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Role of spinal NMDA receptors, protein kinase C and nitric oxide synthase in the hyperalgesia induced by magnesium deficiency in rats

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1 Magnesium (Mg)-deficient rats develop a mechanical hyperalgesia which is reversed by a N-Methyl-D-Aspartate (NMDA) receptor antagonist. Given that functioning of this receptor-channel is modulated by Mg, we wondered whether facilitated activation of NMDA receptors in Mg deficiency state may in turn trigger a cascade of specific intracellular events present in persistent pain. Hence, we tested several antagonists of NMDA and non-NMDA receptors as well as compounds interfering with the functioning of intracellular second messengers for effects on hyperalgesia in Mg-deficient rats.

2 Hyperalgesic Mg-deficient rats were administered intrathecally (10 μl) or intraperitoneally with different antagonists. After drug injection, pain sensitivity was evaluated by assessing the vocalization threshold in response to a mechanical stimulus (paw pressure test) over 2 h.

3 Intrathecal administration of $MgSO_4$ (1.6, 3.2, 4.8, 6.6 μmol) as well as NMDA receptor antagonists such as MK-801 (0.6, 6.0, 60 nmol), AP-5 (10.2, 40.6, 162.3 nmol) and DCKA (0.97, 9.7, 97 nmol) dose-dependently reversed the hyperalgesia. Chelerythrine chloride, a protein kinase C (PKC) inhibitor (1, 10.4, 104.2 nmol) and 7-NI, a specific nitric oxide (NO) synthase inhibitor (37.5, 75, 150 μmol kg^{-1}, i.p.) induced an anti-hyperalgesic effect in a dose-dependent manner. SR-140333 (0.15, 1.5, 15 nmol) and SR-48968 (0.17, 1.7, 17 nmol), antagonists of neurokinin receptors, produced a significant, but moderate, increase in vocalization threshold.

4 These results demonstrate that Mg-deficiency induces a sensitization of nociceptive pathways in the spinal cord which involves NMDA and non-NMDA receptors. Furthermore, the data is consistent with an active role of PKC, NO and, to a lesser extent substance P in the intracellular mechanisms leading to hyperalgesia.

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Abbreviations: AMPA, amino-3-hydroxy-5-methylisooxazole-4-propionic acid; AP-5, D-2-Amino-5-phosphono-valeric acid (competitive NMDA receptor); AUC, area under the curve; CCI, chronic constrictive injury; CNQX, 6-cyano-7-Nitroquinoxaline-2,3-dione (non-NMDA antagonist); CSF, cerebrospinal fluid; DCKA, Dichlorokynurenic acid (competitive NMDA receptor); EAAs, excitatory amino acids; i.p., intraperitoneal; i.t., intrathecal; Mg, magnesium; $MgSO_4$, magnesium sulphate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5, 10-imine maleate (non-competitive NMDA receptor); NMDA, N-Methyl-D-Aspartate; 7-NI, 7-Nitro-Indazole (nNOS inhibitor); NK, neurokinin; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PKC, protein kinase C; SP, substance P

Introduction

The magnesium (Mg)-deficient rat is a new model of hyperalgesia (Dubray *et al*., 1997). After a few days of consummation of a Mg-depleted diet, mechanical hyperalgesia develops in the rat which can be reversed by systemic injection of MK-801, a non-competitive antagonist of N-Methyl-D-Aspartate (NMDA) receptors (Dubray *et al*., 1997). Since many studies, over the last few years, have provided evidence that excitatory amino acid (EAAs) receptors play a significant role in spinal nociceptive transmission and in the sensitization mechanism leading to hyperalgesia (Coderre & Melzack, 1991; Haley *et al*., 1990; Seltzer *et al*., 1991), we assessed in our model the involvement of these EAAs receptors at the spinal level. NMDA and amino-3-hydroxy-5-methylisooxazole-4-propionic acid (AMPA) receptors, identified as ionotropic receptors, belong to the family of EAA receptors (Dickenson, 1997a). When a short painful stimulation is applied, AMPA receptors alone seem to mediate excitatory transmission and to set the baseline level of nociception, while NMDA receptors are not activated, as their channel is plugged by a physiological level of Mg (Mayer *et al*., 1984). However, in chronic pain states, both receptors are probably involved. An intense stimulation of

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primary afferent fibres initially activates AMPA and peptide receptors and, when frequency of stimulation exceeds threshold, the voltage-dependent Mg-block of NMDA receptors is removed, allowing activation of these receptors to take place (Mayer & Westbrook, 1987). As a consequence, the opening of the NMDA receptor channel leads to an influx of calcium (Ca^{2+}) into the neurons, triggering a cascade of intracellular events such as protein kinase C (PKC) activation (Mao *et al*., 1992b; Meller *et al*., 1996) or nitric oxide (NO) production (Meller & Gebhart, 1993) and ultimately immediate-early gene transcription (c-Fos; c-Jun) (Ren & Dubner, 1999; Woolf & Costigan, 1999). NMDA receptors may also be modulated by peptides, like substance P (SP), which is released with glutamate from the primary afferent fibres, to extend and maintain the nociceptive process (Liu *et al*., 1997; Malcangio *et al*., 1998). All these events are responsible for the long-term changes associated with persistent pain (Coderre, 1993; Wong *et al*., 1995). As demonstrated by *in vitro* studies, low Mg concentrations facilitate the opening of the $Na^+ - Ca^{2+}$ channel of NMDA receptors (Mayer *et al*., 1984; Nowack *et al*., 1984; Zhang *et al*., 1996). We may therefore presume that a dramatic decrease in Mg concentration in cerebrospinal fluid (CSF) and in the brain of Mg-deficient rats (Chutkow & Meyers, 1968) may trigger the activation of NMDA receptors. The resulting increase in cytosolic Ca^{2+} level would thereafter elicit a cascade of intracellular events responsible for the sensitization mechanism (Coderre & Melzack, 1991; Haley *et al*., 1990; Ren & Dubner, 1999; Seltzer *et al*., 1991). In this present study, successive steps induced by NMDA receptor activation have been pinpointed as pharmacological targets. The aim of the present work, using intrathecal injection of antagonists, was to evaluate, at the spinal level, the involvement of the NMDA receptor and subsequent intracellular events in the induction of a spinal state of sensitization in the animal model of Mg-deficiency-induced hyperalgesia.

