Agmatine Induces Antihyperalgesic Effects in Diabetic Rats and a Superadditive Interaction with *R*(−)-3-(2-Carboxypiperazine-4-yl)-propyl-1-phosphonic Acid, a *N*-Methyl-D-aspartate-Receptor Antagonist

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ABSTRACT

Agmatine, an endogenous cationic amine resulting from the decarboxylation of L-arginine, produces antihyperalgesic and antiallodynic effects in animal models of chronic neuropathic and inflammatory pain. We examined the effect of agmatine on tactile and thermal allodynia and on mechanical hyperalgesia in streptozocin-induced diabetic rats. To determine its mechanism of action and the potential interest of some of its combinations, the antihyperalgesic effect of agmatine was challenged with α_2-adrenergic imidazoline and opioid-receptor antagonists, and its interaction with the opioid-receptor agonist morphine, the competitive *N*-methyl-D-aspartate receptor antagonist D-CPP [*R*(−)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid], and the nitric-oxide synthase inhibitor L-NAME (L-N^G-nitro-L-arginine methyl ester) were examined. When intrathecally (i.t.) injected (4.4 to 438 nmol/rat), agmatine was ineffective in normal rats but suppressed tactile allodynia (von Frey hair test), thermal allodynia (tail immersion test), and mechanical hyperalgesia (paw-pressure test) in diabetic rats. This spinal antihyperalgesic effect was suppressed by idazoxan (40 μmol/rat i.t.) but not by yohimbine (40 μmol/rat i.t.) or naloxone (0.69 μmol/rat i.v.). In diabetic rats, an isobolographic analysis showed that combinations of i.t. agmatine with i.v. L-NAME or with i.t. morphine resulted in an additive antihyperalgesic effect, whereas the agmatine/D-CPP i.t. combination was superadditive. In summary, the present findings reveal that spinal agmatine produces antiallodynic and antihyperalgesic effects in diabetic neuropathic pain involving, at least for its antihyperalgesic effect, the imidazoline receptors. Moreover, agmatine combined with D-CPP produces an antinociceptive synergy in experimental neuropathy, opening opportunities in the development of new strategies for pain therapy.

Agmatine (AG; 4-(aminobutyl) guanidine) is an endogenous cationic amine obtained from the decarboxylation of L-arginine by the enzyme arginine decarboxylase. The distribution of AG-containing neurons is concentrated in brain regions involved in pain processing (Reis and Regunathan, 2000) and in all areas of the spinal cord gray matter (Fairbanks et al., 2000). AG was described as a neuroprotective agent in experimental models of neurotrauma and excitotoxic disorders (Gilad et al., 1996; Fairbanks et al., 2000; Yu et al., 2000). Antinociceptive properties of AG were also reported in both acute and chronic pain models, in particular, neuropathic pain. Karadag et al. (2003) showed that AG reversed tactile allodynia in spinal nerve ligation and in streptozocin (STZ)-induced diabetes models. Fairbanks et al. (2000) also reported the antiallodynic and antihyperalgesic effect of AG in rats with ligated spinal nerve. In rats with chronic constriction nerve injury, thermal allodynia and hyperalgesia (Aricioglu et al., 2003) and mechanical hyperalgesia (Önal et al., 2003) were dose-dependently reduced by AG.

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ABBREVIATIONS: AG, agmatine, 4-(aminobutyl) guanidine; CL, confidence limit; D-CPP, *R*(−)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid; D, diabetic; DMSO, dimethyl sulfoxide; L-NAME, L-N^G-nitro-L-arginine methyl ester; ME, maximal effect; N, normal; NMDA, *N*-methyl-D-aspartate; NOS, nitric-oxide synthase; STZ, streptozocin; α_2-AD, α_2-adrenergic; MK801, 5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate).

The mechanism of the antinociceptive action of agmatine is not clear yet. Ligand binding and pharmacological experiments demonstrated that AG interacts with α_2-adrenergic (α_2-AD) (Pinthong et al., 1995) and/or imidazoline receptors (Piletz et al., 1995; Raasch et al., 2001; Hou et al., 2003; Roerig, 2003) in spite of its low affinity for these receptors (Önal and Soykan, 2001; Yeçsilyurt and Uzbay, 2001). There is evidence that AG may modulate the action of L-glutamate. AG more weakly interacts with the phencyclidine site than with polyamine sites (Gibson et al., 2002) of the *N*-methyl-D-aspartate (NMDA) receptor and inhibits NMDA receptor-mediated Ca^{2+} currents from cultured hippocampal neurons (Yang and Reis, 1999) and NMDA-evoked firing in dorsal horn neurons in rats, as well as the NMDA-elicited biting and nociceptive behaviors in mice and rats, respectively (Fairbanks et al., 2000). Functional NMDA receptors coupled to neuronal nitric-oxide synthase system are required for the central effects of AG (Aricioglu et al., 2004b). In vitro studies showed that AG competitively inhibits all isoforms of NOS, and most potently, the inducible form (Galea et al., 1996). Furthermore, AG decreases the activity of inducible NOS (Abe et al., 2000) or its protein level (Regunathan and Piletz, 2003). In spite of the accumulating data on the effect of AG, the mechanism of action and the role of AG in painful diabetic neuropathy need to be clarified, and the potential interest of therapeutic associations with AG must be evaluated.

The aim of the present study was i) to assess the spinal antiallodynic and antihyperalgesic effects of AG in STZ-induced diabetic rats, ii) to determine the involvement of α_2-AD, imidazoline, or opioid mechanisms in the AG effect, and overall iii) to assess how new combinations of drugs can interact to relieve pain symptoms. We examined whether AG-induced antinociception could be potentiated by morphine, a conventional opioid analgesic whose efficacy in neuropathic pains is still a contentious issue (Dickenson and Suzuki, 2005), and whether the blockade of the NMDA receptor and NOS activity could be synergistic with AG.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River, Cléon, France) were used. They were housed four per cage under standard laboratory conditions and given food and water ad libitum. Their baseline weight (75–100 g, normal, N; 225–250 g, diabetic, D) was chosen to obtain rats with similar weights (250 g) when the drugs were administered (i.e., 3–4 weeks later). The guidelines of the International Association for the Study of Pain Committee for Research and Ethical Issues concerning animal pain models (Zimmermann, 1983) were followed and approved by the local Ethics committee. Great care was taken, particularly with regard to housing conditions, in order to avoid or minimize discomfort for animals.

Induction of Diabetes

Rats (225–250 g) were rendered diabetic by an i.p. injection of STZ (75 mg/kg) (Zanosar; Upjohn, Saint-Quentin-en-Yvelines, France) dissolved in distilled water. Diabetes was confirmed 1 week later by measurement of tail vein blood glucose levels with glucotide and a reflectance colorimeter (Glucometer 4; Bayer Diagnostics, Puteaux, France). Only rats with a final blood glucose level >14 mM were included in the study. At that time, hyperglycemic rats were subcutaneously injected with insulin (Ultratard, 2 IU/rat) every other day to reduce weight loss and minimize discomfort due to diabetes. This animal model of chronic pain with mechanical, thermal, and chemical hyperalgesia has been described elsewhere (Courteix et al., 1993). Control (N) rats (75–100g) received distilled water only (0.5 ml/kg i.p.) and were allowed to grow up for 3 weeks and were used as weight-matched controls for diabetic rats.

