British Journal of Pharmacology (1998) 125, 175–185
http://www.stockton-press.co.uk/bjp

Attenuation of tolerance to opioid-induced antinociception and protection against morphine-induced decrease of neurofilament proteins by idazoxan and other I_2-imidazoline ligands

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1 Agmatine, the proposed endogenous ligand for imidazoline receptors, has been shown to attenuate tolerance to morphine-induced antinociception (Kolesnikov *et al*., 1996). The main aim of this study was to assess if idazoxan, an α_2-adrenoceptor antagonist that also interacts with imidazoline receptors, could also modulate opioid tolerance in rats and to establish which type of imidazoline receptors (or other receptors) are involved.

2 Antinociceptive responses to opioid drugs were determined by the tail-flick test. The acute administration of morphine (10 mg kg^{-1}, i.p., 30 min) or pentazocine (10 mg kg^{-1}, i.p., 30 min) resulted in marked increases in tail-flick latencies (TFLs). As expected, the initial antinociceptive response to the opiates was lost after chronic (13 days) treatment (tolerance). When idazoxan (10 mg kg^{-1}, i.p.) was given chronically 30 min before the opiates it completely prevented morphine tolerance and markedly attenuated tolerance to pentazocine (TFLs increased by 71–143% at day 13). Idazoxan alone did not modify TFLs.

3 The concurrent chronic administration (10 mg kg^{-1}, i.p., 13 days) of 2-BFI, LSL 60101, and LSL 61122 (valldemossine), selective and potent I_2-imidazoline receptor ligands, and morphine (10 mg kg^{-1}, i.p.), also prevented or attenuated morphine tolerance (TFLs increased by 64–172% at day 13). This attenuation of morphine tolerance was still apparent six days after discontinuation of the chronic treatment with LSL 60101-morphine. The acute treatment with these drugs did not potentiate morphine-induced antinociception. These drugs alone did not modify TFLs. Together, these results indicated the specific involvement of I_2-imidazoline receptors in the modulation of opioid tolerance.

4 The concurrent chronic (13 days) administration of RX821002 (10 mg kg^{-1}, i.p.) and RS-15385-197 (1 mg kg^{-1}, i.p.), selective α_2-adrenoceptor antagonists, and morphine (10 mg kg^{-1}, i.p.), did not attenuate morphine tolerance. Similarly, the concurrent chronic treatment of moxonidine (1 mg kg^{-1}, i.p.), a mixed I_1-imidazoline receptor and α_2-adrenoceptor agonist, and morphine (10 mg kg^{-1}, i.p.), did not alter the development of tolerance to the opiate. These results discounted the involvement of α_2-adrenoceptors and I_1-imidazoline receptors in the modulatory effect of idazoxan on opioid tolerance.

5 Idazoxan and other imidazol(ine) drugs fully inhibited [^{3}H]-(+)-MK-801 binding to N-methyl-D-aspartate (NMDA) receptors in the rat cerebral cortex with low potencies (K_i: 37–190 μM). The potencies of the imidazolines idazoxan, RX821002 and moxonidine were similar, indicating a lack of relationship between potency on NMDA receptors and ability to attenuate opioid tolerance. These results suggested that modulation of opioid tolerance by idazoxan is not related to NMDA receptors blockade.

6 Chronic treatment (13 days) with morphine (10 mg kg^{-1}, i.p.) was associated with a marked decrease (49%) in immunolabelled neurofilament proteins (NF-L) in the frontal cortex of morphine-tolerant rats, suggesting the induction of neuronal damage. Chronic treatment (13 days) with idazoxan (10 mg kg^{-1}) and LSL 60101 (10 mg kg^{-1}) did not modify the levels of NF-L proteins in brain. Interestingly, the concurrent chronic treatment (13 days) of idazoxan or LSL 60101 and morphine, completely reversed the morphine-induced decrease in NF-L immunoreactivity, suggesting a neuroprotective role for these drugs.

7 Together, the results indicate that chronic treatment with I_2-imidazoline ligands attenuates the development of tolerance to opiate drugs and may induce neuroprotective effects on chronic opiate treatment. Moreover, these findings offer the I_2-imidazoline ligands as promising therapeutic co-adjuvants in the management of chronic pain with opiate drugs.

Keywords: Idazoxan; I_2-imidazoline receptors; morphine; pentazocine; tolerance; α_2-adrenoceptor antagonists; N-methyl-D-aspartate receptors; neurofilament proteins; neuroprotection; cytoskeleton

Introduction

Chronic exposure to morphine and other opioid agonists leads to drug tolerance, which is characterized by a decrease in antinociceptive efficacy (Self & Nestler, 1995). Co-administration of morphine with a diversity of drug types, e.g. N-methyl-D-aspartate (NMDA) receptor antagonists (Elliot *et al*., 1995; Trujillo, 1995), inhibitors of NO synthase (Herman *et al*., 1995), calcium channel blockers (Contreras *et al*., 1988) or cholecystokinin antagonists (Kellstein & Mayer, 1991), can blockade or attenuate the development of morphine tolerance, indicating that multiple receptor sites and a variety of

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neurotransmitter systems are capable of modulating opioid tolerance.

Several studies have demonstrated that imidazol(ine)/guanidine compounds elicit central and peripheral effects through the interaction with various non-adrenoceptor sites including the so-called imidazoline receptors (for a review see Bousquet, 1995; French, 1995; Regunathan & Reis, 1996; Molderings, 1997) and various cation channels, including NMDA receptors (Olmos *et al*., 1996). According to differences in their pharmacological profiles, tissue and subcellular distributions, imidazoline receptors have been classified into two main types: I_1- and I_2-imidazoline receptors (Michel & Insel, 1989; Ernsberger, 1992). The I_2-imidazoline receptors are widely distributed and in the brain are expressed on neurones, but mainly on glial cells (Regunathan *et al*., 1993; Olmos *et al*., 1994; Ruggiero *et al*., 1998). The amine agmatine (decarboxylated arginine) has been proposed as an endogenous agonist at imidazoline receptors (Li *et al*., 1994) and to act as a neurotransmitter or neuromodulator for some members of this receptor family (Li *et al*., 1994; Piletz *et al*., 1995). Recently, agmatine has been shown to prevent or attenuate tolerance to morphine and other opioid agonists (Kolesnikov *et al*., 1996), thus suggesting a possible implication of the imidazoline receptor system on the modulation of opioid antinociception and tolerance. The presence of imidazoline receptors also has been detected in brain areas involved in the perception and responses to pain (Ruggiero *et al*., 1998).

Prolonged opiate exposure is associated with marked changes in brain cytoeskeletal proteins, i.e. decreases in neurofilament (NF) proteins (Beitner-Johnson *et al*., 1992; García-Sevilla *et al*., 1997) along with reciprocal increases in glial filament proteins (Beitner-Johnson *et al*., 1993). It has been proposed that such plastic changes may reflect neural injury induced by chronic opiate exposure (Nestler *et al*., 1996). In this sense, agmatine and the imidazoline drug idazoxan have been shown to be neuroprotective in several models of brain injury (Gustafson *et al*., 1989; Maiese *et al*., 1992; Gilad *et al*., 1996).

