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Agmatine enhances cannabinoid action in the hot-plate assay of thermal nociception

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ABSTRACT

Agmatine–cannabinoid interactions are supported by the close association between cannabinoid CB_1 receptors and agmatine immunoreactive neurons and evidence that shared brain mechanisms underlie the pharmacological effects of agmatine and cannabinoid agonists. In the present study, we used the hot-plate assay of thermal nociception to determine if agmatine alters cannabinoid action through activation of imidazoline sites and/or alpha$_2$-adrenoceptors. WIN 55212-2 (1, 2 or 3 mg/kg, i.p.) or CP55,940 (1, 2 or 3 mg/kg, i.p.) administration increased hot-plate response latency. Agmatine (50 or 100 mg/kg, i.p.) was ineffective. Administration of agmatine (50 mg/kg, i.p.) with WIN 55212-2 (1, 2 or 3 mg/kg, i.p.) or CP55,940 (1, 2 or 3 mg/kg, i.p.) produced response-latency enhancement. Regression analysis indicated that agmatine increased the potency of WIN 55212-2 and CP55,940 by 3- and 4.4-fold, respectively, indicating synergy for both drug interactions. Idazoxan, a mixed imidazoline site/alpha$_2$-adrenoceptor antagonist, but not yohimbine (5 mg/kg, i.p.), a selective alpha$_2$-adrenoceptor antagonist, blocked response-latency enhancement produced by a combination of WIN 55212-2 (2 mg/kg) and agmatine. Response-latency enhancement produced by WIN 55212-2 (2 mg/kg) was blocked by SR 141716A (5 mg/kg, i.p.), a cannabinoid CB_1 receptor antagonist; attenuated by idazoxan (2 and 5 mg/kg); and not affected by yohimbine (5 mg/kg). These results demonstrate a synergistic interaction between agmatine and cannabinoid agonists and suggest that agmatine administration enhances cannabinoid action *in vivo*.

1. Introduction

Agmatine is one of the most pharmacologically diverse substances in the mammalian brain (Raasch et al., 1995; Feng et al., 1997; Li et al., 2003; Nguyen et al., 2003). It was initially characterized as an endogenous clonidine-displacing substance of imidazoline sites (Li et al., 1994). In addition to activating imidazoline sites, agmatine displays affinity for alpha$_2$-adrenoceptors and antagonizes glutamatergic NMDA receptors (Loring, 1990; Piletz et al., 1995; Reis and Regunathan, 2000; Fairbanks et al., 2000; Gilad et al., 1996; Auguet et al., 1995). Agmatine also inhibits neuronal nitric oxide synthase and downregulates inducible nitric oxide synthase. In the mammalian brain, agmatine is synthesized by the enzyme arginine decarboxylase and degraded by the enzyme agmatinase (Regunathan et al., 1995; Sastre et al., 1996). Central effects of agmatine include a weak analgesic action, anti-depressant like effects, reduction of seizure-evoked glutamate levels in the frontal cortex, attenuation of neuropathic pain, anti-convulsant effects, improvement of locomotor function following spinal cord injury and blockade of stress- and bacterial endotoxin-evoked hyperthermia (Zomkowski et al., 2005; Kalra et al., 1995; Onal et al., 2003; Feng et al., 2005; Aricioglu and Regunathan, 2005). Agmatine is well known for its interaction with mu opioid receptors. Results reveal that agmatine administration blocks all symptoms of morphine withdrawal, enhances acute morphine analgesia and prevents tolerance to morphine analgesia (Su et al., 2000, 2003; Kolesnikov et al., 1996; Aricioglu-Kartal and Uzbay, 1997; Reis and Regunathan, 2000). Despite the well-documented ability of agmatine to modulate opioid function, its role in cannabinoid function is not yet clear. Prior work suggests that agmatine enhances the hypothermic effect of a cannabinoid agonist, but it is not known if additional cannabinoid-induced actions are modulated by agmatine (Rawls et al., 2006; Compton et al., 1992). In the present study, we investigated the effect of exogenous agmatine on cannabinoid action in the hot-plate assay of thermal nociception and determined whether imidazoline sites and alpha$_2$-adrenoceptors contributed to the agmatine–cannabinoid interaction.

2. Experimental procedures

2.1. Animals

Male Sprague–Dawley rats (Zivic–Miller), weighing 175–200 g, were housed in groups of 3–4 for at least 1 week in an animal room maintained at 22 ± 1 °C and approximately 50 ± 5% relative humidity. Lighting was on a 12/12 h light/dark cycle (lights on at 7:00 and off at

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19:00). Rats were allowed free access to food and water. Animal use procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

2.2. Drug preparation

WIN 55212-2 ([4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenylcarbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one]), CP55,940 ((−)-cis 3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl)-trans-4-(3-hydroxypropyl) cyclohexanol, arachidonylethanolamide (anandamide), agmatine sulfate and yohimbine were purchased from Tocris Bioscience (St. Louis, MO, USA). Idazoxan hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). WIN 55212-2 was dissolved in a 10% cremophor/saline solution and injected intraperitoneally (i.p.). CP55,940 and anandamide were dissolved in 1 part ethanol, 1 part cremophor and 18 parts 0.9% saline (vehicle 1:1:18) and injected i.p. Agmatine, idazoxan and yohimbine were dissolved in physiological saline and injected i.p. SR 141716A ([N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride]) was provided by the National Institute on Drug Abuse (NIDA) and injected intramuscularly (i.m.) in a 20% DMSO/saline solution. All drugs were injected in a volume of 1 ml/kg.

