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Anxiolytic-like effects of group III mGlu receptor ligands in the hippocampus involve $GABA_A$ signaling

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Abstract:
Recent literature data and the results of our earlier pharmacological studies, have provided evidence that antagonists of group I metabotropic glutamate receptors (mGluRs) and agonists of group II mGluRs show anxiolytic-like properties in preclinical and clinical studies. Out of all glutamate receptors, the role of group III mGluRs in anxiety-like states is the least investigated because of the lack of specific pharmacological tools, moreover all group III receptor ligands synthesized so far are not systemically active, so they have to be administered centrally. In the present study, we investigated the anxiolytic-like activity of group III mGlu receptor ligands including a nonselective group III mGlu receptor agonist (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I), group III mGlu receptor antagonist, (*RS*)- -cyclopropyl-4-phosphonophenylglycine (CPPG) and a positive allosteric modulator of mGluR4 (–)-N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC). The Vogel conflict drinking test in rats was used to test the anxiolytic-like effects. The hippocampus was chosen as a site of the injection of drugs, as this brain regions is involved in the regulation of anxiety-related behavior. Intrahippocampal injections (CA1 region of the hippocampus) of PHCCC (12 nmol) but not of CPPG (75 nmol) produced an anxiolytic-like response, moreover, the effect of PHCCC was totally blocked by CPPG. The anxiolytic-like effects of ACPT-I (7.5 nmol) or PHCCC (12 nmol) were significantly attenuated by flumazenil (10 mg/kg), indicating an involvement of GABAergic system in the anxiolytic-like response.

Key words:
ACPT-I, CPPG, PHCCC, anxiety, conflict drinking test, group III mGlu receptors

Introduction

Fifty years of research on anxiety after the initial discovery of benzodiazepines [45] did not result in a breakthrough in the therapy of this disease. Less than 10% of patients with anxiety are treated adequately [12], which is far below the acceptable level. It appears more and more evident that the future research should focus on two major brain transmitters, the inhibitory transmitter GABA and the excitatory one, glutamate. It is rather obvious that the enhancement of GABAergic transmission leads to anxiolysis as exemplified by the efficacy of benzodiazepines. However, benzodiazepines are not free of several adverse effects. Several findings show that substances, which decrease glutamatergic neurotransmission, such as antagonists of ionotropic glutamate receptors, exert anxiolytic-like effects in animals [6], however,

the adverse effect profile precludes these substances from being used in clinical practice.

Another avenue in the search for new anxiolytic drugs may be related to the discovery of metabotropic glutamate receptors [32]. Metabotropic glutamate receptors (mGluRs), an interesting family of eight different receptor subtypes, are classified into three groups according to their amino acid sequence, homology and intracellular coupling [32]. Group I mGlu receptors (mGluR1 and mGluR5) activate the phosphatidylinositol hydrolysis/Ca^{2+} signal transduction pathway; while group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) receptors inhibit adenylyl cyclase signal transduction pathway [7]. The recent data on mGlu receptor ligands, which modulate the function of glutamatergic neurotransmission show, that substances which lead to a decrease in this transmission are possible future anxiolytic drugs. Using a broad variety of tests, anxiolytic-like effects were observed after administration of antagonists of group I mGlu receptors and agonists of group II mGlu receptors [8, 20, 22, 23, 41]. The selective group II mGlu receptor agonist compound LY354740 has progressed into phase II clinical trials for anxiety [46]. Of all glutamate receptors, the role of group III mGluRs in anxiety-like states is the least investigated because of the lack of specific pharmacological tools, moreover, all group III receptor ligands synthesized so far are not systemically active, so they have to be administered centrally. The results of recent studies [30, 47, 48] have demonstrated that selective agonists of these receptors (L-serine-O-phosphate (L-SOP)), (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) and 2-amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric acid (HomoAMPA) showed an anxiolytic-like activity in the Vogel conflict drinking test in rats after intrahippocampal injections; the effects of ACPT-I and HomoAMPA were inhibited by (*RS*)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), group III mGluR antagonist [30, 47]. Further studies have shown that (–)-N- phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), a positive allosteric modulator of mGluR4 [26, 27], exerts anxiolytic-like effects in rats after injections into the rat basolateral nuclei of the amygdala [42].