The present study was, therefore, designed (1) to assess if idazoxan, an α_2-adrenoceptor antagonist (Doxey *et al*., 1983) that also interacts with I_1- and I_2-imidazoline receptors (Bricca *et al*., 1993; Miralles *et al*., 1993) modulates opioid tolerance, (2) to establish which imidazoline receptor type or if other receptors (α_2-adrenoceptors, NMDA receptors) could be implicated in the effects of idazoxan and (3) to assess if idazoxan and other I_2-imidazoline ligands interfere with morphine-induced modulation of immunoreactive neurofilament proteins. Idazoxan was chosen as the reference drug because of its current use in human clinical research (Schmidt *et al*., 1997a,b). A preliminary report of a portion of this study was presented at a meeting of the British Pharmacological Society (Boronat *et al*., 1998).

Methods

Animals and treatments

Male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at $20\pm2^{\circ}$C with a 12 h light/dark cycle. In the chronic treatments (13 days), the drugs were administered i.p. every 12 h and the animals (groups of four) were submitted to the following experimental paradigms. Paradigm one: the animals received 0.9% saline vehicle followed by morphine (10 mg kg^{-1}) 30 min later. Paradigm two: the animals received either idazoxan (10 mg kg^{-1}), RX821002 (10 mg kg^{-1}), RS-15385-197 (1 mg kg^{-1}), moxonidine (1 mg kg^{-1}), LSL 60101 (10 mg kg^{-1}), 2-BFI (10 mg kg^{-1}) or LSL 61122 (valldemossine, 10 mg kg^{-1}) followed by morphine (10 mg kg^{-1}) 30 min later. Paradigm three: the animals were treated as in paradigm two except that morphine was substituted by 0.9% saline vehicle. Paradigm four (saline-treated animals): the animals received two 0.9% saline vehicle injections with a 30 min interval. In another series of chronic treatments, morphine was substituted by pentazocine (10 mg kg^{-1}) in experimental paradigm one, and idazoxan (10 mg kg^{-1}) was administered 30 min before pentazocine as in paradigm two.

In the acute treatments, rats were treated according to the experimental paradigms one and two with a single dose of the different drugs. Morphine was always dosed at 3 mg kg^{-1} and idazoxan, LSL 60101, 2-BFI and LSL 61122 (valldemossine) were administered before morphine as above. Other groups of rats were also treated with a single dose of agmatine (10 mg kg^{-1}, i.p.) before morphine administration.

These drugs were used at doses known to interact with the different target receptors (Ernsberger *et al*., 1993; Olmos *et al*., 1994; Alemany *et al*., 1995; Lalies & Nutt, 1995; Kolesnikov *et al*., 1996; Ventayol *et al*., 1997).

Antinociceptive assay

The antinociceptive (analgesic) response to morphine or pentazocine was measured by the tail-flick (TF) test (D'Amour & Smith, 1941). Ambient temperature of the test room was maintained at $24\pm1^{\circ}$C at all times. The radiant heat source was a projection bulb aimed at the caudal end of the tail (4–6 cm from the tail tip) and adjusted to result in baseline latencies of 3–4 s. The TF latency (TFL) was defined as the time from the onset of radiant heat to tail withdrawal. In the acute and chronic treatments a baseline latency was determined (average of three) for every animal before any drug administration. To minimize tail skin tissue damage, a cut-off time was set as three times the baseline latency (9–12 s). In the chronic treatments, baseline latencies were determined at day 1 and cut-off values for every rat were obtained as above and used in the different time points of the TF test. Baseline latencies were reassessed at the end of chronic treatments (day 13).

TFLs were determined 30 min after the last injection; this time peak for morphine and pentazocine antinociceptive effects had been determined in preliminary studies (Ventayol *et al*., 1997). Five TF trials were done for every rat and time point of the chronic and acute drug treatments. The first two trials were discarded and the rest were performed with (1) 15 s intertrial interval and (2) changes in the position of the tail receiving heat stimulation on each trial. The mean TFL from the last three trials was used in the statistical analysis as the antinociceptive score for that animal and time point. These experiments in rats were performed according to the guidelines of the Universitat de les Illes Balears.

[^{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} M to

10^{-2} M; 11 concentrations). Non-specific binding was determined in the presence of 10^{-4}M ketamine. 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. Analyses 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). All experiments were initially analysed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically by the extra sum of squares principle (F test) as outlined by Munson and Rodbard (1980). The more complex model was accepted if the P value resulting from the F test was less than 0.05.

Immunoblot analysis and quantitation of 68 kDa neurofilament (NF-L) proteins

Immunoblotting of neurofilament proteins of low (68 kDa) molecular weight (NF-L) was done as previously described for other cytoskeletal proteins (Olmos *et al*., 1994) with minor modifications (García-Sevilla *et al*., 1997). After 13 days of chronic drug treatments and 24 h of drug washout the rats were decapitated and brains rapidly removed under ice. Sixty to one hundred mg of brain frontal cortex were homogenized (30 s) with an Ultraturrax homogenizer in 50 volumes of 50 mM Tris-HCl buffer, pH 6.8 containing 2 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM iodoacetamide. The samples were then sonicated (10 s) and centrifuged at 15,000 g for 15 min at 4°C. The supernatant was recovered and the protein content determined by the method of Bradford (1976), using bovine serum albumin as the standard. An aliquot (400 μl) of the supernatant was mixed with 50 μl of 160 mM Tris-HCl, 8% SDS, pH 6.8 and 50 μl of electrophoresis loading buffer (500 mM Tris-HCl, 8% SDS, 30% glicerol, 20% 2-mercaptoethanol, 0.02% bromophenol blue, pH 6.8) and boiled. The samples were then submitted to polyacrylamide gel electrophoresis (SDS–PAGE) in a 10% Laemmli gel (1.5 mm thickness). Proteins were transferred to 0.45 micron nitrocellulose membranes (immunoblotting, Western blotting), and blocked at room temperature for 1 h with phosphate buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 12 mM Na_2HPO_4, 1.38 mM KH_2PO_4, pH 7.4) containing 5% nonfat dry milk, 0.5% bovine serum albumin and 0.2% Tween 20 (blocking solution). The primary antibody, anti-68 kDa NF-L mouse monoclonal IgG1 was then added in fresh blocking solution (1 : 500 dilution) and incubated for 14–16 h at 4°C. After two quick rinses with PBS, membranes were washed (for 10 min at room temperature on each occasion) a further three times with PBS. The secondary antibody, horseradish peroxidase-linked sheep anti-mouse IgG, was then added in fresh blocking solution (1 : 2000 dilution), and incubated for 2 h at room temperature. Immunoreactivity was detected with the Enhanced Chemiluminescence (ECL) Western Blot Detection system (Amersham International plc, U.K.) followed by exposure to Hyperfilm ECL film (Amersham International plc, U.K.). Omission of the primary antibody was used as a negative control; i.e. immunoreactivity was absent under this condition. Films were scanned in the image analyser Bio Image (Millipore, Ann Arbor, MI, U.S.A.).