2.3. Hot-plate experiments

Effect was measured as hot-plate response latency (52.5 °C) after drug treatment. Response was defined by the animal either by licking the forepaws, or hindpaws, or flicking the hindpaws. To avoid tissue damage, animals were exposed to the hot plate (Ugo Basile model 7280, Comerio, Italy) for a maximum of 30 s. Animals were tested initially for baseline latency, and latency was tested again 30, 60, 90 and 120 min post-administration. Data were expressed as:

$$\%\ \text{maximum possible effect} = [(\text{drug response time} - \text{basal response time}) / (30\,\text{s} - \text{basal response time})] \times 100\%.$$

The effect of agmatine by itself was first determined. Rats were injected with agmatine (50 or 100 mg/kg) or saline (Rawls et al., 2007; Su et al., 2000; Regunathan, 2006). For drug combination experiments, an inactive dose of agmatine (50 mg/kg) was administered with WIN 55212-2 (1, 2 or 3 mg/kg), CP55,940 (1, 2 or 3 mg/kg) or anandamide (3 or 7.5 mg/kg). We then investigated a role for imidazoline sites, alpha$_2$-adrenoceptors and cannabinoid CB$_1$ receptors in the agmatine–cannabinoid interaction. Rats were injected with idazoxan (2 mg/kg), a mixed imidazoline site/alpha$_2$-adrenoreceptor antagonist, or yohimbine (5 mg/kg), a selective alpha$_2$-adrenoreceptor antagonist, and then administered a combination of agmatine (50 mg/kg) and WIN 55212-2 (2 mg/kg) 15 min later. A role for cannabinoid CB$_1$ receptors, imidazoline sites and alpha$_2$-adrenoreceptors on response-latency enhancement caused by WIN 55212-2 alone was investigated in a final set of experiments. Rats pretreated with vehicle, SR 141716A (5 mg/kg), idazoxan (2 mg/kg) or yohimbine (5 mg/kg) were injected, 15 min later, with either WIN 55212-2 (2 mg/kg) or vehicle.

2.4. Data and statistical analysis

Time-course data were analyzed using a two-way (group and time) mixed-model analysis of variance (ANOVA) with repeated measures on time followed by pair-wise multiple comparisons incorporating the Bonferroni correction at the different time points. In experiments which measured only the maximal response, a one-way ANOVA was used to determine a significant main effect followed by a Tukey's post-hoc analysis to identify differences between individual groups. In experiments in which only two groups were compared, a Student's *t*-test was used to determine significance. The interaction between agmatine and WIN 55212-2 or CP55,940 was analyzed using dose–response data obtained from the hot-plate test (response latency determined 30 min post-injection). Analysis of drug combinations to distinguish synergism from simple additivity followed the procedure described previously (Tallarida, 2001). In cases in which one of the two drugs is inactive (i.e., agmatine), its presence in a simply additive combination has no effect on the dose–effect curve of the active drug (i.e., WIN 55212-2 or CP55,940). Therefore, the analysis is one in which the dose–effect curve of the active drug is statistically compared before, and after, the addition of the inactive agent. Values of $P<0.05$ were considered to be statistically significant in all cases.

3. Results

3.1. Effect of agmatine on hot-plate response latency

The effect of agmatine (50 and 100 mg/kg) by itself on the hot-plate response latency is presented in Fig. 1. A two-way ANOVA on the individual response latencies revealed that there was not a significant drug interaction ($F_{2,\,20}=3.054$, $P>0.05$), time interaction ($F_{3,\,60}=2.050$, $P>0.05$), or drug time interaction ($F_{6,\,60}=0.3097$, $P>0.05$).

3.2. Effect of agmatine and cannabinoid co-administration on hot-plate response latency

The effect of a fixed, inactive dose of agmatine (50 mg/kg) on the increase in response latency caused by progressively increasing doses of WIN 5521-2 (1, 2 and 3 mg/kg) is displayed in Fig. 2. This dose of agmatine (50 mg/kg), which by itself did not alter the response latency (Fig. 1), enhanced the increase in response latency caused by each dose of WIN 55212-2 (1, 2 and 3 mg/kg) ($P<0.05$, Student's *t*-test, Fig. 2a–c). Using the data in Fig. 2a–c, we compared the dose–response relation of the active agent, WIN 55212-2, and the dose–response relation of WIN 55212-2 in combination with the inactive agent, agmatine (Fig. 2d). These two dose–response data sets, using the effect level of response latency 30 min post-administration, were used to construct regression lines (effect on log dose) in Fig. 2d. Regression analysis revealed a pronounced leftward shift in the combination's regression line (Fig. 2d). Because the lines did not differ significantly in slope ($P>0.05$), this shift was expressed in terms of relative potency (R), which is defined as the ratio of the amount of each drug required to produce the same effect (i.e., the ratio of ED_{50} values for the active drug alone and the active drug in combination with the inactive agent). R, computed with the assistance

