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Modulation by α-difluoromethyl-ornithine and aminoguanidine of pain threshold, morphine analgesia and tolerance

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Abstract

The effects of α-difluoromethyl-ornithine (DFMO) and aminoguanidine, which might influence the metabolism of endogenous agmatine, on pain threshold, morphine analgesia and tolerance were investigated in mice. In the mouse acetic acid writhing test, intracerebroventricular (i.c.v.) injection of DFMO or aninoguanidine significantly elevated the pain threshold as indicated by a decrease in the number of writhings. DFMO or aminoguanidine obviously increased the analgesic effect of morphine in the mouse acetic acid writhing test and the mouse heat radiation tail-flick assay. These effects of DFMO and aminoguanidine were antagonized by idazoxan (3 mg/kg, i.p.), which is a selective antagonist of the imidazoline receptor. In the mouse heat radiation tail-flick assay, aminoguanidine significantly prolonged the tail-flick latency of animals, suggesting that the pain threshold was elevated. Furthermore, both DFMO and aminoguanidine enhanced morphine analgesia and inhibited acute morphine tolerance in the mouse heat radiation tail-flick assay. Neither DFMO nor aminoguanidine inhibited the activity of nitric oxide synthase in different brain areas in mice in vivo. These results indicate that the substances involved in the metabolism of endogenous agmatine could modulate the pain threshold, morphine analgesia and tolerance, indicating the possible role of endogenous agmatine in the pharmacological effects of morphine.

Keywords: α-Difluoromethyl-ornithine; Aminoguanidine; Agmatine; Morphine; Analgesia; Tolerance

1. Introduction

Agmatine in mammalian tissues has been postulated to be an endogenous ligand for imidazoline receptors (Li et al., 1994). Numerous experiments have shown that agmatine can enhance morphine analgesia, and inhibit tolerance to and substance dependence on morphine in mice and rats in vivo. All these effects of agmatine are based on activation of imidazoline receptors and are antagonized by the imidazoline receptor selective antagonist idazoxan (Kolesnikov et al., 1996; Li et al., 1999a,b). Furthermore, our previous research has demonstrated that the imidazoline receptor antagonist idazoxan is able to decrease the pain threshold, inhibit the analgesic effect of morphine, promote the development of tolerance to morphine and induce the abstinence syndrome in morphine-dependent mice and rats (Su et al., 2000). The density of imidazoline receptors expressed in different brain regions in rats decreases significantly after chronic morphine treatment (Su et al., 2001). All these results suggest the possible role of endogenous agmatine and imidazoline receptors in the modulation of pain and the pharmacological effects of opioids. So it is reasonable to presume that endogenous agmatine in the central nerve system (CNS) can modulate the effects of opioids by activation of imidazoline receptors.

L-arginine is turned into agmatine by L-arginine decarboxylase (L-ADC), to nitric oxide (NO) by nitric oxide synthase (NOS), into ornithine by arginase, and ornithine is further turned into putrescine by L-ornithine decarboxylase (Reis and Regunathan, 1998, 2000). If we managed to block one of the three pathways, it is possible that the metabolism of L-arginine through the other two pathways would be increased relatively. α-Difluoromethyl-ornithine (DFMO) is an inhibitor of both L-ornithine decarboxylase and arginase (Selamnia et al., 1998) and a stimulator of L-ADC (Hernandez and Schwarcz de Tarlovsky, 1999), so the quantity of endogenous agmatine might be increased by DFMO. Agmatine is degraded by diamine oxidase. Aminoguanidine is an

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inhibitor of diamine oxidase, and so administration of aminoguanidine might also increase endogenous agmatine levels in mammals (Holt and Baker, 1995). Based on above, DFMO and aminoguanidine may exert the same effects on the pharmacological actions of opioids such as exogenous agmatine.

Furthermore, NOS, which participates in morphine analgesia and the development of tolerance to and physical dependence on morphine, is also involved in the metabolism of agmatine and can be inhibited competitively by agmatine in vitro and in vivo (Galea et al., 1996; Babey et al., 1994; Pataki and Telegdy, 1998; Pelligrino et al., 1996; Garthwaite and Boulton, 1995). However, whether DFMO and aminoguanidine have any effects on the activity of NOS is not clear. Thus, it is still not sure whether DFMO and aminoguanidine modulate the pain threshold and the pharmacological effects of opioids and, if so, whether the mechanisms underlying the effects of DFMO and aminoguanidine are related to the actions of endogenous agmatine in brain.

