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# Effect of agmatine on the development of morphine dependence in rats: potential role of cAMP system

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**Abstract**

Agmatine is an endogenous amine derived from arginine that potentiates morphine analgesia and blocks symptoms of naloxone-precipitated morphine withdrawal in rats. In this study, we sought to determine whether treatment with agmatine during the development of morphine dependence inhibits the withdrawal symptoms and that the effect is mediated by cAMP system. Exposure of rats to morphine for 7 days resulted in marked naloxone-induced withdrawal symptoms and agmatine treatment along with morphine significantly decreasing the withdrawal symptoms. The levels of cAMP were markedly increased in morphine-treated rat brain slices when incubated with naloxone and this increase was significantly reduced in rats treated with morphine and agmatine. The induction of tyrosine hydroxylase after morphine exposure was also reduced in locus coeruleus when agmatine was administered along with morphine. We conclude that agmatine reduces the development of dependence to morphine and that this effect is probably mediated by the inhibition of cAMP signaling pathway during chronic morphine exposure.


*Keywords:* Agmatine; Morphine; Dependence; Withdrawal; cAMP; Tyrosine hydroxylase

## 1. Introduction

Agmatine is an amine formed by the decarboxylation of L-arginine by the enzyme arginine decarboxylase (ADC). While long recognized to be synthesized and stored in plants, bacteria, and invertebrates (Tabor and Tabor, 1984), agmatine and its biosynthetic enzyme were discovered in mammals, originally in rat brain (Li et al., 1994), later in other tissues and serum (Feng et al., 1997; Lortie et al., 1996; Raasch et al., 1995). Agmatine binds to imidazoline and α2-adrenoceptors and proposed as an endogenous ligand for imidazoline receptors (Li et al., 1994; Piletz et al., 1995). Agmatine has been shown to modulate transmitter/hormone release (Li et al., 1994; Kalra et al., 1995), and possibly act as a neurotransmitter/modulator in brain (Regunathan et al., 1995; Reis and Regunathan, 2000). Agmatine exerts a wide range of biologic activities on several organ systems, including the CNS. In various chronic pain models, agmatine attenuates thermal, tactile allodynia and has an anti-inflammatory effect (Fairbanks et al., 2000; Regunathan and Piletz, 2003; Onal et al., 2004; Karadag et al., 2003). Agmatine has a weak analgesic effect in tail flick test and enhances morphine-induced antinociception (Kolesnikov et al., 1996; Yesilyurt and Uzbay, 2001). Furthermore, it reduces tolerance to morphine (Fairbanks and Wilcox, 1997; Li et al., 2002) and attenuates behavioral signs of morphine abstinence syndrome in vitro and in vivo (Aricioglu-Kartal and Uzbay, 1997; Li et al., 1998; Aricioglu et al., 2003a,b).

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The beneficial effects of agmatine in potentiating morphine analgesia and at the same time reducing the withdrawal symptoms have been clearly documented (Aricioglu-Kartal and Uzbay, 1997; Li et al., 2002; Li et al., 1998). While the interaction with α2-adrenoceptors has been proposed as the mechanism for the potentiation of analgesic effect of morphine (Kolesnikov et al., 1996; Yesilyurt and Uzbay, 2001), the mechanisms for the reduction in dependence/withdrawal symptoms are not clear. Agmatine does not bind to any type of opioid receptors, thus, ruling out the possibility of interaction at the receptor level (Bradley and Headley, 1997). In previous studies, agmatine was injected at the time of inducing withdrawal with naloxone and it has been proposed that agmatine acts by inhibiting *N*-METHY D-ASPARTATE receptors to reduce withdrawal symptoms (Aricioglu-Kartal and Uzbay, 1997; Li et al., 2002). However, it is not known whether agmatine will reduce dependence and withdrawal by interfering with the development of dependence, if administered along with morphine. There is at least one in vitro study suggesting that agmatine could interfere with molecular adaptive changes to chronic morphine exposure (Li et al., 1999b). Therefore, the objectives of this study are to investigate whether (a) chronic administration of agmatine during morphine exposure reduces withdrawal symptoms; (b) agmatine interferes with super activation of cAMP system that leads to reduce dependence and withdrawal in rats; and (c) agmatine interferes with the activation of locus coeruleus neurons during chronic morphine exposure as measured by the expression tyrosine hydroxylase.

