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Interaction Between L-Type Calcium Channels and Antagonist of Cannabinoid System on Anxiety in Male Rat

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Background: The elevated plus-maze (EPM) has been broadly used to investigate anxiolytic and anxiogenic compounds. There is little information about the effect of interaction between calcium channels and cannabinoid system on the phenomenon of anxiety.
Objectives: This study aimed to examine the effects of acute and chronic coadministration of AM251, as cannabinoid CB1 receptor antagonist, and Verapamil, as L-type Ca^{2+} channels blocker, on EPM test in rats.
Materials and Methods: The data were obtained from male Wistar rat, weighing 220 to 260 g. Animals were allocated to five groups: Control, Verapamil, AM251, acute Verapamil + AM251, and chronic (injection for 8 days) Verapamil + AM251 groups. The percentage of entries into the open arms of the EPM, the time spent in the open arms, and the number of entries into the closed arms during ten minutes was recorded.
Results: Intraperitoneally (IP) injection of AM251 before EPM trial decreased open arms exploration and open arm entry. On the other hand, Verapamil increased open arms exploration and open arm entry. Combined injection of Verapamil and AM251 had conflicting effects on the responses of each of these two compounds alone. AM251 and Verapamil had no effects on the number of closed arm entries.
Conclusions: IP injection of CB1 receptor antagonist might have an anxiogenic profile in rat, whereas calcium channel blocker attenuated the anxiogenic effect of AM251. Our results suggest that there is an interaction between functions of L-type Ca^{2+} channels and cannabinoid system in anxiety.

*Keywords:*Verapamil; L-Type Calcium Channels; AM251; Rat; Anxiety

1. Background

Anxiety is among the most common psychologic disorders with high worldwide prevalence (1). Pharmacologic studies, clinical investigations, and in recent years, analyses of genetically-modified mice have implicated a remarkable diversity of mechanisms in the etiology, modulation, and treatment of anxiety (2). The neurobiological underpinnings of anxiety disorder has been studied in both animal and human models, and it is widely accepted that dysregulation of brain regions and structures are associated with anxiety (3). A variety of neurotransmitter mechanisms such as GABAergic, serotonergic, noradrenergic, and endocannabinoid systems contribute to the regulation of anxiety behavior (4). Cannabinoid system is affected with cannabinoid drugs derived from *Cannabis sativa* and exogenesis cannabinoid agents (5). The psychoactive constituents are hashish, 9-tetrahydrocannabinol (THC), cannabidiol, and marijuana (6). Several levels of evidence suggest that the endocannabinoid system plays a role in the regulation of mood or anxiety (7). Therefore, the cannabinoid system can be seen as one of the key regulatory elements of anxiety behavior (8). Cannabinoids are produced throughout the brain and cannabinoid CB1 receptors are particularly well-represented in the cortex (entorhinal and cingulate), hippocampus, lateral septum, nucleus accumbens, amygdala, and peri-aqueductal gray area (PAG) (2). Cannabinoid receptor agonists/antagonists have been shown to exert anxiolytic effects in some studies (9) but anxiogenic effects in others (10-13). Furthermore, CB1 receptor agonists are reported to induce biphasic effects, with lower doses being anxiolytic and higher doses being anxiogenic (14). Through using CB1 receptor knockout mice, several studies have reported anxiogenic responses in classical anxiety paradigms such as elevated plus-maze (8).

Calcium ion is the most common signal transduction element in neurons and its entry is tightly regulated by two major classes of voltage-gated calcium channels (VGCCs): the high-voltage activated (HVA) (L, P/Q, and N-type) and the low-voltage activated (LVA) (T-type) calcium channels. It has been suggested that calcium channel affects anxiety-related behaviors (15). Furthermore, it has

been shown that nimodipine, flunarizine, and Verapamil, which are L-type VDCC antagonists, blocked nicotine-induced and amphetamine-induced withdrawal signs, including increased anxiety and depression-like state, after seven or 14 days of spontaneous cessation of drug administration in mice at the doses that did not have any effects in naive mice in those behavioral paradigms by themselves (16). Verapamil hydrochloride is a calcium channel blocker (CCB) of the phenylalkylamine group that binds with high affinity to the α1-subunit of the L-type calcium channel complex (17).

There is a variety of animal tests for the investigation of anxiolytic or anxiogenic effects of substances (18, 19). Behavior in the elevated plus-maze (EPM) is a model of anxiety for rodents and it might serve as a new basis for developing anxiolytic agents and investigating psychologic and neurochemical factors of anxiety (20-22). Rats were allowed to explore two elevated open and two elevated closed arms of the EPM apparatus that has been confirmed to be applicable to rats and mice. According to Barrett, an anxiolytic effect is suggested when the drug increases open arms entries without altering the total number of arm entries (19-21, 23). An increase of the time and the proportion of the entrances into the open arms without a changed locomotor activity is regarded as a powerful marker for an anxiolytic substance effect (22). Locomotor activity of the animals was assessed by measuring the number of entries into closed arms and total distance travelled by the animal (24-27).