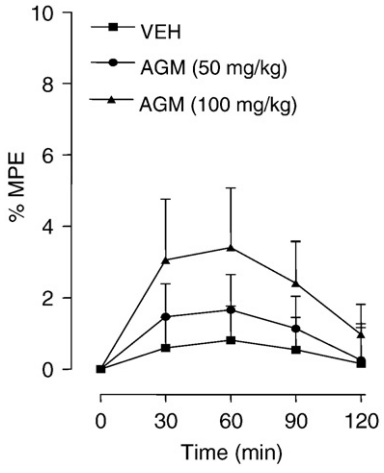

Fig. 1. Effect of agmatine (AGM) on reaction latency in the hot-plate assay. AGM (50 or 100 mg/kg) or vehicle (VEH) was injected at 0 min. Reaction latencies were recorded for each rat at 30, 60, 90 and 120 min following injection and data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE).

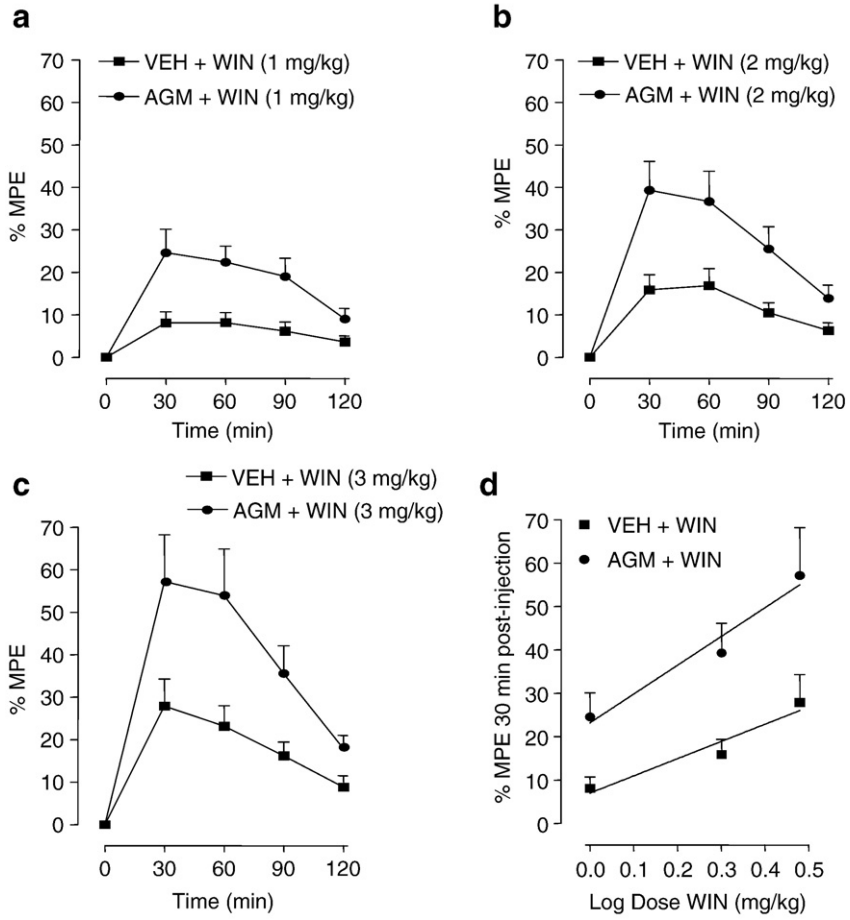

Fig. 2. Effect of agmatine (AGM) and WIN 55212-2 (WIN) on reaction latency in the hot-plate assay. A fixed dose (50 mg/kg) of AGM was administered with WIN 55212-2 (1, 2 or 3 mg/kg) at 0 min. Reaction latencies were recorded for each rat at 30, 60, 90 and 120 min following injection and data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE). A Student's *t*-test on the individual data sets revealed that AGM enhanced the antinociception caused by each dose of WIN: (a) 1 mg/kg, ($P<0.05$, 95% CL −18.31 to −1.254); (b) 2 mg/kg, ($P<0.05$, 95% CL −24.88 to −1.441); (c) 3 mg/kg, ($P<0.05$, 95% CL −34.03 to −1.335). (d) Regression analysis conducted on the data sets presented in (a)–(c) indicated that the combination dose–effect curve for AGM plus WIN was elevated significantly ($P<0.05$) above the curve for WIN alone and revealed a shift measured as relative potency, $R=2.72$ (95% confidence limits, 1.609 to 7.367).

of Pharm Tools Pro (The McCary Group, Elkins Park, PA), was found to be 2.72, with 95% confidence limits (1.609 to 7.367). This value of *R*, significantly greater than unity, indicates synergism for the interaction between WIN 55212-2 and agmatine.

