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Effects of novel anxiolytic 4-butyl-alpha-agarofuran on levels of monoamine neurotransmitters in rats

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Abstract

4-Butyl-α-agarofuran (AF-5) is a new compound derived from α-agarofuran, a constituent extracted from *Aquillaria agallocha* Roxb. Our previous research has shown that AF-5 has significant antianxiety activity in several animal models. In this study, an antianxiety effect was observed in a social interaction test after acute treatment with AF-5 (0.5–4.0 mg/kg, i.p.) in rats. Using high-performance liquid chromatography (HPLC)-electrochemical detection (ECD), we further investigated the effects of AF-5 on monoamine neurotransmitters both in rat brain tissues and in striatum dialysates. After acute administration of AF-5 (5.0 mg/kg, i.p.), serotonin (5-hydroxytryptamine, 5-HT) tissue levels significantly decreased by 26.3%, 30.4%, and 17.4% of the vehicle–control levels, in the striatum, cortex, and midbrain, respectively. The dopamine level decreased by 34.7% in the striatum and 19.0% in the midbrain, while in the hypothalamus, it increased to 156.6%. The epinephrine level decreased by 34.6% in the cortex. In cerebral microdialysis perfusates from rat striatum, the extracellular dopamine level declined stepwise after treatment with AF-5 (10.0 mg/kg, i.p.). By 200 min postinjection, the dopamine level reached a minimum, about 40% of the baseline value. At the same time, the extracellular levels of 5-hydroxyindolacetic acid, 3-4-dihydroxyphenylacetic acid, and homovanillic acid increased significantly, the maximum values were 150%, 145%, and 175% above baseline, respectively. This study suggests that AF-5 is a potent anxiolytic agent, and that its beneficial action may be related to its effects on central monoamine neurotransmitters.

Keywords: 4-Butyl-α-agarofuran (AF-5); Anxiety; Monoamine neurotransmitter; Behavior; Tissue concentration; Microdialysis

1. Introduction

Anxiety disorders are inappropriate or pathological anxiety aroused by various stressors, such as physiological and environmental changes (Wang et al., 1990). Nowadays, with increasing competition, anxiety disorders have already become one of the most widespread psychiatric diseases, causing considerable distress to individuals, families, and society. Their incidence in Chinese adults is 3%, while in Americans it is 7.1% (Jiang et al., 1995; Boulenger and Lavallee, 1993).

4-Butyl-α-agarofuran (AF-5; $C_{18}H_{30}O$, MW=262.4; Fig. 1), one of the α-agarofuran derivatives from the Chinese traditional medicine Gharu-wood (*Aquillaria agallocha* Roxb.), is a completely new compound synthesized by Guo et al. (2002) in our institute. In preclinical pharmacological research, AF-5 has shown specific and significant antianxiety activity in several animal models, with a higher potency and a lower toxicity than diazepam and buspirone (Li et al., 2001; Guo et al., 2002; Liu et al., 2003). However, its mechanisms of action have not been elucidated yet.

The etiology and pathogenesis of anxiety disorders are complicated and still unclear. Currently, many reports suggest that the occurrence of anxiety is associated with

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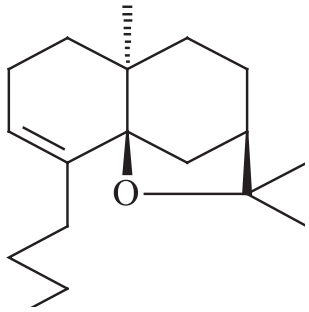

Fig. 1. Chemical structure of AF-5.

the dysfunction of central monoamine neurotransmitters. Modulation of these neurochemicals forms the basis of the actions of anxiolytic drugs. Furthermore, serotonin (5-hydroxytryptamine, 5-HT) and dopamine are crucial neurotransmitters in the central nerve systems (CNS). Their major metabolites in the brain are 5-hydroxyindolacetic acid, 3-4-dihydroxyphenylacetic acid, and homovanillic acid. Therefore, an effect of AF-5 on central monoamine neurotransmitters might underlie its anti-anxiety mechanisms.

In this study, we first tested the anxiolytic effect of acute administration of AF-5 in a social interaction test in rats, and then, using high-performance liquid chromatography (HPLC)-electrochemical detection (ECD), we investigated the effects of AF-5 on monoamine levels in brain homogenates and cerebral microdialysis perfusates. The aim is to determine the neurochemical mechanisms of the antianxiety activity of AF-5.

