dependence of the block was consistent with the presence of a binding site for antazoline located within the NMDA channel pore with an IC_{50} of 10–12 μM at 0 mV.

6 It is concluded that imidazol(ine) drugs and agmatine are neuroprotective against glutamate-induced necrotic neuronal cell death *in vitro* and that this effect is mediated through NMDA receptor blockade by interacting with a site located within the NMDA channel pore.

Keywords: Imidazoline; agmatine; neurotoxicity; neuroprotection; glutamate; N-methyl-D-aspartate receptors; cerebellar granule cells

Abbreviations: G-LH buffer, modified Locke-HEPES (M-LH) buffer containing 10 mM glucose; K^+_{ATP}, ATP-sensitive K^+ channels; LSL 60101, 2-(2-benzofuranyl)imidazole; LSL 60129, 2-(1,4-benzodioxan-6-yl)-4,5-dihydroimidazole; LSL 61122, 2-styryl-2-imidazoline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; MTT_R, reduced MTT; NMDA, N-methyl-D-aspartate; PCP, phencyclidine; RX821002, 2-methoxy idazoxan

Introduction

Several studies have demonstrated that imidazol(ine)/guanidine compounds display nanomolar affinities for various non-adrenoceptor sites, the so-called imidazoline receptors, and that the interaction with these recognition sites elicits various central and peripheral effects (for a review see Bousquet, 1995; French, 1995; Regunathan & Reis, 1996; Molderings, 1997). The amine agmatine (decarboxylated arginine) has been proposed as an endogenous agonist at imidazoline receptors (Li *et al*., 1994). Independently of their affinities on these receptors, imidazol(ine)/guanidine compounds also interact (in the micromolar range) with the monoamine oxidase enzymes (Ozaita *et al*., 1997) and various cation channels. These compounds block ATP-sensitive K^+ channels (K^+_{ATP} channels) in pancreatic β-cells and rat insulinoma (RIN) cells and this leads to the stimulation of insulin release (Jonas *et al*., 1992; Olmos *et al*., 1994; Berdeu *et al*., 1997; Proks & Ashcroft, 1997). Imidazol(ine) drugs inhibit the acetylcholine-induced secretion of catecholamines in adrenal chromaffin cells (Ohara-Imaizumi & Kumakura, 1992) by blocking nicotinic acetylcholine receptors (Musgrave *et al*., 1995). These compounds also interact with 5-HT_3 receptors in N1E-115 cells inhibiting the veratridine-induced influx of guanidinium to these cells (Molderings *et al*., 1996). Finally, an interaction with red cell Gardos channels (Coupry *et al*., 1996) and with rat brain N-methyl-D-aspartate (NMDA) receptors (Olmos *et al*., 1996) has also been demonstrated.

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Glutamate, the major excitatory neurotransmitter in the brain, is also a potent excitotoxin, inasmuch as prolonged exposure of most cultured neurones to micromolar concentrations of this aminoacid leads to neurotoxicity (Choi, 1988). In this sense, glutamate exposure of primary cultures of cerebellar granule cells is a well characterized model of neurotoxicity which results from the overstimulation of the NMDA receptor (Lysko *et al*., 1989; Schramm *et al*., 1990; Berman & Murray, 1996), leading to alterations in Ca^{2+} homeostasis (Milani *et al*., 1991). In this context, imidazol(ine) drugs and agmatine have been shown to be neuroprotective in brain injuries of necrotic (Gustafson *et al*., 1990; Maiese *et al*., 1992; Gilad *et al*., 1996) and apoptotic neuronal cell death (Olmos *et al*., 1999) in which glutamate-mediated neurotoxicity is clearly involved as the mechanism determining cell death. Thus, the present study was designed (1) to assess the potential neuroprotective effect of several imidazol(ine) drugs and agmatine on glutamate-induced necrosis and on apoptosis induced by low extracellular K^+ in cultured cerebellar granule cells and (2) to seek whether imidazol(ine) drugs could exert neuroprotective effects through NMDA receptor blockade. A preliminary report of a portion of this study has been previously presented in abstract form (DeGregorio-Rocasolano *et al*., 1999).

Methods

Cerebellar granule cell cultures

Primary cultures of granule cells were prepared from cerebella of 7-day-old Wistar rat pups as previously described (Lysko *et al*., 1989; Boje *et al*., 1993; Fossom *et al*., 1995). Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies. Dissociated cells were plated in 24-well plastic plates (2 cm^2) previously coated with poly-L-lisine hydrobromide (10 $\mu g\ ml^{-1}$, MW > 300,000) and the density was adjusted to give approximately 5×10^5 cells per cm^2. Cells were cultured in Eagle's Basal Medium with the following additions: 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 0.1 mg ml^{-1} gentamicin, and 25 mM KCl.

The replication of non-neuronal cells was prevented by adding cytosine arabinoside (10 μM) 18–24 h after plating. The cultures were incubated at 37°C in 5% CO_2 in air saturated with water vapour. Conditioned media from naive cultures were reserved for later use. The cells were used for experiments after 8–11 days in culture.

Glutamate induction of necrotic neuronal death

In cerebellar granule cells depleted of energy resources, glutamate induces a rapid necrosis, whereas development of the apoptotic programme of neuronal death requires the integrity of mitochondrial function (Dessi *et al*., 1993; Ankarcrona *et al*., 1995). Thus, cultured neurones were washed once with 0.75 ml aliquots of modified Locke-HEPES (M-LH) buffer without magnesium and glucose (in mM: NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 1.3, and HEPES 10, (pH 7.35) and then preincubated in the same buffer for 25 min; thereafter the imidazol(ine) drugs or agmatine (see Table 1 for their chemical structures) were added for 15 min where appropriate. This preincubation time (40 min) in M-LH buffer depleted cerebellar granule cells of energy resources and rendered them susceptible to L-glutamate toxicity (Lysko *et al*., 1989). Neurotoxicity was then induced by a 30 min incubation with L-glutamate in M-LH buffer in the presence or absence of experimental drugs. Each experimental condition was replicated in at least three independent preparations. The cultures were washed twice with 0.75 ml aliquots of M-LH buffer containing 10 mM glucose (G-LH buffer) at the end of this incubation period. Conditioned media (0.75 ml) was added to the cultures and the cultures were returned to the incubator.