Nociceptive Test Procedure

Mechanical Hypersensitivity. Rats were placed individually on an elevated plastic mesh (1-cm^2 perforations) in a clear plastic cage and were adapted to the testing environment for at least 15 min. von Frey hairs (Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL) with calibrated bending forces (1.479, 2.041, 3.630, 5.495, 8.511, 11.749, and 15.136*g*) were used to deliver punctate mechanical stimuli of varying intensity. Filaments exerting a force above 15.136*g* were not used as they produced lifting of the paw. Starting with the lowest filament force (1.479*g*), von Frey hairs were applied from below the mesh floor to the plantar surface of the hind paw, with sufficient force to cause slight bending against the paw, and held for 1 s. Each stimulation was applied five times with an interstimulus interval of 4 to 5 s. Care was taken to stimulate random locations on the plantar surface. A positive response was noted if the paw was robustly and immediately withdrawn. Paw-withdrawal threshold was defined as the minimal pressure required to elicit a withdrawal reflex of the paw, at least one time on the five trials. Voluntary movement associated with locomotion was not considered as a withdrawal response. If no response was noted to any trial, the process was repeated with the following force hair, and the filament that produced a positive response was noted as the threshold. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to von Frey hair application. The 15.136-g hair was selected as the upper limit cut-off for testing.

Thermal Allodynia. The tail of the rat was immersed in a water bath maintained at 42°C [a temperature that is normally innocuous in normal rats (Courteix et al., 1993)] until tail withdrawal or signs of struggle were observed (cut-off time, 15 s). Because this test involves handling of the animals, 1 day before the experiment, the experimenter would handle the rats in the testing environment until they would sit quietly in the hand for 15 s (which corresponds to the cut-off time), two or three times depending on their capacity to be quiet. On the day of the experiment, rats were again handled by the experimenter for 15 s above the water bath to get the rat used to the condition of the test. No rats showed aversive reaction during handling. The tail of the rat then was immersed into the water. The reaction time (i.e., the time necessary to observe the withdrawal of the tail from the bath) was measured two to three times to obtain two consecutive values that differed no more than 10% and respecting an interval of at least 15 min between two measures. The tail of the rat was immediately dried with a soft cellulose paper to avoid tail cooling between two measures. A shortened duration of immersion indicated allodynia.

Mechanical Hyperalgesia. Rats underwent the paw-pressure test. Nociceptive thresholds, expressed in grams, were measured using an Ugo Basile analgesimeter (Bioseb, France) by applying an increasing pressure to the left hind paw until a squeak (vocalization threshold) was elicited (maximal pressure applied was 450 g). Because this test involves animal handling, the experimenter was able to get the rat used to being handled as follows. Three days before the experiment, rats were used to being handled without escaping from the hand of the experimenter for 20 s, two or three times depending on their capacity to be quiet. On the day of the experiment, rats were again handled two to three times by the experimenter for 20 s, and simultaneously, the Ugo Basile apparatus was started for the rat to get used to the noise of the apparatus. No rats showed aversive reaction during handling. The paw of the rat then was placed under the tip, and the progressive pressure was applied until the rat vocalized. The vocalization threshold was measured three or four times to obtain two consecutive values that differed no more than 10% and respecting an interval of at least 10 min between two measures.

Drugs and Chemicals

Agmatine [4-(aminobutyl) guanidine, M_r 228.3] and D-CPP [$R(-)$-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid, M_r 252.2, a competitive NMDA-receptor antagonist] were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), dissolved in oxygen-free distilled water the day before the experiment, and stored in appropriate aliquots at −20°C. L-NAME (L-N^G-nitro-L-arginine methyl ester, M_r 269.7, a nonselective NOS inhibitor) and naloxone (M_r 363.8, a nonselective opioid-receptor antagonist) were purchased from Sigma-Aldrich and dissolved in saline (0.9% NaCl) on the day of the experiment. Yohimbine hydrochloride (M_r 395.41, an α_2-AD-receptor antagonist) and idazoxan hydrochloride (M_r 240.69, a mixed imidazoline/α_2-AD-receptor antagonist) were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (30% DMSO) the day before the experiment and stored in appropriate aliquots at −20°C. Morphine hydrochloride (M_r 375.8) was purchased from the Coopération Pharmaceutique Française (Melun, France) and dissolved in distilled water on the day of the experiment.

Injections

AG, D-CPP, morphine, yohimbine, idazoxan, DMSO, and distilled water were injected i.t. in a volume of 10 μl in the subarachnoid space between L_5 and L_6 using a 30 G × ½-inch needle and a 50-μl Hamilton syringe as described by Mestre et al. (1994). Intrathecal injection is stressful for rats and, according to IASP recommendation, requires anesthesia. Thus, before injection, rats were slightly anesthetized with volatile isoflurane (3.5%) and recovered 5 min after the removal from the anesthesia chamber. The eventual effect of anesthesia could be observed in the control groups (distilled water injection). Naloxone, L-NAME, and saline were administered intravenously via a caudal vein in a volume of 1 ml/kg.

Experimental Design

Tests took place 3 to 4 weeks after the injection of STZ (D rats) or distilled water (N rats). At that time, D rats were selected as 1) allodynic [rats displaying a reaction to innocuous tactile (von Frey hair test) or thermal (tail-immersion test) stimuli] and 2) hyperalgesic (rats in which the reduction in nociceptive pain thresholds to paw pressure was above 15% of the value obtained before the STZ injection).

Different animals were used for each condition. The D allodynic or hyperalgesic animals underwent the von Frey hair test or the tail-immersion test or the paw-pressure test before drug injection. Once the two stable threshold values were obtained, drugs were injected and thresholds were determined according to the following experiments:

Experiment A: Effect of AG on Tactile and Thermal Allodynia in Diabetic Rats. AG (4.4, 43.8, or 438.0 nmol/rat) or distilled water (10 μl/rat) was injected i.t. The withdrawal thresholds to von Frey hair application or the reaction time to tail immersion were then determined in different animals every 15 min for 60 min.

Experiment B: Effect of AG on Mechanical Hyperalgesia in Diabetic Rats. Study of the Involvement of α_2-AD, Imidazoline, and Opioid Receptor in Diabetic Rats. AG (4.4, 21.9, 43.8, 109.5, and 219.0 nmol/rat) or distilled water (10 μl/rat) was injected i.t. in D and N rats. The vocalization thresholds to paw pressure were measured at 15, 30, 45, 60, 90, and 120 min after the injections.

Yohimbine (40 μmol/rat i.t.), idazoxan (40 μmol/rat i.t.), or 30% DMSO (10 μl/rat, i.t.) was injected just before and naloxone (0.69 μmol/kg i.v.) or saline (1 ml/kg i.v.) was injected 15 min before AG i.t. (219.0 nmol/rat) or distilled water (10 μl/rat i.t.) was injected in D rats. The vocalization thresholds then were measured at 15, 30, 45, 60, and 90 min after the second injection.