2. Objectives

Despite available data about the effect of cannabinoid system and calcium channels role on anxiety phenomenon, the interaction of these two systems on anxiety have not been studied. In addition, it is not clear whether the effect of cannabinoid system on anxiety in part is a result of its effect on L-type calcium channels. Therefore, in this study the effects of cannabinoid antagonist compound (AM251) and L-type CCB (Verapamil), either alone or in combination, on anxiety were investigated using acute and chronic models of administration in rats.

3. Materials and Methods

3.1. Animals

Male Wistar rats weighting 220 to 260 g were purchased from Pasteur Institute (Tehran, Iran). They were kept at 20 ± 2℃ in a 12-hour light/12-hour dark cycle with food and water supply ad libitum. Animals were acclimated to laboratory conditions for one week before the experiments. Each rat was used only once. All research and animal care procedures were approved by the Veterinary Ethics Committee of the Hamadan University of Medical Sciences and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985). A total of 50 male rats were allocated to five groups of ten as follows: 1) Control, 2) Verapamil, 3) AM251, 4) Acute Verapamil + AM251, 5) Chronic Verapamil + AM251.

3.2. Drugs

AM251 (1 mg/kg; Sigma, USA) as CB1 receptor antagonist Verapamil (25 mg/kg; Sigma, Germany) as CCB were used. Physiologic saline (0.9% sodium chloride) and dimethylsulfoxide (DMSO) (Sigma, USA) were used as the vehicle (control group). All drugs were prepared freshly and administered intraperitoneally (IP) in a volume of 0.1 mL per 10 g of body weight. In acute groups, all substances were administrated 30 minutes before EPM test.

3.3. Elevated Plus-Maze Test

Anxiolytic activity of substances was measured using the EPM test. This test has been widely validated to measure anxiety in rodents (20-23). Briefly, for rats, the apparatus consisted of two open arms (50 × 10 × 1 cm each), two enclosed arms (50 × 10 × 50 cm each), and a central platform (10 × 10 cm), arranged in such a way that the two arms of each type were opposite to each other. The maze was elevated 50 cm above the floor. Rats were placed in the center of the maze facing the open arms. The Rats explored the maze and their behaviors were monitored by digital camera above the maze for ten minutes. After each test, the apparatus was cleaned with 10% ethanol to eliminate any remaining odors. The time spent in the open arms, the number of entries into the open arms, and percentage of entries into the open arms were calculated (21, 22). In acute groups, animals were tested 30 minutes after IP injection of AM251, Verapamil, or combination of both substances. In chronic groups, animals were tested following eight days of treatment with assigned substances.

3.4. Statistical Analysis

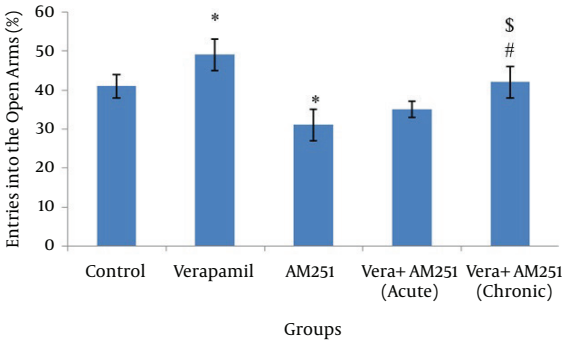
All the results were presented in terms of mean ± standard error of means (SEM). The data were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Differences were considered significant at $P < 0.05$.

4. Results

4.1. Effects on the Percentage of Entries in Open Arms

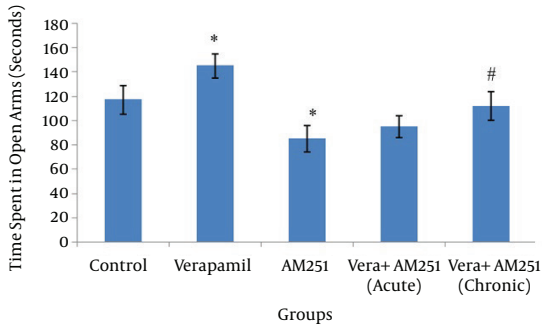
The effects of AM251, Verapamil, and AM251+ Verapamil on the percentage of entries in open arms are shown in Figure 1. One-way ANOVA showed that there was a significant difference between experimental groups in percentage of entries into open arms. Tukey's post-hoc test revealed significant reduction in percentage of