Low K^+ induction of apoptotic neuronal death

Cerebellar granule cells maintained in serum-free medium with 25 mM K^+ undergo an apoptotic death when switched to 5 mM K^+ (D'Mello *et al*., 1993; Ishitani *et al*., 1997). To investigate the effects of imidazol(ine) drugs on apoptosis induced by low extracellular K^+, cerebellar granule cells were washed twice and maintained in serum-free Eagle's Basal Medium containing 5 mM K^+ (low $[K^+]_{out}$) for 24 h in the presence or absence of drugs where indicated. Other cell cultures were washed identically and maintained in serum-free Eagle's Basal Medium with 25 mM K^+ (high $[K^+]_{out}$) in the presence or absence of drugs until neurotoxicity assays were performed.

Assessment of neurotoxicity and neuroprotection

Neurotoxicity was quantitatively determined 24 h after treatment using a tetrazolium salt colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Mosmann, 1983). Mitochondrial enzymes have the capacity to transform MTT into a reduced insoluble formazan product (MTT_R), thus, the MTT assay provides an indication of mitochondrial metabolic function. Decreased MTT metabolism 24 h after neurotoxic treatment indicates loss of functional mitochondria in viable neurons because results obtained with this technique correlate well with cell viability estimated by staining with fluorescein diacetate (Lysko *et al*., 1989) or propidium iodide (Ankarcrona *et al*., 1995). Cells were incubated for 30 min at 37°C with 1 ml of G-LH buffer containing 0.4 μM MTT. The buffer was aspirated and replaced with 300 μl of dimethyl sulphoxide to extract the blue formazan complex. The entire extract was transferred to a 96-well plate and the optical density of samples was measured. The MTT_R formation was calculated as the difference between the optical densities measured at 490 and 630 nm. To normalize for differences in the absorbance values among different culture plates and assays, values were expressed relative to unexposed control cells (see below).

Per cent neurotoxicity was defined as the per cent decrease in the formation of MTT_R in cells exposed to L-glutamate with respect to unexposed control cells and was calculated as follows:

$$\%\ \text{Neurotoxicity} = 100 - \text{G100} \qquad (1)$$

where $G = MTT_R$ in L-glutamate exposed cells/MTT_R in control cells.

Per cent neuroprotection was defined as the per cent increase in the formation of MTT_R in cells exposed to L-glutamate plus experimental drugs with respect to cells exposed to L-glutamate and was calculated as follows:

$$\%\ \text{Neuroprotection} = \frac{100(\text{T100} - \text{G100})}{100 - (\text{G100})} \qquad (2)$$

where $T = MTT_R$ in L-glutamate plus experimental drugs exposed cells/MTT_R in control cells.

Data from neurotoxicity and neuroprotection curves were fitted to a four-parameter logistic equation by computer-

assisted non-linear regression using the Prism programme (GraphPAD, San Diego, CA, U.S.A.).

[^{3}H]-(+)-MK-801 binding assays and analyses of binding data

Radioligand binding assays with [^{3}H]-(+)-MK-801 and preparation of P_2 membrane fractions from the rat parieto-occipital cortex were done as previously described (Olmos *et al*., 1996). Drug competition studies were performed in a total volume of 500 μl, containing 400 μl of membrane suspension and [^{3}H]-(+)-MK-801 (4×10^{-9} M), in the absence or presence of various concentrations of the competing drugs ($10^{-6}-10^{-2}$ M; 11 concentrations). Non-specific binding was determined in the presence of 10^{-4} M ketamine. Glutamate and glycine were not included in the assays because the content of endogenous aminoacids contaminating the crude (non washed) membrane preparations is sufficient to occupy the transmitter recognition sites of the NMDA receptors and cause partial enhancement of [^{3}H]-(+)-MK-801 binding (see Foster & Wong, 1987 for details). Furthermore, the addition of 10 μM glutamate to rat brain synaptic membrane preparations did not significantly change the affinity of agmatine for [^{3}H]-(+)-MK-801 binding sites (Anis *et al*., 1990). The mixture was incubated for 45 min at 23°C and then subjected to rapid filtration through Whatman GF/C filters using a Brandel 48 R cell harvester (Biomedical Research and Development Laboratories, U.S.A.). The filters were then rinsed twice with 5 ml of ice-cold incubation buffer and counted for radioactivity by liquid scintillation spectrometry at 50% efficiency. Analysis of competition experiments as well as the fitting of data to the appropriate binding models were performed by computer-assisted non-linear regression using the EBDA-LIGAND programmes (Munson & Rodbard, 1980; McPherson, 1985).

Electrophysiology of NMDA receptor

Experiments were carried out on the cell line HEK-293. Cells were grown in Dulbecco's modified Eagle Medium supplemented with 10% foetal calf serum, 1% glutamine and 1% penicillin/streptomycin. Cell-transfection was done as previously described (Villarroel *et al*., 1998) by electroporating the cDNAs encoding for the NR1-1a and NR2C subunits of the NMDA receptor together with the cDNA encoding for Green Fluorescent Protein (GFP) (1 : 2 : 1 ratio) for visualization of positively transfected cells and then seeded in plastic Petri dishes containing 2 ml of medium. Non-transfected cells do not express any kind of glutamate receptor. After 3–5 h the medium was replaced with a glutamine-free medium containing the NMDA receptor glycine site antagonist 7-chlorokynurenic acid (100 μM) to prevent NMDA-receptor-mediated cell death. The cDNAs employed were a generous gift of Dr S. Nakanishi (University of Kyoto).

HEK-293 cells were recorded 1 day after transfection. Currents activated by 200 μM L-glutamate in the presence of 20 μM glycine were measured at −70 mV, unless otherwise indicated, in the whole-cell configuration of the patch-clamp technique using an EPC7 amplifier. The borosilicate glass electrodes had resistances of 3–6 MΩ and the series resistance was compensated by 30–60% when the current responses exceeded 400 pA. Solutions were delivered using a fast perfusion system (Lerma *et al*., 1998). The composition of the extracellular solution was (in mM): NaCl 160, KCl 2.5, $CaCl_2$ 0.5, glucose 10, HEPES 10 and glycine 0.02 (pH 8.4 with NaOH; 325 mOsM). An alkaline pH was used to evoke larger currents. The composition of the intracellular solution was (in mM): $CsCH_4SO_3$ 126, CsCl 10, $MgCl_2$ 5, $CaCl_2$ 0.5, EGTA 10 and HEPES 10 (pH 7.3 with CsOH; 310 mOsM).