After scanning, standard curves were constructed using samples from saline-treated rats. In these curves, the total protein loaded in at least five wells (4–18 μg) was plotted against the integrated optical density (IOD). A linear relationship (correlation coefficient; $r = 0.98 - 0.99$) between the amount of protein loaded in the gel and the IOD was found all over the range of protein content used. For the quantitation of NF-L protein immunoreactivity, samples from saline-treated and drug-treated rats were loaded in the same gel as well as the standard curve and, for every sample, a theoretical amount of protein loaded in the gel (Pt) was obtained by intrapolation of its IOD in the standard curve. The percentage of NF-L immunoreacivity of a given sample with respect to the standard (saline-treated) samples was calculated as $(Pt/Pr) \times 100$; where Pr is the real amount of protein loaded in the gel well. This quantitation procedure was repeated at least five times for each rat brain sample in different gels. The mean intra- and inter-assay coefficients of variation were 7 and 10%, respectively.

Statistics

TFLs represent the mean ± s.e.mean of four rats. Multiple *post hoc* comparisons between different experimental paradigms at the same time point and within the same experimental paradigm between different time points of chronic treatment were done using one-way analysis of variance (ANOVA) followed by Scheffé's test. In NF-L immunoblot quantitation experiments, results after treatments are expressed as per cent of values of saline-treated rats, as described above, and represent the means ± s.e.mean. Statistical comparisons were done as above. The level of significance was chosen as $P = 0.05$.

Materials and drugs

Anti-68 kDa NF-L mouse monoclonal antibody and horseradish peroxidase-linked sheep anti-mouse IgG antibody were purchased from Amersham International plc (U.K.). [^{3}H]-(+)-MK-801 (23.9 Ci mmol) 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.); 2-BFI (2-(2-benzofuranyl)-2-imidazoline) HCl (synthesized by Dr Plá as LSL 61103 at S.A. Lasa Laboratorios, Spain); idazoxan HCl (synthesized by Dr F. Geijo at S.A. Lasa Laboratorios, Spain); ketamine HCl (Sigma Chemical Co., U.S.A.); LSL 60101 [2-(2-benzofuranyl)imidazole HCl] and LSL 61122 [2-styryl-2-imidazoline HCl, valldemossine] (synthesized by Dr F. Geijo at S.A. Lasa Laboratorios, Spain); morphine HCl (Unión Químico-Farmacéutica S.A.E., Madrid, Spain), moxonidine HCl (Beiersdorf, Hamburg, Germany), pentazocine HCl (Laboratorios Fides, Barcelona, Spain), RX821002 HCl (2-methoxy idazoxan) (synthesized by Dr F. Geijo at S.A. Lasa Laboratorios, Spain) and RS-15385-197 HCl ((8a*R*, 12a*S*, 13a*S*)-3-methoxy-12-methanesulfonyl-5,6,8a,9,10,11,12,12a,13,13a - decahydro - 8H - isoquino [2,1-g]-naphthyridine) (Syntex, Palo Alto, U.S.A.). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

Effects of idazoxan on the antinociceptive responses to morphine and pentazocine

The acute administration of morphine (10 mg kg^{-1}, i.p.) 30 min after a saline injection resulted in cut-off times for

TFLs (9–12 s) (Figures 1, 2 and 4). Chronic morphine (10 mg kg^{-1}) administration to rats receiving saline injections resulted in development of tolerance to its antinociceptive action, as demonstrated by the marked and significant ($P<0.05$) decline in TFLs at days 10 and 13 of treatment compared with day 1 (Figures 1, 2 and 4).

When idazoxan (10 mg kg^{-1}) was given chronically 30 min before morphine no significant differences in TFLs were found between days 1–13 of treatment, thus indicating almost complete prevention of μ-opioid tolerance by idazoxan (TFLs increased by 37–143% with respect to saline-morphine treated rats; $P<0.05$) (Figure 1a). Idazoxan alone had no effects upon TFLs when compared with rats receiving saline injections (Figure 1a).

The acute administration of pentazocine (10 mg kg^{-1}), a mixed κ/δ-opioid receptor agonist and μ-antagonist, also resulted in increased TFLs (cut-off values on day 1) and, similarly to morphine, its initial antinociceptive response was lost at days 10 and 13 of chronic treatment (Figure 1b). Idazoxan (10 mg kg^{-1}) given chronically 30 min before pentazocine significantly attenuated tolerance at days 10 and 13 of treatment (TFLs increased by 56 and 71%, respectively, $P<0.05$) (Figure 1b).

Chronic treatment with idazoxan plus morphine or pentazocine did not alter the subsequent ability of rats to respond to the TF test, as revealed by the lack of significant differences in baseline TFLs between saline-saline (3.8 ± 0.3 s), saline-morphine (3.5 ± 0.4 s), idazoxan-morphine (4.3 ± 0.2 s), saline-pentazocine (3.3 ± 0.1 s) and idazoxan-pentazocine (2.9 ± 0.2 s) at the end of the chronic treatments (day 13). These baseline values did not differ from those obtained at the beginning of each chronic treatment. These results indicated that these chronic drug treatments did not alter the antinociceptive threshold.

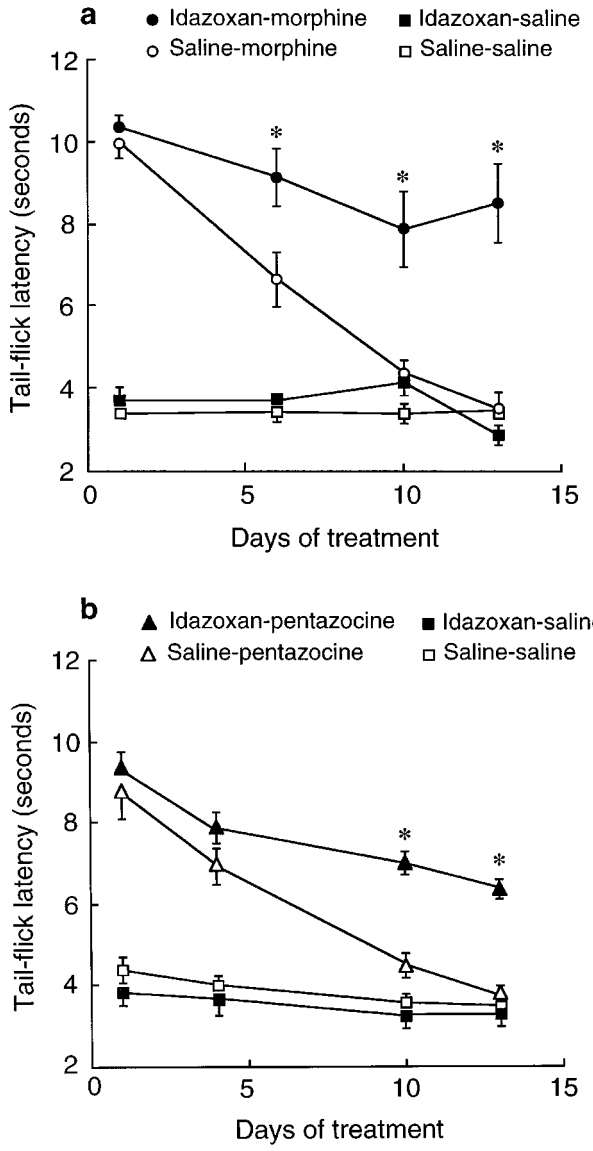

Figure 1 Effects of idazoxan on the antinociceptive responses to (a) morphine and (b) pentazocine. Drugs were administered at 10 mg kg^{-1}, i.p. every 12 h. Idazoxan was always administered 30 min before the opiate or saline injections. Tail-flick tests were performed 30 min after the last injection at the indicated days. Points represent means ± s.e mean of four rats. *At least $P<0.05$ (ANOVA followed by Scheffé's test) when compared with the corresponding saline-morphine or saline-pentazocine treated rats.