2. Materials and methods

2.1. Animals and drugs

Male Wistar rats, weighing 230–270 g, were used in this study. The rats were individually housed with food and water available ad libitum. The experiments were performed in accordance with the guidelines established by the European Community for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences.

AF-5 was provided by the Department of Synthesis of our institute with a purity of 99.8%. It was dissolved in 0.9% saline after being dispersed with Tween-80. Drugs and saline, which served as vehicle–control, were administered to rats in a volume of 2 ml/kg body weight. Epinephrine, dopamine, 5-HT, 3-4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindolacetic acid, and isoproterenol (IP) were purchased from Sigma (USA). These chemicals were dissolved in double-distilled water to be used as a mixed standard and were frozen at −70 °C immediately.

2.2. Social interaction test

The social interaction test apparatus was a square wooden box (60×60×40 cm) lit with a strong light (300 lux). A miniature video camera was placed vertically above the box, and rats were observed on a monitor in an adjacent room. The experimenter remained outside the test room during the test, and the test arena was wiped down with 10% ethanol between each test session.

On the experimental day, under high-intensity light conditions, rats were allocated to pairs; each pair was from the same treatment group (AF-5 or vehicle) and did not differ in weight by more than 10 g. Ten minutes after drug administration (i.p.), rats were placed in the opposite corners of the box facing the walls, and the social interaction test began. Each social interaction test lasted for 9 min. The duration of the social interaction (including sniffing, following, mutual grooming, boxing, and wrestling), which provided the measure of anxiety, was scored by an observer blind to the drug treatment. (File, 1980; Tucci et al., 2003; File and Seth, 2003).

2.3. Chemical assays

Epinephrine, dopamine, 5-HT, and their metabolites were determined by HPLC (Shimadzu, LC-6A, Japan) with ECD (BAS Amperometric Detector, CC-4, USA) systems. A reverse-phase column (Dikma, diamond, C-18 ODS, 250×4 mm, USA) was used for separation. The working electrode potential of the detector was set at 760 mV relative to an Ag–AgCl reference electrode. The composition of the mobile phase was 0.1 M acetate-citrate buffer at pH 3.7, containing 15% methanol, 1.09 mM octyl sodium sulphate acid, 0.4 mM dibutylamine, and 0.2 mM EDTA. The flow rate was 1.2 ml/min.

2.4. Effects of AF-5 on levels of monoamines and their metabolites in tissue homogenate of several brain regions of rat

Rats were divided into two groups randomly: vehicle group and AF-5 group. Each animal was acutely administered AF-5 (5.0 mg/kg, i.p.) or saline, and was decapitated 30 min later. Brains were quickly removed and frozen. Different regions were separated (striatum, cortex, hippocampus, midbrain, hypothalamus, dorsal thalamus) and weighed, then immediately put into an appropriate volume of 0.4 M perchloric acid solution, which included a constant concentration of IP as internal standard. After homogenization and centrifugation (4 °C, 15 000×g, 10 min), supernatants were collected into tubes containing 1/2 volume of K^+ solution (20 mM potassium citrate, 300 mM K_2HPO_4, 2 mM EDTA), then incubated at 0 °C for 10 min. After centrifugation (4 °C, 15 000×g, 10 min), 20 μl of tissue homogenate supernatant was injected directly into the HPLC system; the mixed standard was used as reference (Zhang et al., 1987).

2.5. Effects of AF-5 on the extracellular levels of monoamines and their metabolites in the striatum of anesthetized rats

2.5.1. Surgery and microdialysis procedure

The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and were placed in a stereotaxic frame. The skull was exposed, and a burr hole was drilled to accommodate the probe. A straight-type dialysis probe (CMA/10, 4 mm long, 0.5 mm o.d., CMA, Sweden) was implanted into the caudate putamen of the striatum with the following coordinates: AP 0.4 mm, LR 3.0 mm, DV 7.0 mm from the bregma (Paxinos and Watson, 1986). The animal was kept under anesthesia by intraperitoneal injection of sodium pentobarbital (40 mg/kg) every 90 min throughout the experiment. The probe was connected to a microinfusion pump (SAGE syringe pump model 352) and perfused at a constant flow rate of 1.5 μl/min with artificial cerebrospinal fluid (ACSF) solution (146 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.9 mM Na_2HPO_4, 0.1 mM NaH_2PO_4, pH 7.4). After a 1-h equilibration period, 40-min dialysate samples were consecutively collected. Two basal samples, the mean of which was taken as basal extracellular level (baseline value), were collected before drug administration. AF-5 was given to rats at 10.0 mg/kg (i.p.), and the vehicle-treated rats were used as controls. The perfusate from the striatum (caudate putamen) was injected directly into the HPLC-ECD system and immediately analyzed for dopamine, 5-HT, 3-4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid.