In the present study, we investigated the possible effects of DFMO and aminoguanidine on pain threshold, morphine analgesia and tolerance in mice and whether these effects were related to the action of endogenous agmatine or the activity of NOS.

2. Materials and methods

2.1. Animals

Male and female Kunming mice (Beijing Animal Center, China) weighing 18–22 g were used in all experiments. Animals were maintained on a 12 h light–dark cycle and given ad libitum access to food and water, strictly in compliance with the guidelines established for the use of experimental animals by the European Community.

2.2. Drugs and treatments

DFMO hydrochloride was purchased from Merck KgaA (Darmstadt Germany). L-N^G-nitro arginine methyl ester (L-NAME) and aminoguanidine hydrochloride were obtained from Sigma (St. Louis, MO, USA). Idazoxan was produced by Research Biochemicals International (Natick, MA, USA). Morphine hydrochloride and acetic acid were produced by Qinghai Pharmaceutical Factory and Beijing Chemical Plant, respectively. The NOS assay kit was bought from Biotinge-Tech (Beijing, China). DFMO was administered by intracerebroventricular (i.c.v.) injection 2 h before morphine administration in a volume of 10 μl/20 g (Genedani et al., 1989). Aminoguanidine was given i.c.v. 30 min before morphine administration in a volume of 10 μl/20 g. Idazoxan (3 mg/kg) was administered intraperitoneally (i.p.) 15 min prior to DFMO or aminoguanidine administration. Morphine was given subcuteneously (s.c.) 30 min prior to pain threshold determination.

2.3. Antinociceptive tests

2.3.1. Acetic acid writhing test

Mice were injected with 0.4 ml of 0.6% acetic acid (i.p.). The number of writhings, characterized by a wave of contraction of the belly followed by extension of the hind limbs, was counted within 15 min after i.p. injection of acetic acid. Groups of 15 mice per treatment were used, and each animal was used for only one treatment (Su et al., 2000).

2.3.2. Heat radiation tail-flick assay

Nociceptive threshold was determined using a tail-flick analgesia meter (Columbus Instruments). Mice were allowed to adapt to the testing environment for at least 1 h prior to any treatment. The tail-flick latency was defined as the time from the onset of radiant heat to tail withdrawal; tail-flick latency cut-off was between 2 and 4 s. Each animal served as its own control, and the latency of the response was measured both before and after drug administration. The analgesic activity of drugs was evaluated as possible maximal analgesic percentage (PMAP)= [(latency after medication − baseline latency)/(16 s − baseline latency) × 100%]. Groups of 15 mice per treatment were used, and each animal was used only for one treatment. The behavioral experiments were performed with the approval of the Ethics Committee of Animal Experiment in Beijing, China.

2.4. Acute tolerance test

In the heat radiation tail-flick assay, in order to set up an acute tolerance model, mice were pretreated with a single dose of morphine (100 mg/kg s.c.). The mice were pretreated with saline (i.c.v.), DFMO (i.c.v.) 2 h or aminoguanidine (i.c.v.) 30 min prior to the single dose of morphine (s.c.). Each animal served as its own control, and the analgesic effect of morphine (5 mg/kg, s.c.) was evaluated as the PMAP both before and 6 h after morphine (100 mg/kg). Groups of 15 mice per treatment were used, and each animal was used only for one treatment (Li et al., 1999b).

2.5. NOS activity assay

In present study, DFMO, aminoguanidine and L-NAME were given i.c.v. in a volume of 10 μl/20 g. NOS activity in the mouse brain following drug treatment was determined as a measure of concentration of NO, using a direct NOS assay kit. Mice were killed by decapitation 2 h after DFMO exposure and 30 min after aminoguanidine and L-NAME. The brain was dissected on ice into cerebrum, thalamus and cerebellum, and then the tissues were homogenized in 10 volumes (w/v) of cold saline. The homogenates were centrifuged at 6000 × g for 10 min at 4 °C and the supernatants were collected for

assay. The protein concentration of the supernatant was determined by Coomassie brilliant blue method. NO concentration was determined colorimetrically at a wavelength of 530 nm, following the instructions of the kit. The activity of NOS is expressed as NO nmol per min per mg of protein.

2.6. Data analysis

The results are expressed as the means ± S.E.M. One-way analysis of variance (ANOVA) was used to analyze the statistical significance of differences among groups.