Effects of selective I_2-imidazoline ligands on the antinociceptive response to morphine

The concurrent chronic administration of LSL 60101 (10 mg kg^{-1}), a rather selective I_2-ligand, and morphine (10 mg kg^{-1}, 13 days) significantly attenuated morphine tolerance at days 8, 10 and 13 of treatment (TFLs increased by 48, 62 and 64%, respectively, $P<0.05$) (Figure 2a). On the evening of day 13, the treatments were discontinued and all the experimental groups were subjected to a 6-day drug-free period. When the rats that had been treated with LSL 60101-morphine were challenged on day 20 with morphine (10 mg kg^{-1}), they continued to show a greater antinociceptive effect, when compared to animals that had been treated with saline-morphine (TFL increased by 41%; $P<0.05$), which demonstrated a persistence in the attenuation of morphine tolerance in rats treated with LSL 60101-morphine as well as a persistence of tolerance in rats treated with saline-morphine (Figure 2a). No significant differences were found in baseline TFLs between saline-morphine and LSL 60101-morphine treated rats at days 13 and 20 (data not shown). LSL 60101 alone did not modify TFLs (Figure 2a).

The implication of I_2-imidazoline receptors in the protective effects of idazoxan and LSL 60101 on morphine tolerance was reinforced by the effects of two more selective and potent I_2-imidazoline drugs: 2-BFI and LSL 61122 (valldemossine). The concurrent chronic treatment of 2-BFI (10 mg kg^{-1}) and morphine (10 mg kg^{-1}, 13 days) completely prevented morphine tolerance, i.e. no significant differences in TFLs were found between days 1–13 of treatment (TFLs increased by 84–172% with respect to saline-morphine treated rats) (Figure 2b). Similarly, the concurrent chronic treatment of LSL 61122 (valldemossine) (10 mg kg^{-1}) and morphine (10 mg kg^{-1}, 13 days) markedly attenuated morphine tolerance at days 8, 10 and 13 of treatment (TFLs increased by 75, 102 and 94%, respectively, $P<0.05$) (Figure 2c). No significant differences were found in baseline TFLs between saline-morphine, 2-BFI-morphine and LSL 61122-morphine at the end of the chronic treatments (day 13) (data not shown).

The attenuation of morphine tolerance in rats concurrently treated with morphine and idazoxan or other I_2-imidazoline selective drugs could have resulted from a non-specific drug interaction, and not from a blockade in the development of morphine tolerance *per se*. This possibility was tested by using a lower dose of morphine (3 mg kg^{-1}, i.p.) with which TFLs did not achieve cut-off values (defined as three times the baseline value) (Figure 3). When idazoxan, LSL 60101, 2-BFI, LSL 61122 (valldemossine) and agmatine were administered

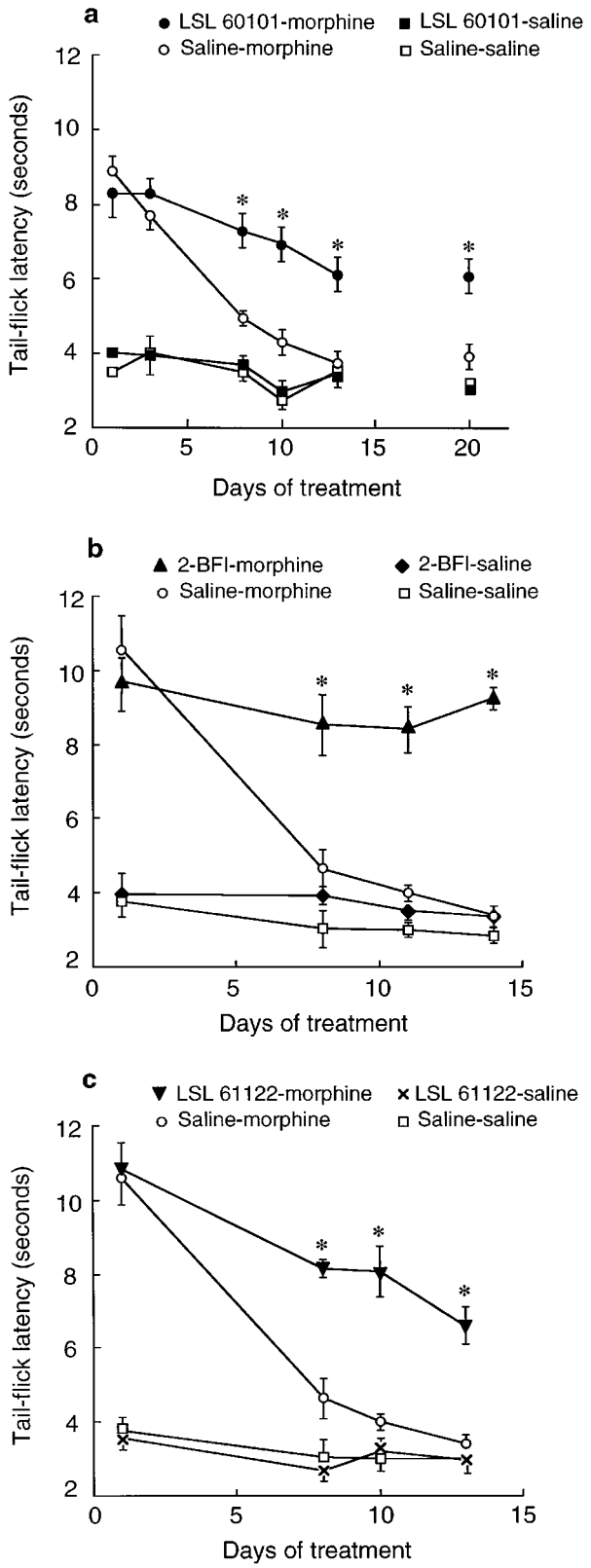

Figure 2 Effects of drugs selective for I_2-imidazoline receptors: (a) LSL 60101, (b) 2-BFI and (c) LSL 61122 on the antinociceptive responses to morphine. Drugs were administered at 10 mg kg^{-1}, i.p. every 12 h and always 30 min before morphine (10 mg kg^{-1}, i.p.) or saline injections. Tail-flick tests were performed 30 min after the last injection at the indicated days. Points represent means ± s.e.mean of four rats. *At least $P<0.05$ (ANOVA followed by Scheffé's test) when compared with the corresponding saline-morphine treated rats.

i.p. at 10 mg kg^{-1} 30 min before morphine (3 mg kg^{-1}) no potentiation or attenuation of the acute (30 min) antinociceptive effect of morphine was observed (Figure 3). These results further suggested that the prolongation of antinociception in rats chronically treated with morphine and I_2-imidazoline ligands could be due to a blockade of morphine tolerance.

Prevention of morphine tolerance by I_2-imidazoline ligands: lack of involvement of α_2-adrenoceptors and I_1-imidazoline receptors

Idazoxan is an imidazoline α_2-adrenoceptor antagonist that also displays nanomolar affinity for I_1-imidazoline receptors (Bricca *et al*., 1993; Codd *et al*., 1995). Thus, the possible involvement of these receptors in mediating the effect of idazoxan was first studied.