Methods

Animals

Male Wistar rats (Charles River, France), weighing 90–100 g, were used in all experiments. Rats were housed ($n=4$/cage) in standard laboratory conditions with free access to food and water. The cages were equipped with a grid to prevent rats from eating their stools as that can modify the Mg-deficiency induction.

Induction of the Mg-deficiency in rats

Animals were randomly divided into two groups, subsequently, for a 9-day period. The two groups of rats (depleted or normal) were allowed to feed *ad libitum*, either with the synthetic depleted diet (Mg-deficient rats) or the control diet (control rats). Deionized water was also provided *ad libitum* during the same period.

The synthetic depleted diet contained (g kg^{-1}): casein (200), sucrose (650), alphacel (50), choline bitartrate (2), D-L methionine (3), AIN-76A vitamins mix (10), Mg-free mineral mix (35), corn oil (50) (ICN Biomedicals, Orsay, France). The control diet was similar but with the addition of MgO (1.67 g kg^{-1}) to the mineral mix. The components were mixed together in an industrial mixer for 2 h. Finally, Mg concentrations in each diet, determined by flame atomic absorption spectrometric analysis (Perkin Elmer 400, Norwalk, CT, U.S.A.), were 35 and 980 mg kg^{-1} for the depleted and control diets, respectively.

Three additional groups of animals ($n=5$/group), submitted to the same depleted diet conditions than rats used for the behavioural studies, were used to check the Mg plasma level. Animals were anaesthetized with sodium pentobarbital (40 mg kg^{-1} of body weight, i.p.). Blood was collected from the abdominal aorta into heparinized tubes. Plasma obtained after low-speed centrifugation ($2000 \times g$ for 15 min) were stored in at $-20^{\circ}C$ until analysis. Mg plasma level was assessed by atomic absorption flame spectrometry (Perkin Elmer 400, Norwalk, CT, U.S.A.).

Nociceptive test

The antinociceptive effect of the tested compounds was assessed by using the paw pressure test previously described by Randall & Sellito (1957). An increasing mechanical pressure was exerted by an analgesimeter (Apelex type 003920, Ugo Basil, Italy) on the left hind paw, until vocalization was elicited. This vocalization threshold was expressed in grams corresponding to the pressure applied. The cut-off value corresponded to the maximum pressure (500 g) allowed by the device after calibration for these experiments.

Behavioural testing

Behavioural observation allowed evaluating stereotypes (head weaving and circling, jerky movements, classed as absent or present) and global motor activity.

Drugs and chemicals

The following compounds have been tested: A non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist: dizocilpine maleate or (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5, 10-imine maleate (MK-801) (RBI, Sigma-Aldrich Co., Saint Quentin Fallavier, France) was dissolved in 0.9% NaCl (saline) on the day of experiment. Magnesium sulphate ($MgSO_4$) (Sigma-Aldrich Co., Saint Quentin Fallavier, France) was dissolved in saline. Two competitive NMDA receptor antagonists: D-2-amino-5-phosphono-valeric acid (AP-5) binding to the glutamate site (ICN Pharmaceuticals France, Orsay, France), was dissolved in saline and 5,7-dichlorokynurenic acid (DCKA) binding to the glycine site (RBI, Sigma-Aldrich Co., Saint Quentin Fallavier, France), was dissolved in 50% dimethyl sulphoxide (DMSO, Sigma-Aldrich Co., Saint Quentin Fallavier, France) (vehicle). A potent amino-3-hydroxy-5-methylisooxazole-4-propionic acid (AMPA)/kainate receptor selective antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI, Sigma-Aldrich Co., Saint Quentin Fallavier, France) was dissolved in 50% DMSO. A potent PKC inhibitor, chelerythrine chloride (RBI, Sigma-Aldrich Co., Saint Quentin Fallavier, France) was dissolved in 10% DMSO. A selective inhibitor of brain nitric oxide synthase (nNOS), 7-nitro-indazole (7-NI) (RBI, Sigma-Aldrich Co., Saint Quentin Fallavier, France)

had to be dissolved in arachis oil, justifying the intraperitoneal (i.p.) route. A neurokinin $(NK)_1$ receptor antagonist, SR-140333 (Sanofi Recherche, Montpellier, France) was dissolved in 15% v v^{-1} DMSO +85% v v^{-1} saline and a NK_2 receptor antagonist, SR-48968 (Sanofi Recherche, Montpellier, France) was dissolved in saline.

Injections

All drugs, except 7-NI, were injected intrathecally (i.t.) in the subarachnoid space between L5 and L6 vertebras according to Mestre *et al*. (1994), under a transient volatile anaesthesia with 3.5% isoflurane for 2 min. Behavioural testing commenced following a 5 min recovery period. The volume of the administered drugs was 10 μl. 7-NI was injected intraperitoneally in a volume of 5 ml kg^{-1}. 7-NI is a very lipophilic compound and crosses easily the blood-brain barrier (Babbedge *et al*., 1993; MacKenzie *et al*., 1994).

Injections (i.t. or i.p.) of saline or vehicle were systematically performed in a control group of Mg-deficient rats (saline or vehicle-treated group). In order to avoid any uncontrollable environmental influence that could induce a change in the behavioural response, treatments were administered blind, with randomization procedure, using the method of equal complete blocks of animals. For each experiment, one block includes a number of animals equal to the number of treatments performed, each animal receiving a different treatment. The number of blocks corresponds to the effect of each treatment group ($n=6-8$) of the experiment.

Experimental design

According to previous experiments with this model (Dubray *et al*., 1997), each experiment took place 9 days after the introduction of the Mg-deficient diet, when the mechanical hyperalgesia was clearly in place. At this time, in Mg-deficient or control matched age rats, the control values for hindpaw vocalization, taken as the mean of two consecutive stable values which do not differ more than 10%, were recorded just before injection of the tested drug at T0. Then, the effect of the drugs was assessed by recording the vocalization threshold at 15, 30, 45, 60, 90 and 120 min after injection. The experiments were carried out in a quiet room by the same investigator. We took care to avoid any discomfort of the animals. The protocol of the test was monitored by a local ethical committee. These experiments were conducted according to the guidelines of the Committee for Research and Ethical Issue of the I.A.S.P. (Zimmerman, 1983).