Taking into account the above observations, the present study was designed to further evaluate the effects of PHCCC, which is an allosteric modulator of mGlu4 receptors [27], and CPPG, which is a potent antagonist of group II/III mGlu receptors (with 20 fold selectivity for group III mGlu receptors) [50], in the Vogel conflict drinking test in rats. In the second part of this study the interaction between the agonist ACPT-I and antagonist of group III mGlu receptor and PHCCC have also been examined. Finally we decide to find whether the GABAergic system is implicated in the anxiolytic-like effects of ACPT-I and PHCCC. To solve this problem, we investigated the influence of flumazenil, a benzodiazepine (BZD) receptor antagonist, on the anticonflict activity of ACPT-I and PHCCC. To test the anxiolytic-like effects, the hippocampus was chosen as a site for the injection of drugs, because it is involved in the regulation of anxiety-related behavior [18]. The results of our studies indicated that the anxiolytic-like effects of ACPT-I or PHCCC after intrahippocampal administration in rats might occur *via* interactions with mGlu4 receptor and that the GABAergic system was involved in the modulation of the anxiolysis mediated by the above-mentioned substances.

Materials and Methods

Animals

Male Wistar rats, weighing 250 ± 20 g, were used in the study. The animals were kept in cages individually (40 × 27 × 15 cm), on a natural day-night cycle and at a room temperature of 19–21°C, with free access to food and tap water before the experiment. All the experiments were performed in the light phase of the natural light-dark cycle (from November till May) between 9 a.m. and 2 p.m. All experimental procedures were approved by the Animal Care and Use Committee at the Institute of Pharmacology, Polish Academy of Sciences in Kraków.

Intrahippocampal injections

The rats were anesthetized with intramuscular injection of ketamine (100 mg/kg) and xylazine (65 mg/kg) in a 0.9% NaCl. A socket with two stainless steel guide cannualae (0.4 mm o.d., 0.3 mm i.d., 8.0 mm long) was implanted stereotaxically 2 mm above the CA1 region of the dorsal hippocampus (A 5.2 mm, L 2.0 mm, H 7.3 mm from the interaural line) [31], and was fixed to the skull with stainless steel screws and dental acrylic cement. Seven days later the rats were

subjected to behavioral testing. Solutions were administered bilaterally over 60 s. The injection needle remained in place for an additional 30–60 s before it was removed and replaced with a stylet.

Conflict drinking test (Vogel test)

A modification of the method of Vogel [52] was used. On the first day of the experiment, the rats were adapted to the test chamber for 10 min. After the adaptation period, the animals were deprived of water for 24 h and were then placed in the test chamber for 10 min with free access to the drinking bottle.

Afterwards, they were allowed a 30-min free-drinking session in their home cage. After another 24-h water deprivation, the rats were again placed in the test chamber and were allowed to drink for 30 s. Immediately afterwards, drinking attempts were punished with an electric shock (0.5 mA). The impulses were delivered every 2 s (timed from the moment when a preceding shock was delivered) between the grid floor and the spout of the drinking bottle. Each shock lasted 1 s and if the rat was drinking when an impulse was released, it received a shock. The number of shocks accepted throughout a 5-min experimental session was recorded by an experimenter who observed a behavioral reaction (e.g., body jerks) of rats to the electric shock.

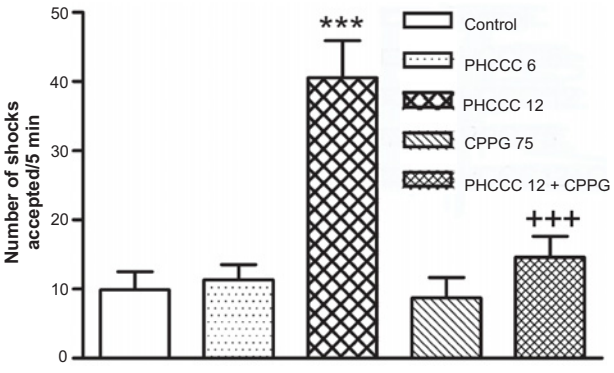

Fig. 1. Effects of PHCCC and CPPG in the conflict drinking Vogel test in rats after intrahippocampal injection. The compounds were administered into the hippocampus (*ihp*) 10 min or 20 min (CPPG) before the test. Values are expressed as the means ± SEM of the number of shocks accepted during 5-min experimental session; n = 7–8 rats per group. Symbols indicate differences in Newman-Keuls Multiple Comparison Test. *** $p < 0.001$ comparing to control, +++ $p < 0.001$ *vs.* PHCCC (12 nmol)-treated rats