To evaluate channel blockade at different potentials, the current in response of a voltage ramp from −70 to +70 mV of 450 ms of duration was fitted to the Boltzman equation:

$$I = \frac{(V - V_{rev})G}{1 + e^{(V-V_{0.5})/S}} \quad (3)$$

where V is the membrane potential, V_{rev} is the reversal potential, G is the conductance, $V_{0.5}$ is the voltage at which 50% of the current is blocked at a given blocker concentration, and S is the slope factor. The values for G, $V_{0.5}$ and S were allowed to vary during the fitting procedure, while V_{rev} was fixed to a value estimated by a linear fit of the I–V relation on the control ramp. The parameters were used to generate idealized I–V relations, that in turn were used to generate idealized relations of the blocker effect at different potentials. This procedure was repeated at different concentrations of the blocker, and the concentration-response relation at a given potential was fitted to the equation:

$$I = \frac{1}{1 + \left(\frac{IC_{50}}{[B]}\right)^n} \quad (4)$$

where IC_{50} is the concentration of the compound that produces 50% blockade, [B] is the drug concentration, and n is the Hill coefficient.

The relation of the IC_{50} with voltage was fitted to the Woodhull model (Woodhull, 1973) with the linear equation:

$$pIC_{50} = pIC_{50}(0) + \frac{z\delta.FV}{RT} \quad (5)$$

where pIC_{50} is the negative logarithm of the IC_{50}, $IC_{50}(0)$ is the IC_{50} value at 0 mV, RT/F = 25.4 mV at 22°C and $z\delta$ is the product of the charge of the blocker and the fraction of the electrical distance within the plane of the membrane.

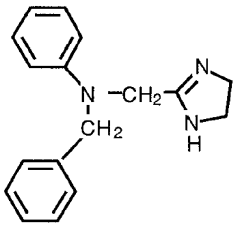
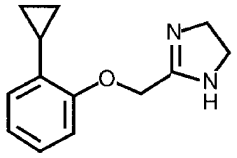
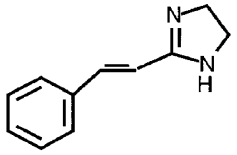
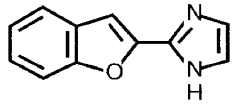
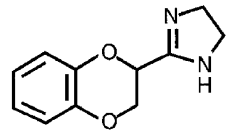
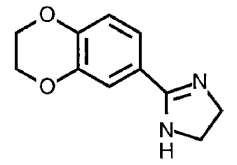
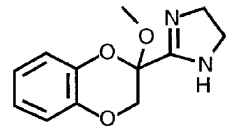
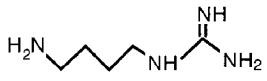
Statistics

Results are expressed as mean ± s.e.mean. Student's unpaired *t*-test was used for the statistical evaluations. Correlation coefficients were calculated by the method of least squares. The level of significance was $P = 0.05$.

Drugs

[^{3}H]-(+)-MK-801 (23.9 Ci mmol^{-1}) was supplied by New England Nuclear Du Pont (U.S.A.). Other drugs (and their sources) included: agmatine sulphate (Aldrich Chemical Co., U.S.A.), antazoline HCl (Sigma Chemical Co., U.S.A.); 7-chlorokynurenic acid (Tocris Cookson Ltd, U.K.); cirazoline HCl (Synthélabo Recherche, France); L-glutamic monosodium salt (Sigma); glycine (Tocris Cookson Ltd, U.K.); idazoxan HCl, LSL 60101 [2-(2-benzofuranyl)imidazole HCl], LSL 60129 [2-(1,4-benzodioxan-6-yl)-4,5-dihydroimidazole HCl] and LSL 61122 [2-styryl-2-imidazoline HCl, valldemossine] (synthesized by Dr F. Geijo at S.A. Lasa Laboratorios, Barcelona, Spain); (+)-MK-801 (dizocilpine) maleate (RBI, Natick, U.S.A.); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma) and RX821002 (2-methoxy idazoxan) HCl (synthesized by Dr F. Geijo at S.A. Lasa Laboratorios). Cell culture media were obtained from Gibco-BRL (Life technologies Ltd, U.K.). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Table 1 Potencies of various imidazol(ine) drugs and agmatine protecting against glutamate-induced neurotoxicity and affinities for NMDA receptors

Drug	*Chemical structure*	*Protection against glutamate neurotoxicity* EC_{50} (μM)	*Affinity for NMDA receptors* [^{3}H]-(+)-MK-801 binding K_i (μM)
Antazoline		13	13
Cirazoline		44	54
LSL 61122		54	37
LSL 60101		75	132
Idazoxan		90	134
LSL 60129		101	54
RX821002		106	190
Agmatine		196	219

The neuroprotective effects of imidazol(ine) drugs and agmatine were assessed in cultured cerebellar granule cells exposed to 20 μM L-glutamate. Affinities for NMDA receptors were assessed in competition experiments against [^{3}H]-(+)-MK-801 binding to membranes from rat cerebral cortex. EC_{50} values were determined directly by simultaneous non-linear regression analysis (Prism programme) of data from eight wells. Binding parameters (K_i values) for NMDA receptors were determined by simultaneous analysis of 2–4 independent experiments for each drug using the EBDA-LIGAND programmes. K_i values of LSL 61122, LSL 60101, idazoxan and RX821002 for NMDA receptors were taken from Boronat *et al.* (1998). For LSL 60101, Hill slopes were significantly higher than unity ($P<0.001$, F-test). For agmatine, Hill slopes were significantly lesser than unity ($P<0.001$, F-test), however, the K_i representing the affinity for all the sites labelled by the radioligand was taken for convenience in the correlation analysis (Figure 5). Standard errors for EC_{50} and K_i values were less than 5% of the reported mean values. See text and Figures 2 and 4 for further details.