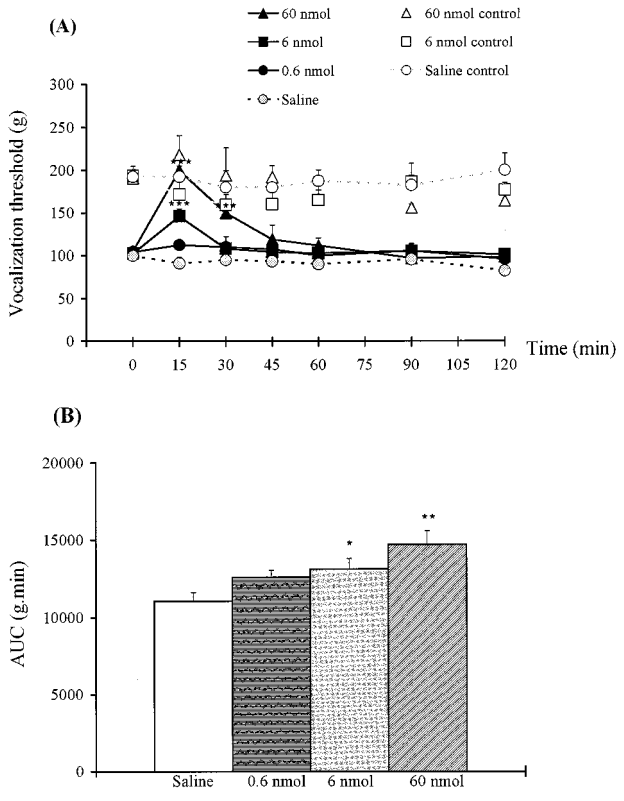

Figure 1 Effect of intrathecal injections of a non-competitive NMDA receptor antagonist, MK-801 (0.6, 6.0, 60 nmol rat^{-1}, $n=8$/dose) or saline (10 μl rat^{-1}, $n=8$) on the time-course of the vocalization threshold of Mg-deficient rats submitted to the paw pressure test (A). AUC was calculated throughout the period of 120 min for each dose in Mg-deficient rats (B). Results are expressed as means$\pm$s.e.mean. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared to the saline-treated group of Mg-deficient rats.

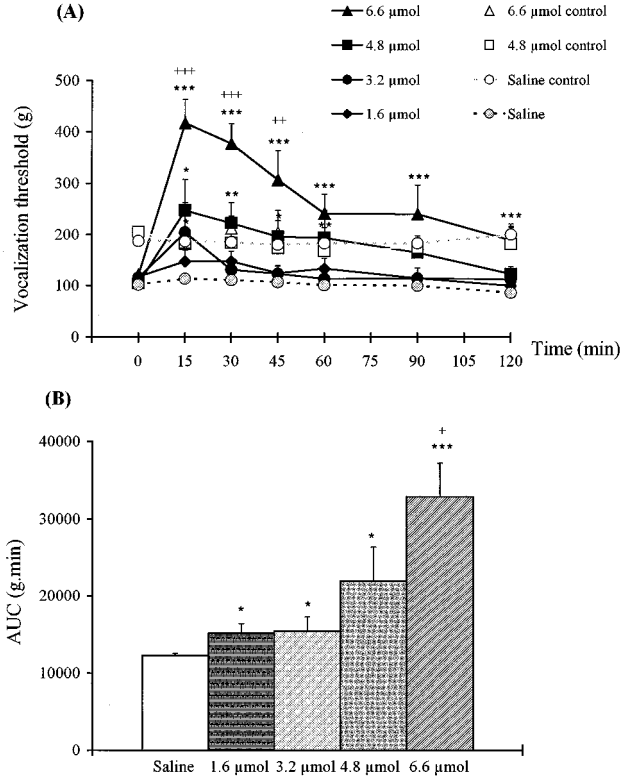

Figure 2 Effect of intrathecal injections of $MgSO_4$ (1.6, 3.2, 4.8, 6.6 μmol rat^{-1}, $n=8$/dose) or saline (10 μl rat^{-1}, $n=8$) on the time-course of the vocalization threshold of Mg-deficient and control rats submitted to the paw pressure test (A). AUC was calculated throughout the period of 120 min for each dose in Mg-deficient rats (B). Results are expressed as means$\pm$s.e.mean. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared to the saline-treated group of Mg-deficient rats; $+P<0.05$, $++P<0.01$, $+++P<0.001$; compared to the saline-treated group of control rats.

Three experiments were performed

Experiment A: Effects of intrathecal $MgSO_4$ and antagonists to EAA receptors on the hyperalgesia induced by Mg-deficiency in rats $MgSO_4$ (1.6, 3.2, 4.8 or 6.6 μmol rat^{-1}), MK-801 (0.6, 6.0 or 60 nmol rat^{-1}) and AP-5 (10.2, 40.6 or 162.3 nmol rat^{-1}) were injected i.t. ($n=8$/dose) in Mg-deficient and control rats. Because some intrathecal doses of these drugs have been described to induce side-effects like stereotypies and/or motor dysfunction ($MgSO_4$: 7.6 μmol rat^{-1}, Ishizaki *et al*., 1999; MK-801: 60 nmol rat^{-1}, Coderre & Van Empel, 1994; AP-5: 101.4 nmol rat^{-1} and 152.2 nmol rat^{-1}, Coderre & Van Empel, 1994 and Nishiyama, 2000, respectively), the highest doses used in Mg-deficient rats were tested in normal rats (control group). DCKA (0.97, 9.7 or 97 nmol rat^{-1}, i.t.) ($n=7$/dose), a selective antagonist of glycine site (Corbett & Dunn, 1993; Kehne *et al*., 1991) and CNQX (0.9, 8.6 or 86 nmol rat^{-1}, i.t.) ($n=6$/dose) (Coderre & Van Empel, 1994) reported at these dose as no inducing side effect, were tested only in Mg-deficient rats.

Experiment B: Effects of inhibitors of PKC and nNOS on the hyperalgesia induced by Mg-deficiency in rats Chelerythrine chloride (1, 10.4 or 104.2 nmol rat^{-1}, i.t.) ($n=8$/dose) and 7-NI (37.5, 75 or 150 μmol kg^{-1}, i.p.) ($n=7$/dose) were injected in Mg-deficient and control rats.