The concurrent chronic administration of RX821002 (10 mg kg^{-1}), the 2-methoxy derivative of idazoxan and also a potent α_2-adrenoceptor antagonist, and morphine (10 mg kg^{-1}, 13 days) did not attenuate the development of morphine tolerance (Figure 4a). Similar negative results were obtained when the highly potent and selective non-imidazoline α_2-adrenoceptor antagonist, RS-15385-197 was administered (1 mg kg^{-1}) before morphine in the chronic treatment (Figure 4b). These α_2-adrenoceptor antagonists did not modify TFLs when given alone (Figures 4a, b). The concurrent chronic administration of moxonidine (1 mg kg^{-1}), a mixed I_1-imidazoline receptor and α_2-adrenoceptor agonist before morphine (10 mg kg^{-1}, 13 days) did not prevent the development of morphine tolerance (Figure 4c). Moxonidine alone did not alter TFLs (Figure 4c).

No significant differences were found in baseline TFLs between saline-morphine, RX821002-morphine, RS-15385-197-morphine and moxonidine-morphine at the end of the chronic treatments (day 13) (data not shown). Together, these results discounted the involvement of α_2-adrenoceptors and I_1-imidazoline receptors in the modulatory effect of idazoxan on opioid tolerance.

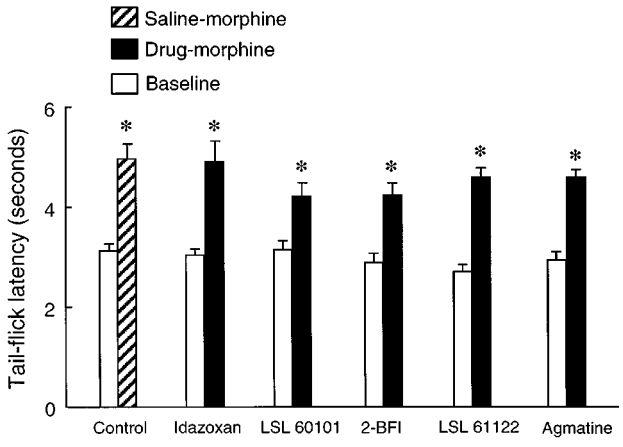

Figure 3 Effects of idazoxan and other drugs selective for I_2-imidazoline receptors on the antinociceptive response to a single administration of morphine. Imidazol(ine) drugs and agmatine were administered i.p. at 10 mg kg^{-1} 30 min before morphine (3 mg kg^{-1}, i.p.). Control rats received saline injections and then morphine (3 mg kg^{-1}, i.p.). Tail-flick (TF) tests were performed 30 min after morphine administration. Columns represent means ± s.e.mean of four rats. *$P<0.05$ when compared with baseline TFLs. No significant differences ($P>0.05$) were found between drug-morphine treated rats and control rats in TFLs after drug treatments (ANOVA followed by Scheffé's test).

Prevention of morphine tolerance by I_2-imidazoline ligands: lack of involvement of NMDA receptors

Imidazol(ine) drugs have been shown to interact with the phencyclidine site of NMDA receptors (Olmos *et al.*, 1996). Antagonists of these receptors have been demonstrated to prevent or attenuate the development of tolerance to the antinociceptive effects of opiates (see Introduction). The possibility that idazoxan could prevent opioid tolerance through a mechanism involving blockade of NMDA receptors was assessed using radioligand receptor binding.

Idazoxan and other imidazol(ine) drugs fully inhibited [^{3}H]-(+)-MK-801 binding to membranes from rat cerebral cortex (Figures 5a, b). The competition curves for LSL 60101 and moxonidine were very steep, and non-linear analysis revealed Hill slopes (n_H) higher than unity ($n_H = 3.6 \pm 0.9$ and 2.7 ± 0.9, respectively, $P < 0.001$; F test) (Figure 5b and Table 1). On the other hand, competition curves for agmatine were shallow and non-linear analysis indicated that, for this compound, a two-site fit was significantly better than a one-site fit. Agmatine displayed medium ($K_i = 8.6$ μM) and low affinity ($K_i = 840$ μM) for these two sites on NMDA receptors (Figure 5b and Table 1). The significant deviation from unity of Hill slopes for the competition curves of LSL 60101, moxonidine and the amine agmatine against [^{3}H]-(+)-MK-801 binding, suggests that these drugs may also bind to other sites on NMDA receptors (e.g. the poliamine site), and that this interaction can be influenced by both positive (Hill slopes higher than unity for LSL 60101 and moxonidine) and negative (Hill slopes lesser than unity for agmatine) interactions.

Potencies of all the drugs tested against [^{3}H]-(+)-MK-801 binding were in the micromolar range (Figures 5a, b and Table 1). The potencies of the imidazolines idazoxan, its 2-methoxy derivative, RX821002, and moxonidine were similar (Table 1), but only idazoxan was able to prevent opioid tolerance (see above). These results suggested that although imidazol(ine) drugs are able to interact with NMDA receptors, there is no relation between the potency on these receptors and the ability to attenuate opioid tolerance.

Effect of chronic morphine treatment on NF-L proteins in brain: reversal by idazoxan and LSL 60101

Chronic morphine treatment in rats and opiate addiction in humans are associated with down-regulation of immunoreactive NF protein levels in brain (Beitner-Johnson *et al.*, 1992; García-Sevilla *et al.*, 1997). Since I_2-imidazoline receptors modulate opioid tolerance it was interesting to test if these novel receptors could also interfere in the morphine-induced regulation of NF proteins levels.

NF-L immunoreactivity was measured at the end of chronic treatments (13 days plus 24 h washout) in brain of rats in which antinociceptive scores (TFLs) had been determined previously. The experiment in Figure 2a was repeated, only until day 13, with similar results on TFLs (data not shown), then the animals were killed and their frontal cortex dissected for quantitation of NF-L immunoreactivity. A specific band for NF-L protein was found in the frontal cortex after immunoblotting (Figure 6a); the relative molecular mass (Mr = 68 kDa) was that described for the monomeric form of this cytoskeletal protein (Lee & Cleveland, 1996).