## 2. Materials and methods

### 2.1. Animals

All procedures in this study are in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (USA) and the declaration of Helsinki. Male Sprague–Dawley rats were used for morphine exposure with approved protocol by the University of Mississippi Medical Center. Rats were housed in a quiet, temperature (20±2 °C)- and humidity (60±3%)-controlled room in which a 12/12-h light–dark cycle was maintained (0700–1900 h light). Rats were fed standard lab chow and tap water ad libitum during the study. The animals were housed under these conditions for at least 4 days prior to being used for experiments.

### 2.2. Drugs used in the study

Naloxone HCl and Agmatine sulfate were purchased from Sigma (USA). Morphine and placebo pellets were obtained from National Institute on Drug Abuse. Agmatine and naloxone were dissolved in saline and injected to rats intraperitoneally (i.p.) in a volume of 0.1 ml/100 g.

### 2.3. Morphine dependence/withdrawal

Animals divided into three groups ($n$=6 for each group). The first two groups received one morphine pellet containing 75 mg morphine base that was implanted subcutaneously in the scapular area under light halothane anesthesia on day 1. Immediately after implantation, they received saline or agmatine (10 mg/kg, i.p.) and the injection was repeated after 7 h. Rats received two more pellets 3 days after the first implantation and saline or agmatine injections were continued twice daily for 7 days. The third group received placebo pellets and received 10 mg/kg agmatine i.p. twice daily for 7 days. On day 8, all rats received naloxone (2 mg/kg, i.p.) to precipitate morphine withdrawal syndrome. Just after naloxone injection, rats were placed in Plexiglas boxes (base area: 25×30 cm, height: 35 cm) and morphine withdrawal signs, such as jumping, wet dog shakes, writhing, teeth chattering, defecation, diarrhea, tremor and ptosis, were observed, evaluated for 15 min. All signs were counted except ptosis, tremor, diarrhea (1–3) and teeth chattering (1–10) which were qualitatively rated. All experiments were carried out at the same day and in the light period of the day. Withdrawal was rated according to Gellert and Holtzman's (1978) rating scale. This scale consists of graded signs and checked signs. Graded signs (counted) were assigned a weighting factor from 1 to 4 which was based on the frequency of their appearance, while checked signs (rated) were assigned values of 2–7 independent of the frequency of their appearance. The statistical analysis was performed for signs of morphine abstinence as follows. Jumping, wet dog shakes, writhing and defecation were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. Diarrhoea, teeth chattering, tremor and ptosis were analyzed by Kruskal–Wallis followed by Tukey's multiple comparison test. Withdrawal scores were complied using one-way ANOVA followed by Neuman–Keuls individual means comparisons. The criterion for significance was set at $p<0.05$.

### 2.4. cAMP production in rat brain slices after morphine exposure

Male Sprague–Dawley rats were (six animals in each group) implanted s.c. morphine pellets (75 mg each) or control placebo pellets as described in the above experiment. After 8 days, animals were sacrificed and frontal cortical slices (300 μm) were prepared as described earlier using MacIlwaine's brain slicer (Regunathan and Reis, 1994). The slices were preincubated in oxygenated physiological buffer (Krebs Ringer) for 30 min at 37 °C and then incubated with buffer, naloxone (100 μM) or agmatine (100 μM) in fresh buffer for 15 min in the presence of isobutyl methyl xantine (phospodiesterase inhibitor). The incubation was stopped by the addition of 95% ethanol, the tissue was homogenized and centrifuged to remove proteins. The