Experiment C: Effects of intrathecal antagonists to NK receptors on the hyperalgesia induced by Mg-deficiency in rats SR-140333 (0.15, 1.5 or 15 nmol rat^{-1}, i.t.) ($n=7$/dose), a NK1 receptor antagonist, and SR-48968 (0.17, 1.7 or 17 nmol/rat, i.t.) ($n=8$/dose), a NK2 receptor antagonist, were injected in Mg-deficient rats.

All doses of drugs used in our experiments were chosen, according to the literature data, showing that they are able to induce an anti-hyperalgesic effect in different behavioural pain models (see references above and in Discussion).

Expression of results and statistical analysis

Data for the vocalization thresholds are expressed as mean ± s.e.mean (standard error of the mean). Statistical significance was assessed using a two-way analysis of variance (ANOVA) followed by a PLS Fischer's test to compare at each time point during the experiment, the value for the drug-treated group with the saline or vehicle-treated groups both in Mg-deficient and in control rats. The significance levels were: ***$P<0.001$; **$P<0.01$; *$P<0.05$. To measure a global effect of the drugs, area under the time-course curve

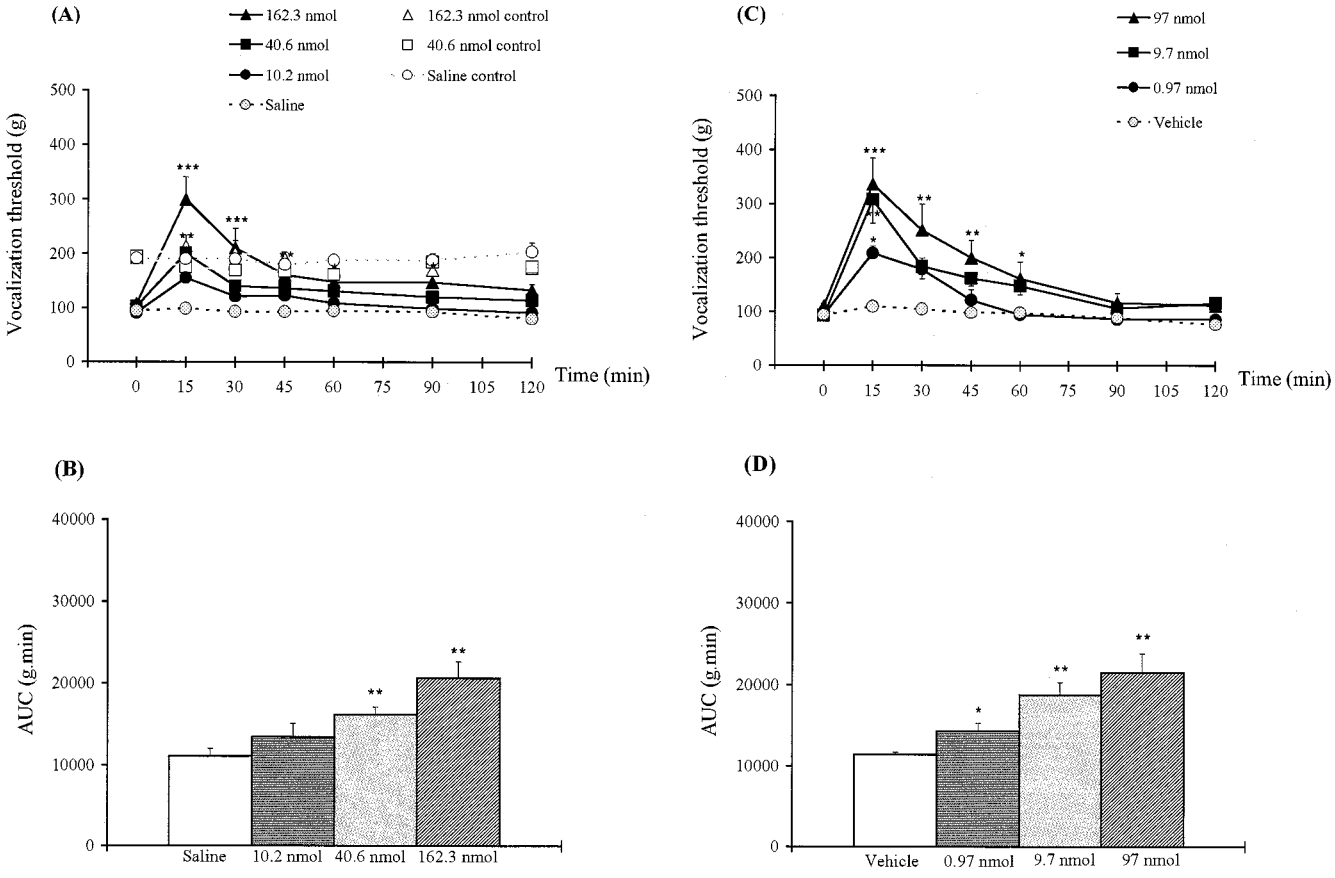

Figure 3 Effect of intrathecal injections of two competitive NMDA receptor antagonists, AP5 (10.2, 40.6, 162.3 nmol rat^{-1}, $n=8$/dose) or saline (10 μl rat^{-1}, $n=8$) (A) and DCKA, (0.97, 9.7, 97 nmol rat^{-1}, $n=7$/dose) or vehicle (10 μl rat^{-1}, $n=7$) (C) on the time-course of the vocalization threshold of Mg-deficient and control rats submitted to the paw pressure test. AUC was calculated throughout the period of 120 min for each dose in Mg-deficient and control rats (B,D). Results are expressed as means ± s.e.mean. *$P<0.05$, **$P<0.01$ and ***$P<0.001$; compared to the saline or vehicle-treated groups of Mg-deficient rats.

(AUC) was calculated throughout the 120 min of the experimental period. Statistical analysis (*t*-test) was conducted between the drug-treated group and the saline or vehicle-treated group.