Chronic treatment (13 days) with morphine (10 mg kg^{-1}) induced a marked decrease (49%; $P < 0.001$) in NF-L immunoreactivity in the frontal cortex, when compared with rats that had received saline injections (Figures 6a, b). In contrast, chronic treatment (13 days) with idazoxan (10 mg kg^{-1}) and LSL 60101 (10 mg kg^{-1}) did not modify significantly the levels of NF-L proteins in brain (Figures 6a, b). Interestingly, the concurrent chronic treatment (13 days) of idazoxan (10 mg kg^{-1}) or LSL 60101 (10 mg kg^{-1}) and

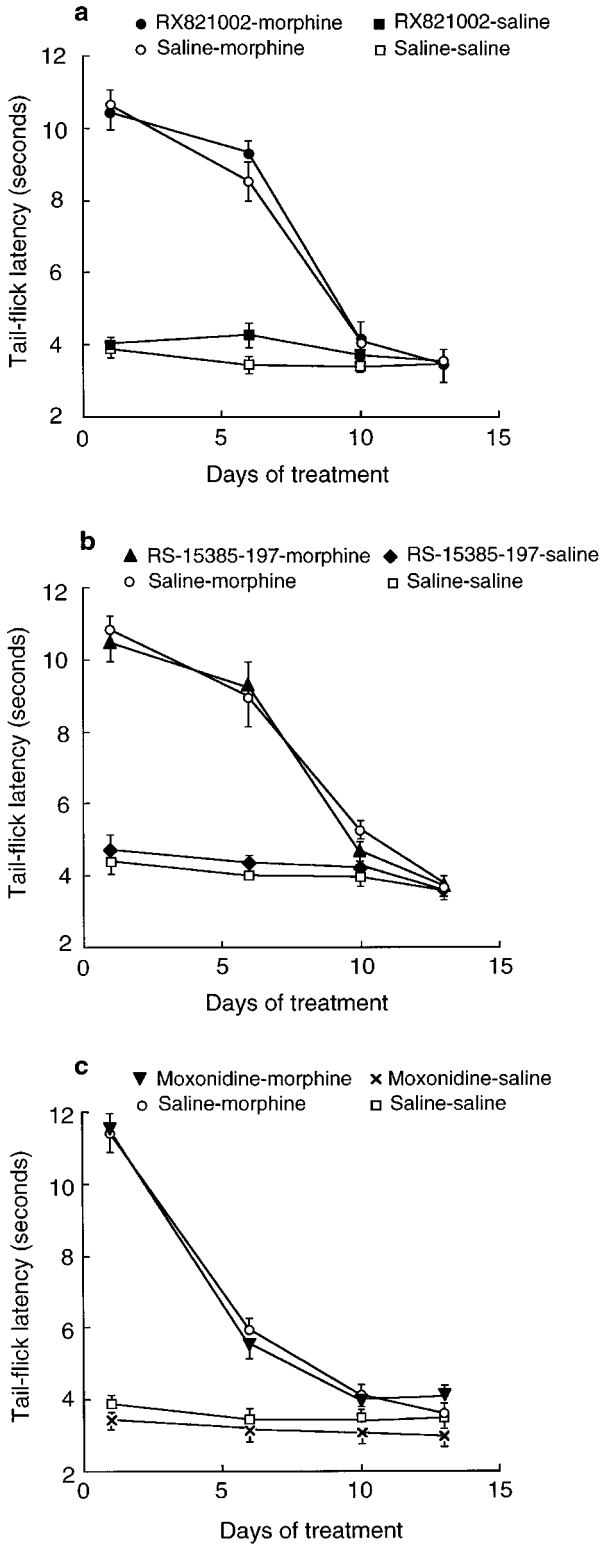

Figure 4 Effects of drugs selective for α_2-adrenoceptors and moxonidine on the antinociceptive responses to morphine. (a) RX821002 (10 mg kg^{-1}), (b) RS-15385-197 (1 mg kg^{-1}) and (c) moxonidine (1 mg kg^{-1}) were administered i.p. every 12 h and always 30 min before morphine (10 mg kg^{-1}, i.p.) or saline injections. Tail-flick tests were performed 30 min after the last injection at the indicated days. Points represent means ± s.e.mean of four rats.

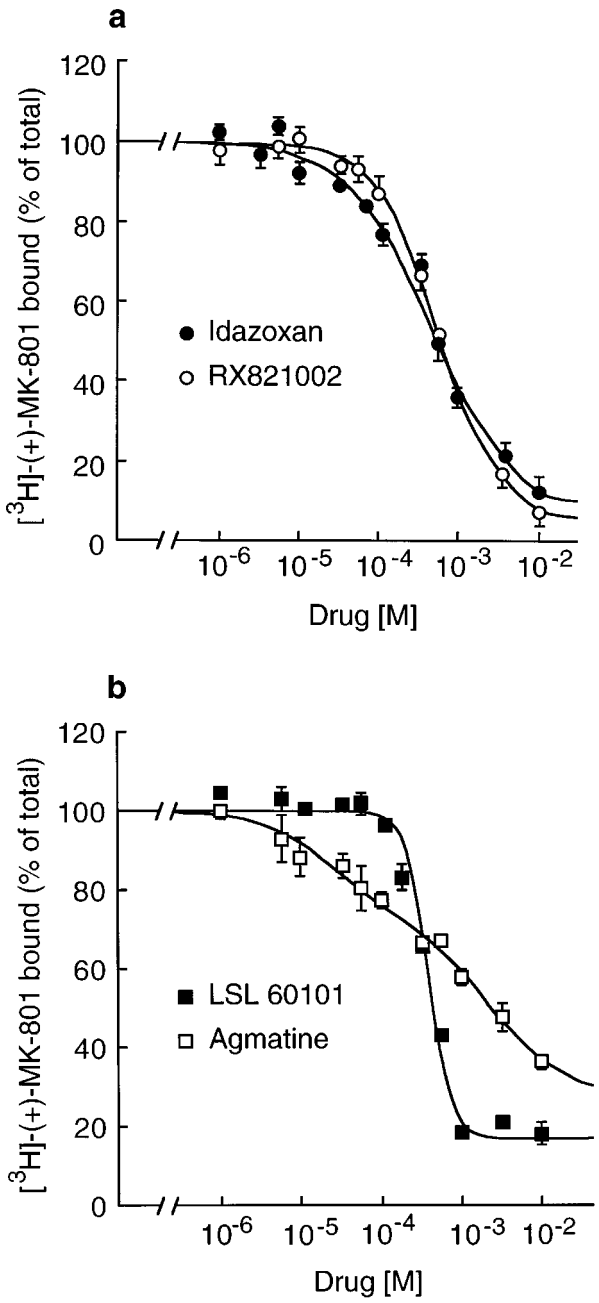

Figure 5 Inhibition of binding of [^{3}H]-(+)-MK-801 to NMDA receptors in rat cerebral cortex by (a,b) imidazol(ine) drugs and (b) agmatine. Cortical membranes were incubated at 23°C for 45 min with [^{3}H]-(+)-MK-801 (4×10^{-9} M) in the absence or presence of various concentrations of the competing drugs. Total control binding was about 25,000 d.p.m. Data shown are means ± s.d. or s.e.mean of two to four independent experiments per drug. See Table 1 for K_i values and other details.

morphine, completely reversed the morphine-induced decrease in NF-L immunoreactivity (Figure 6a, b).