Experiment C: Assessment of the Interaction of AG with Morphine, D-CPP, and L-NAME on Mechanical Hyperalgesia in Diabetic Rats. We used the isobolographic method as described by Tallarida (1992). To do so, D rats received morphine i.t. (2.7, 13.3, 26.6, and 133 nmol/rat), L-NAME i.v. (1.25, 6.25, 12.50, and 18.75 μmol/rat), or D-CPP i.t. (10, 25, 100, and 250 μmol/rat) to determine the ED_{50} or the ED_{25} of each drug, defined as the dose of a drug that produces, respectively, 50 or 25% of the maximal effect before the combination experiment. The doses studied in combination then were selected according to the potency ratio (R) of each drug: $ED_{50\ (morphine)}/ED_{50\ (AG)} = 26.5/319.5$ (i.e., 0.0828) or $ED_{25\ (D\text{-}CPP)}/ED_{25\ (AG)} = 0.103/36.5$ (i.e., 0.0028) or $ED_{25\ (l\text{-}NAME)}/ED_{25\ (AG)} = 2.660/0.0365$ (i.e., 72.81).

Then drugs were administered at fixed proportions (p1 and p2) corresponding to: $p_{drug\ (\%)} = ED_{25\ or\ 50\ (drug)}/(ED_{25\ or\ 50\ (drug)} + ED_{25\ or\ 50\ (AG)})$. Thus, AG was coadministered with the following: morphine at $p_1 = ED_{50\ (morphine)}/(ED_{50\ (AG)} + ED_{50\ (morphine)}) = 26.5/(26.5 + 319.5)$ (i.e., 7.65%) and $p_2 = ED_{50\ (AG)}/(ED_{50\ (AG)} + ED_{50\ (morphine)}) = 319.5/(319.5 + 26.47)$ (i.e., 92.35%); D-CPP at $p_1 = ED_{25\ (D\text{-}CPP)}/ED_{25\ (AG)} + ED_{25\ (D\text{-}CPP)} = 0.103/(0.103 + 36.5)$ (i.e., 0.28%) and $p_2 = ED_{25\ (AG)}/ED_{25\ (AG)} + ED_{25\ (D\text{-}CPP)} = 36.5/(0.103 + 36.5)$ (i.e., 99.72%); and L-NAME at $p_1 = ED_{25\ (l\text{-}NAME)}/ED_{25\ (AG)} + ED_{25\ (l\text{-}NAME)} = 2.660/(0.0365 + 2.660)$ (i.e., 98.65%) and $p_2 = ED_{25\ (AG)}/ED_{25\ (AG)} + ED_{25\ (l\text{-}NAME)} = 0.0365/(0.0365 + 2.660)$ (i.e., 1.35%).

After the drug injections, animals were submitted to the paw-pressure test every 15 or 30 min for 120 min. Each experiment was performed blind using different animals and in randomized blocks to avoid chronobiological effects and to assess the effect of different treatments under the same environmental conditions (n = 6 to 8 rats according to treatment).

Data Analysis

Results are expressed as mean ± S.E. of raw data. A nonparametric Friedman repeated one-way analysis of variance was performed followed by multiple comparison procedures (Tukey's or Bonferroni's t test). The statistics software used was SigmaStat for Windows. The significance level was set at $P < 0.05$.

To examine the dose-response relationship of the different drugs on mechanical hyperalgesia, data were converted to percentage of maximal effect (% ME):

$$\%\ \mathrm{ME} = \frac{\text{Postdrug threshold} - \text{predrug threshold}}{450 - \text{Predrug threshold}} \times 100 \qquad (1)$$

The ED_{50} or ED_{25}, the 95% confidence limit (CL), and the mean ± S.E. were calculated by computer-assisted analysis of the graded dose-response curves using a custom Microsoft Excel macro program based on the method described by Tallarida (1992).

Isobolographic analysis was performed according to Tallarida (1992). First, the potency of individual drugs was determined. The ED_{50} or ED_{25} of AG was plotted on the ordinate, and the morphine ED_{50}, D-CPP ED_{25}, or L-NAME ED_{25} was plotted on the abscissa. A theoretical simple additive line for a combination of both drugs was then generated by connecting the ED_{50} for AG with that of morphine and by connecting the ED_{25} for AG with that of D-CPP or L-NAME. For each combination, the ED_{50} or ED_{25} of the mixture (ED_{50mix}, ED_{25mix}) and the mean ± S.E. of the mixture were calculated by linear regression of the dose-response curve and resolved into its component parts according to the dose ratio. The potency and 95% CL of both drugs were compared using a t test, with the theoretical additive value (ED_{50add} or ED_{25add}) obtained from the $ED_{50/25}$ for each combined drug according to the formula $ED_{50/25add} = ED_{50/25(drug)}/(p_1 + Rp_2)$, where R is the potency ratio of the drug to AG ($r = ED_{50/25(drug)}/ED_{50/25(AG)}$); p_1 is the proportion of the drug in the total dose, and p_2 is the proportion of AG in the total dose. No significant difference between the ED_{50mix} and the ED_{50add} or between the ED_{25mix} and the ED_{25add} suggests a simple additive effect of both drugs, whereas when ED_{50mix} or ED_{25mix} is significantly less than ED_{50add} or ED_{25add}, a superadditive effect of the combination is indicated.

Results

Clinical Status of Animals

The specificity of painful reactions observed in D rats was questioned because of the general health alteration of the animals. In this work and in the previous studies, we paid attention to the general health status of the animals. Loss greater than 10% of the initial body weight, loss of activity, or piloerection was one of the criteria to justify the removal of the animals. In the present study, rats were treated with insulin and tested at 3 or 4 weeks after diabetes was induced. Only 8% of D rats was excluded from the experiment. The mean body weight of D rats maintained in the study 3 to 4 weeks after STZ injection was 256.9 ± 2.8 versus 245.6 ± 0.8 g (n = 205 rats) before STZ injection.

Effect of AG on Tactile and Thermal Allodynia in Diabetic Rats

The general behavior of diabetic rats was unaffected by the i.t. injection of AG or distilled water.

Tactile Allodynia (von Frey Hair Test). Before the injection of STZ, the withdrawal threshold to von Frey hair application was higher than 15.136 g, (Fig. 1). Four weeks after STZ injection, 52% (21/40) of diabetic rats showed tactile allodynia characterized by a reduction in paw-withdrawal thresholds to 4.11 ± 0.43 g (n = 21). After doses of 43.8 (n = 7) and 438.0 nmol/rat (n = 7), AG significantly increased paw-withdrawal thresholds from 15 to 30 or 60 min after the injection. A maximal effect was observed at 30 min with 43.8 nmol/rat (paw-withdrawal threshold: 13.71 ± 0.99 g) and 438.0 nmol/rat (paw-withdrawal threshold: 13.20 ± 0.68 g) and corresponded to a reversal of diabetes-induced tactile allodynia.

Thermal Allodynia (Tail-Immersion Test at 42°C). A significant reduction in reaction time to tail immersion was obtained in 69% (31/45) of diabetic rats 4 weeks after STZ injection (before STZ: reaction time >15 s; week 4 after diabetes induction: reaction time = 11.01 ± 0.21 s, n = 31) (Fig. 2).