Results

In our present study, after 9 days of experimental diet, Mg-deficient rats displayed the expected decrease in Mg plasma concentration as previously described (Chutkow & Meyers, 1968; Dubray *et al*., 1997; Malpuech-Brugère *et al*., 2000) (0.17 ± 0.01 mmol l^{-1}). This concentration was largely lower than this obtained in control rat (0.81 ± 0.02 mmol l^{-1}) in our previous study (Dubray *et al*., 1997).

Effects of antagonists to EAA receptors and $MgSO_4$ on the hyperalgesia induced by Mg-deficiency in rats

The hyperalgesia observed in Mg-deficient rats was dose-dependently reversed by intrathecal injection of MK-801 ($60-0.6$ nmol rat^{-1}) (Figure 1A). A significant ($P<0.001$) anti-hyperalgesic effect was observed with the two highest doses (6 and 60 nmol rat^{-1}) in Mg-deficient rats, 15 min after drug injection (200.0 ± 14.5 and 146.0 ± 8.5 g respectively, in comparison with the saline-treated group, 91.1 ± 6.5 g). No significant effect was observed with the low dose (0.6 nmol rat^{-1}) of MK-801. The AUC of the vocalization threshold confirmed the dose-dependent antinociceptive effect of MK-801 (Figure 1B). However, in control rats, we did not observe any changes in either the behaviour of the animals or their vocalization threshold.

The intrathecal injection of $MgSO_4$ ($6.6-1.6$ μmol rat^{-1}) produced a dose-dependent elevation of the vocalization threshold in the Mg-deficient group of animals (Figure 2A,B). At the highest dose (6.6 μmol rat^{-1}), $MgSO_4$ induced a significant ($P<0.001$) antinociceptive effect which peaked at 15 min after injection (417.1 ± 40.9 g for the $MgSO_4$-treated group *vs* 113.6 ± 4.1 g for the saline-treated group) and persisted throughout the experiment. At doses of 4.8 and 3.2 μmol rat^{-1}, a significant anti-hyperalgesic effect was also observed, with a maximum effect ($P<0.05$) occurring 15 min after injection. The dose of 1.6 μmol rat^{-1} did not significantly alter the vocalization threshold. At the highest dose (6.6 μmol rat^{-1}), three out of eight animals presented for 15 min some signs of a bilateral motor weakness of the hind limbs but recovered fully thereafter. In contrast to their effects on the hyperalgesia in the Mg-deficient group, it should be noted that the two highest doses of $MgSO_4$ did not change the vocalization threshold in control rats yet could

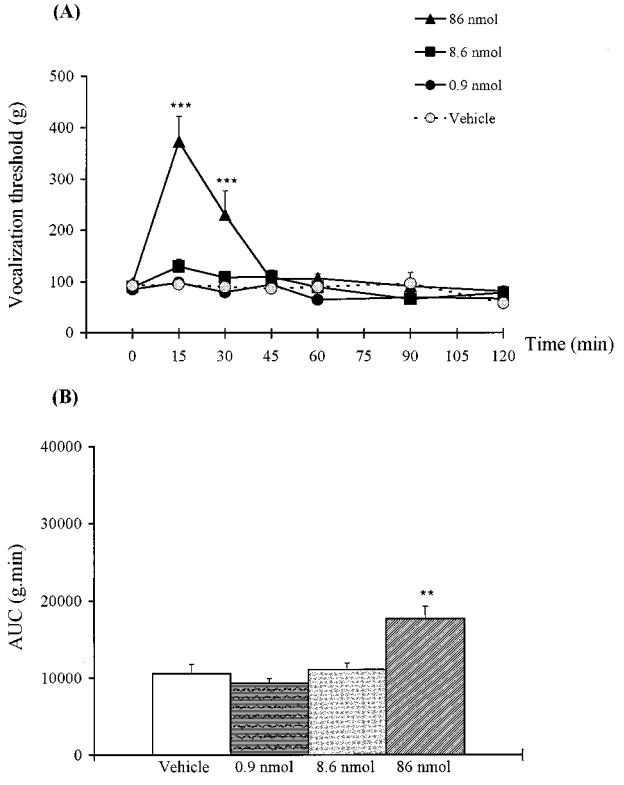

Figure 4 Effect of intrathecal injections of a competitive AMPA receptor antagonist, CNQX (0.9, 8.6, 86 nmol rat^{-1}, $n=6$/dose) or vehicle (10 μl rat^{-1}, $n=6$) on the time-course of the vocalization threshold of Mg-deficient rats submitted to the paw pressure test (A). AUC was calculated throughout the period of 120 min for each dose in Mg-deficient rats (B). Results are expressed as means$\pm$s.e.mean. $^{**}P<0.01$ and $^{***}P<0.001$; compared to the vehicle-treated group of Mg-deficient rats.

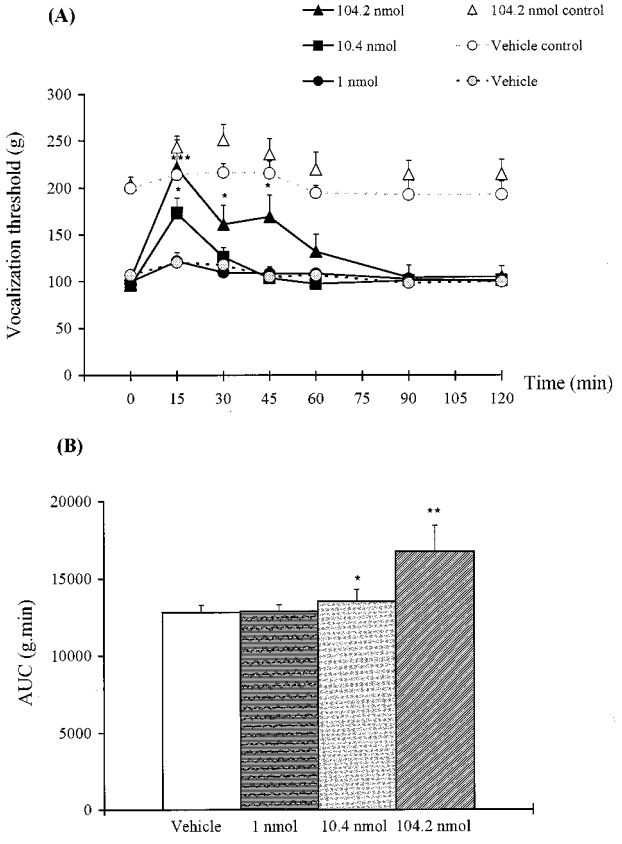

Figure 5 Effect of intrathecal injections of a PKC inhibitor, chelerythrine chloride (1, 10.4, 104.2 nmol rat^{-1}, $n=8$/dose) or vehicle (10 μl rat^{-1}, $n=8$) on the time-course of the vocalization threshold of Mg-deficient and control rats submitted to the paw pressure test (A). AUC was calculated throughout the period of 120 min for each dose in Mg-deficient rats (B). Results are expressed as means$\pm$s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$; compared to the vehicle-treated groups of Mg-deficient rats.

cause motor effects. Thus, the positive effects on hyperalgesia in Mg-deficient group of animals are not due to motor effects.