2.5.2. Histology

After the microdialysis experiments, the brains were removed, and the probe implantation sites were verified histologically. Only experiments in which the probes were found to be correctly located were included.

2.5.3. In vitro recovery experiments

To examine the recovery of monoamines through the dialysis probe, in vitro recovery experiments were performed. The dialysis probe was immersed in ACSF solution containing certain concentrations of dopamine, 5-HT, 3-4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindolacetic acid before the in vivo microdialysis procedure. The probe was perfused at a rate of 1.5 μl/min at room temperature, and the samples were collected at 40-min intervals. The concentrations were measured with the HPLC-ECD system. The recovery of dopamine, 5-HT, 3-4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid was 17.1%, 24.2%, 36.1%, 34.7%, and 42.6%, respectively.

2.6. Statistical analysis

Statistical analysis of data from the social interaction test was achieved by using one-way analysis of variance (ANOVA), followed by comparison of the AF-5 groups and the vehicle group with Fisher's least-square difference (LSD) test.

Differences between the AF-5 group and the vehicle group regarding monoamine levels in each individual brain region were assessed by means of Student's *t*-test.

For the microdialysis experiment, the average concentration of each monoamine during the period preceding drug treatment was used as the baseline value, and individual data are expressed as a percentage of this value. The vehicle-treated rats were used as control. Data for the time courses were analyzed by two-way ANOVA, with treatment as a "between groups" variable and time as a "within groups" variable.

The significance levels for all statistical tests were set at $P<0.05$.

3. Results

3.1. Social interaction test

Data for social interaction are shown in Fig. 2. During the whole test session, systemic administration of AF-5 significantly prolonged the total time spent in social interaction ($F[4,45]=126.8$, $P<0.001$). This effect was dose-dependent (0.5–2.0 mg/kg). At dose of 2.0 mg/kg, AF-5 exerted its maximum effect ($P<0.01$ vs. vehicle), and at a higher dose (4.0 mg/kg) its action decreased slightly.

3.2. Effects of AF-5 on levels of monoamines and their metabolites in tissue homogenates of several brain regions

After acute administration of AF-5, 5-HT levels were significantly lower than control values in several brain

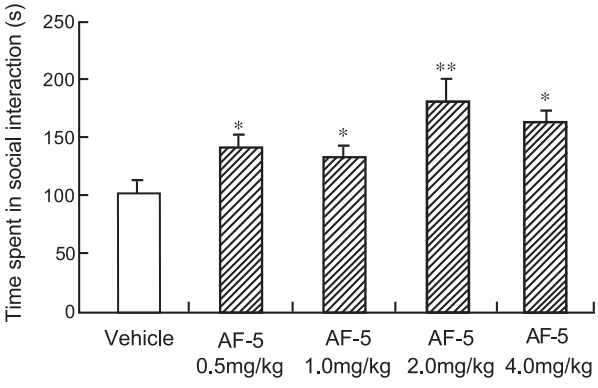

Fig. 2. Time spent in social interaction test of rats tested under high-intensity light and unfamiliar test conditions after acute administration of vehicle or AF-5 (0.5, 1.0, 2.0, 4.0 mg/kg, i.p.). The test lasted 9 min. Data are means±S.E.M. of interaction time (n=10). The values are compared with the value of the vehicle-treated group. *$P<0.05$, **$P<0.01$ vs. vehicle.

regions (Fig. 3A), especially in rat striatum, cortex, and midbrain, where levels were decreased by 26.3%, 30.4%, and 17.4% of control level ($P<0.05$, vs. vehicle), respectively. The dopamine levels (Fig. 3B) decreased by 34.7% and 19.0% in rat striatum and midbrain ($P<0.05$ vs. vehicle), but increased to 156.6% in rat hypothalamus ($P<0.01$ vs. vehicle). AF-5 at this dose had no notable effect on the concentrations of the major metabolites (3-4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid). We also observed that the cortical level of epinephrine was reduced by 34.6% ($P<0.05$ vs. vehicle), as shown in Fig. 3C.