Drug treatment

Flumazenil (purchased from Hoffman-La Roche; Basel, Switzerland) was suspended in a 1% aqueous solution of Tween 80 and administered intraperitoneally (*ip*), 60 min before the tests. ACPT-I, synthesized at Faust Pharmaceuticals (Strasbourg, France), CPPG was from Tocris Cookson Ltd, Bristol, UK, (–)-N-phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxamide (PHCCC) was a gift from Dr Fabrizio Gasparini (Novartis, Basle, Switzerland). Compounds: ACPT-I, CPPG and PHCCC were dissolved in sterile saline with the addition of a minimal amount of 0.1 M NaOH (pH = 7.2) and injected into the CA1 region of the hippocampus in a volume of 0.5 µl/site 10 min before the test. Control rats received vehicle.

Statistical analysis

The obtained data were presented as the means ± SEM, and evaluated by a one-way analysis of variance, followed by Dunnett's or Newman-Keuls multiple comparison tests, where $p < 0.05$ was considered significant. GraphPad Prism 4.0 program was used.

Results

The effects of intrahippocampal injections of group III mGlu receptor ligands in conflict drinking test

Intrahippocampal injections of mGlu4 receptor enhancer PHCCC (12 nmol) produced an anxiolytic-like response, which was blocked by mGlu III receptor antagonist CPPG (75 nmol) [$F(4,39) = 13.98$, $p < 0.001$, Fig. 1]. A simultaneous administration of PHCCC (6 nmol) and ACPT-I (3.75 nmol) at sub-threshold doses did not cause any statistically significant effects [$F(3,28) = 0.2337$, ns, Fig. 2].

The anxiolytic-like effect of ACPT-I (7.5 nmol) was significantly attenuated by flumazenil (10 mg/kg), [$F(3,25) = 8.352$, $p < 0.005$], indicating an involvement of GABAergic system in the anxiolytic like response (Fig. 3). Flumazenil (10 mg/kg) also inhibited the anxiolytic-like effects of PHCCC (12 nmol) (Fig. 4).

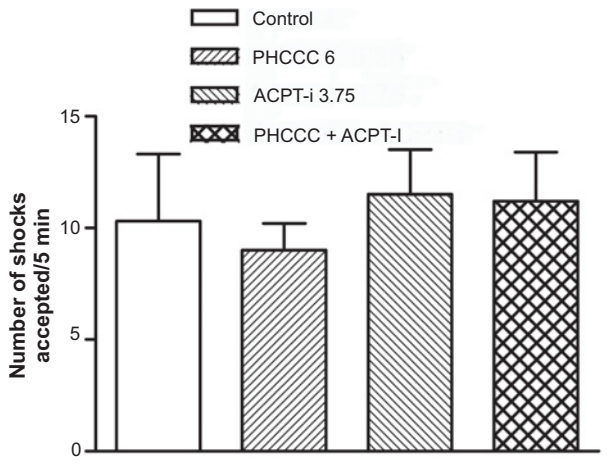

Fig. 2. Effects of PHCCC alone or in combination with ACPT-I in the conflict drinking Vogel test in rats after intrahippocampal injection. The compounds were administered into the hippocampus (*ihp*) 10 min or 20 min (CPPG) before the test. Values are expressed as the means ± SEM of the number of shocks accepted during 5-min experimental session; n = 7–8 rats per group.