Agmatine (50 mg/kg) had a similar effect on CP55,940 (1 or 2 mg/kg) ($P<0.05$, Fig. 3a–b). Regression analysis comparing the dose–response relation of CP55,940 and dose–response relation of CP55,940 in combination with agmatine revealed a pronounced leftward shift in the combination's regression line (Fig. 3d). The regression lines did not differ significantly in slope ($P>0.05$) and *R* was found to be 4.39, with 95% confidence limits of 3.192 to 5.596, indicating the interaction was synergistic. The increase in response latency produced by the highest dose (3 mg/kg) of CP55,940 was also enhanced in the presence of agmatine, but the effect did reach statistical significance ($P>0.05$) (Fig. 3c). Agmatine (50 mg/kg) did not significantly enhance the increase in response latency following anandamide (3 or 7.5 mg/kg, i.p.) administration ($P>0.05$) (Fig. 4).

3.3. Role of imidazoline sites and alpha$_2$-adrenoreceptors in response-latency enhancement produced by co-treatment with agmatine and WIN 55212-2

Idazoxan (imidazoline site/alpha$_2$-adrenoreceptor antagonist) and yohimbine (selective alpha$_2$-adrenoreceptor antagonist) were used to determine whether imidazoline sites and alpha$_2$-adrenoreceptors mediated response-latency enhancement caused by co-exposure to agmatine (50 mg/kg) and WIN 55212-2 (2 mg/kg) (Fig. 5). A one-way ANOVA on peak response latency revealed a significant main effect ($F_{3,\ 32}=6.139$, $P<0.0001$) (Fig. 5). The response latency of rats co-exposed to agmatine (50 mg/kg) and WIN 55212-2 (2 mg/kg) was significantly greater than rats treated with WIN 55212-2 (2 mg/kg) by itself ($P<0.05$). Pretreatment with idazoxan abolished normal response-latency enhancement in rats co-exposed to agmatine (50 mg/kg) and WIN 55212-2 (2 mg/kg) ($P<0.05$). Conversely, pretreatment with yohimbine (5 mg/kg) did not reduce enhanced response latency produced by agmatine (50 mg/kg)/WIN 55212-2 (2 mg/kg) co-administration ($P>0.05$).

3.4. Role of cannabinoid CB$_1$, imidazoline sites and alpha$_2$-adrenoreceptors in response-latency enhancement caused by WIN 55212-2

Effects of three drugs – SR 141716A, idazoxan and yohimbine – on response-latency enhancement caused by WIN 55212-2 (2 mg/kg) are presented in Fig. 6. A one-way ANOVA on peak response latency revealed a significant main effect ($F_{7,\ 36}=4.767$, $P<0.0001$) for the data set. WIN 55212-2 (2 mg/kg) increased response latency (34.7 ± 7.5) compared to vehicle ($P<0.05$). Neither idazoxan (2 mg/kg) nor yohimbine (5 mg/kg) significantly increased response latency compared to vehicle ($P>0.05$). In drug combination experiments,