The dose of 4.4 nmol/rat (n = 7) of AG did not affect the reaction time, but doses of 43.8 (n = 8) and 438 nmol/rat (n = 8) significantly increased the reaction time 15 to 30 min after the injection. The maximal effect obtained at 15 min (15 ± 0 and 14.33 ± 0.17 s after 43.8 and 438 nmol/rat, respectively) corresponded to a reversal of thermal allodynia.

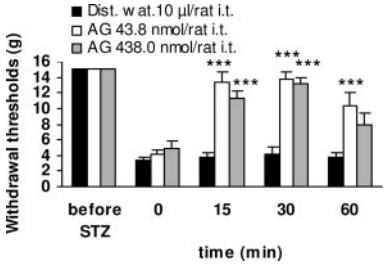

Fig. 1. Time course of the effect of distilled water (10 µl/rat i.t.) and AG (43.8 or 438 nmol/rat i.t.) on paw-withdrawal thresholds of D rats to the application of von Frey filaments. Withdrawal thresholds measured before (0) and after drug injection are expressed in grams. Data are means ± S.E. from seven rats. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus corresponding predrug values (time 0).

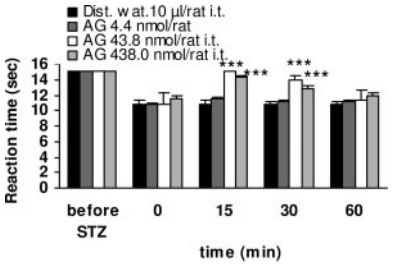

Fig. 2. Time course of the effect of distilled water (10 µl/rat i.t.) and AG (4.38, 43.8, or 438 nmol/rat i.t.) on tail withdrawal of D rats submitted to the tail-immersion test in warm water (42°C). Reaction times measured before (0) and after drug injection are expressed in seconds. Data are means ± S.E. from seven or eight rats. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus corresponding predrug values (time 0).

Effect of AG on Mechanical Hyperalgesia: Evidence for the Involvement of I_2-Imidazoline Receptor in Diabetic Rats

STZ injection significantly reduced vocalization thresholds 3 weeks after the induction of diabetes [before STZ: 298.1 ± 3.5 g; week 3 after diabetes induction:162.4 ± 5.1 g in 73% (44/60) of the diabetic rats].

In N rats, AG (43.8 and 219.0 nmol/rat) failed to increase vocalization thresholds (results not shown). In D rats, after doses of 21.9, 43.8, 109.5, and 219.0 nmol/rat, AG significantly and dose-dependently increased vocalization thresholds 15 to 60 min after the injection (Fig. 3a). A complete suppression of diabetes-induced hyperalgesia was observed after 219.0 nmol/rat. The maximal score (293 ± 11 g) obtained at 30 min corresponded to an increase of 170.6 ±

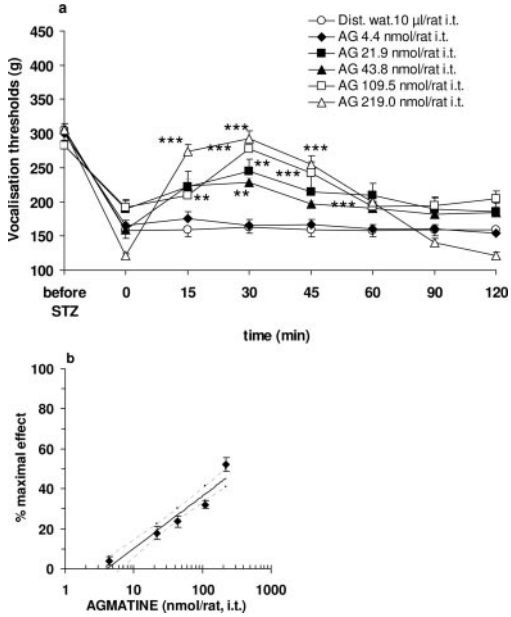

Fig. 3. a, time course of the effect of distilled water (10 µl/rat i.t.) and AG (4.38–219 nmol/rat i.t.) on paw-pressure-induced vocalization thresholds in D rats. Vocalization thresholds measured before (0) and after drug injection are expressed in grams. b, spinal effect of agmatine on paw-pressure-induced vocalization thresholds in D rats. Results are expressed as percentage of the maximal effect. The dashed line represents the 95% Cl. Data are means ± S.E. from six to eight rats. The absence of an error bar means that the value of S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus corresponding predrug values (time 0).

10.2 g. The ED_{50} and ED_{25} values and the 95% confidence interval (in parentheses) corresponding to this antihyperalgesic effect were 319.5 (215.7–546.4) and 36.5 nmol/rat (28.1–47.1), respectively (Fig. 3b).

The injection of the α_2-AD receptor antagonist yohimbine (Fig. 4a) or of the nonselective opioid antagonist naloxone (Fig. 4b) did not modify the antihyperalgesic effect of AG. The maximal effect obtained with each combination (325.7 ± 28.7 and 312.9 ± 24.1 g, respectively) was similar to that observed with the association of AG/30% DMSO (340 ± 23.1 g) (Fig. 4a) or AG + saline (330 ± 25.4 g) (Fig. 4b). Idazoxan, the nonselective antagonist of imidazoline/α_2-AD receptors, significantly reduced the antihyperalgesic effect of AG. The maximal effect obtained with the combination of AG + idazoxan (252.5 ± 18.4 g) was not different from the combination of distilled water/30% DMSO (242.1 ± 16.8 g) (Fig. 4c).