3. Results

3.1. Effect on pain threshold

In the mouse acetic acid writhing test, both DFMO and aminoguanidine reduced nociceptive sensitivity. The number of writhings was about 23 for control mice in the 15-min period after administration of acetic acid. Both DFMO and aminoguanidine (i.c.v.) significantly decreased the number of writhings in a dose-dependent manner ($P<0.05$, $n=15$). Idazoxan i.p. given alone had no effect on the tail flick latency and number of writhings. The analgesic effects of both DFMO and aminoguanidine could be antagonized by idazoxan (3 mg/kg, i.p.) injected 15 min before administration of the two drugs. In the heat radiation tail-flick assay, aminoguanidine (i.c.v.) prolonged the tail-flick latency from 3 to 4.9s ($P<0.05$, $n=15$); however, idazoxan (i.p.) did not inhibit the effect of aminoguanidine. DFMO (i.c.v.) had no effect on the pain threshold in this animal model (Table 1).

Table 1
Effects of DFMO, aminoguanidine (AMG) and idazoxan on pain threshold in the mouse acetic acid writhing test and heat radiation tail-flick assay

Drugs	Dose (mg/kg)	Writhing number/15 min	Tail-flick latency (s)
Saline	–	23.6 ± 2.8	3.0 ± 1.1
Idazoxan	3	24.7 ± 3.0	2.8 ± 0.9
DFMO	0.1	21.2 ± 3.2	3.0 ± 0.6
	0.25	10.5 ± 2.3[a]	2.9 ± 0.7
	0.5	8.7 ± 2.1[a]	4.6 ± 1.6
Idazoxan + DFMO	3 + 0.5	17.7 ± 2.9[b]	
AMG	0.05	19.2 ± 10.8	3.1 ± 0.8
	0.125	9.8 ± 2.1[a]	3.4 ± 0.9
	0.25	7.5 ± 1.4[a]	4.9 ± 0.7[a]
Idazoxan + AMG	3 + 0.25	13.9 ± 2.0[c]	5.0 ± 0.6

The mice received saline (i.c.v.), idazoxan (i.p.), DFMO (i.c.v.) and AMG (i.c.v.) alone, and idazoxan 15 min prior to DFMO and AMG, respectively. The number of writhings was counted and tail flick latency was determined 2 h after DFMO administration or 30 min after AMG or 15 min after idazoxan administration. Each value represents the mean ± S.E.M. for 15 mice in each group.

DFMO: α-difluoromethyl-ornithine; AMG: aminoguanidine; M: morphine.

[a] $P<0.05$ compared with saline, one-way ANOVA test.

[b] $P<0.05$ compared with DFMO, one-way ANOVA test.

[c] $P<0.05$ compared with AMG, one-way ANOVA test.

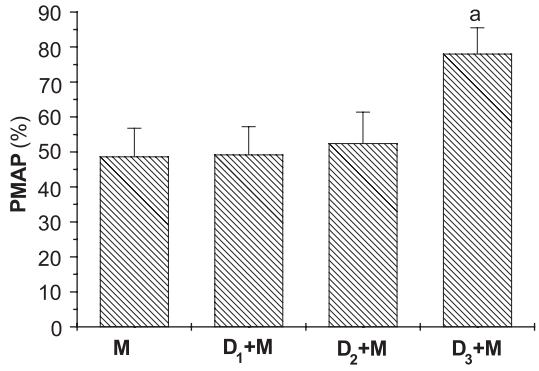

Fig. 1. Effects of DFMO (i.c.v.) on morphine (s.c.) analgesia in the mouse heat radiation tail-flick assay. The mice received morphine 2.5 mg/kg alone (M) or morphine 2.5 mg/kg plus different doses of DFMO ($D_1=0.125$, $D_2=0.25$ and $D_3=0.5$ mg/kg). DFMO was administered 2 h before morphine injection. Possible maximal analgesic percentage (PMAP) was evaluated 30 min after morphine administration. Each value represents the mean ± S.E.M. for 15 mice in each group. [a]$P<0.05$ compared with morphine. One-way ANOVA test.