Figure 1. The Effects of AM251, Verapamil, Acute AM251 + Verapamil, and Chronic AM251 + Verapamil on the Percentage of Entries into Open Arms During the Ten-Minute Test Session



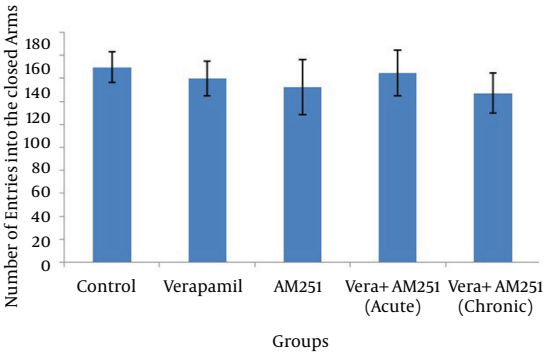
Data represent means ± SEM; *, significant difference in compare with control (P < 0.05); #, significant difference in compare with AM251 (P < 0.05); and $, significant difference in compare with acute AM251 + Verapamil (P < 0.05).

Figure 2. The Effects of AM251, Verapamil, Acute AM251+ Verapamil, and Chronic AM251+ Verapamil Administration on the Time Spent in Open Arms During the Ten-Minutes Test Session



Data represent mean ± SEM; *, significant difference in comparison with control (P < 0.05); and #, significant difference in compare with AM251 (P < 0.05).

Figure 3. The Effects of AM251, Verapamil, Acute AM251+ Verapamil, and Chronic AM251+ Verapamil Administration on the Number of Closed Arm Entries During the Ten-Minutes test Session



Data represent mean ± SEM.

entries into the open arms after administration of AM251 (P < 0.05) whereas Verapamil treated groups showed a significant increases in percentage of entries into open arms (P < 0.05) in comparison with control group. Considering the involvement of both cannabinoid system and calcium channels in anxiety and the overlap of the cannabinoid system and calcium channels in the central nervous system, we investigated the effects of AM251 and Verapamil coadministration on animal's behavior in EPM. Our results indicated that chronic coadministration of AM251 + Verapamil had different effects on the percentage of entries, which was higher than AM251 and AM251+ Verapamil acute groups (P < 0.05 for both).

4.2. Effects on the Time Spent in Open Arms

Rats in AM251 group showed significant decrease in the time spent in open arms in comparison with control group (P < 0.05). Verapamil administration significantly enhanced the time spent in open arms in comparison to the control groups (P < 0.05). Chronic coadministration of AM251 + Verapamil had different effects on the time spent in open arms, which was higher than that in AM251 group (P < 0.05) (Figure 2).

4.3. Effects on the Closed Arms Entry

The number of entrance into closed arms did not exhibit significant changes by administration of AM251, Verapamil, and AM251+ Verapamil in comparison to control group(P > 0.05) (Figure 3).

5. Discussion

The results showed that although administration of Verapamil attenuated anxiety behavior in rats, treatment with AM251 led to anxiogenic behavior. L-type CCB and cannabinoid receptor antagonist did not have any effect on the locomotion of rats in EPM.

Our result was similar to previous investigations showing that blockade of the endogenous cannabinoid by CB1 antagonist could induce anxiety-like responses in rats (28). In that regard, it has been reported that cannabinoids have anxiolytic properties in various rodent model (12, 29-33). Likewise, systemic activation of CB1 receptors produced anxiolytic effects in EPM (34, 35). However, no effect by CB1 receptors has been reported in the light-dark box, fear conditioning, and EPM (31, 36-38). The anxiogenic effects of CB1 receptors have been reported in both systemic and intra-hippocampal in plus-maze and hole board testes (39, 40). Some of the cannabinoid receptor agonists produce anxiolytic effects in the plus maze at low doses (35) and produce an anxiogenic profile in higher doses (41). Furthermore, it has been shown that THC and other CB1 receptor agonists exert a bidirectional influence on anxiety responses according to the administrated dosage (42-47). The biphasic effects of cannabinoids on anxiety-