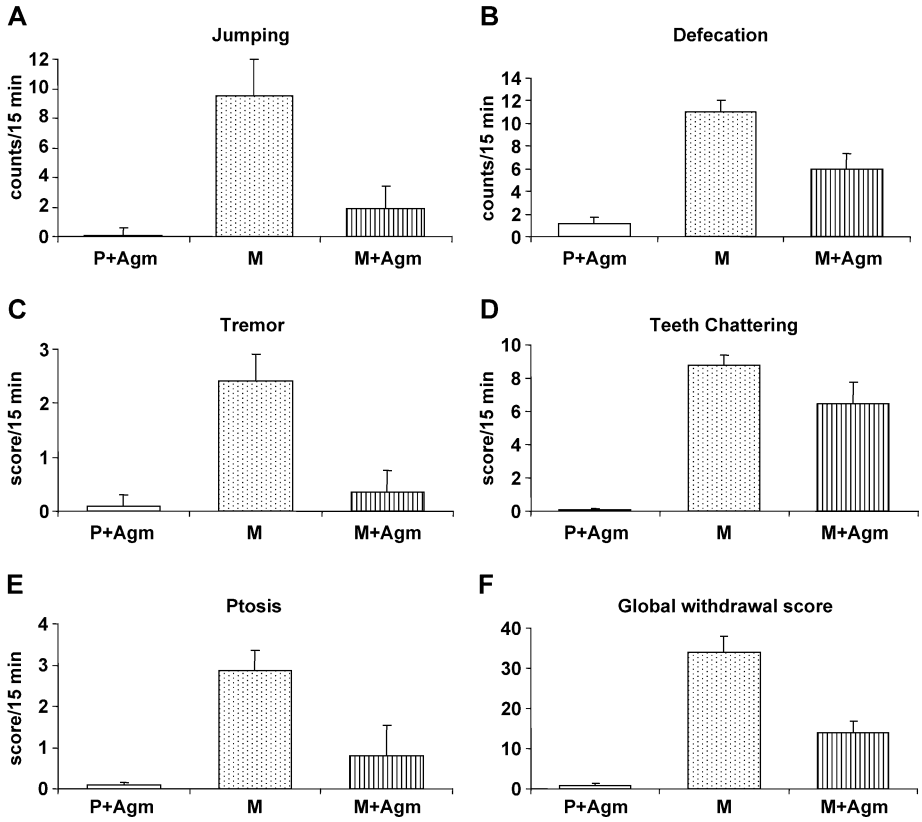


Fig. 1. The effects of agmatine (Agm) on naloxone precipitated morphine withdrawal. Rats were implanted with placebo (P) or one morphine (M) pellet (75 mg morphine base) on day 1 and two more pellets on day 3. After 7 days, withdrawal was induced on the eighth day by injecting naloxone (2 mg/kg, i.p.). Agmatine (10 mg/kg, i.p.) was injected twice daily for 7 days and no agmatine was injected on the eighth day, the day of inducing withdrawal. Panels A through E indicate mean withdrawal frequencies induced by naloxone that was observed for 15 min for each group ($n$=6). *$p$<0.05 compared to morphine group as determined by analysis of variance. Panel F illustrates the global withdrawal scores of the three groups of rats. This score consists of graded signs (based on the frequency) and checked signs (independent of the frequency) and was calculated using all symptoms of withdrawal. *$p$<0.05 compared to morphine group.

ethanol extract was evaporated under vacuum and the residue was dissolved in cAMP assay buffer. The cAMP levels were determined as described earlier (Regunathan and Reis, 1994) using commercially available enzyme immunoassay (EIA) kit (Assay Design Laboratories, Ann Arbor, MI). The protein amount in each slice was determined after the incubation and results are expressed as nmol cAMP/mg protein.

### 2.5. TH expression in morphine-treated rat brain

Male Sprague–Dawley rats were (six animals in each group) implanted s.c. morphine pellets (75 mg each) or control placebo pellets and treated with agmatine as described in the above experiment. All rats were sacrificed on the eighth day and brain regions including the frontal cortex, striatum and locus coeruleus were prepared for immunoblot analysis. The tissues homogenized in HEPES buffer (pH 7.4) and centrtifuged at 1000×$g$. The supernatant was solubilized in sodium dodecyl sulphate sample buffer for polyacrylamide gel electrophoresis. The separated proteins was transferred to polyvinyldene difluoride membrane and exposed to monoclonal antibody to TH (Chemicon Int., Temecula, CA) at 1/1000 dilution or a polyclonal antibody to β-actin (Chemicon Int.). The membranes were processed for immunoblot analysis using enhanced chemiluminescence (ECL) method. The immunoreactive band was visualized using Kodak Image Station and quantitated using the imaging software.