3.2. Influence of morphine analgesia

In mouse heat radiation tail-flick assay, morphine (2.5 mg/kg) exhibited an obvious analgesic effect. DFMO enhanced morphine analgesia in a dose-dependent manner. In the DFMO (i.c.v.) group, the PMAP of morphine (2.5 mg/kg) was increased from 48.7% to 78.1% ($P<0.05$, $n=15$). Aminoguanidine also enhanced morphine analgesia dose dependently. In the presence of aminoguanidine (0.25 mg/kg, i.c.v.), the PMAP of morphine (2.5 mg/kg) was increased from 33.7% to 59% ($P<0.05$, $n=15$) (Figs. 1 and 2).

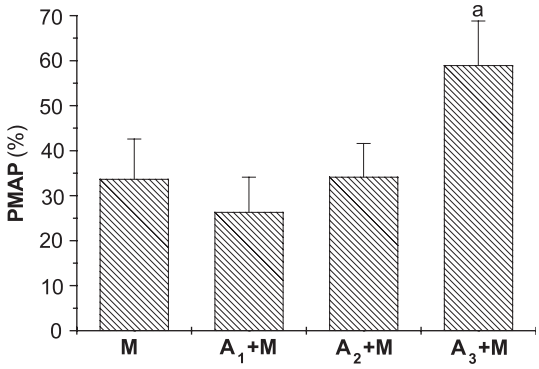

Fig. 2. Effects of aminoguanidine (i.c.v.) on morphine (s.c.) analgesia in the mouse heat radiation tail-flick assay. The mice were given morphine 2.5 mg/kg alone (M) or different dose of aminoguanidine ($A_1=0.05$, $A_2=0.125$ and $A_3=0.25$ mg/kg) plus morphine 2.5 mg/kg. Aminoguanidine was administered 30 min prior to morphine injection. Possible maximal analgesic percentage (PMAP) was evaluated 30 min after morphine administration. Each value represents the mean ± S.E.M. for 15 mice in each group. [a]$P<0.05$ compared with morphine. One-way ANOVA test.

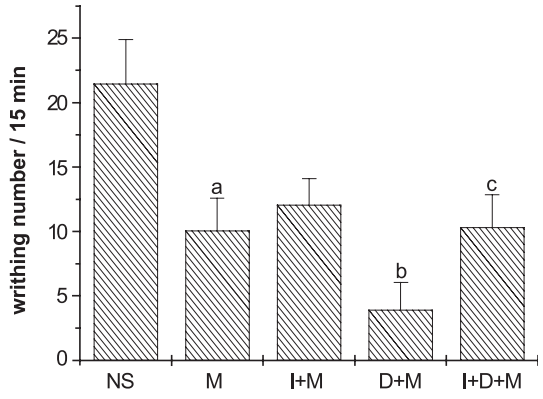

Fig. 3. Effects of DFMO and idazoxan on morphine analgesia in the mouse acetic acid writhing test. The mice were given saline (N.S.), morphine (0.5 mg/kg, s.c.) (M), DFMO (0.25 mg/kg, i.c.v.) 2 h prior to morphine (D + M), and idazoxan (3 mg/kg i.p.) 15min prior to DFMO and then 2 h prior to morphine (I + D + M). Each value represents the mean ± S.E.M. for 15 mice in each group. [a]$P < 0.05$ compared with N.S., [b]$P < 0.05$ when compared with M, [c]$P < 0.05$ compared with D + M. One-way ANOVA test.

In the mouse acetic acid writhing test, morphine (0.5 mg/kg, s.c.) significantly reduced the number of writhings. Coadministration of DFMO (0.25 mg/kg, i.c.v.) and aminoguanidine (0.125 mg/kg i.c.v.) with morphine further reduced the number of writhings. Idazoxan (3 mg/kg i.p.) given alone had no effect on morphine analgesia; however, the enhancing effect of DFMO and aminoguanidine on morphine analgesia in the mouse acetic acid writhing test was inhibited by i.p. idazoxan 3 mg/kg (Figs. 3 and 4).

3.3. Acute tolerance

In the mouse heat radiation tail-flick assay, a single s.c. dose of morphine (100 mg/kg) induced acute tolerance, indicated by the decrease in analgesic effect of morphine (5 mg/kg). The PMAP of morphine (5 mg/kg s.c.) was reduced from 42% to 16% ($n = 15$, $P < 0.05$). Administration of DFMO (0.25 mg/kg i.c.v.) or aminoguanidine (0.125 mg/kg i.c.v.) prior to administration of morphine (100 mg/kg s.c.) inhibited this decrease. In these groups, the PMAP of morphine (5 mg/kg s.c.) after pretreatment of the mice with a single dose of morphine had no significant difference compared with that before pretreatment, indicating that tolerance was blocked (Table 2).