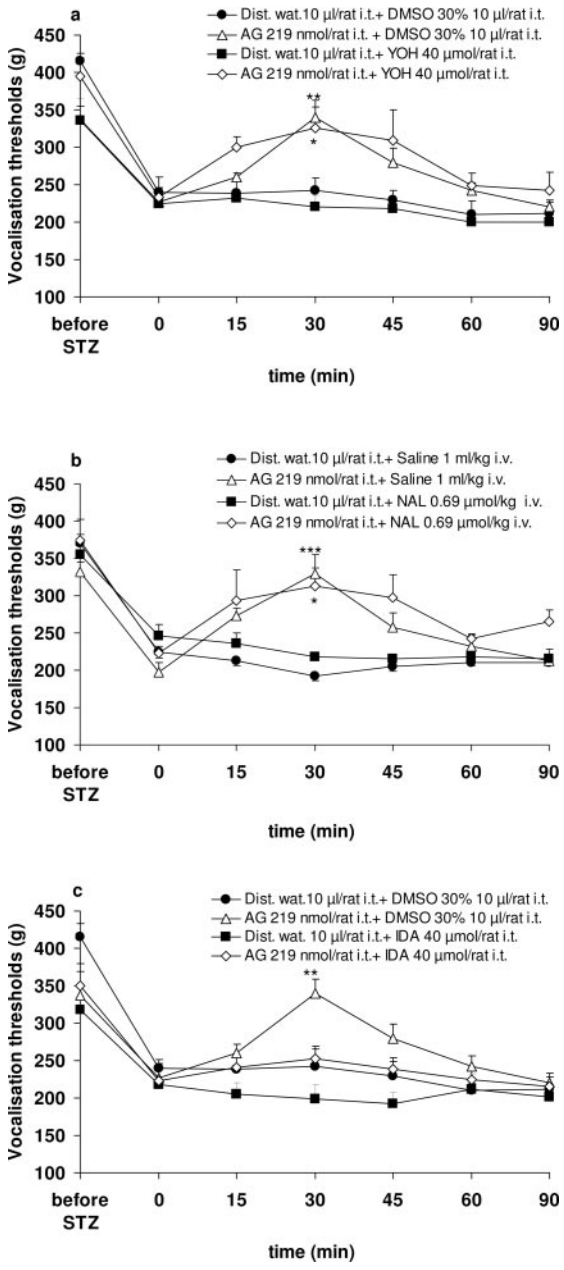

Fig. 4. Time course of the effect of saline (0.9% NaCl, 1 ml/kg i.v.), 30% DMSO (10 µl/rat i.t.), yohimbine (YOH) (40 µmol/rat i.t.) (a), naloxone (NAL) (0.69 µmol/rat i.v.) (b), or idazoxan (IDA) (40 µmol/rat i.t.) (c) on the paw-pressure-induced vocalization thresholds after AG or distilled water in D rats. Results are expressed in grams. Data are means ± S.E. from six to eight rats. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus corresponding predrug values (time 0).

Experiment C: Assessment of the Interaction of AG with Morphine, D-CPP, and L-NAME on Mechanical Hyperalgesia in Diabetic Rats. To perform the isobolographic analysis, the ED_{50} value for morphine or the ED_{25} values for D-CPP and L-NAME were first determined from the dose-response curves.

Intrathecal injections of morphine (2.7, 13.3, 26.6, and 133.0 nmol/rat) significantly and dose-dependently increased the vocalization thresholds, resulting in a suppression of mechanical hyperalgesia (data not shown). The ED_{50} (95% CL) value for morphine was 26.5 nmol/rat (15.6–47.5) (Fig. 5a).

Intrathecal injections of D-CPP (0.010, 0.025, 0.099, and 0.248 nmol/rat) significantly and dose-dependently increased the paw-pressure induced vocalization thresholds resulting in a suppression of hyperalgesia (data not shown). The ED_{25} (95% CL) value for D-CPP was 0.103 nmol/rat (0.048–0.528) (Fig. 5b).

Intravenous injections of L-NAME (1.25–18.75 µmol/rat) significantly and dose-dependently increased the vocalization thresholds resulting in a suppression of mechanical hyperalgesia (data not shown). The ED_{25} value (95% CL) for L-NAME was 2.660 µmol/rat (0.15–5.93) (Fig. 5c).

Assessment of the Interaction AG/Morphine. Morphine and AG were coadministered at fixed proportions of $p_1 = 7.65\%$ and $p_2 = 92.35\%$, determined as described previously. Because the peak effects of AG (i.t.) and morphine (i.t.) occurred at 30 min, the injection of morphine was given immediately after the injection of AG. AG and morphine were coadministered i.t. at the combined doses of nanomoles per rat of AG/morphine: 11.0:0.9, 33.1:2.7, 99.2:8.2, and 198.3:16.4, which corresponds to the total doses of 11.9, 35.8, 107.4, and 214.7 nmol/rat. The coinjection of AG with morphine at all of the doses studied did not produce any abnormal reaction. The two highest doses of the combination significantly increased the vocalization thresholds at 30 and 45 min after the injections (Fig. 6a). The highest dose of the combination suppressed the hyperalgesia (preSTZ threshold: 349.7 ± 30.8 g) with a maximal score elevation of 160.3 ± 29.6 g at 45 min corresponding to a vocalization threshold of 352.5 ± 30.4 g.

The total ED_{50mix} for the combination of AG/morphine was 146.3 nmol/rat, representing 135.1 nmol of AG/rat and 11.2 nmol of morphine/rat (plotted at $1.12.10^{-8}$ and $1.35.10^{-7}$) (Fig. 6b). The theoretical additive ED_{50add} for the combination of AG/morphine, calculated as described previously, was 173.2 nmol/rat (plotted at $1.32.10^{-8}$ and $1.60.10^{-7}$). The *t* test applied to the potency ratio between the total ED_{50mix} and the ED_{50add} for the theoretical additive point showed no significant difference ($t = 0.390$), indicating that the combination was only additive.

Assessment of the Interaction AG/D-CPP. D-CPP and AG were coadministered at fixed proportions of $p_1 = 0.28\%$ and $p_2 = 99.72\%$, determined as described previously. Because the peak effects of AG (i.t.) and D-CPP (i.t.) occurred at 30 min, the injection of D-CPP was given immediately after the injection of AG. AG and D-CPP were coadministered i.t.

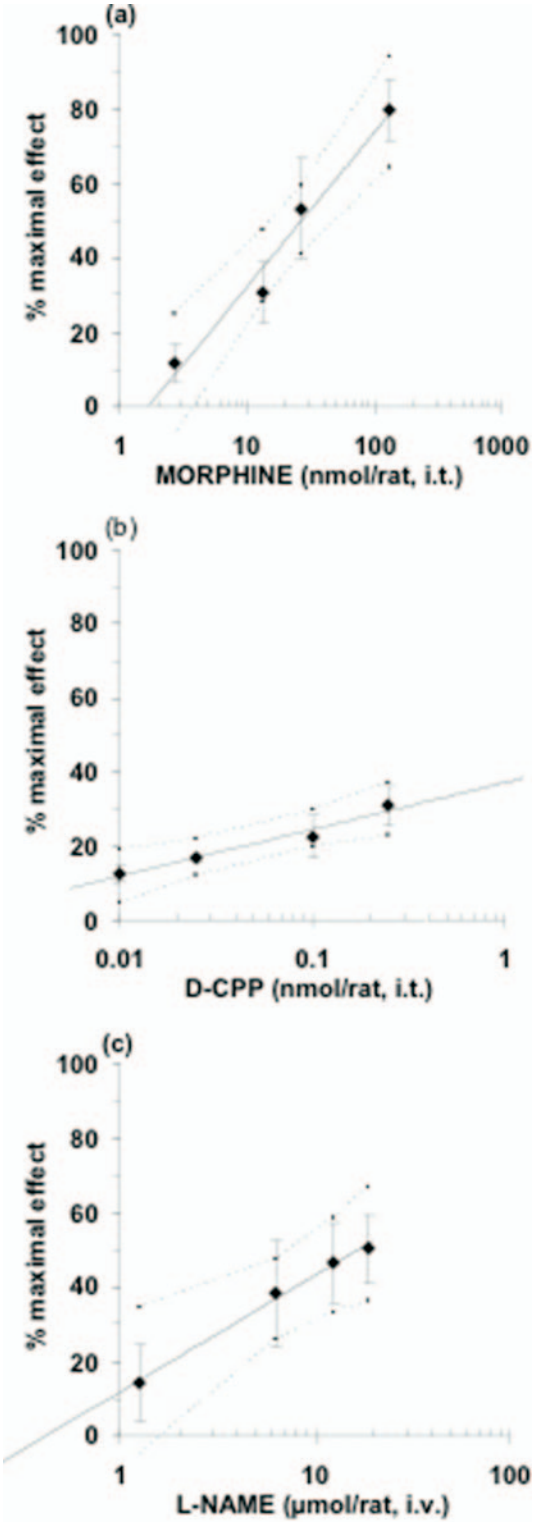

Fig. 5. Spinal effect of morphine (2.7–133 nmol/rat i.t.) (a), D-CPP (0.01–0.25 nmol/rat i.t.) (b), and L-NAME (1.25–18.75 µmol/rat i.v.) (c) on paw-pressure-induced vocalization thresholds in D rats. Results are expressed in percentage of the maximal effect. Data are means ± S.E. from six to eight rats. The dashed line represents 95% CL. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol.