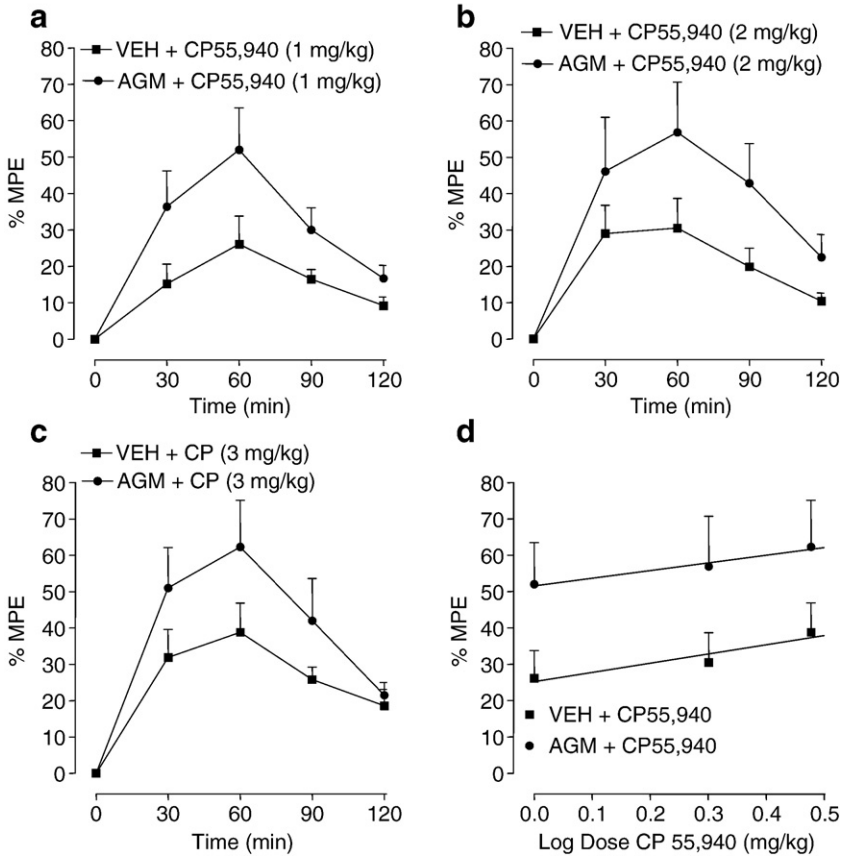

Fig. 3. Effect of agmatine (AGM) and CP55,940 on reaction latency in the hot-plate assay. A fixed dose (50 mg/kg) of AGM was administered with CP55,940 (1, 2 or 3 mg/kg) at 0 min. Reaction latencies were recorded for each rat at 30, 60, 90 and 120 min following injection and data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE). A Student's *t*-test on the individual data sets revealed that AGM significantly enhanced antinociception caused by: (a) 1 mg/kg of WIN ($P<0.05$, 95% CL −26.51 to −0.7299) and (b) 2 mg/kg of WIN ($P<0.05$, 95% CL −28.58 to −2.860) but not by (c) 3 mg/kg of WIN ($P<0.05$, 95% CL −25.16 to 0.4798). (d) Regression analysis conducted on the data sets presented in (a)–(c) indicated that the combination dose–effect curve for AGM plus CP55,940 was elevated significantly ($P<0.05$) above the curve for CP55,940 alone and revealed a shift measured as relative potency, $R=4.39$ (95% confidence limits, 3.192 to 5.596).

pretreatment with SR 141716A (5 mg/kg) or idazoxan (2 mg/kg) reduced response-latency enhancement produced by WIN 55212-2 (2 mg/kg) ($P<0.05$). Yohimbine (5 mg/kg) did not alter the WIN 55212-2 (2 mg/kg)-induced increase in response latency ($P>0.05$). Because 2 mg/kg of idazoxan was effective, we tested additional doses of the imidazoline/alpha$_2$-adrenoreceptor antagonist against WIN 55212-2 (2 mg/kg) (Fig. 7). When administered at doses of 2 or 5 mg/kg, idazoxan reduced the enhanced response latency caused by WIN 55212-2 (2 mg/kg) ($P<0.05$). Lower doses (0.5, 1 mg/kg) were ineffective ($P>0.05$).

4. Discussion

Despite the pharmacological diversity of agmatine, only a few studies have investigated its effect on non-opioid drug action. In the present study, we investigated the effect of agmatine on cannabinoid action. Because agmatine immunoreactive neurons located in the midbrain are associated closely with cannabinoid CB_1 receptors and overlapping mechanisms contribute to the pharmacological effects of agmatine and cannabinoid agonists (Tsou et al., 1998; Pettit et al., 1998; Ruggiero et al., 1998; Raasch et al., 1995; Meng et al., 1998; Welch et al., 1995; Welch and Stevens, 1992; Rawls et al., 2006), we hypothesized that exogenous agmatine would modulate cannabinoid action in conscious rats. This is, in fact, what we found. Combined administration of agmatine and a cannabinoid agonist (WIN 55212-2 or CP55,940) produced response-latency enhancement in the hot-plate assay of thermal nociception. A mixed imidazoline site/alpha$_2$-adrenoreceptor antagonist idazoxan, but not a selective alpha$_2$-adrenoreceptor antagonist yohimbine, abolished reaction latency enhancement produced by co-administration of WIN 55212-2 and agmatine. Response-latency enhancement produced by WIN 55212-2 was attenuated by idazoxan or a cannabinoid CB_1 receptor antagonist, SR 14171A. Taken together, these findings suggest that agmatine increases cannabinoid agonist action through a mechanism involving imidazoline sites.

Agmatine and cannabinoid agonists produced different effects on response latency, with agmatine lacking effect and cannabinoids producing enhancement. Prior work shows that agmatine displays weak analgesic activity in some animal models of acute and chronic pain such as the mouse acetic acid writhing test and rat 4% saline writhing test (Li et al., 1999) and produces dose-dependent anti-hyperalgesia in chronic pain models such as inflammatory pain and neuropathic pain (Fairbanks et al., 2000; Su et al., 2000, 2003). Agmatine is ineffective in nociceptive experimental models such as the radiant heat tail-flick test and hot-plate assay. In contrast to agmatine, cannabinoid agonists reduce pain sensitivity in a number of nociceptive assays, including the tail-flick and hot-plate assays of thermal nociception and acetic acid writhing and formalin tests of noxious pain (Bicher and Mechoulam, 1968; Dewey et al., 1972; Novelli et al., 1983; Martin, 1985; Martin and Lichtman, 1998; Fox and Bevan, 2005; Lever and Rice, 2007). Since antinociceptive doses of WIN 55212-2, CP55,940 and anandamide can impair motor coordination in the rotarod assay, it was unclear in our experiments whether the increase in hot-plate response latency produced by cannabinoids was due to antinociception, motor impairment or a mix of the two (Fox et al., 2001; but see Liang et al., 2007).