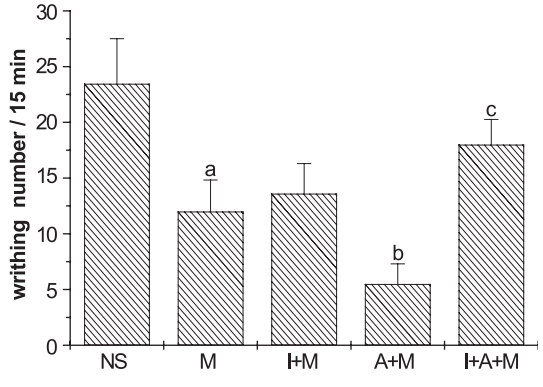

Fig. 4. Effects of aminoguanidine and idazoxan on morphine analgesia in the mouse acetic acid writhing test. The mice were given saline (N.S.), morphine (0.5 mg/kg, s.c.) alone (M), aminoguanidine (0.125 mg/kg, i.c.v.) 30 min prior to morphine (A + M), and idazoxan (3 mg/kg i.p.) 15 min prior to aminoguanidine and 30 min prior to morphine (I + A + M). Each value represents the mean ± S.E.M. for 15 mice in each group. [a]$P < 0.05$ compared with N.S., [b]$P < 0.05$ compared with M, [c]$P < 0.05$ compared with A + M. One-way ANOVA test.

Table 2
Effect of DFMO and aminoguanidine (AMG) on tolerance induced by acute treatment of morphine in the heat radiation tail-flick assay

Drugs	Dose (mg/kg)	$PMAP_1$	$PMAP_2$
M	5	42.4 ± 8.0	16.1 ± 3.6[a]
DFMO + M	0.25 + 5	36.4 ± 7.5	27.6 ± 6.7
AMG + M	0.25 + 5	47.7 ± 10.4	40.4 ± 9.5

The mice were i.c.v. treated with saline, DFMO 2 h or AMG, 30 min prior to a single dose of morphine (100 mg/kg s.c.). The analgesic effect of morphine (5 mg/kg s.c.) was evaluated by $PMAP_1$ (before morphine 100 mg/kg pretreatment) and $PMAP_2$ (6 h after the morphine 100 mg/kg pretreatment). Each value represents the mean ± S.E.M. for 15 mice in each group.
DFMO: α-difluoromethyl-ornithine; AMG: aminoguanidine; M: morphine.
[a] $P < 0.05$ compared with $PMAP_1$, one-way ANOVA test.

3.4. NOS activity

NOS activity, expressed as the concentration of NO (nmol per min per mg of protein), was 15.6 ± 0.9 in the cerebrum, 15.7 ± 1.6 in the thalamus, and 17.4 ± 1.5 in the cerebellum of control mice. Administration of L-NAME (i.c.v.) inhibited significantly NOS activity in the cerebrum and cerebellum. However, DFMO and aminoguanidine (i.c.v.) did not influence NOS activity in the cerebrum, thalamus or cerebellum compared with control (Table 3).

Table 3
The effects of L-NAME, DFMO and aminoguanidine (AMG) on NOS activity in mouse brain

Drugs	NOS activity (nmol per min per mg of protein)		
	Cerebrum	Thalamus	Cerebellum
Saline	15.6 ± 0.9	15.7 ± 1.6	17.4 ± 1.5
L-NAME	11.0 ± 1.2[a]	14.0 ± 1.0	13.6 ± 0.9[a]
DFMO	18.7 ± 1.6	17.0 ± 0.7	18.8 ± 0.5
AMG	17.6 ± 1.0	15.5 ± 1.3	17.7 ± 1.4

The mice were pretreated with saline (i.c.v), DFMO (0.5 mg/kg i.c.v.) 2 h or AMG (0.5 mg/kg i.c.v.) 30 min or L-NAME (0.5 mg/kg i.c.v.) 30 min prior to the assay. Each value represents the mean ± S.E.M. of five samples in each group.
DFMO: α-difluoromethyl-ornithine; AMG: aminoguanidine; L-NAME: L-N^G-nitro arginine methyl ester.
[a] $P < 0.05$ compared to saline, one-way ANOVA test.