## 3. Results

### 3.1. Effect of agmatine on morphine withdrawal

Three groups of rats, morphine, morphine+agmatine and placebo+agmatine, were prepared for behavioral experiments. Rats that received morphine pellets and saline injections showed clear signs of withdrawal after the injection of naloxone (2 mg/kg) as measured by several symptoms including jumping, tremor, ptosis, defecation and teeth chattering (Fig. 1). In the second group of rats that received morphine and agmatine for 7 days, the intensity of naloxone-precipitated withdrawal symptoms were significantly lower ($p$<0.05). The largest effect of agmatine was observed in jumping behavior [$F$(2,15)=9.889; $p$<0.05; Fig.

1A]. Agmatine also significantly decreased the intensity of defecation (Fig. 1B), weight loss [$F(2,15)=26.88$, 12.43, respectively, $p<0.05$] and tremor [Fig. 1C; $H(2,15)=14.37$, $p<0.05$]. Agmatine produced slight but nonsignificant effect on teeth chattering (Fig. 1D), diarrhoea, ptosis (Fig. 1E) and wet dog shakes in the study [$H(2,15)=13.78$, 12.28, 13.22 and $F(2,15)=10.16$, respectively; $p>0.05$]. In placebo group that received agmatine, naloxone did not cause any behavioral changes, indicating that agmatine by itself has no effect in naive rats. The global withdrawal score was calculated using all symptoms of withdrawal, including jumping, wet dog shakes, teeth chattering, tremor, defecation, diarrhoea, abnormal posture, facial fasciculation, salivation, swallowing movements, ptosis, penile erection, ejaculation, irritability and weight loss. While morphine-dependent rats showed as score of 34.71±2.1, the cotreatment with agmatine resulted in a score of 15.84±1.8 that was significantly lower ($p<0.05$) (Fig. 1F).

### 3.2. Effects of agmatine in rat brain slices

Chronic exposure to morphine has been shown to exhibit adenylate cyclase superactivation in rat brain slices (Copeland et al., 1989) and this model has been used to study the biochemical changes during morphine dependence/withdrawal. To determine whether agmatine interferes with this sustained activation of adenylate cyclase in rat brain, we measured the production of cAMP in brain cortical slices in vitro after chronic exposure to morphine. Cortical slices, prepared from placebo-, morphine- or morphine+agmatine-treated rats, were exposed to basal buffer (Kreb Ringer Buffer), agmatine (100 μM) or naloxone (100 μM) for 15 min and cAMP levels were measured in slices. As shown in Fig. 2, naloxone produced a large increase in cAMP levels in morphine-treated rat brain slices compared to placebo-treated rats. This effect of naloxone was significantly lower in morphine+agmatine-treated rats. It was also observed that basal cAMP levels were slightly higher in morphine-treated rats compared to placebo rats probably due to some spontaneous withdrawal of morphine in vitro. Agmatine (100 μM) had no direct effect on cAMP levels in control or morphine-treated rat brain slices (Fig. 2).

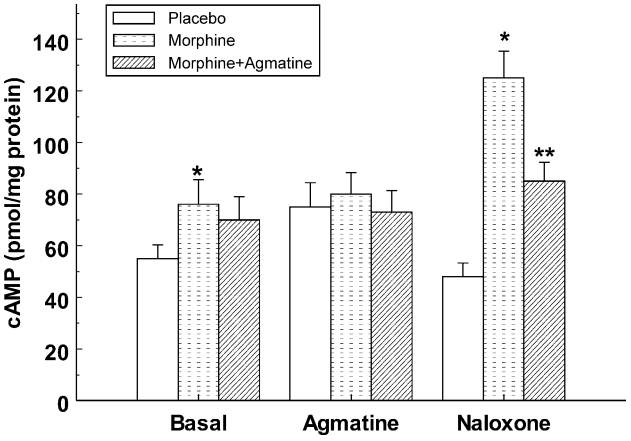


Fig. 2. Effect of agmatine on cAMP production in morphine-treated rat brain. Rats were exposed to morphine pellets (75 mg) for 7 days (one pellet on day 1 and two more pellets on day 3) and sacrificed on the eighth day. Brain cortical slices were prepared and cAMP production was measured in vitro. The levels of cAMP was measured in cortical slices (300 μm) in vitro after incubating with agmatine, norepinephrine or naloxone (100 μM each) for 15 min. The values are from 6 rats in each group and in vitro incubations were done in triplicates. *$p<0.001$ compared to placebo group; **$p<0.001$ compared to morphine group.