Discussion

This study demonstrates that the concurrent chronic administration of morphine or pentazocine with idazoxan and other I_2-imidazoline receptor selective ligands, such as LSL 60101, 2-BFI and LSL 61122 (valldemossine), prevented or attenuated

Table 1 Affinity of various drugs for I_2-imidazoline receptors (labelled by [^{3}H]-2-BFI binding) and for N-methyl-D-aspartate (NMDA) receptors (labelled by [^{3}H]-(+)-MK-801 binding) in the rat cerebral cortex

Drug	*I_2-Imidazoline receptors [^{3}H]-2-BFI binding* K_i *(μM)*	*NMDA receptors [^{3}H]-(+)-MK-801 binding* K_i *(μM)*
LSL 61122	0.0001	37
2-BFI	0.007	112
Idazoxan	0.014	134
LSL 60101	0.350	132*
Moxonidine	5	164*
RX821002	45	190
RS-15385-197	200	262
Agmatine	294	8.6*

Binding parameters (K_i values) for I_2-imidazoline receptors were taken from Miralles *et al.* (1993); Alemany *et al.* (1995) and Ozaita *et al.* (1997). For LSL 61122 (valldemossine), 2-BFI, idazoxan and LSL 60101 the K_i value represents the affinity for the high affinity site of brain I_2-imidazoline receptors. Binding parameters (K_i values) for NMDA receptors were determined by simultaneous analysis of two to four independent experiments for each drug using the EBDA-LIGAND programmes. *Denotes Hill slope significantly different from unity in the competition curves. For LSL 60101 and moxonidine, Hill slopes were significantly higher than unity ($P < 0.001$; *F*-test). For agmatine, Hill slope were significantly lesser than unity and a two-site fit was better than a one-site fit ($P < 0.001$; *F*-test); the K_i value represents the affinity for the site with the best potency which accounts for a 38% of total [^{3}H]-(+)-MK-801 binding to NMDA receptors. See text and Figure 5a and b for further details.

the development of opioid tolerance. Moreover, idazoxan and LSL 60101 also had neuroprotective effects on morphine-induced decreases of immunolabelled 68 kDa neurofilament (NF-L) proteins in the rat frontal cortex.

Lack of involvement of α_2-adrenoceptors, I_1-imidazoline receptors and NMDA receptors in the effect of idazoxan

The effect of idazoxan could not be attributable to its antagonistic activity on α_2-adrenoceptors because other potent α_2-antagonists such as RX821002 (Galitzky *et al.*, 1990; Miralles *et al.*, 1993) and RS-15385-197 (Brown *et al.*, 1993) did not modify morphine antinociception or the development of tolerance to the opioid.

I_1-imidazoline receptors also do not seem to be implicated in the effects of idazoxan, because moxonidine, a mixed I_1/α_2-agonist (Ernsberger *et al.*, 1993; Likungu *et al.*, 1996) did not affect the antinociceptive effect of morphine or the development of tolerance to the opioid. Moxonidine given alone did not have antinociceptive or hyperalgesic effects. Together, these results suggest that the activation of I_1-imidazoline receptors is not involved in morphine-induced antinociception and tolerance.

The NMDA receptors, a class of excitatory aminoacid receptors, have an important role in the development of opioid tolerance and dependence (Elliot *et al.*, 1995; Trujillo, 1995). Competitive and non-competitive NMDA antagonists have been proved to prevent or attenuate the development of morphine tolerance (Trujillo & Akil, 1991; Tiseo *et al.*, 1994). In this context, imidazoline drugs have been shown to interact weakly with NMDA receptors in the rat brain (Olmos *et al.*, 1996; present results). However, the current results indicate

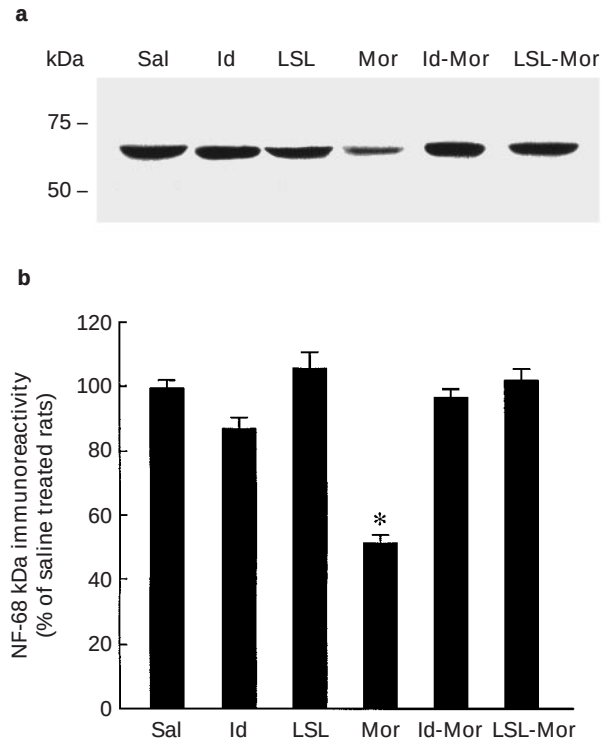

Figure 6 (a) Representative immunoreactive bands using antisera against NF-L proteins ($M_r = 68$ kDa) in the frontal cortex of rats after saline (Sal) or chronic (13 days) treatments with idazoxan (Id), LSL 60101 (LSL), morphine (Mor), idazoxan plus morphine (Id-Mor) and LSL 60101 plus morphine (LSL-Mor). Imidazol(ine) drugs were administered i.p. at 10 mg kg^{-1} every 12 h and always 30 min before morphine (10 mg kg^{-1}, i.p.). Samples from the frontal cortex were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes (immunoblotting), incubated with the specific primary and secondary antibodies and visualized by the Enhanced Chemiluminescence (ECL) method. The apparent molecular mass of NF-L protein was determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. All samples were run in the same gel. The amount of total protein loaded per well was as follows (in μg): 11 (Sal); 8 (Id); 10 (LSL); 11 (Mor); 7 (Id-Mor) and 10 (LSL-Mor). (b) NF-L immunoreactivity after the various chronic treatments expressed as a percentage of that in saline-treated rats (see Methods). Columns represent means ± s.e.mean of four to eight rats per treatment. $^*P < 0.001$ when compared with any other treatment (ANOVA followed by Scheffé's test).

that there is not a relation between potency of imidazol(ine) drugs on NMDA receptors and their ability to protect against the development of morphine tolerance, which makes it unlikely that protection against morphine tolerance by imidazol(ine) drugs may involve blockade of NMDA receptors.

Involvement of I_2-imidazoline receptors in the attenuation of opioid tolerance and neuroprotection after chronic morphine treatment

In addition to idazoxan, for many years the prototypical ligand for I_2-imidazoline receptors (Miralles *et al*., 1993; French, 1995), other more selective and/or potent I_2-imidazoline receptor ligands such as LSL 60101 (Alemany *et al*., 1995); 2-BFI (Hudson *et al*., 1995; Alemany *et al*., 1997) and LSL 61122 (Ozaita *et al*., 1997), also protected or attenuated morphine tolerance. It is unlikely that the effects of these imidazol(ine) drugs involve a direct interaction with opioid receptors; first, because idazoxan is not active on these receptors (Doxey *et al*., 1983) and the μ-agonist methadone displays low affinity against [^{3}H]-idazoxan binding to I_2-imidazoline receptors in the rat cerebral cortex ($K_i = 12$ μM; unpublished results from this laboratory), and second, because no potentiation or attenuation of morphine antinociception was observed when these I_2-ligands and agmatine were administered before morphine (3 mg kg^{-1}, i.p.) to naive rats. These results indicate that there is not synergy between I_2-imidazoline receptors and opioid receptors. In addition, the lack of effects on TFLs of the I_2-ligands in the absence of morphine, suggests that activation/blockade of I_2-imidazoline receptors does not induce antinociceptive or hyperalgesic effects. Therefore, chronic treatment with I_2-imidazoline ligands appears to modulate the development of tolerance to opiate drugs.