4. Discussion

In the mouse acetic acid writhing test, DFMO and aminoguanidine significantly increased the pain threshold and enhanced the analgesic effect of morphine. In the mouse heat radiation tail-flick assay, DFMO and aminoguanidine increased the PMAP of morphine and inhibited the development of acute tolerance to morphine. Neither DFMO nor aminoguanidine influenced the activity of NOS. Some effects of DFMO and aminoguanidine were antagonized by idazoxan, suggesting the involvement of imidazoline receptors and the possible relationship with endogenous agmatine.

Agmatine, ornithine and NO are synthesized from the same substrate L-arginine, catalyzed by L-ADC, arginase and NOS, respectively (Selamnia et al., 1998). Inhibition of one metabolic pathway of L-arginine might increase activity in the other two metabolic pathways. DFMO is an inhibitor of L-ornithine decarboxylase and arginase (Selamnia et al., 1998) and a stimulator of L-ADC (Hernandez and Schwarcz de Tarlovsky, 1999). The current results demonstrate that DFMO was able to elevate the pain threshold, and enhance morphine analgesia and inhibit acute morphine tolerance, effects which were sensitive to the imidazoline receptor antagonist idazoxan, but it was not able to inhibit NOS activity. All these results indicate that DFMO modulation of morphine actions might be related to an increase in the concentration of agmatine in the CNS. Aminoguanidine treatment may also result in the accumulation of endogenous agmatine in cells through inhibition of diamine oxidase, which degrades agmatine to guanido butanoic acid. Thus, it is reasonable to suppose that DFMO and aminoguanidine might have the same effects on pain threshold and morphine analgesia as exogenous agmatine. The present study proved this hypothesis, and the results are consistent with the pharmacological actions of exogenous agmatine in vivo (Li et al., 1999a,b).

However, the results reported by Genedani et al. (1989) were quite different from those of our study. They found that DFMO (50 μg/rat i.c.v.) inhibited the analgesic effect of morphine (15 mg/kg i.p.) significantly in rats and had no effect on the pain threshold. The discrepancy may result from the different analgesic animal model and different doses of DFMO (0.25 mg/kg i.c.v.) and morphine (0.5 mg/kg s.c.) used in the present experiment. We have no direct evidence suggesting that DFMO and aminoguanidine affect the concentration or the distribution of endogenous agmatine in different brain regions, and thus endogenous agmatine concentrations after DFMO and aminoguanidine treatment should be determined in the future.

Many studies indicated that NOS inhibitors enhanced morphine analgesia and inhibited tolerance to morphine, so we needed to analyze the possible role of DFMO and aminoguanidine on NOS activity. Unlike the NOS inhibitor L-NAME, DFMO had no inhibitory effect on NOS activity in this study, indicating that the modulation by DFMO of the pain threshold and morphine analgesia may not be related to a direct inhibition of NOS activity. Aminoguanidine is considered to be an NOS inhibitor and mostly interacts with inducible NOS, which is not normally present in the brain and is expressed on demand during a variety of inflammatory conditions (Corbett et al., 1992; Hasan et al., 1993; Moss et al., 1995). We did not observe an inhibitory effect of aminoguanidine on NOS activity in the present study. However, we can still not rule out that the effects of aminoguanidine on the pain threshold and morphine analgesia are due to the inhibition of NOS in the spinal cord since a study by Hu et al. (1996) indicated that intrathecal administration of aminoguanidine significantly prevented the nociception induced by injection of formalin into the hindpaws of rats, an effect that was attributed to its inhibition of NOS activity. In addition, the effect of aminoguanidine on mouse tail-flick latency was not prevented by idazoxan in our experiment, a result which is not consistent with that of the mouse acetic acid writhing test, which suggests that aminoguanidine has a complicated mechanisms of action. No matter what, the effects of aminoguanidine on pain sensitivity and morphine analgesia are at least partly due to its inhibitory effect on the metabolism of endogenous agmatine.

Since DFMO can result in the depletion of polyamines in the CNS (Slotkin et al., 1982), it is also possible that DFMO exerts its effect through the inhibition of polyamine synthesis. However, in other experiments from our laboratory, we proved that polyamines, including spermidine and spermine, did not affect the pain threshold and morphine analgesia (Su et al., 2003).

In conclusion, our present results indicate that compounds which influence the metabolism of endogenous agmatine could modulate the pain threshold and morphine actions, and provide further experimental data to validate the hypothesis that endogenous agmatine and imidazoline receptors might constitute a new modulatory system for opioid function.

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