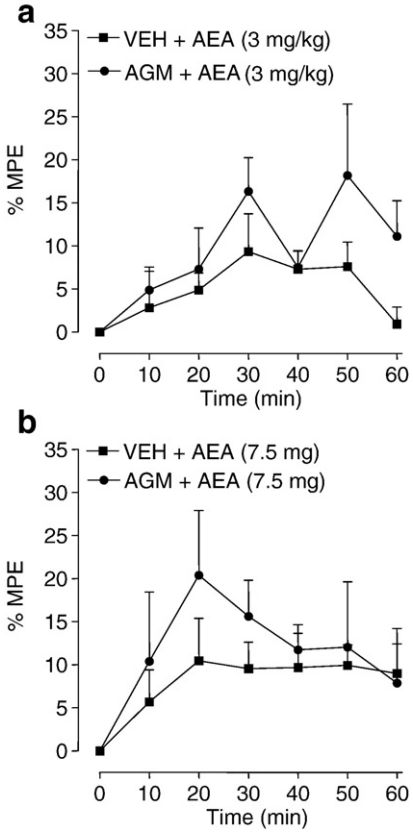

Fig. 4. Effect of agmatine (AGM) and anandamide (AEA) on reaction latency in the hot-plate assay. A fixed dose (50 mg/kg) of AGM was administered with AEA (3 or 7.5 mg/kg) at 0 min. Reaction latencies were recorded for each rat at 10, 20, 30, 40, 50 and 60 min following injection and data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE). A Student's *t*-test on the individual data sets revealed that AGM did not significantly enhance antinociception caused by either dose of AEA.

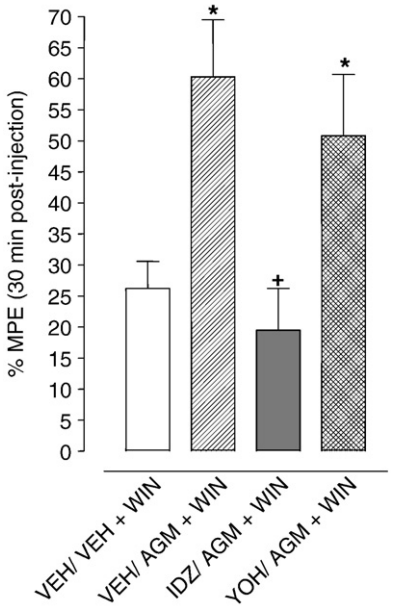

Fig. 5. Effect of pretreatment with idazoxan (IDZ) or yohimbine (YOH) on hot-plate response-latency enhancement produced by co-administration of agmatine (AGM) and WIN 55212-2 (WIN). Following determination of predrug (baseline) latency, rats were pretreated with vehicle (VEH), IDZ (2 mg/kg) or YOH (5 mg/kg). Fifteen minutes later rats were injected with a combination of AGM (50 mg/kg) plus WIN (2 mg/kg) or VEH plus WIN (2 mg/kg). Reaction latency was determined 30 min later. Data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE). $^{*}P<0.05$ compared to VEH / VEH + WIN group; $^{+}P<0.05$ compared to VEH / AGM + WIN group.

Co-treatment with agmatine and WIN 55212-2 resulted in response-latency enhancement. Agmatine caused about a 3-fold leftward shift in the WIN 55212-2 dose–response curve, thus indicating synergy for the drug interaction. The ineffectiveness of agmatine by itself indicates the synergy between WIN 55212-2 and agmatine is not based on the spontaneous action of agmatine. Rather, it is more likely that the synergy resulted from an interaction between two separate components — cannabinoid CB_1 receptor system and agmatine and its action system. In the case of cannabinoid CB_1 receptors, SR 141716A blocked the increase in response latency caused by WIN 55212-2, thus confirming a role for cannabinoid CB_1 receptor activation in the response (Ledent et al., 1999; Zimmer et al., 1999; De Vry et al., 2004; Palazzo et al., 2001; Monhemius et al., 2001). In the case of agmatine, we could not antagonize its effects in isolation because it was inactive when administered by itself. However, it is known that agmatine produces the biological effects through at least four mechanisms: imidazoline site activation; alpha$_2$-adrenoreceptor activation; NMDA receptor antagonism; and nitric oxide synthase inhibition (Loring, 1990; Piletz et al., 1995; Reis and Regunathan, 2000; Feng et al., 2005; Fairbanks et al., 2000; Gilad et al., 1996; Reis et al., 1998; Rawls et al., 2006). This evidence led us to conjecture that one, or more, of those mechanisms mediated the agmatine–cannbinoid synergy observed in our experiments. Thus, we tested the effect of the agmatine/cannabinoid agonist combinations in the presence of a mixed imidazoline site/alpha$_2$-adrenoreceptor antagonist (idazoxan) or selective alpha$_2$-adrenoreceptor antagonist (yohimbine). Experiments revealed that idazoxan, but not yohimbine, blocked response-latency enhancement in rats co-exposed to agmatine and WIN 55212-2. The positive effect of idazoxan, coupled with the ineffectiveness of yohimbine, suggests imidazoline site activation contributed to the enhancement of cannabinoid action by agmatine. Our results cannot exclude the possibility that agmatine enhanced cannabinoid action by antagonizing NMDA receptors or inhibiting nitric oxide synthase, but evidence that cannabinoid-evoked analgesia in mice is inhibited by NMDA receptor antagonism and