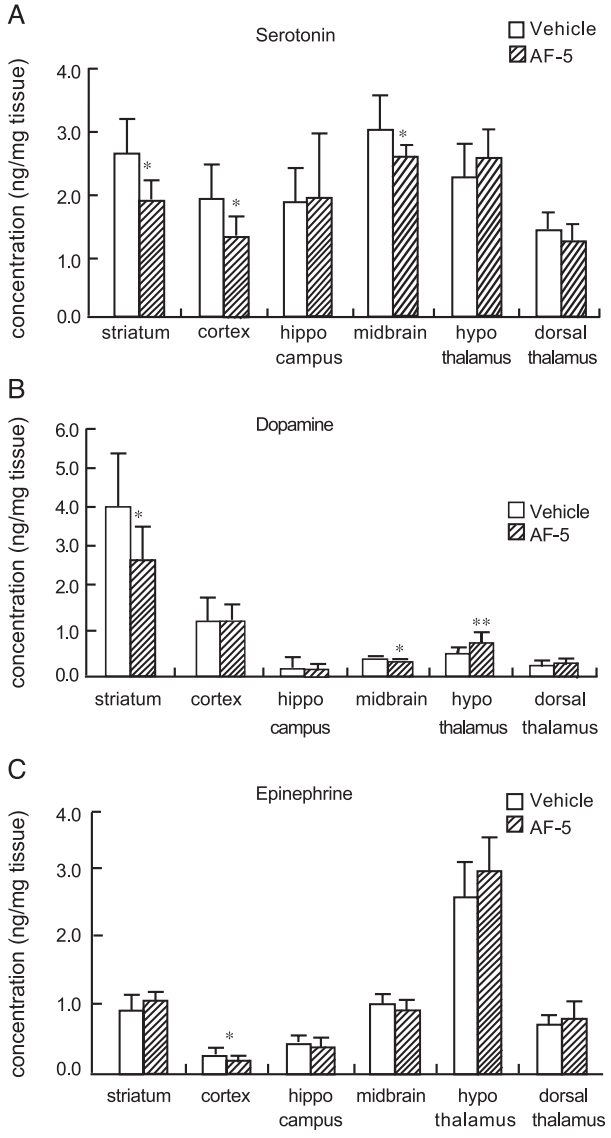

Fig. 3. Effect of AF-5 (5.0 mg/kg, i.p.) on concentrations of monoamines in several brain regions of rats. Data are means±S.E.M. of tissue concentrations of 5-HT (A), dopamine (B), and epinephrine (C) ($n=7$). The values are compared with the corresponding values of the vehicle-treated group. *$P<0.05$, **$P<0.01$ vs. vehicle.

3.3. Effect of AF-5 on the extracellular levels of monoamines and their metabolites in striatum of anesthetized rats

The effect of acute administration of AF-5 on extracellular monoamine levels in rat striatum is shown in Fig. 4. Vehicle-treated rats were used as controls, and all monoamines levels in the control dialysates differed little during the entire perfusion period. After AF-5 administration, the extracellular dopamine level (Fig. 4A) in the striatum decreased stepwise, beginning immediately after injection, and during the first 40 min postinjection, the dopamine level decreased by 25% of the baseline value ($P<0.05$ vs. vehicle). By 200 min postinjection, the dopamine level reached a minimum, about 40% of the baseline value ($P<0.01$ vs. vehicle). Two-way ANOVA of data, obtained after injection of AF-5, showed a significant effect of treatment ($F[1,6]=10.6$, $P=0.02$), but no effect of time ($F[5,18]=0.79$, $P=0.57$) or interaction between treatment and time ($F[5,18]=0.17$, $P=0.97$).

The extracellular levels of 3-4-dihydroxyphenylacetic acid (Fig. 4B), homovanillic acid (Fig. 4C), and 5-hydroxyindolacetic acid (Fig. 4D) increased after injection of AF-5, the maximum values were 145%, 175%, and 150% above baseline, respectively. The data were also examined by two-way ANOVA and showed a significant effect of treatment on levels of the three metabolites (3-4-dihydroxyphenylacetic acid, $F[1,6]=11.2$, $P=0.02$; homovanillic acid, $F[1,6]=25.1$, $P<0.001$; 5-hydroxyindolacetic acid, $F[1,6]=48.8$, $P<0.001$), but a different effect of time (3-4-dihydroxyphenylacetic acid, $F[5,18]=0.4$, $P=0.84$; homovanillic acid, $F[5,18]=0.96$, $P=0.46$; 5-hydroxyindolacetic acid, $F[5,18]=4.08$, $P=0.005$). (The 5-HT concentration in dialysates was too low to be detected.)