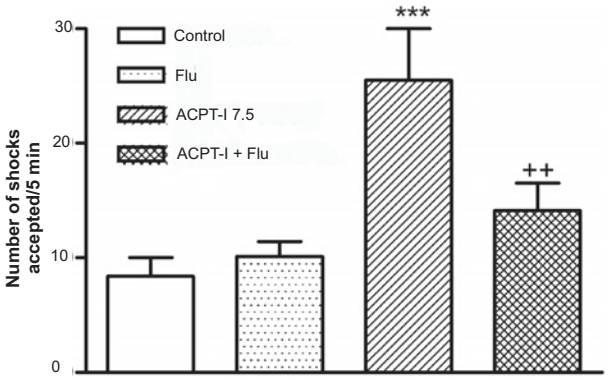

Fig. 3. Effects of flumazenil (Flu) on the anxiolytic-like action of ACPT-I in the conflict drinking Vogel test in rats. Flu (10 mg/kg) was administerd *ip*. The compounds were administered into the hippocampus (*ihp*) 10 min or 20 min (CPPG) before the test. Values are expressed as the means ± SEM of the number of shocks accepted during 5-min experimental session; n = 7–8 rats per group. *** $p < 0.001$ comparing to control, ++ $p < 0.01$ *vs*. ACPT-I (7.5 nmol)-treated rats

Discussion

The hippocampus is known to be one of the brain structures involved in anxiety [18]. Glutamate, the most prevalent transmitter in the brain, is very important to the physiology of the hippocampus [51]. Hippocampal neurons are rich in mGlu receptors including group III mGluRs [4, 39].

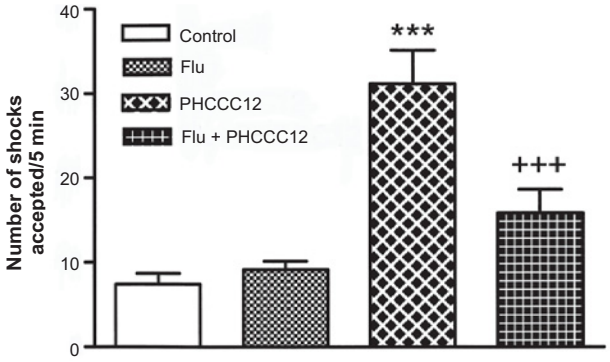

Fig. 4. Effects of flumazenil (Flu) on the anxiolytic-like action of PHCCC in the conflict drinking Vogel test in rats. Flu (10 mg/kg) was administered *ip*, 30 min before the test. PHCCC was administered into the hippocampus (*ihp*) 10 min before the test. Values are expressed as the means ± SEM of the number of shocks accepted during 5-min experimental session; n = 7–8 rats per group. *** $p < 0.001$ comparing to control, +++ $p < 0.001$ vs. PHCCC (12 nmol)-treated rats

Our earlier results show that intrahippocampal injections of ACPT-I, a non selective group III mGlu receptor agonist-induced a clear anxiolytic-like response conforming our earlier findings [30]. The specificity of the effect was confirmed by the ability of CPPG, a group III mGlu receptor antagonist [50] to inhibit the effect of the agonist in a dose dependent manner. The antagonist itself was not effective [30]. ACPT-I is a nonselective group III mGlu receptor agonist with micromolar potencies at mGlu4,6,8 receptors and milimolar at mGlu7 receptors [1]. A question arises which receptors are responsible for the anxiolytic-like effect of ACPT-I in this structure. The earlier data demonstrated that mGlu8 receptor agonists were free of anxiolytic effects after central administration. Neither (*RS*)-4-phosphonophenylglycine (*RS*-PPG), an agonist of group III mGlu receptors with preferential affinity for mGlu8 receptors [16] nor the most selective mGluR8 agonist (*S*)-3,4-DCPG [49] produced an anxiolytic-like effect after injection into the CA1 region of the hippocampus in the conflict drinking Vogel test in rats [30, 43]. Since the group 6 mGlu receptors are localized in retina [36] and express very low density in the brain, including the hippocampus, it is difficult to attribute the effect of ACPT-I to stimulation of that receptor subtype. The effectiveness of ACPT-I can, therefore, be related to the simulation of mGlu4 or/and of mGlu7 receptors which are both widely distributed in the hippocampus [4, 38].