Results

Neuroprotective effects of imidazol(ine) drugs and agmatine against L-glutamate-induced neurotoxicity in cultured cerebellar granule cells

Thirty minutes exposure of energy deprived cerebellar granule cells to L-glutamate (1–100 μM) caused a concentration-dependent neurotoxicity, as revealed 24 h later by a decrease in the ability of these cells to metabolize MTT to formazan (Figure 1a). L-glutamate induced neurotoxicity with E_{max} and EC_{50} values of 75% and 5 μM, respectively (Figure 1a). Concurrent exposure of cells with L-glutamate and the non-competitive NMDA receptor antagonist MK-801 (1 μM) prevented (93%, $P<0.001$) the neurotoxicity induced by 20 μM (EC_{100}) L-glutamate (Figure 1b); showing that NMDA receptors mediate the neurotoxic effects of L-glutamate in energy deprived cerebellar granule neurons.

When cerebellar granule cells were exposed to 20 μM L-glutamate in the presence of various imidazol(ine) drugs and agmatine, these compounds fully prevented L-glutamate-induced neuronal death with different potencies (Figure 2 and Table 1). In the absence of L-glutamate, exposure of granule cells to imidazol(ine) drugs and agmatine (up to 500 μM) did not modify MTT reduction with respect to unexposed control cells (data not shown).

Lack of protective effects of imidazol(ine) drugs in cerebellar granule cells exposed to low potassium concentrations

The potential neuroprotective effect of various imidazol(ine) drugs was tested in a model of apoptotic neuronal death that

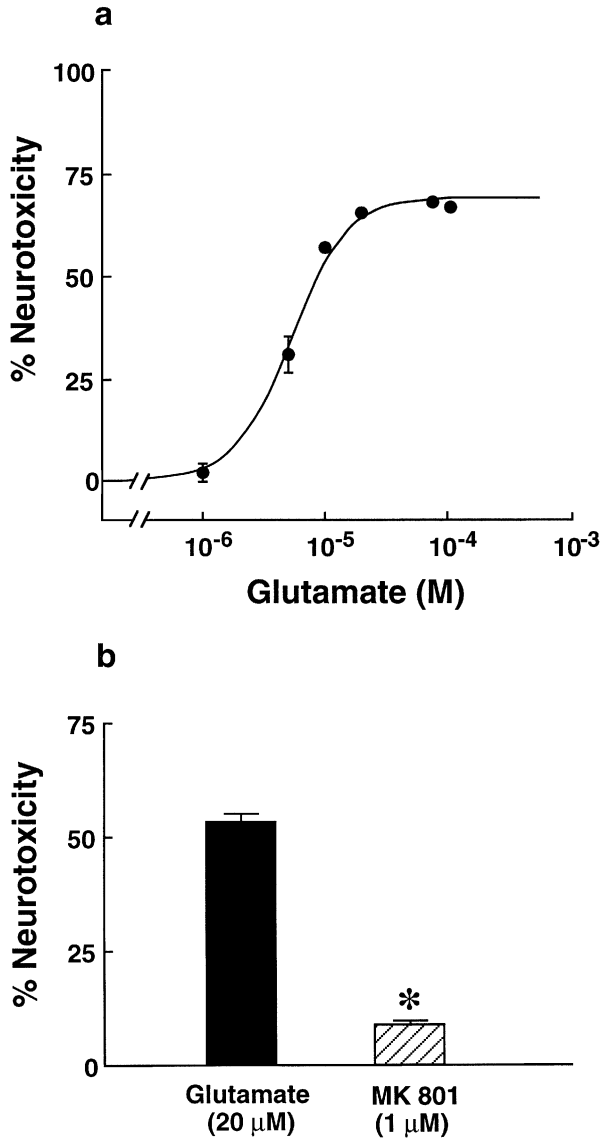


Figure 1 (a) Neurotoxic effect of L-glutamate on cerebellar granule cells as measured by the concentration-dependent inhibition in the formation of reduced MTT relative to unexposed control cells. Neurons were depleted of energy resources by 40 min preincubation in a medium without magnesium and glucose and then incubated for 30 min in the presence of different concentrations of L-glutamate. Neurotoxicity was determined 24 h later as described in Methods. Data shown are mean±s.e.mean of eight wells. (b) Prevention by MK-801 (dizocilpine) of the neurotoxic effect of an EC_{100} of L-glutamate in cerebellar granule cells. Data shown are mean± s.e.mean of eight wells. *$P<0.001$ as compared with cells exposed to L-glutamate (Student's *t*-test).

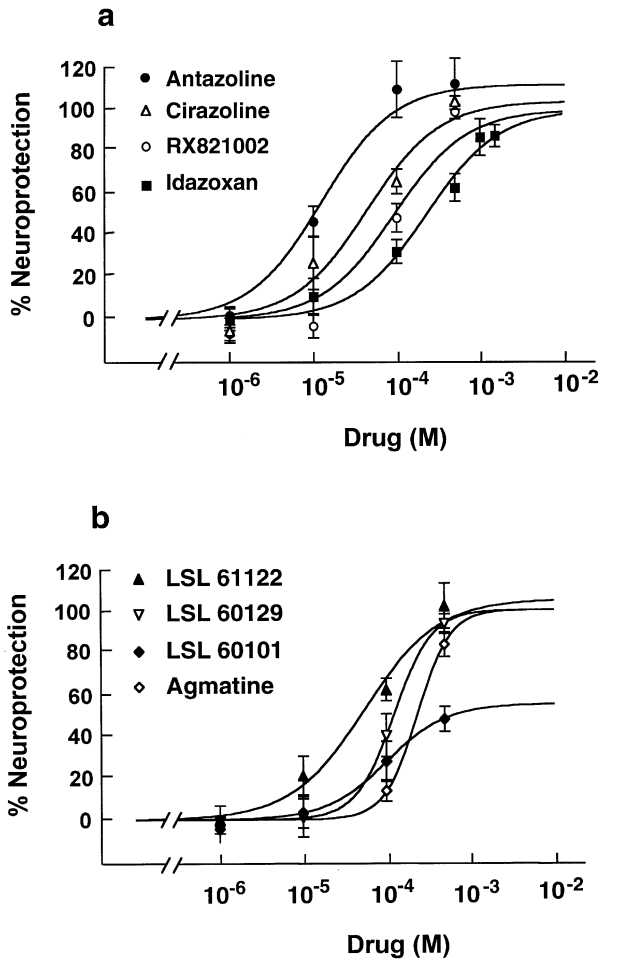


Figure 2 Neuroprotective effects of (a) antazoline, cirazoline, RX821002 and idazoxan and (b) LSL 61122, LSL 60129, LSL 60101 and agmatine against the neurotoxic effect of 20 μM L-glutamate in cerebellar granule cells, as measured by the concentration-dependent increase in the formation of reduced MTT relative to L-glutamate exposed cells. Neurons were depleted of energy resources by 40 min preincubation in a medium without magnesium and glucose and then incubated for 30 min in the presence of L-glutamate. Imidazol(ine) drugs were added during the last 15 min of the preincubation time and maintained throughout the L-glutamate exposure. Neuroprotection was determined 24 h later as described in Methods. Points represent mean±s.e.mean of eight wells. See Table 1 for EC_{50} values.