4. Discussion

Anxiety disorders, which have a complicated underlying process, present in a number of forms. Pharmacotherapy remains the most widespread and efficacious treatment, and the development of new antianxiety drugs is a very active field.

Previous studies have shown that AF-5 has definite anxiolytic effects, assessed in the elevated plus-maze test, open field test, light/dark test, tail-suspension test, and operant conditioned response test (Li et al., 2001; Guo et al., 2002; Liu et al., 2003). Our present study confirmed the above results. Pretreatment with AF-5 significantly decreased the time spent in social interaction at doses from 0.5 to 4.0 mg/kg, without causing a concomitant marked reduction in locomotor activity in rats.

In the social interaction test, we chose the test conditions of high-intensity light combined with an unfamiliar arena, thereby generating the highest level of anxiety in the test rats (File and Seth, 2003). The antianxiety effect of acute administration of AF-5 in this model was dose-dependent

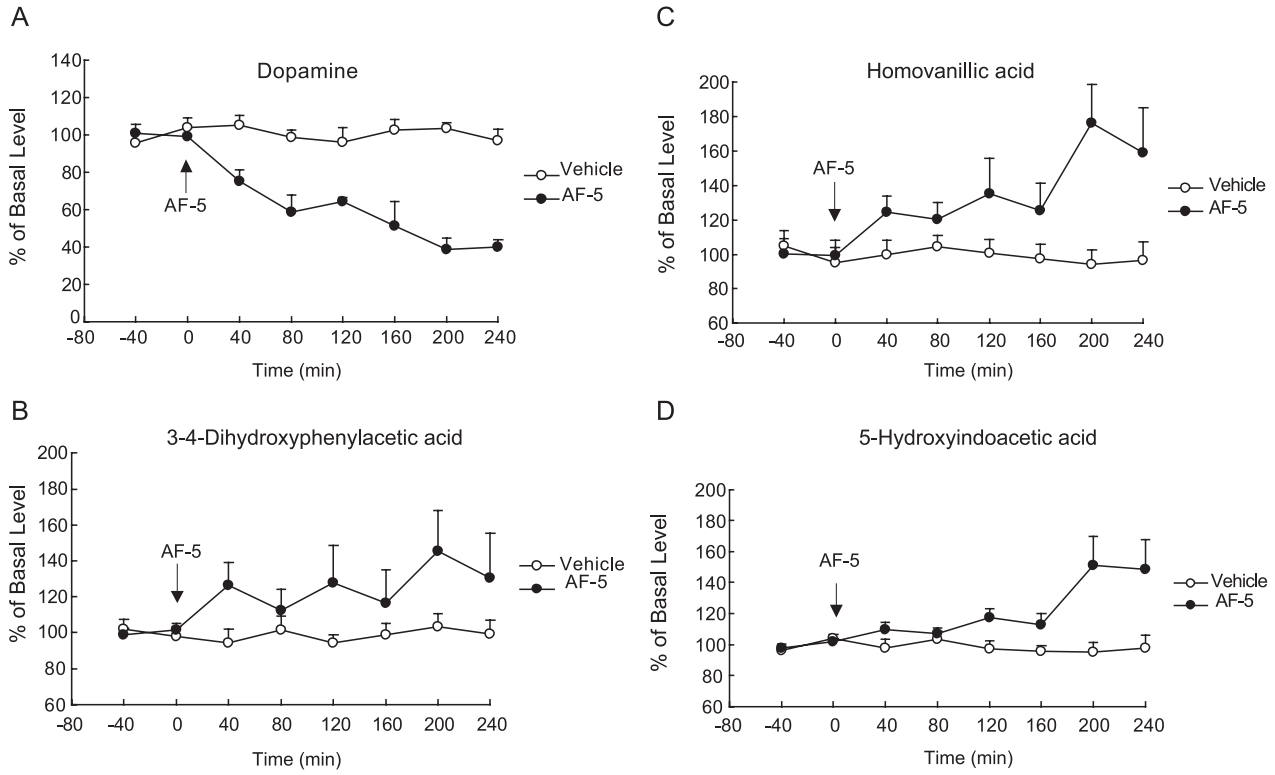

Fig. 4. Effect of AF-5 on the extracellular levels of dopamine (A), 3-4-dihydroxyphenylacetic acid (B), homovanillic acid (C), and 5-hydroxyindolacetic acid (D) in striatum of anesthetized rats (n=4). Rats were acutely treated with vehicle or AF-5 (10.0 mg/kg, i.p.) at time zero. Data are means±S.E.M. of dopamine, 3-4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid levels, expressed as a percentage of the baseline value (100%). The values are compared with the corresponding value of the vehicle-treated group.

from 0.5 to 2.0 mg/kg, and at dose of 2.0 mg/kg the efficacy was maximum. At a higher dose (4.0 mg/kg), its effect weakened slightly, because the sedative action of AF-5 became relatively stronger, although the sedative action was much lower than that of diazepam (Guo et al., 2002).

Clinical and animal studies have provided evidence to support the involvement of central neurochemical systems, such as neurotransmitters, neuromodulators, and neural endocrine, in anxiety disorders (Revelli et al., 1998; Van de Kar and Blair, 1999). Especially, changes in monoamine transmitters have been further studied and remain the focus of attention.

In the current theory of anxiety, the central dopaminergic system is considered the crucial factor. Dazzi et al. (2001) reported that foot shock or administration of an anxiogenic drug both produced a marked increase in cortical dopamine output in normal rats, and chronic treatment with imipramine completely inhibited these changes. Our current results are consistent with this report. Treatment with AF-5 significantly reduced tissue concentrations of dopamine in homogenates of striatum, midbrain, and cortex. To clarify the changes in the dopaminergic system, we studied the extracellular dopamine level in the striatum. Using microdialysis, we found that the extracellular dopamine level declined significantly after AF-5 was given. At the same time, the levels of 3-4-dihydroxyphenylacetic acid and homovanillic acid, the major metabolites of dopamine, increased significantly. Therefore, we can speculate that the mechanism involved in our results may be associated with a facilitating effect of AF-5 on the extracellular metabolism of dopamine.