The intrathecal injection of two competitive antagonists of NMDA receptors, AP5 (162.3-10.2 nmol rat^{-1}) and DCKA (97–0.97 nmol rat^{-1}) produced dose-dependent increases in vocalization threshold in Mg-deficient rats (Figure 3). At the highest doses, AP5 (162.3 and 40.6 nmol rat^{-1}) induced a significant anti-hyperalgesic effect which peaked at 15 min after the injection (300.0±40.9 and 200.0±14.4 g, respectively). At these doses, neither change in the vocalization threshold, nor behavioural effect was observed in control rats (Figure 3A,B). No significant effect was observed with the low dose (10.2 nmol rat^{-1}) of AP5. The AUC of the vocalization threshold confirmed the dose-dependent effect of AP5 (Figure 3B).

DCKA (0.97, 9.7 and 97 nmol rat^{-1}) produced a significant dose-dependent effect on hyperalgesia in Mg-deficient rats, which peaked 15 min after injection. The maximal values were 208.6±13.0 g ($P<0.05$ *vs* the vehicle-treated group, 110.0±6.8 g), 308.0±34.1 g ($P<0.01$) and 336.7±48.3 g ($P<0.001$), respectively (Figure 3C). This dose-effect with DCKA was confirmed with the AUC evaluation (Figure 3D). No side-effect was observed at any of the doses used in our study.

At the highest dose (86 nmol rat^{-1}), CNQX, an antagonist of the AMPA receptors, produced a significant ($P<0.001$) increase in the vocalization threshold in Mg-deficient rats (Figure 4A). The maximal value, 15 min after the injection, was 373.3±39.4 g ($P<0.001$ *vs* 95.5±10.9 g for the vehicle-treated group). This antinociceptive effect was confirmed with the AUC value (Figure 4B). The low doses (8.6 and 0.9 nmol rat^{-1}) of CNQX as well as the vehicle, did not change the time-course of the vocalization threshold in Mg-deficient rats (Figure 4A).

Effects of inhibitors of PKC and nNOS on the hyperalgesia induced by Mg-deficiency in rats

Chelerythrine chloride (104.2–1 nmol rat^{-1}) reversed, dose-dependently, hyperalgesia in Mg-deficient rats (Figure 5A). The highest doses (104.2 and 10.4 nmol rat^{-1}) induced a significant ($P<0.001$ and $P<0.05$, respectively) anti-hyperalgesic effect which peaked 15 min after the injection (221.6±29.7 g and 173.8±16.0 g, respectively). No effect was observed with the lowest dose of chelerythrine (1 nmol rat^{-1}). These results were confirmed with the AUC assessment (Figure 5B). It was important to note that the highest doses of chelerythrine and vehicle did not modify the time-course of the vocalization threshold in control rats (Figure 5A).

7-NI (150–37.5 μmol kg^{-1}, i.p.) modified the vocalization threshold (Figure 6A). At the highest doses, 7-NI produced a significant ($P<0.001$) anti-hyperalgesic effect which peaked at 30 min for the dose of 150 μmol kg^{-1} (185.7±16.4 g) and 15 min for the dose of 75 μmol kg^{-1} (184.9±11.7 g). This anti-hyperalgesic effect was confirmed by the AUC values (Figure 6B). The dose of 37.5 μmol kg^{-1} and the vehicle did not change the vocalization threshold in Mg-deficient rats. Furthermore, neither the dose of 150 μmol kg^{-1} nor the vehicle perturbed the time-course of the vocalization threshold in control groups (Figure 6A).

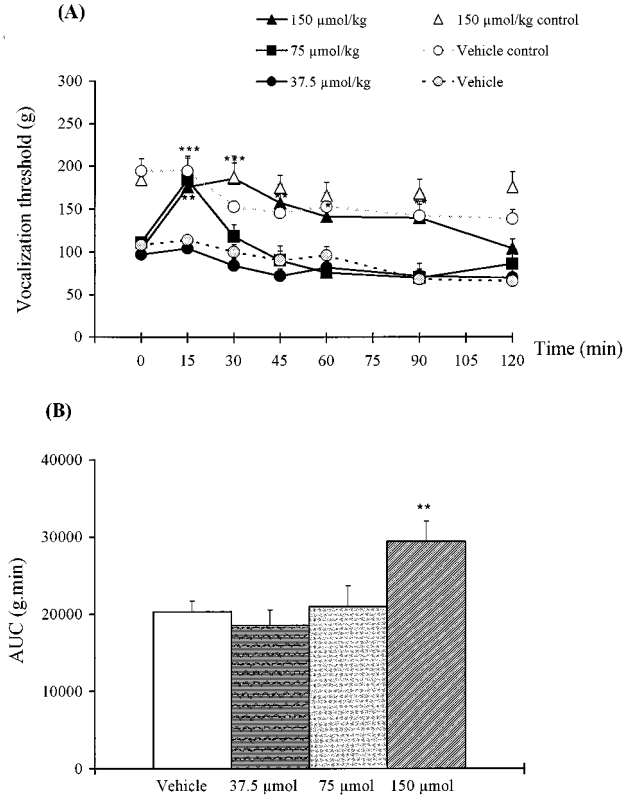

Figure 6 Effect of intraperitoneal injections of a nNOS inhibitor, 7-NI (37.5, 75, 150 μmol kg^{-1}, $n=7$/dose) or vehicle (5 ml kg^{-1}, $n=7$) on the time-course of the vocalization threshold of Mg-deficient and control rats submitted to the paw pressure test (A). AUC was calculated throughout the period of 120 min for each dose in Mg-deficient rats (B). Results are expressed as means±s.e.mean. $*P<0.05$, $**P<0.01$ and $***P<0.001$; compared to the vehicle-treated groups of Mg-deficient rats.