These findings are in agreement with a previous study showing that I_2-imidazoline receptors are not involved in the antinociceptive action of clonidine (Monroe *et al*., 1995), but contrast with others reporting hyperalgesic responses to the high affinity I_2-ligand RS 45041 (Houghton & Westlund, 1996), or reduction in the nociceptive responses of dorsal horn neurones after spinal administration of the I_2-ligand BU-224 (Diaz *et al*., 1997). Also in this context, a single dose of agmatine (10 mg kg^{-1}, s.c.) enhanced morphine antinociception in naive mice (Kolesnikov *et al*., 1996), but no effect was observed in the present study when agmatine (10 mg kg^{-1}, i.p.) was administered before morphine to naive rats (Figure 3). Recently, agmatine (10–200 mg kg^{-1}, i.v.) did not enhance the potency of the μ-opioid receptor agonist fentanyl (Bradley & Hadley, 1997). This result, together with the low affinity of agmatine for I_2-imidazoline receptors in the rat cerebral cortex (Table 1), raises the question as to whether the previously reported analgesic effects of agmatine (Kolesnikov *et al*., 1996) are mediated or not through I_2-imidazoline receptors.

In the present study, chronic morphine (10 mg kg^{-1}, i.p. every 12 h for 13 days) was associated with a marked decrease (49%) in the immunoreactivity of NF-L proteins (Lee & Cleveland, 1996). Similar reductions of NF-L (47%) were reported in brains of opioid addicts (García-Sevilla *et al*., 1997). In a previous study in rats, chronic morphine also was shown to induce a decrease in the immunoreactive levels of NF-L in the brain, which represented a real decrease because similar or yet higher reductions were obtained when the proteins were quantitated after Coomassie blue staining (Beitner-Johnson *et al*., 1992). NF proteins are intrinsic determinants of axonal caliber (Hoffman *et al*., 1984) and reduced levels of NF are associated with decreased axonal caliber and conduction velocity (Sakaguchi *et al*., 1993). Direct evidence for impairment of axonal transport following chronic morphine treatment has been demonstrated (Beitner-Johnson & Nestler, 1993a). Chronic morphine also has been shown to reduce the size, and alter the shape, of specific dopamine neurones in brain (Sklair-Tavron *et al*., 1996; Nestler *et al*., 1996). It has been proposed that these structural and functional changes may reflect neural injury induced by chronic opiate exposure (Nestler *et al*., 1996). In fact, neuronal damage induced by various procedures has been shown to also result in down-regulation of NF proteins (Gold *et al*., 1985; Liuzzi & Tedeschi, 1992). Recently, exposure of neuronal cultures from embryonic chick brain and specific cell lines to μ- and κ-opioid agonists increased their vulnerability to death by an apoptotic mechanism (Dawson *et al*., 1997; Goswami *et al*., 1998).

Chronic treatment with morphine is associated with increased synaptosomal calcium content (Guerrero-Munoz *et*

al., 1979; Welch & Olson, 1991). Elevation of intraneuronal calcium and calcium-dependent activation of nitric oxide synthase are common pathways for both opioid tolerance and neuronal death, and drugs that are active protecting against opioid tolerance (e.g. NMDA receptor antagonists, calcium channel blockers and NO synthase inhibitors; see Introduction) are also active protecting against brain injury (Buisson *et al*., 1992; Casanovas *et al*., 1996). In this context, agmatine and the imidazoline drug idazoxan have been shown to be neuroprotective in several models of brain injury (Gustafson *et al*., 1989; Maiese *et al*., 1992; Gilad *et al*., 1996). In the present study, the concurrent administration of morphine with idazoxan or the more selective I_2-imidazoline ligand, LSL 60101, not only attenuated tolerance to the opioid but also completely protected, in the same animals, against morphine-induced decreases of NF-L proteins in brain. These results suggest that, as demonstrated for other receptor systems, attenuation of opioid tolerance and neuroprotection by I_2-imidazoline ligands might be related. Although the signal transduction associated with I_2-imidazoline receptors are not fully understood, their preferential subcellular localization on mitochondrial outer membranes (Tesson & Parini, 1991; Tesson *et al*., 1991) makes it possible that activation of I_2-imidazoline receptors could be related to increased mobilization of mitochondrial calcium stores, thus reducing synaptosomal calcium content and, in this way, opioid tolerance and the neurotoxic effects of chronic opiate exposure.

Chronic administration of I_2-imidazoline drugs is associated with astrocytic hyperplasia with parallel increases in the levels of the astrocytic marker glial fibrillary acidic protein (GFAP) and in glial I_2-imidazoline receptors (Olmos *et al*., 1994; Alemany *et al*., 1995). In contrast, morphine exposure suppresses the growth of astrocytes (Hauser & Stiene-Martin, 1991; Stiene-Martin & Hauser, 1993). Also, opioid addiction induces down-regulation of glial I_2-imidazoline receptors in the human frontal cortex (Sastre *et al*., 1996). In this context, the concurrent administration of morphine and I_2-imidazoline ligands might counteract the inhibitory effects on glial growth of chronic opiate exposure. Since astrocytes are important to regulate synaptic density (Meshul *et al*., 1987), the growth of astrocytes could modulate the synaptic plasticity that seems to be associated with chronic morphine exposure (Nestler *et al*., 1996). Such morphological changes could explain the long-lasting effects of LSL 60101 in preserving the antinociceptive effect of morphine, even after a 6-day drug-free period (Figure 2a).

Activation of astroglia by I_2-imidazoline ligands could also contribute to prevent or attenuate both the tolerance and the neurotoxic effects of chronic opiate administration. Firstly, astrocytes are a site of synthesis and storage of agmatine (Regunathan *et al*., 1995). Increased agmatine production by activated astroglia may have paracrine effects on neighbouring neurones, leading to attenuation of morphine tolerance and neuroprotection, as demonstrated for the exogenously administered agmatine (Kolesnikov *et al*., 1996; Gilad *et al*., 1996). Secondly, activated astrocytes produce several neurotrophic factors (Oderfeld-Nowak *et al*., 1992) and it has been demonstrated that administration of specific neurotrophic factors can reverse some of the effects of chronic morphine exposure (Bershow *et al*., 1995); this may also counteract the decreased levels of these factors observed after chronic morphine administration (Beitner-Johnson & Nestler, 1993b). Thirdly, astrocytic hyperplasia may be associated with increased glial glutamate uptake (Dingledine & McBain, 1994), reducing in this way the increased NMDA receptor activity that is associated with the development of tolerance to chronic morphine (Elliot *et al*., 1995).

In conclusion, the present results demonstrate that chronic treatment with I_2-imidazoline ligands attenuates the development of tolerance to opiate drugs and may induce neuroprotective effects on chronic opiate treatment. These findings offer the I_2-imidazoline ligands as promising therapeutic co-adjuvants in the management of chronic pain with opiate drugs.

This study was supported by DGICYT Grant PB94-0002 Mod C. M.A.B. was supported by a fellowship from the Universitat de les Illes Balears. We are grateful to Consell Insular de Mallorca for support, Dr R.V. Rial for supplying the tail-flick apparatus and the various pharmaceuticals firms for gifts of drugs. J.A.G.-S. is a member of the Institut d'Estudis Catalans.

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(Received January 1, 1998
Revised June 1, 1998
Accepted June 5, 1998)