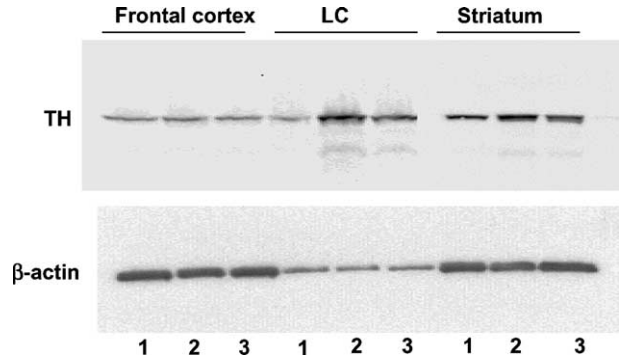


Fig. 3. Effect of agmatine on TH expression in morphine-treated rat brain. Rats were treated with placebo, morphine (one 75 mg pellet on day 1 and two more pellets on day 3) or morphine+agmatine (10 mg/kg, i.p., twice daily for 7 days) and brain regions were analyzed by immunoblot for the expression of TH. Lanes: 1, placebo; 2, morphine; 3, morphine+agmatine. The same samples were also used to determine the expression of β-actin as the control housekeeping protein. This is a representative data from one set of rats that was replicated in four animals in each group.

### 3.3. Effects of agmatine on TH expression in rat brain

Western blot analysis of brain regions from rats, exposed to morphine or placebo pellets and received agmatine, were carried out using TH antibody (mouse monoclonal from Chemicon). In all animals, TH expression was higher after morphine exposure in locus coeruleus and striatum but not in frontal cortex compared to placebo rats. As shown in Fig. 3 from one set of rats, agmatine treatment significantly reduced the higher TH expression in LC, slightly reduced in striatum and had no effect on frontal cortex TH expression. The analysis of the density of protein bands from all animals indicated that morphine exposure increased the TH expression in LC and striatum by three- and twofold, respectively, and agmatine treatment along with morphine reduced the levels of TH in LC by about 50% and in striatum by about 20%. As shown in Fig. 3, β-actin expression was not altered, indicating the uniform loading transfer and specificity of the TH response.

## 4. Discussion

While several previous studies have reported the effects of agmatine in reducing morphine withdrawal symptoms (Aricioglu-Kartal and Uzbay, 1997; Li et al., 2002), the

molecular mechanisms of this action is not clear. In this study, we report that exogenously injected agmatine interferes with the development of dependence to morphine by reducing the upregulation of cAMP system in rat brain.

Following the discovery of agmatine in mammalian brain, several studies observed that agmatine could potentiate the analgesic effect of morphine while reducing the symptoms of dependence and withdrawal (Aricioglu-Kartal and Uzbay, 1997; Li et al., 1998; Li et al., 2002; Aricioglu et al., 2003a,b). As agmatine does not bind to opiate receptors, these actions are not mediated by direct effect on opiate receptors (Bradley and Headley, 1997). The abilities of agmatine to bind to α2-adrenoceptors (Li et al., 1994; Pinthong et al., 1995a; Pinthong et al., 1995b; Piletz et al., 1995) and to block NMDA receptor channels (Yang and Reis, 1999) or inhibit nitric oxide synthase (NOS; Galea et al., 1996; Li et al., 1999a; Demady et al., 2001) were considered as potential mechanisms for these actions. We have recently reported that acute administration of agmatine just before withdrawal failed to reverse centrally mediated withdrawal symptoms to morphine in neuronal NOS (nNOS)-deficient transgenic mice while it was preventing peripheral withdrawal symptoms (Aricioglu et al., 2004). These findings suggested that functional NMDA receptors coupled to nNOS system is required for the central effects of agmatine. The activation of α2-adrenoceptors by agonists, like clonidine, inhibits dependence and withdrawal. While agmatine was discovered by its ability to bind to α2-adrenoceptors (Li et al., 1994), several subsequent functional studies reported that agmatine is not an agonist at this site (Pinthong et al., 1995a,b; Jurkiewicz et al., 1996). Moreover, administration of α2-adrenergic agonists, like clonidine, while blocking opiate withdrawal, also causes sympathetic inhibition and reduction in arterial pressure (Gatti et al., 1988; Hieble and Kolpak, 1993). Agmatine, administered i.c.v. or i.p., does not lower arterial pressure in several animal models (Sun et al., 1995; Szabo et al., 1995; Raasch et al., 2002), thus ruling out the possibility of α2-adrenoceptor activation in this action of agmatine. Therefore, agmatine appears to display unique effect different from those agents bind to the same receptors.