at the combined doses (nanomoles/rat) of AG/D-CPP: 3.72:0.011, 7.45:0.022, 37.23:0.108, and 74.46:0.216, which corresponds to the total doses of 3.734, 7.468, 37.341, and 74.679

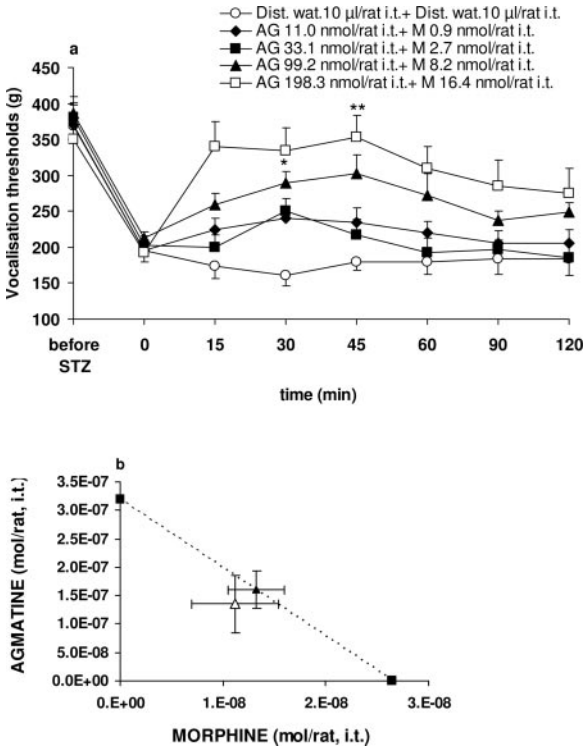

Fig. 6. a, time course of the effect of the combination of AG and morphine (M) on paw-pressure-induced vocalization thresholds in D rats. The treatments administered were as follows: distilled water (10 µl/rat i.t.) + distilled water (10 µl/rat i.t.) or the combination of AG i.t./M i.t. at the following combined doses (nanomoles/rat): 11.0:0.9, 33.1:2.7, 99.2:8.2, and 198.3:16.4. Results are expressed in grams. Data are means ± S.E. from six to eight rats. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus corresponding predrug values (time 0). b, isobologram for the effect of a combination of agmatine and morphine on paw-pressure-induced vocalization thresholds in D rats. The dashed line represents the theoretical additive interaction. The interception of the dashed line on the ordinate and abscissae is the observed ED_{50} values for agmatine and morphine alone, respectively. The solid symbol represents the ED_{50mix} for the combination of agmatine/morphine. The ED_{50add} is represented by the open symbol. The means ± S.E. for morphine and agmatine are resolved into morphine (abscissa scale) and agmatine (ordinate scale) components and shown by horizontal and vertical bars, respectively. The absence of a bar means that the value of the S.E. is smaller than the size of the symbol.

nmol/rat. The coinjection of i.t. AG with i.t. D-CPP at all of the doses studied did not produce any abnormal reaction. With the except of the lower dose, all of the doses of the combination significantly and dose-dependently increased the vocalization thresholds the 15th or 30th min after the injections (Fig. 7a). The highest dose of the combination suppressed the hyperalgesia and exerted an antinociceptive effect (361.9 ± 30.3 g versus preSTZ thresholds of 306.6 ± 22.0 g), with a maximal score elevation of 158.4 ± 29.1 g at 30 min.

The total ED_{25mix} for the combination of AG/D-CPP was 5.295 nmol/rat, representing 5.280 nmol of AG and 0.015 nmol of D-CPP (plotted at $1.5.10^{-11}$ and $0.53.10^{-8}$) (Fig. 7b). The theoretical additive ED_{25add} for the combination calculated as described previously was 18.31 nmol/rat (plotted at $5.17.10^{-11}$ and $1.83.10^{-8}$) (Fig. 7b). The t test applied to the potency ratio between the total ED_{25mix} and the ED_{25add} for the theoretical additive point showed a significant difference ($t = 2.827$) ($P < 0.01$), indicating that the combination was superadditive (Fig. 7b).

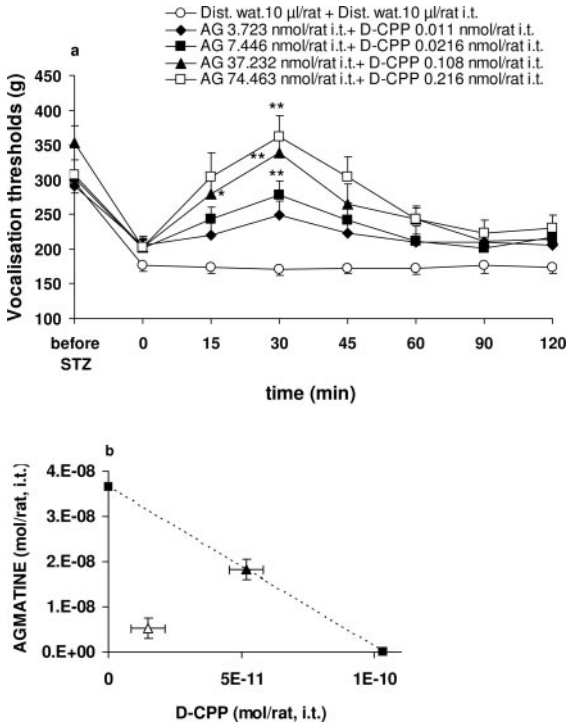

Fig. 7. a, time course of the effect of the combination of AG and D-CPP on paw-pressure-induced vocalization thresholds in D rats. The treatments administered were as follows: distilled water (10 µl/rat i.t.) + distilled water (10 µl/rat i.t.) or the combination of AG i.t./D-CPP i.t. at the following combined dose (nanomoles/rat): 3.72:0.011, 7.45:0.022, 37.23:0.108, and 74.46:0.216. Results are expressed in grams. Data are means ± S.E. from seven or eight rats. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus corresponding predrug values (time 0). b, isobologram for the effect of a combination of agmatine and D-CPP on paw-pressure-induced vocalization thresholds in D rats. The dashed line represents the theoretical additive interaction. The interception of the dashed line on the ordinate and abscissae is the observed ED_{25} values for agmatine and D-CPP alone, respectively. The solid symbol represents the ED_{25mix} for the combination of agmatine/D-CPP. The ED_{25add} is represented by the open symbol. The means ± S.E. for D-CPP and agmatine are resolved into D-CPP (abscissa scale) and agmatine (ordinate scale) components and shown by horizontal and vertical bars, respectively. The absence of a bar means that the value of the S.E. is smaller than the size of the symbol.