Effects of intrathecal antagonists to NK receptors on the hyperalgesia induced by Mg-deficiency in rats

At the highest dose (15 nmol rat^{-1}), SR 140333, a NK_1 antagonist, was able to induce a significant ($P<0.05$) anti-hyperalgesic effect which appeared 15 min after injection (170.0±12.7 g) (Figure 7A). This significant effect was confirmed by calculating the AUC ($P<0.01$ *vs* the vehicle-treated group) (Figure 7A).

The intrathecal injection of NK_2 antagonist, SR 48968 induced only a weak significant anti-hyperalgesic effect with 17 nmol rat^{-1} (158.6±12.9 g), at 15 min after injection ($P<0.05$, *vs* the vehicle treated group, 115.0±4.2 g) (Figure 7). Although a small effect was observed with the paw pressure test, no effect was shown with the AUC (Figure 7D), suggesting that SR 48968 did not markedly reverse the hyperalgesia in Mg-deficient rats.

Discussion

In accordance with our previous results (Dubray *et al.*, 1997), the present data show that Mg-deficiency in the rat is associated with a mechanical hyperalgesia. In parallel, no generalized change in behaviour (e.g. increase in spontaneous motor activity, tremors) which could induce false-positve

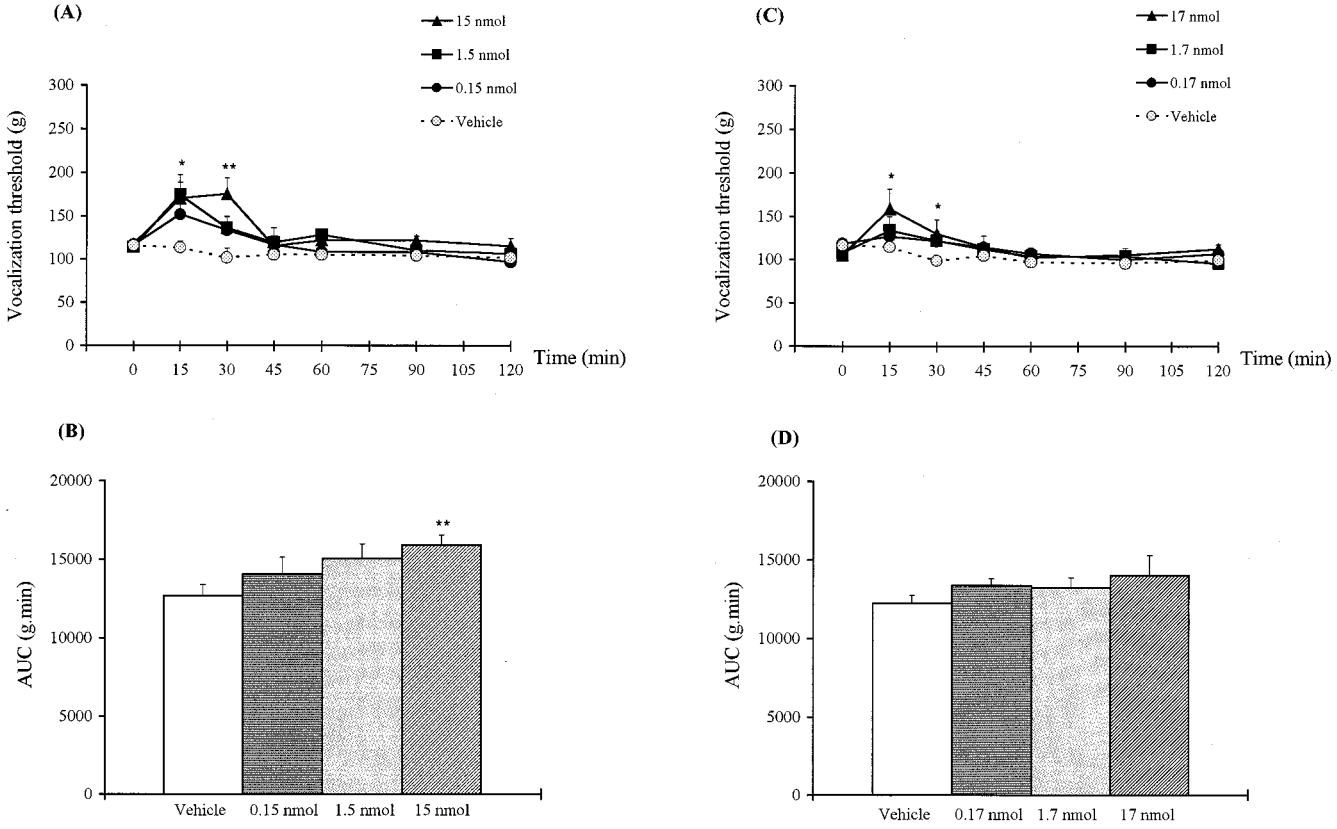

Figure 7 Effect of intrathecal injections of NK1 (SR-140333; 0.15, 1.5, 15 nmol rat^{-1}, $n=7$/dose) (A) and NK2 (SR-48968; (0.17, 1.7, 17 nmol rat^{-1}, $n=8$/dose) (C) receptor antagonists or vehicle (10 μl rat^{-1}) on the time-course of the vocalization threshold of Mg-deficient rats submitted to the paw pressure test. AUC was calculated throughout the period of 120 min for each dose in Mg-deficient rats (B,D). Results are expressed as means$\pm$s.e.mean. *$P<0.05$ and **$P<0.01$; compared to the vehicle-treated groups of Mg-deficient rats.