Thus, the action of agmatine at membrane receptors may not be fully responsible for its ability to reverse morphine dependence and withdrawal. In earlier behavior studies, agmatine was administered just before the injection of naloxone to induce withdrawal and agmatine effectively blocked the withdrawal symptoms (Aricioglu-Kartal and Uzbay, 1997). In the present study, we administered agmatine along with morphine during chronic exposure for 7 days and withdrawal was induced by naloxone injection without any preinjection of agmatine at that time. Still, agmatine substantially reduced naloxone-induced withdrawal symptoms in these rats. Therefore, it is conceivable that agmatine blocks the events leading to hyperexcitable state of the neurons during chronic morphine exposure and that, unlike NMDA receptor agonists, direct inhibition of glutamatergic neurotransmission may not be the only mechanism for this action. It is also important to point out that in all animal studies reported, agmatine has no effect on normal behavior, motor activity, cardiovascular parameters or any other toxic effects in normal animals in doses up to 100 mg/kg. Based on these findings and other reports that agmatine directly activates G proteins (Ferry and Landry, 2002) and inhibits the superactivation (Li et al., 1999b), we have developed the hypothesis that agmatine may be acting intracellularly at signal transduction pathway to modulate the neuronal excitability. The cellular and molecular mechanisms for the dependence and withdrawal to opiate have been fairly well documented. The electrophysiological and neurochemical studies indicated hyperexcitable state of neurons that occur due to cycle of molecular events of higher phosphorylation and gene expression resulting in adaptive upregulation of cAMP system after chronic morphine abuse (De Vries and S.T., 2002; Nestler, 2002). Such an upregulation of cAMP system has been shown in in vitro model systems, including NG108-15 cells, cells transfected with: μ-opioid receptors as well as in vivo animal models (Copeland et al., 1989; Mehta and Strada, 1994; Avidor-Reiss et al., 1995; Guitart and Nestler, 1996). The resulting higher cAMP causes increased protein kinase A (PKA) activity which phosphorylates several target proteins, including TH and cAMP response element binding protein (CREB). The phosphorylated CREB subsequently acts as transcription factor, increasing the expression of several proteins, including adenylate cyclase and TH (Lane-Ladd et al., 2002). We have shown that agmatine, when treated along with morphine, inhibits naloxone-induced increase in cAMP in rat brain slices in vitro. By inhibiting the overproduction of cAMP, agmatine could be interfering with downstream events, such as activation of PKA and CREB. The major regions that control the reinforcing and reward behavior to opiates are striatum, LC and prefrontal cortex. As agmatine is enriched in these brain regions, this could suggest a role for an endogenous system in modulating opiate dependence and withdrawal. One such downstream marker is TH that is induced in LC during chronic morphine exposure (De Vries and S.T., 2002; Nestler, 2002). Agmatine treatment along with morphine reduced the induction of TH protein expression after chronic morphine treatment in LC confirming the downstream effects of the inhibition of cAMP upregulation. Thus, the inhibition of the persistent activation of cAMP system by agmatine results in decreased phosphorylation and activation of several neural proteins, including ion channels, TH and transcription factors, which may contribute to the lower withdrawal symptoms to morphine.

The most fascinating aspect of agmatine, an endogenous molecule, is its ability to potentiate the analgesic effect of morphine and at the same time to reduce the morphine dependence and withdrawal. The findings from this and previous reports suggest that increasing endogenous agmatine could offer novel therapeutic advantage in morphine

analgesia and dependence. For example, combining agmatine with morphine during pain management, while reducing the effective dose of morphine, will also prevent the development of dependence to morphine.

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