does not involve activation of the NMDA receptor. Switching culturing conditions of cerebellar granule cells from a medium containing high (25 mM) KCl concentrations to a medium containing low (5 mM) KCl resulted in a decrease in cell viability measured by a 64% decrease in MTT reduction in control cells (Figure 3). Exposure of cells to the imidazol(ine) drugs cirazoline, LSL 60101 and LSL 61122 did not modify significantly the loss in MTT metabolism (Figure 3).

Involvement of NMDA receptor blockade in the neuroprotective effects of imidazol(ine) drugs and agmatine

The above results suggested that prevention of L-glutamate-induced necrotic neuronal death by imidazol(ine) drugs and agmatine in cerebellar granule cells could be mediated by NMDA receptor blockade. To further address this question, the ability of the imidazol(ine) drugs tested and agmatine to interact (agonist or antagonist properties) with NMDA receptors was tested.

Imidazol(ine) drugs and agmatine fully inhibited [^{3}H]-(+)-MK-801 binding to membranes from rat cerebral cortex with potencies in the micromolar range (Figure 4 and Table 1). The obtained profile of drug affinity for [^{3}H]-(+)-MK-801 binding sites on NMDA receptors was very similar to that previously obtained for the effects of the same drugs preventing L-glutamate-induced neurotoxicity. In fact, there was a good correlation ($r=0.90$) between the pEC_{50} values for the neuroprotective effects of these drugs and the pK_i values obtained in competition curves against [^{3}H]-(+)-MK-801 binding (Table 1 and Figure 5). These results suggested that a direct interaction of imidazol(ine) drugs and agmatine with the phencyclidine/MK-801 site of the NMDA receptor could block the neurotoxic effects of L-glutamate. This question was addressed by testing if the imidazoline drug antazoline (the most potent) and agmatine (the least potent) could inhibit glutamate-induced currents.

Whole-cell patch clamp recordings were performed in HEK-293 cells transfected to express the NR1-1a/NR2C subunits of the NMDA receptor. In the absence of Mg^{2+}, antazoline (3 μM) reduced the glutamate-induced current by approximately 45% at -70 mV when applied in the external solution containing 200 μM glutamate and 20 μM glycine (Figure 6a). The effect was reversible upon the washing off of antazoline (not shown). The block was most potent at hyperpolarizing membrane potentials and less effective at positive voltages (Figure 6b). Thus, the IC_{50} at -70 mV was 3.6 μM and increased to 215 μM at $+70$ mV (Figure 6c and d). The plot showing the IC_{50} values for antazoline (logarithmic scale) obtained at different voltages presented two linear portions, with the inflection point close to the current reversal potential. The slope values, representing the effective valences ($z\delta$), were 0.19 and 0.55 (Figure 6d).

Agmatine also reduced the glutamate-induced current in HEK-293 cells. As expected from the potency inhibiting [^{3}H]-

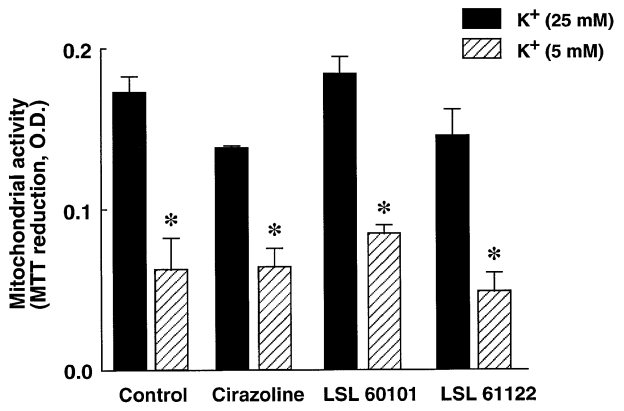


Figure 3 Effect of cirazoline, LSL 60101 and LSL 61122 on neurotoxicity induced by switching from serum-free high $[K^+]_{out}$ (25 mM) to serum-free low $[K^+]_{out}$ (5 mM) medium in cerebellar granule cells. Cells were maintained in serum-free Eagle's Basal Medium containing either 25 or 5 mM K^+ for 24 h in the presence (100 μM) or absence (control) of drugs where indicated. Neurotoxicity was measured using the MTT reduction (mitochondrial activity) assay. Data shown are mean$\pm$s.e.mean of absorbance (optical density, O.D.) units from eight wells. *$P<0.05$ as compared with cells maintained in high $[K^+]_{out}$ (25 mM) medium (Student's t-test). No significant differences were found between control cells and cells exposed to drugs and maintained in low $[K^+]_{out}$ (5 mM) medium.