hyperalgesia, was observed as previously noted (unpublished data). Spinal NMDA receptors are shown to be involved in this hyperalgesia, confirming the established role of this receptor in pathophysiological pain processes, as described in many electrophysiological studies using neuropathic or inflammatory models (Stanfa & Dickenson, 1999; Suzuki *et al*., 2001). (1) MK-801 inhibited the mechanical hyperalgesia in a dose-dependent manner, whereas it had no effect in the control group. In several models of neuropathic pain, both systemic (Davar *et al*., 1991; Malcangio & Tomlinson, 1998; Smith *et al*., 1994; Wei & Pertovaara, 1999) and intrathecal MK-801 (Chaplan *et al*., 1997; Mao *et al*., 1992a, c; Sotgiu & Biella, 2000; Yamamoto & Yaksh, 1992a) also reduce hyperalgesia; it is effective in the NMDA dependent phase of the formalin test (Coderre & Melzack, 1992; Näsström *et al*., 1992; Yamamoto & Yaksh, 1992b) and in other inflammatory pain models (Ren *et al*., 1992). (2) The intrathecal administration of $MgSO_4$ in deficient rats induced an anti-hyperalgesic effect which illustrates that the hyperalgesia induced by Mg-deficiency is reversible and confirms, on the basis of the Mg-block of the channel of the ionotropic NMDA receptor, the involvement of spinal NMDA receptors in this hyperalgesia. The highest dose produced a transient paralysis in some in Mg-deficient rats as reported by Ishizaki *et al*. (1999) and Karasawa *et al*. (1998); the limited duration of the motor dysfunction means it cannot account for the anti-hyperalgesia. Our results are consistent with other behavioural studies: Ishizaki *et al*. (1999) and Takano *et al*. (2000), showing that similar intrathecal doses of $MgSO_4$ suppress the NMDA receptor-dependent phase 2 of the formalin test; Begon *et al*. (2000) and Xiao & Bennett (1994), showing that systemic or intrathecal Mg injections have an anti-hyperalgesic effect in animal models of neuropathic pain. (3) The anti-hyperalgesic effect of AP-5 or DCKA (competitive antagonists of glutamate and glycine site of NMDA receptors, respectively), which have been shown to abolish NMDA induced wind-up of dorsal horn nociceptive neurons (Dickenson & Aydar, 1991; Dickenson & Sullivan, 1990; Haley *et al*., 1990; Rygh *et al*., 2001), further supports the involvement of these receptors in the hyperalgesia induced by Mg-deficiency. Although Mg-deficiency very likely facilitates the opening of the NMDA receptor channel, this mechanism is clearly still dependent on the presence of glutamate and glycine at their respective binding sites.

Moreover, it should be noted that AP5, DCKA, and the higher doses of $MgSO_4$ were also anti-nociceptive in the deficient animals. There was an overshoot of the response to the paw pressure test above control values. This would suggest that the depletion of Mg causes a sequence of events that allows the NMDA receptor to participate in the basal neuronal activity, as well as contributing to hyperalgesia. Exogenous MK-801 however, was only anti-hyperalgesic. This difference between the drug and the cation may arise from variations in the potency, efficacy, voltage-dependency

and unblocking kinetics (MacDonald & Nowack, 1990). Furthermore, though MK-801 and Mg induce a channel blockade, the two have different sites of action.

Concerning AMPA receptors, we have shown that only the highest intrathecal dose of CNQX increased significantly the nociceptive threshold in Mg-deficient rats. These results are in agreement with those obtained by Näsström *et al.* (1992) in mice (5, 10, 50 nmol mice^{-1}) in the formalin test. However, high doses of CNQX lose specificity and block the strychnine-insensitive glycine site of NMDA receptors (Birch *et al.*, 1988), which could contribute to the observed anti-hyperalgesic effect of CNQX.

The dose-dependent anti-hyperalgesic effect induced by intrathecal injection of chelerythrine, a PKC inhibitor, suggests that this enzyme, one of the key intracellular events after NMDA activation (Coderre, 1993; Mayer & Miller, 1990), is involved in Mg-deficiency-induced hyperalgesia. Interestingly, similar doses of chelerythrine are effective in various models of hyperalgesia (intrathecal NMDA injection, Meller *et al.*, 1996; formalin test, Yashpal *et al.*, 1995 or intraplantar injection of bee venom, Li *et al.*, 2000) which confirms that Mg-deficiency induces an hyperalgesia due to a similar PKC-dependent mechanism. A recent electrophysiological and behavioural study on PKCγ-null mice, confirms that PKC is strongly involved in the long-term NMDA-dependent activity, and is notably required for the hyperexcitability in deep cells of spinal cord (Martin *et al.*, 2001), which is in line with the prolonged hyperalgesia observed in Mg-deficient rats.

The anti-hyperalgesic effect of 7-NI, a specific neuronal NOS inhibitor (MacKenzie *et al.*, 1994; Moore *et al.*, 1993a, b), in Mg-deficient rats, is evidence for a pivotal role of NO in this hyperalgesia. NO is thought to be produced in response to NMDA receptor activation (Garthwaite *et al.*, 1989), and plays an important role in nociceptive processing in the spinal cord (Meller & Gebhart, 1993). Stanfa *et al.* (1996) demonstrated that 7-NI blocked the wind-up of dorsal horn neurons after carrageenan inflammation. In the formalin test, NOS inhibitors suppress the chemical second phase (Haley *et al.*, 1992; Malmberg & Yaksh, 1993). Therefore, if we consider that the Mg-deficiency produces an activation of spinal NMDA receptors and as a consequence induces a cascade of events involving NOS, we can assume that NO participates in a positive feedback mechanism enhancing the release of glutamate and SP from primary afferent terminals (Garry *et al.*, 1994; Sorkin, 1993).

The limited reduction of mechanical hyperalgesia in Mg-deficient rats by SR-140333 and to a lesser extent by SR-48968, suggests a weak involvement of these NK_1 and NK_2 receptors and their endogenous ligands in hyperalgesia due to Mg-deficiency. These two compounds were shown more effective than here, on mechanical hyperalgesia observed in diabetic and mononeuropathic rats (Coudore-Civiale *et al.*, 1998), suggesting pathophysiological differences in the spinal involvement of SP and neurokinin A according to the aetiology of the hyperalgesia.

Conclusion

The present data clearly show, for the first time, that Mg-deficiency-induced hyperalgesia is linked to the activation of spinal NMDA receptors and to the cascade of ensuing intracellular changes. They consolidate the hypothesis that this hyperalgesia results from altered regulation of these receptors by Mg-deficiency, and suggest a sensitization of spinal dorsal horn neurons needing to be further explored by an electrophysiological approach.

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