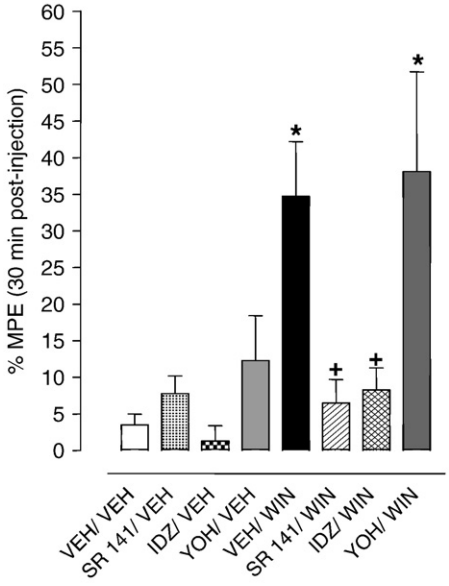

Fig. 6. Effect of pretreatment with idazoxan (IDZ), yohimbine (YOH) or SR 141716A (SR 141) on reaction latency enhancement produced by WIN 55212-2 (WIN). Following determination of predrug (baseline) latency, rats were pretreated with vehicle (VEH), IDZ (2 mg/kg), YOH (5 mg/kg) or SR 141 (5 mg/kg). Fifteen minutes later rats were injected with VEH or WIN (2 mg/kg) and reaction latency was determined 30 min later. Data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE). $^{*}P<0.05$ compared to VEH + VEH group; $^{+}P<0.05$ compared to VEH + WIN group.

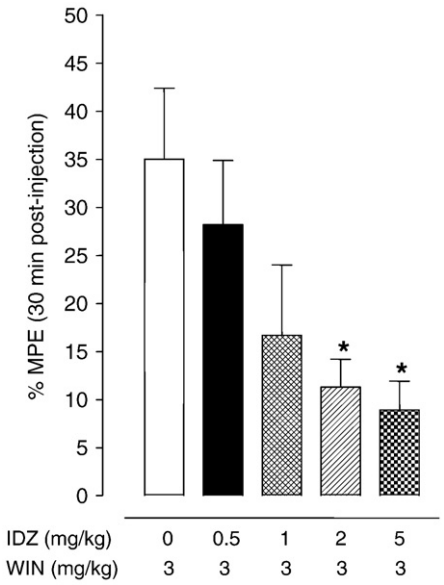

Fig. 7. Dose-related effect of idazoxan (IDZ) on reaction latency enhancement produced by WIN 55212-2 (WIN). Following determination of predrug (baseline) latency, rats were pretreated with vehicle (VEH) or IDZ (0.5, 1, 2 or 5 mg/kg). Fifteen minutes later rats were injected with WIN (2 mg/kg), and reaction latency was determined 30 min later. Data were expressed as the mean ± S.E.M. of the percentage of maximal possible effect (% MPE). $^{*}P<0.05$ compared to VEH (i.e., IDZ [0 mg/kg]) + VEH group.

unaffected by nitric oxide synthase inhibition (Spina et al., 1998; Thorat and Bhargava, 1994), effects that are both different from the enhancing effect of agmatine demonstrated here, do not support a major role for either substrate in the agmatine–cannabinoid interaction.

Co-treatment with agmatine and anandamide did not produce response-latency enhancement, although a nonsignificant trend toward an interaction was observed when agmatine was administered with the highest dose of anandamide. It is possible that a significant interaction would have been observed if agmatine had been administered with even higher doses of anandamide, but those doses were not tested here because they produce side effects such as sedation, catalepsy and hypothermia. Anandamide, compared to CP55,940 and WIN 55212-2, produced response-latency enhancement that was both less in magnitude and shorter in duration, a finding likely related to its brief duration of action following systemic administration owing to rapid inactivation by the enzyme fatty acid amide hydrolase (FAAH) (Deutsch and Chin, 1993). Thus, it is conceivable that experimental approaches in which agmatine is administered with methanandamide, a stable anandamide analogue which displays increased resistance to enzymatic hydrolysis (Abadji et al., 1994), or a combination of anandamide and a FAAH inhibitor, a strategy which extends the duration of action of anandamide by slowing its rate of catabolism, may result in significant response-latency enhancement.