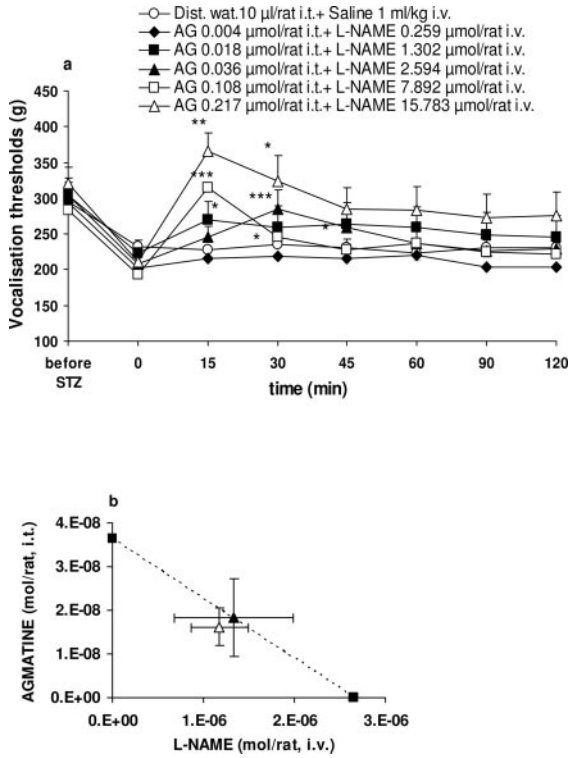

Fig. 8. a, time course of the effect of the combination of AG and L-NAME on paw-pressure-induced vocalization thresholds in D rats. The treatments administered were as follows: distilled water (10 µl/rat i.t.) + saline (0.9% NaCl) (0.25 ml/rat i.v.) or the combination of AG i.t./L-NAME i.v.) at the following combined doses (micromoles/rat): 0.004:0.259, 0.018:1.302, 0.036:2.594, 0.108:7.892, and 0.217:15.783. Results are expressed in grams. Data are means ± S.E. from six to eight rats. The absence of an error bar means that the value of the S.E. is smaller than the size of the symbol. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus corresponding predrug values (time 0). b, isobologram for the effect of a combination of agmatine and L-NAME on paw-pressure-induced vocalization thresholds in D rats. The dashed line represents the theoretical additive interaction. The interception of the dashed line on the ordinate and abscissae is the observed ED_{25} values for agmatine and L-NAME alone, respectively. The solid symbol represents the ED_{25mix} for the combination of agmatine/L-NAME. The ED_{25add} is represented by the open symbol. The means ± S.E. for L-NAME and agmatine are resolved into L-NAME (abscissa scale) and agmatine (ordinate scale) components and shown by horizontal and vertical bars, respectively. The absence of a bar means that the value of the S.E. is smaller than the size of the symbol.

Assessment of the Interaction AG/L-NAME. L-NAME and AG were coadministered at fixed proportions of $p_1 = 98.65\%$ and $p_2 = 1.35\%$, determined as described previously. Because the peak effects of AG (i.t.) and L-NAME (i.v.) occurred at 30 and 45 min, respectively, the injection of L-NAME was given 15 min before the injection of AG. Intrathecal AG and i.v. L-NAME were coadministered at the combined doses µM/rat of AG/L-NAME: 0.004:0.259, 0.018:1.302, 0.036:2.594, 0.108:7.892, and 0.217:15.783, which corresponds to the total doses of 0.263, 1.320, 2.63, 8, and 16 µmol/rat. The coinjection of i.t. AG with i.v. L-NAME at all of the doses studied did not produce any abnormal reaction. With the except of the lower dose, all of the doses of the combination significantly and dose-dependently increased the vocalization thresholds from the 15th or 30th min to the 30th or 45th min after the second injection (Fig. 8a). The highest dose of the combination totally reversed the hyperalgesia and exerted an antinociceptive effect (366.4 ± 25.3 g versus preSTZ thresholds of 320.4 ± 23.3 g), with a maximal score elevation of +155.4 ± 23.3 g at 15 min.

The total ED_{25mix} for the combination of AG/L-NAME was 1.194 µmol/rat, representing 0.0162 µmol of AG/rat and 1.178 µmol of L-NAME/rat (plotted at $1.18.10^{-6}$ and $1.62.10^{-8}$) (Fig. 8b). The theoretical additive ED_{25add} for the combination of AG/L-NAME, calculated as described previously, was 1.348 µmol/rat (plotted at $1.33.10^{-6}$ and $1.83.10^{-8}$) (Fig. 8b). The *t* test applied to the potency ratio between the total ED_{25mix} and the ED_{25add} for the theoretical additive point showed no significant difference ($t = 0.218$), indicating that the combination was only additive.

Discussion

The present results indicate that i.t. AG suppresses tactile and thermal allodynia and mechanical hyperalgesia in D rats while exerting no effect on mechanical nociception in healthy rats, showing that the effect of AG only occurs in the condition of neuropathy. This is in agreement with previous results reporting that AG reduced tactile allodynia in the spinal nerve ligation and the STZ-induced diabetic neuropathy in rats

(Karadag et al., 2003), and thermal hyperalgesia in rats with chronic constriction nerve injury (Aricioglu et al., 2003). No abnormal behavioral or motor effects were observed with any of the AG doses tested as already shown with cumulative doses lower than 1095 nmol (Horvàth et al., 1999).

The kinetics of the antiallodynic effect showed a maximal activity of 15 to 30 min after the injection and a long-term duration (60 min) consistent with the persistence of AG in the central nervous system for hours to days (Roberts et al., 2005). Interestingly, 438 nmol of AG had the same maximal effect as did 43.8 nmol on allodynia tests, in accordance with the naturally expected asymptote of a dose-response curve.

No antinociceptive effect of the amine was observed in healthy animals, suggesting that central sensitization is required for AG to induce its effect. Likewise, no effect of AG was reported in naive animals stimulated with von Frey filaments (Karadag et al., 2003) and in the warm water tail-immersion test or against substance P-evoked nociceptive behavior (Fairbanks et al., 2000) or in response to thermal stimulus of noninflamed paw in carrageenan-treated rats (Horvàth et al., 1999). However, a previously published study (Hou et al., 2003) indicates that similar i.t. doses of AG (22–219 nmol) suppresses the nociceptive discharges of parafascicular neurons elicited by tail pinch in healthy rats. One of the reasons for this discrepancy might be due to experimental conditions (anesthetized versus unanesthetized rats) and to the nature of the response (neuronal discharge versus behavioral response).