The anxiolytic-like effects of PHCCC, an allosteric modulator of mGlu4 receptors [26, 27], which also af-

ter injection into amygdala produced anxiolytic-like effect [42] support the view that mGlu4 receptors might be responsible for the anxiolytic-like effect of ACPT-I. PHCCC is also an mGlu1 receptor antagonists [2], and blockade of this receptor type also leads to anxiolysis [24], however, the ability of group III mGlu antagonist CPPG to counteract the action of PHCCC supports notion of the involvement of group III receptors in that phenomenon. The fact that the combined administration of ACPT-I together with PHCCC (both agents were injected at a very low, not effective doses) did not cause anxiolytic-like effect, might mean the same receptor subtype (i.e. mGlu4 receptor) is engaged in the anxiolysis in the hippocampus. There are no pharmacological data concerning the involvement of mGlu7 receptors in anxiety, however, in mGlu7 knockout mice an anxiolytic-like effects were demonstrated [9]. Whether mGlu7 receptor antagonists might produce anxiolytic effect remains to be determined.

Stimulation of group III mGlu receptors suppresses excitatory synaptic transmission is several brain regions [3, 10, 11, 17, 19, 29] including CA1 neurons in the hippocampus [5]. The mechanism of the anxiolytic-like effects of agonists of group III mGluR may be related to their ability to inhibit glutamate release *via* the stimulation of presynaptic group III mGluR autoreceptors. Thus, the reduction of stimulatory glutamatergic neurotransmission *via* the activation of group III mGlu receptors may produce consequences similar to those occurring after the enhancement of GABAergic neurotransmission by benzodiazepines, which are the most commonly used anxiolytic drugs [35].

There is another possible explanation of the mechanism of anxiolytic-like action of ACPT-I and PHCCC. Both group II and group III of mGlu receptors are predominantly localized presynaptically on the glutamatergic terminals, and provide a negative feedback to modulate the glutamate release. However, several data have also shown that some mGlu receptors are present on non-glutamatergic neurons, such as GABAergic terminals, where they suppress GABA release when they are activated [36, 51]. For example, the release of GABA in the hippocampus is regulated by presynaptic mGlu heteroreceptors [21] including group III mGlu receptors, stimulation of which suppressed GABA release [37]. Electrophysiological studies have demonstrated a heteroreceptor role of group III mGlu4 and mGlu7 receptors on GABA terminals [28, 33, 53]. In our study, benzodiazepine receptor antagonist flumazenil inhibited the action of ACPT-I and PHCCC, indicating that the GABAergic system was involved in the modulation of the anxiolysis induced by both agents. Anxiolytic-like effects of many classes of drugs, both typical like diazepam and others including serotonergic [13] or noradrenergic [40] ligands as well as the NMDA receptor antagonists [34] are blocked by flumazenil. This indicates that the GABAergic system is involved in the modulation of the anxiolysis mediated by the above mentioned substances. Flumazenil is also known to reverse the anxiolytic-like effects of group II mGlu receptor agonist LY354740 [14]. LY354740 is an agonist of group II mGlu receptors, with predominant presynaptic localization. It is, therefore, feasible that the anxiolytic effect of both agonists of group II and group III mGlu receptors involves GABAergic neurotransmission.

It has been reported [38] that the selective group III mGlu receptor agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4) more strongly depresses GABAergic transmission to interneurons than to pyramidal neurons, implying that GABAergic transmission among interneurons is modulated by glutamate spillover from excitatory afferent terminals. Depression of GABAergic input to identified hippocampal neurons by group III metabotropic glutamate receptors in the rat [25] was also described. Electron microscopic immunocytochemistry confirmed that mGlu4, mGlu7a and mGlu8a receptors occur in the presynaptic active zone of GABAergic terminals on the interneurons [25]. In the hippocampus interneuronal network consisting of subsequent inhibitory GABAergic neurons exists [15]. If the interneurons represent another GABAergic neuron, the inhibition of GABA release *via* stimulation of presynaptic mGlu receptor might cause a disinhibition of that interneuron and an increase in inhibitory transmission. This might lead to the increased GABAergic neurotransmission and to anxiolysis.

A different explanation for the anxiolytic-like action of ACPT-I might be based on its interaction with group I mGlu receptors. Stimulation of group III mGlu receptors inhibits EPSPs induced by the stimulation of group I mGlu receptors [28]. Therefore, it may cause effects similar the mGlu 1 receptor antagonists, which exert anxiolytic-like effects in animals [24, 44].

Irrespectively of the nature of the anxiolytic effect of agonists of group III mGlu receptors, our results in-

dicate that such compounds might be used as anxiolytic drugs in the future.

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