Our data suggest that the cannabinoid effect depends partially on active imidazoline sites. Idazoxan attenuated the WIN 55212-2-induced response-latency enhancement whereas yohimbine was ineffective. These data reveal that imidazoline sites play a permissive role in response-latency augmentation caused by a cannabinoid agonist. The mechanism is unknown, but one possibility is that cannabinoid CB_1 receptor activation by WIN 55212-2 triggers downstream activation of imidazoline sites, with concomitant activation of both CB_1 receptors and imidazoline sites required for WIN 55212-2 to produce maximal response-latency enhancement. *In vitro* experiments using human heart tissue indicate commonalities between cannabinoid CB_1 receptors and imidazoline sites (Molderings et al., 1999). Further, radioligand binding experiments using rat brain cortex membranes reveal that imidazoline site ligands inhibit binding of the radioligand [^{3}H]SR 141716A to its specific binding sites (Molderings et al., 1997). This prior evidence that imidazoline sites and cannabinoid receptors are related with respect to their pharmacological properties supports a functional role for cannabinoid CB_1 receptor-imidazoline site crosstalk in conscious animals (Molderings et al., 1999; Rawls et al., 2007). What is unclear is the nature of the crosstalk, especially regarding whether cannabinoid CB_1 receptors alter imidazoline site activity by regulating the concentration of endogenous imidazoline ligands such as anandamide, beta-carbolines (e.g., harman and harmane), and imidazoleacetic acid-ribotide (Head and Mayorov, 2006). Future studies examining the effects of the cannabinoid system on endogenous imidazoline ligands will provide additional insight into the mechanism of CB_1-imidazoline site crosstalk. It is also unclear which imidazoline site subtype, I1, I2 or atypical I3 (non-I1/I2), mediated the effects observed in our experiments (Wu et al., 2007). I1 imidazoline receptors are located in plasma membranes and display high affinity to clonidine and moxonidine (Eglen et al., 1998). I2 imidazoline receptor shows high affinity to other imidazolines or guanidine, which is located in mitochondrial outer membranes and presents a novel recognition site on monoamine oxidase (Piletz et al., 1995; Eglen et al., 1998). Finally, I3 imidazoline sites are located in the pancreas and regulate insulin secretion (Morgan and Chan, 2001). Identifying the specific imidazoline site responsible for the enhancing effect of agmatine in our study is hampered by a lack of commercially available agents that selectively antagonize imidazoline I1 and I2 sites and mice lacking I1 and I2 sites. For example, idazoxan, which completely prevented response-latency enhancement caused by co-treatment with agmatine and WIN 55212-2, binds to both imidazoline I1 and I2 sites. When more selective imidazoline site antagonists become available, future studies will identify the specific site that agmatine activates to enhance cannabinoid potency.

The agmatine–cannabinoid interaction may depend as much on administration route as the particular pain state (i.e., nociceptive versus inflamed or nerve-injured) (Nguyen et al., 2003; Fairbanks et al., 2000). Agmatine is thought to have a central site of action and alters phenomena that are primarily centrally mediated, but its polar nature limits blood–brain barrier penetrability. Tissue levels of agmatine attained in the CNS after central administration are higher and of longer duration than levels following systemic administration of thousand-fold higher amounts (Roberts et al., 2005; Nguyen et al., 2003). The marked influence that administration route has on the pharmacological effects of agmatine is likely due to both its limited CNS penetrability and documented interactions with multiple cellular targets. An example of how the effects of agmatine vary with administration route is provided by studies conducted in pithed spontaneously hypertensive rats, where agmatine decreases blood pressure and heart rate after intravenous administration; increases blood pressure without affecting heart rate following i.c.v. injection; and increases heart rate without altering blood pressure following administration into the fourth ventricle (Sun et al., 1995; Schäfer et al., 1999; Raasch et al., 2002). Future studies will determine if the effect of agmatine on cannabinoid action varies with administration route (systemic, i.c.v., or intrathecal). Since cannabinoid agonists exert antinociceptive and anti-hyperalgesic effects at the peripheral and central (spinal and supraspinal) levels in acute and chronic pain models (Iversen and Chapman, 2002; Pertwee, 2001; Barinaga, 2001), future studies will also be directed at identifying a site of action for the agmatine–cannabinoid synergy.

In summary, exogenous agmatine produced approximately a 3- and 4.4-fold shift in the dose–response curves of WIN 55212-2 and CP55,940, respectively. These results suggest that agmatine, in addition to its well known enhancement of morphine analgesia and blockade of morphine dependence, modulates cannabinoid function *in vivo* (Kolesnikov et al., 1996; Li et al., 1999, 2003; Reis and Regunathan, 2000; Regunathan, 2006; Su et al., 2000, 2003; Rawls et al., 2006, 2007). More research and better pharmacological tools are needed to determine whether endogenous agmatine modulates cannabinoid action.

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