The antihyperalgesic effect of AG probably involves spinal imidazoline receptors (directly and/or indirectly) but not the spinal α_2-AD, because i.t idazoxan but not i.t. yohimbine inhibited its effect on mechanical hyperalgesia. The activation of imidazoline receptors by AG has been shown to suppress nociceptive inputs of healthy rats (Hou et al., 2003); however, there are also arguments against the involvement of imidazoline receptors in the AG-induced inhibition of NMDA behavior using the selective imidazoline type 1-receptor antagonist efaroxan (Fairbanks et al., 2000).

The lack of effect of the α_2-AD receptor antagonist yohimbine upon AG antihyperalgesia, which confirms previous data (Bradley and Headley, 1997), suggests that the inhibitory effect of idazoxan may be selective for imidazoline receptors in spite of its greater selectivity (5–50-fold) for α_2-adrenoreceptors over imidazoline binding sites (see Head and Mayorov, 2006). Contrary to the present results, systemic yohimbine has been shown to block the effect of AG in formalin-evoked tonic pain in mice (Önal and Soykan, 2001) and to decrease the anticonvulsant effect of AG on pentylenetetrazole-induced seizures in mice (Demehri et al., 2003). Furthermore, the morphine-enhancing activity of central AG has been shown to be attenuated by yohimbine (Yeçsilyurt and Uzbay, 2001; Roerig, 2003). The reasons for this discrepancy are not known; it may be due to the test employed or the route of injection of the antagonist. The results reported here using the i.t. route demonstrated the lack of involvement of spinal α_2-AD receptors, at least for mechanical hyperalgesia.

The involvement of the opioid system in the antihyperalgesic effect of AG has been explored by blocking the opioid receptors with naloxone and completed by combining AG with morphine. The choice of the systemic route of administration of the antagonist was made to block both spinal and supraspinal opioid receptors. In the present study, naloxone failed to suppress the antihyperalgesic effect of AG, suggesting that the effect of AG does not directly involve opioid receptors, thus confirming previous binding experiments (see Su and Qin, 2003). Furthermore, the coadministration of AG with morphine is additive. Some groups have reported that AG (Horvàth et al., 1999; Yeþilyurt and Uzbay, 2001; Roerig, 2003) or substances involved in the metabolism of endogenous AG (Lu et al., 2003) enhanced the analgesic effect of morphine; however, others have shown that intrathecal or intracerebral AG did not potentiate morphine analgesia but prevented opiate tolerance (Kitto and Fairbanks, 2006). Moreover, AG was able to reduce fentanyl-evoked self-administration (dependence) (Morgan et al., 2002) and suppress the morphine abstinence syndrome (Aricioglu et al., 2004a,b). With regard to the effect of AG on morphine analgesia, the experimental design of the present work was very different from that of these other studies. First, the experimental pain models did not concern neuropathic pain, because carrageenan-induced inflammation in rats (Horvàth et al., 1999) or healthy mice (Yeçsilyurt and Uzbay, 2001; Lu et al., 2003; Roerig, 2003) were employed. Second, the drugs were administered by different routes in those studies: s.c. for morphine; i.p. for AG (Yeçsilyurt and Uzbay, 2001); i.c.v., i.t., or s.c. for AG; and i.t. for morphine (Roerig, 2003). Third, the nociceptive tests employed involved the thermal tail-flick (Yeþilyurt and Uzbay, 2001; Roerig, 2003) and Hargreaves tests (Horvàth et al., 1999). Fourth, in these experiments, the opiate sometimes induced analgesia when administered alone (Yeçsilyurt and Uzbay, 2001) but in doses higher (1–10 μg i.t. or 50–500 pmol i.t.) than those used here (Horvàth et al., 1999; Roerig, 2003). Finally, none of these studies performed an isobolographic analysis to examine the interaction between AG and morphine.

The NMDA receptor-NOS system is involved in the reorganization of the central nervous system resulting from chronic pain conditions. AG has been shown to inhibit NOS (Galea et al., 1996) and antagonize NMDA receptors (Yang and Reis, 1999; Fairbanks et al., 2000). The present work and previous studies showed that L-NAME, a nonselective NOS inhibitor, suppressed hyperalgesia or allodynia (Levy et al., 2000; Lui and Lee, 2004) in experimental models of neuropathy. However, the present results demonstrated that the antinociceptive effect of the coadministration of AG with L-NAME is only additive. This is in line with the study of Karadag et al. (2003), reporting that the coadministration of AG and L-NAME or 7-nitroindazole did not influence the antiallodynic effect of AG in the spinal nerve ligation and the STZ-induced diabetes models. The results reported here showed a lack of potentiation of the antihyperalgesic effect of AG coadministered with L-NAME, suggesting that the two drugs may share a common mechanism of action (i.e., inhibition of NOS activity). This is in accordance with in vitro studies (Galea et al., 1996; Abe et al., 2000; Regunathan and Piletz, 2003) and consistent with in vivo findings (Fairbanks et al., 2000; Roberts et al., 2005) showing that AG inhibited NMDA-evoked nociceptive behavior (NOS-independent) and thermal hyperalgesia (NOS-dependent) with differential potency.

In spinal cord, the activation of neuronal nitric-oxide synthase is a mechanism triggered by a high Ca^{2+} influx through NMDA receptors. Therefore, besides blocking the NO production, we questioned the blockade of the NMDA receptor in the effect of AG. The coadministration of the NMDA receptor-com-

petitive antagonist D-CPP with AG results in a synergistic antihyperalgesic effect, indicating that the drugs act through different binding sites. Moreover, literature data suggest that AG is not a competitive antagonist but could interact with a site located in the channel pore (Yang and Reis, 1999) and/or the polyamine site (Gibson et al., 2002). The present results are also in line with the lack of potentiation of the antiallodynic effect of AG, with subthreshold doses of the noncompetitive NMDA receptor antagonist MK801 in neuropathic pain (Karadag et al., 2003). A similar receptor "block-based" effect of AG has been suggested to explain its antidepressant-like effect in mice (Li et al., 2003) or its neuroprotective effect in rats (Feng et al., 2002). The higher antinociceptive effect obtained when AG is coadministered with D-CPP than with L-NAME could be explained by the suppression of both the intracellular increase of calcium and the consecutive activation of transduction systems implying multiple enzymatic activities (NOS, kinases, etc.). In this regard, it has recently been reported that ketamine (an uncompetitive NMDA antagonist) intrathecally coadministered with (±)CPP led to a superadditive antinociceptive effect in monoarthritic rats (Pelissier et al., 2007), thus opening the possibility that synergy between AG and D-CPP had been the result of interaction at different binding sites of the NMDA receptor and not the consequence of post-NMDA receptor events. Further research is required to clarify this aspect.

In conclusion, the present findings indicate that AG suppresses tactile and thermal allodynia and mechanical hyperalgesia in diabetic neuropathic pain and involves imidazoline receptors for its effect on mechanical hyperalgesia. Regarding the antihyperalgesic effect in response to mechanical stimulus, the combination of AG with morphine or L-NAME produces additive antihyperalgesia, suggesting a probable common mechanism of action of both drugs with AG, whereas D-CPP strongly potentiated the antihyperalgesic effect of AG by some mechanisms, which need to be elucidated. The good tolerability allows consideration of AG as a potential therapeutic agent in the management of neuropathic pain alone or combined with low doses of analgesic drugs.

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