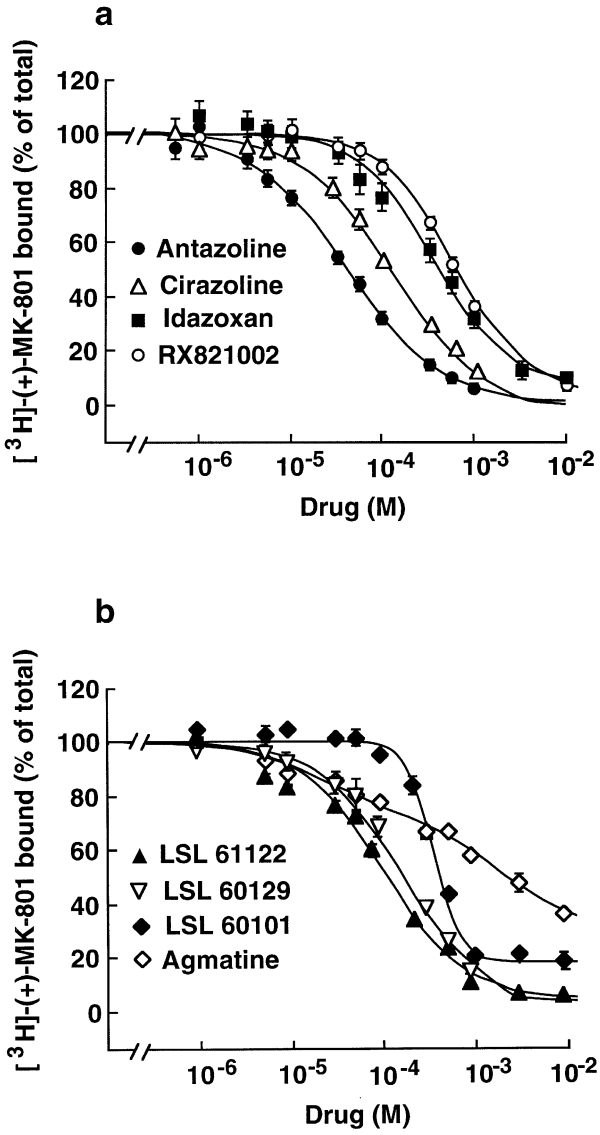


Figure 4 Inhibition of binding of [^{3}H]-(+)-MK-801 by (a) antazoline, cirazoline, idazoxan and RX821002 and by (b) LSL 61122, LSL 60129, LSL 60101 and agmatine in the rat cerebral cortex. Membranes were incubated at 23°C for 45 min with [^{3}H]-(+)-MK-801 (4×10^{-9} M) in the absence or presence of different concentrations of the competing drugs. Data shown are mean$\pm$s.e.mean of 2–4 independent experiments performed in triplicate and expressed as a percentage of total control binding (about 12,000 d.p.m.). See Table 1 for K_i values.

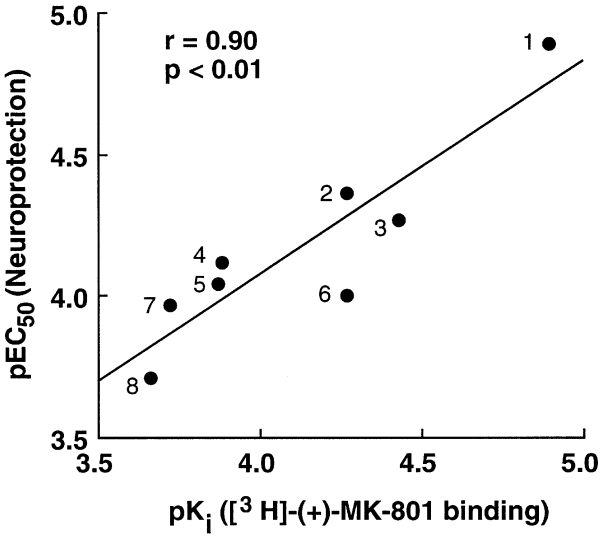


Figure 5 Correlation between the potency (expressed as $pK_i = -\log K_i$) of several imidazol(ine) drugs and agmatine inhibiting the binding of [^{3}H]-(+)-MK-801 to NMDA receptors in the rat brain and the potency (expressed as $pEC_{50} = -\log EC_{50}$) of the same compounds protecting against L-glutamate neurotoxicity in cerebellar granule cells (data taken from Table 1). The data were best described by the equation $y = 0.75x + 1.06$ ($r = 0.90$; $P < 0.01$). The identification of drugs is as follows: (1) antazoline, (2) cirazoline, (3) LSL 61122, (4) LSL 60101, (5) idazoxan, (6) LSL 60129, (7) RX821002 and (8) agmatine.

(+)-MK-801 binding, agmatine was less potent than antazoline and a 47% reduction of glutamate-induced current was achieved in the presence of 200 μM agmatine at -70 mV (Figure 7a). The effect was also reversible upon the washing off of agmatine (not shown) and larger at hyperpolarizing membrane potentials than at positive voltages (Figure 7b and c). A remarkable difference between the action of agmatine and antazoline was revealed upon returning to the holding potential after a voltage ramp to $+70$ mV (Figures 6a and 7a). The current after the voltage ramp remained at the same level when it was blocked with agmatine (Figure 7a). In contrast, in the presence of antazoline there was an increased inward current that slowly returned to the original levels after the voltage ramp. The rates of this 'after-ramp' current relaxations were faster as the concentration of antazoline increased (Figure 6a).

Discussion

This study demonstrates that imidazol(ine) compounds and agmatine fully protected cultured cerebellar granule cells against glutamate-induced toxicity. In the present experimental conditions, cerebellar granule cells are rendered susceptible to glutamate neurotoxicity by glucose deprivation. In energy depleted cerebellar granule cells, glutamate induces a loss of mitochondrial membrane potential (Lysko *et al*., 1989). Overstimulation of the NMDA receptor by glutamate leads to