related responses have been extensively documented in rodents. In agreement with human evidence, preclinical studies have elucidated that the acute administration of low doses CB1 receptor agonists elicits anxiolytic-like effects in avoidance tasks (12, 35, 48). Conversely, high concentrations of the same compounds are generally associated with the opposite outcomes (31, 49, 50). In human studies, it has been reported that consumption of modest amounts of cannabis and CB1 receptor agonists would result in euphoria, relaxation, heightened perception, sociability, and creativity while moderate to high doses have been reported to elicit phobia, agitation, panic, dysphoria, and cognitive impairments (42-47). In line with these premises, early studies showed a robust anxiolytic effect of low-dose nabilone in comparison with placebo (51, 52). Additionally, the few available reports on the clinical outcomes of recreational cannabinoids showed that a moderate consumption of these substances was generally associated with euphoria and disinhibition (53), but the abuse of these substances was associated with high levels of anxiety and mood disturbances (54-56). Now, it knows that cannabinoids exert their actions via CB1 receptors in the central nervous system (57, 58). These receptors are localized in brain regions, i.e. prefrontal cortex, nucleus accumbens, amygdala, and hippocampus, which are involved in emotion and anxiety behavior (59, 60). Compounds such as cannabidiol and synthetic CB1 receptor agonists produce anxiolytic behavior via activation cannabinoid receptors (5, 61). Anxiety is increased by genetic and pharmacologic inhibition of the CB1 receptor (12, 29, 35, 62). Martin et al. showed that CB1 knockout mice were more anxious than wild types were in EPM (63). Nevertheless, contradictory results have been reported under different experiment conditions (41, 64, 65). It is hypothesis that test conditions, differences in agonists, various doses, and the treatment are responsible for contentious effects of cannabinoid compounds. For example, various doses (31, 39), test conditions (31, 36, 38-40), and kind of knocked-out mice (33, 63) are important factors in the observed behavioral effects in the experiment.

Calcium is an important signaling molecule in neurons and as such, neuronal free [Ca^{2+}] is highly regulated. Brief, controlled elevations in cytoplasmic Ca^{2+} levels occur during physiologic processes such as neurotransmitter release (66-69). More importantly, elevated levels of intracellular Ca^{2+} are thought to activate numerous Ca^{2+}-dependent processes that lead to cell death and blockage of Ca^{2+} channels might play a key role in preventing these events (70). Several evidence showed that L-type Ca^{2+} channels modulate several neuronal processes. It has been shown that blockade of L-type Ca^{2+} channels could affect the actions of endogenous or exogenous cannabinoid compounds in acute and chronic models of seizure which were performed by pentylenetetrazole and electrical stimulation of amygdala, respectively (71). The Ca^{2+} influx is a necessary step in both neurotransmitter release and synthesis of endocannabinoids (72). In contrast, Ca^{2+} influx inhibition leads to reduction in endocannabinoid synthesis (71). Secretion of neuromodulators has been reported to be dependent to L-type voltage-dependent calcium channels (78-80). On the other hand, it has been demonstrated that the L-type Ca^{2+} channels exist at presynaptic terminals of central synapses and are activated by membrane depolarization while the Ca^{2+} influx through L-type Ca^{2+} channels does not directly trigger transmitter release as effectively as N-type and P/Q-type Ca^{2+} channels (73-76). On contrary, other studies have shown that the L-type Ca^{2+} channels selectively contribute to presynaptic facilitation and potentiation (77-79). Moreover, it has been reported that the synthetic cannabinoid receptor agonist, HU210, inhibited the capsaicin-induced influx of Ca^{2+}. The inhibitory effects of HU210, in general, are consistent with several reports of cannabinoid inhibition of capsaicin-evoked responses (80). Moreover, an increase of intracellular Ca^{2+} concentrations, which might activate the Ca^{2+}-dependent N-acyltransferase (NAT) (81). NAT controls the rate-limiting step in anandamide synthesis (82). In amygdala kindling, which was considered as a chronic model of seizure, the cannabinoid receptor agonist WIN55, 212-2 showed protective effects based on measured seizure parameters. Moreover, while Verapamil administration did not change seizure parameters in this model of temporal lobe epilepsy, co-administration of Verapamil and WIN55, 212-2 attenuated the protective effect of WIN55, 212-2 against amygdala-kindled seizures in rat (71). It was shown to have no major side effects although there are controversial reports on mnemonic effect of chronic and acute administration of Verapamil (83). Coadministration of either Verapamil or diltiazem with URB597 significantly attenuated the antiseizure effect of the cannabinoid compound. In addition, coadministration of Verapamil and arachidonyl-2-chloroethylamide (ACEA) diminished the protective effect of ACEA in pentylenetetrazole-induced seizure (53).

In conclusion, Verapamil and AM251 produced anxiolytic-like and anxiogenic-like effects, respectively. It has been shown that the calcium channel modulate cannabinoid output that causes changes in anxiety level; nonetheless, in contemporary studies of the consumption of two substances, CCB could change the production of endocannabinoids, which in turn results in the enhancement of anxiolytic effect. This finding suggests that the potentiation of cannabinoid system might be considered as a beneficial strategy for the treatment of anxiety. Future investigations are essential for better understanding the interactive effects and neurobiological mechanisms of action of endocannabinoid system and calcium channel on properties of anxiety.

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