Changes in the serotonergic system were also observed in our study. 5-HT is found throughout the CNS in high levels and participates in modulating mood. According to the classic serotonin hypothesis, anxiety is usually associated with increased endogenous 5-HT, and anxiolytics tend to decrease endogenous 5-HT (Rex et al., 1993; Rex et al., 1994; Clement and Chapouthier, 1998). Some scientists proposed that an ascending 5-HT neural pathway, originating from the dorsal raphe nucleus and innervating the frontal cortex and amygdala, could facilitate the generation of anxiety by releasing 5-HT (Deakin and Graeff, 1991). There is considerable unambiguous evidence to support this theory. Administration of a 5-HT-releasing agent (fenfluramine) or 5-HT agonist (*meta*-chlorophenyl-piperazine) was shown to induce anxiogenic actions in patients with panic disorder, as well as in animal models (Charney et al., 1987; Targum, 1990; Tao et al., 2002). The selective 5-HT_{1A} receptor agonist (buspirone) showed anxiolytic effects by markedly decreasing the concentration of 5-HT (Matos et al., 1996). Our present studies are consistent with these reports. After administration of AF-5, the levels of 5-HT in

homogenates of several brain regions (striatum, cortex, and midbrain) decreased significantly. In the striatum microdialysis experiment, although the extracellular 5-HT concentration in dialysates was too low to be detected, the extracellular level of 5-hydroxyindolacetic acid increased just like the levels of dopamine metabolites, thus indicating that the 5-HT level might decrease concomitantly with an increase in its metabolism.

The theory concerning the role of the central epinephrine system in anxiety is that increased epinephrine output can lead to anxiety via what might be called excessive or dysfunctional arousal. Inhibition of such changes can attenuate anxiety (Taraka et al., 2000). Similarly in our study, an inhibitory effect on cortical epinephrine level was observed after administration of AF-5. It might be another pathway by which AF-5 acts in anxiety disorders.

In summary, our behavioral studies confirmed the potent anxiolytic effect of AF-5 in the social interaction test in rats. Our neurochemical studies suggested that this effect was mediated by modulation of the levels of monoamine neurotransmitters, mostly by decreasing the levels of 5-HT, dopamine, and epinephrine in the central nervous system.

Acknowledgments

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