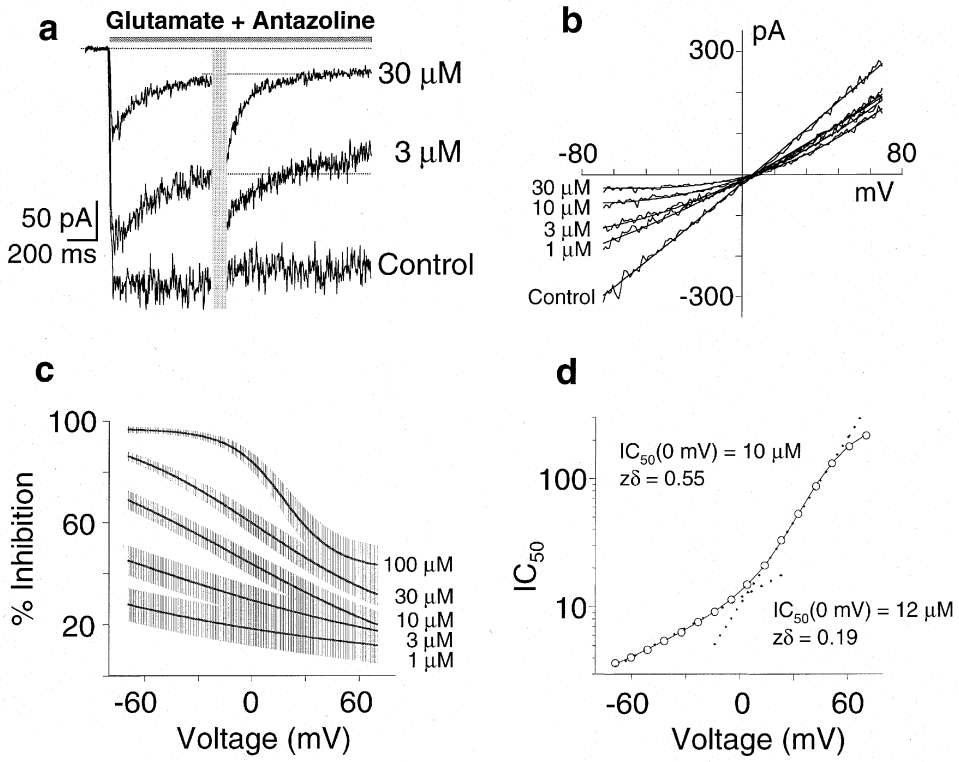


Figure 6 NMDA receptor channel blockade by antazoline in HEK-293 cells transfected to express the NR1-1a and NR2C subunits of the NMDA receptor. (a) Response to 200 μM glutamate in the absence (control) or presence of the indicated concentrations of antazoline. The response returned to control levels upon removal of antazoline (not shown). The current traces were interrupted by a voltage ramp to $+70$ mV (shaded bar). On depolarization the channel was partially relieved of antazoline blockade. On returning to the -70 mV holding voltage the channel was blocked again, causing the observed relaxations of the traces on the right. (b) Ramp I–V curves in the absence (control) or the presence of the indicated concentrations of antazoline. Solid lines represent fits to the Boltzman equation. (c) Extent of current reduction at different voltages in the presence of the indicated concentrations of antazoline. This relationship was generated from the fitted relation in (b) (see Methods). The vertical lines represent the s.e.mean of at least four independent determinations. (d) The logarithm of the IC_{50} values for the inhibition of glutamate-induced currents are plotted as a function of voltage. The relation presented two linear portions that were fitted to the Woodhull model; the best fit parameters for IC_{50} at 0 mV and $z\delta$ are indicated on the figure.

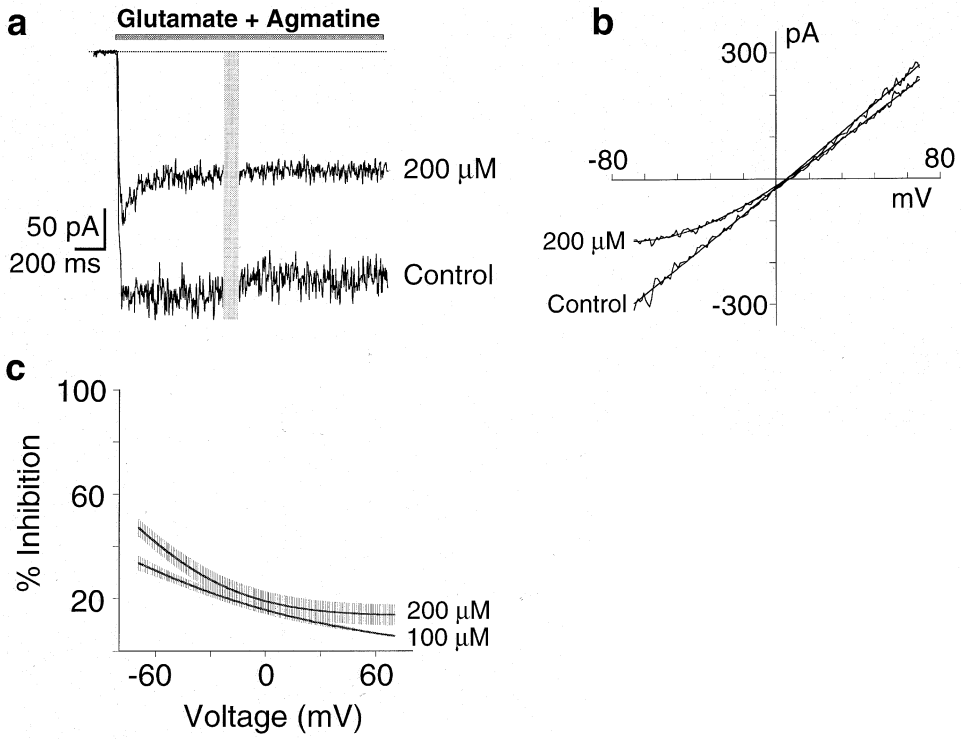


Figure 7 NMDA receptor channel blockade by agmatine in HEK-293 cells transfected to express the NR1-1a and NR2C subunits of the NMDA receptor. (a) Responses to 200 μM glutamate without (control) or with 200 μM agmatine. The response returned to control levels upon removal of agmatine (not shown). The current trace is interrupted by a voltage ramp to +70 mV (shaded bar). (b) Ramp I–V curves obtained in the absence (control) or the presence of 200 μM agmatine in the same cell as in (a). Solid lines represent fits to the Boltzman equation. These data were obtained from the same cell as in Figure 6a. (c) Extent of current reduction at different voltages in the presence of the indicated concentrations of agmatine. This relationship was generated from the fitted relation in (b) (see Methods). The vertical lines represent the s.e.mean of at least four independent determinations.

a Ca^{2+} overload; the inability to maintain osmotic integrity *via* ion exchange provokes cell lysis and cells die by necrosis (Ankarcrona *et al*., 1995). In the present study, the NMDA receptor antagonist MK-801 prevented glutamate-induced toxicity in cultured cerebellar granule cells, as previously described (Lysko *et al*., 1989; Berman & Murray, 1996), thus reinforcing the involvement of NMDA receptors in the neurotoxic effect of glutamate.

Recently, it has been demonstrated that after NMDA receptor stimulation there is a fast mitochondrial Ca^{2+} uptake, suggesting that Ca^{2+} influx through NMDA receptors has a privileged access to mitochondria (Peng & Greenamyre, 1998). The fact that the I_2 subtype of imidazoline receptors is mainly located on mitochondrial membranes (Tesson *et al*., 1991), might suggest that the neuroprotective effect of the drugs tested could be mediated by activation of I_2 receptors that would increase mitochondrial Ca^{2+} influx, and reduce excessive cytosolic Ca^{2+} accumulation. However, no correlation exists between the potency of the tested imidazol(ine) drugs and agmatine on rat brain I_2 imidazoline receptors and the potency protecting against glutamate toxicity ($r=0.17$, $P=0.69$). These results indicate that activation of I_2-imidazoline receptors is not involved in the neuroprotective effects of these drugs against glutamate-induced neurotoxicity.

Because a strong correlation ($r=0.90$, $P<0.01$) was found between the affinity of the imidazol(ine) drugs and agmatine for NMDA receptors and the potency of these compounds in protecting against glutamate-induced neuronal death, the neuroprotective effects of these drugs can be explained by a direct action at NMDA receptors. This interpretation is reinforced by two facts: (1) the lack of effect of the drugs tested in a model of apoptotic neuronal death (low K^+ exposure) in which NMDA receptors are not involved and (2) the ability of antazoline and agmatine to directly block glutamate-induced currents in HEK-293 cells transfected to express the NR1-1a/NR2C subunits of the NMDA receptor. In this sense, whole cell patch-clamp experiments indicated that antazoline and agmatine behave as concentration- and voltage-dependent antagonists of the NMDA receptor. Based on the analysis of voltage dependence of the block, the action of antazoline is consistent with the mechanism of an open channel blocker (Figure 6). Also in this sense, agmatine (100 μM) has been recently shown to block NMDA currents in rat hippocampal neurones by interacting with a site located within the NMDA channel pore and the guanidino group of agmatine has been identified as the responsible moiety for blockade of the NMDA receptor channel (Yang & Reis, 1999). These results, together with the ability of imidazol(ine) drugs and agmatine to inhibit the binding of [^{3}H]-(+)-MK-801 to the phencyclidine site of NMDA receptors (present study; Olmos *et al*., 1996; Boronat *et al*., 1998) and the similar chemical structures of antazoline, the most potent imidazoline drug tested, and phencyclidine, suggest that the binding site for imidazol(ine) drugs and agmatine could overlap with that of the phencyclidine (PCP) site. Indeed, the IC_{50} value of antazoline estimated at 0 mV (10–12 μM) was very close to the K_i value (13 μM) obtained in competition curves against [^{3}H]-(+)-MK-801 binding to rat cerebral cortex membranes. Our electrophysiological analysis indicates that antazoline interacts directly with the NMDA receptor channel pore. The Woodhull model assumes that there is not interaction between a channel blocker and permeant ions (Woodhull, 1993). However, we observed a slight deviation of our data from this model (Figure 6d). The simplest explanation is that when

the net current is outward (many ions flow in the outward direction) the permeating ions expel the blocker from its binding site on the pore (Antonov *et al*., 1998) or alternatively, that divalent cations interact with the blocker binding site within the pore (Lerma *et al*., 1991). The neuroprotective effect of imidazoline drugs and agmatine against glutamate-induced neurotoxicity is specific and related to NMDA receptor blockade, since neurotoxicity in cerebellar granule cells by other mechanisms not involving glutamate receptor activation cannot be prevented by glutamate receptor antagonists (Dargent *et al*., 1996) nor imidazol(ine) drugs.

The potencies (micromolar range) of imidazol(ine) drugs and agmatine at blocking NMDA receptors ([^{3}H]-(+)-MK-801 binding) are in good agreement with the potencies of the same compounds at blocking other cation channels, such as K^+_{ATP} channels (Jonas *et al*., 1992; Shepherd *et al*., 1996), nicotinic acetylcholine (Musgrave *et al*., 1995) and 5-HT$_3$ receptors (Molderings *et al*., 1996). Furthermore, as also demonstrated for these cation channels, imidazol(ine) drugs possessing two benzene rings (e.g. antazoline) are more potent on the NMDA receptor than others with only one aromatic ring (e.g. idazoxan) and these, in turn, are more potent than other molecules lacking an aromatic ring (e.g. agmatine) (Table 1). Hydrocarbon substituents on the benzene ring (e.g. cirazoline) increase the affinity of imidazol(ine) drugs for the NMDA receptor, as demonstrated for the interaction of these drugs with other cation channels (see Sakuta & Okamoto, 1994 for comparison). These findings, together with the observation that there are good correlations between the profiles of drug affinity of imidazol(ine) compounds at blocking different cation channels (K^+_{ATP} channels, NMDA and 5-HT$_3$ receptors) (Olmos *et al*., 1996; Molderings, 1997), reinforce the hypothesis of the existence of a common imidazol(ine)/agmatine binding site on all these cation channels (Olmos *et al*., 1996).

The present results indicate that NMDA receptor blockade by imidazol(ine) drugs and agmatine is anti-excitotoxic in an *in vitro* model of necrotic neuronal death. The imidazoline drug idazoxan and agmatine can also exert neuroprotection after focal or global ischaemia (Gustafson *et al*., 1989; 1990; Maiese *et al*., 1992; Gilad *et al*., 1996), two *in vivo* models of necrotic neuronal death in which clear protective effects of NMDA receptor antagonists have been demonstrated (Meldrum, 1990; Holt, 1997). However, the relatively low affinity of idazoxan and agmatine for NMDA receptors and the fact that the extent of agmatine transport into the brain is unknown, make it difficult that the weak interaction of these drugs with NMDA receptors would also explain the survival-promoting effects found *in vivo*. In fact, it has been suggested that the neuroprotective effect of idazoxan after global ischaemia in the rat is related to the hypothermic effects of this drug (Craven & Conway, 1997). Also in this context, competitive and non-competitive NMDA receptor antagonists have been proved to attenuate the development of tolerance to morphine-induced antinociception (Trujillo & Akil, 1991; Tiseo *et al*., 1994). Although the same effect has been observed after concurrent chronic treatment of morphine with various imidazol(ine) drugs, no relationship between the potency of the drugs tested on NMDA receptors and their ability to attenuate morphine tolerance was found (Boronat *et al*., 1998).

In conclusion, the present study indicates that imidazol(ine) drugs and agmatine are neuroprotective in an *in vitro* model of glutamate-induced necrotic neuronal death and that this effect is mediated through NMDA receptor blockade. Further studies are needed to establish the possible *in vivo* functional implications of these findings.

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