Role of the Cholecystokinin System in Anxiolytic Activity of Dipeptide GB-115

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We studied the effect of dipeptide GB-115, a retroanalogue of cholecystokinin-4 with anxiolytic properties, on the behavior of outbred rats and BALB/c and C57Bl/6 mice induced by cholecystokinin-4 receptor agonists and yohimbine. Anxiogenic agents were shown to cause anxiety in rats and C57Bl/6 mice (with an active response to stress) in the open field test and elevated plus maze test, but did not modulate the behavior of BALB/c mice exhibiting a freezing response to emotiogenic exposure. Activation of cholecystokinin-4 type 2 receptors abolished the antianxiety effect of GB-115 in BALB/c mice. This dipeptide prevented the development of cholecystokinin-4-induced anxiety in C57Bl/6 mice and outbred rats. α_2-Adrenoceptor antagonist yohimbine did not modulate the effects of GB-115 in BALB/c mice. GB-115 did not prevent the development of yohimbine-induced anxiety in C57Bl/6 mice. Our results confirm the data on phenotype-specific activity of GB-115. We conclude that cholecystokinin-4 and GB-115 have a common pharmacological target.

Key Words: *dipeptide GB-115; anxiolytic; cholecystokinin; cholecystokinin-4; anxiogenic agent*

Neuropeptide cholecystokinin (CCK) plays a role in the neurobiological mechanisms of stress via the interaction with peripheral (CCK_1) and central receptors (CCK_2). Tetrapeptide CCK (CCK-4) activates CCK_2 receptors, which causes anxiety and panic response [7]. Previous experiments showed that stress and anxiogenic drugs increase the content of CCK in the frontal cortex of the brain [5]. Functional inactivation of the central CCK system holds promise for the prevention of mental symptoms, including anxiety and panic disorders. This mechanism mediates the anxiolytic effect of various chemical compounds.

A series of N-phenalkanoyl-substituted tryptophan-containing dipeptides with CCK-positive and CCK-negative properties was synthesized at the V. V. Zakusov Institute of Pharmacology (Russian Academy of Medical Sciences) on the basis of the Shemyakin–Ovchinnikov–Ivanov topochemical principle for construction of new peptide compounds [1]. An inverse structure of the peptide chain relative to the endogenous tetrapeptide CCK-4 (Trp-Met-Asp-Phe) can provide antagonistic (L-configuration of amino acid residues) or agonistic activity (D-configuration of amino acid residues). Pharmacological study of the series of more than 20 dipeptides has focused on glycyl-L-tryptophan amide-N-(6-phenylhexanoyl) (compound GB-115) ehibiting the highest anxiolytic activity [1].

It was hypothesized that the antianxiety effect of CCK_2 receptor antagonists requires the so-called "substrate" (*i.e.*, high level of anxiety and fear) [15]. Taking into account these data, we studied the effect of a CCK receptor antagonist GB-115 on the behavior of outbred and inbred animals in the standard tests for anxiety after treatment with anxiogenic agents CCK-4, GB-104, and yohimbine.

MATERIALS AND METHODS

Experiments were performed on inbred male BALB/c and C57Bl/6 mice (20-22 g; Pushchino nursery) and

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outbred male rats (190-220 g; Stolbovaya nursery, Russian Academy of Medical Sciences). The animals were maintained in a vivarium of the V. V. Zakusov Institute of Pharmacology under standard conditions. They were housed in cages (10 mice per cage and 6 rats per cage) under natural light/dark regimen and had free access to water and standard pelleted feed for 10 days before the start of the study. Experiments were conducted in the fall-winter period at 9:00-13:00.

The study was performed with dipeptides GB-115 ($Ph(CH_2)_5CO$-Gly-*L*-Trp-NH_2; 0.025 and 0.05 mg/kg; CCK-negative activity) and GB-104 ($Ph(CH_2)_4CO$-Gly-*D*-Trp-NH_2; 0.02 mg/kg; CCK-positive activity). These compounds were synthesized at the V. V. Zakusov Institute of Pharmacology [1]. We also used a benzodiazepine tranquilizer diazepam (1.0 mg/kg; Sigma), CCK_2 receptor agonist CCK-4 (4.0 μg/kg; Russian Cardiology Research and Production Complex), α_2-adrenoceptor antagonist yohimbine (5.0 mg/kg; Sigma), and distilled water (control). The substances were injected intraperitoneally in a dose of 0.1 ml per 10 g body weight (for mice) or 0.1 ml per 100 g body weight (for rats). The scheme of treatment appeared as follows: (1) control; (2) anxiogenic agent; (3) GB-115 or diazepam (15 min after administration of the anxiogenic agent); and (4) GB-115 or diazepam. The doses were chosen from the results of experimental studies and published data on the efficiency of these agents.

The emotional stress exposure was modeled in the open field (OF) test as described elsewhere [2]. Before OF testing, all animals were maintained in darkness for 1 min and then placed into one of the peripheral squares. The behavior of animals was observed for 3 min. The following parameters were recorded: number of crossed peripheral segments (peripheral activity); number of crossed central segments and entries into the center (central activity); number of vertical rearing postures (vertical activity); and total locomotor activity (sum of the peripheral, vertical, and central activities).

Anxiety of animals was studied in the elevated plus maze (EPM) test [2,12]. The mice were tested in EPM with transparent walls, which allowed us to evaluate reliably the behavioral differences between the strains and anxiogenic effect of pharmacological agents [10]. The animals were placed into the central area. The main spatial and temporal parameters (time spent in the open arms; and number of entries into the open and closed arms) were recorded for 300 sec. Increasing the time spent in the open arms and number of entries into the open arms (with no change in locomotor activity; *i.e.*, total number of entries into the open and closed arms) was considered as a criterion of the anxiolytic effect. The time spent in the open arms and number of entries into the open arms were calculated as follows:

$$\text{Time spent in the open arms} = \frac{\text{Time spent in the open arms (sec)}}{300 \text{ sec}} \times 100\% \quad (\mathbf{1});$$

and

$$\text{Number of entries into the open arms} = \frac{\text{Number of entries into the open arms}}{\text{Total number of entries into the open and closed arms}} \times 100\% \quad (\mathbf{2}).$$

The results were analyzed by one-way analysis of variance (ANOVA) and nonparametric Mann–Whitney *U* test for independent samples. Intergroup differences were evaluated by Dunnett's test (when the analysis of variance demonstrated a significant effect).

RESULTS

CCK-4 significantly decreased ($p<0.01$) total locomotor activity of C57Bl/6 mice with active response to stress, but had no effect on this parameter in high-anxiety BALB/c mice exhibiting a greater freezing response as compared to C57Bl/6 mice (21.2±3.6 and 133.1±6.3 sec, respectively; $p<0.001$). Single administration GB-115 did not modulate the behavior of C57Bl/6 mice, but produced the anxiolytic effect on BALB/c mice. It was manifested in the increase of locomotor activity (criterion of anxiety), which is consistent with the results of previous experiments [2]. Preactivation of CCK_2 receptors abolished the anti-anxiety effect of dipeptide GB-115 in BALB/c mice, which illustrates functional antagonism between CCK-4 and GB-115. These data suggest that anxiolytic activity of the CCK dipeptide analogue depends on its interaction with CCK_2 receptors (Table 1).

Administration of CCK-4 to rats was followed by a significant decrease in the time spent in the open arms ($p<0.05$) and number of entries into the open arms. These changes are typical of anxiety. The anxio-

TABLE 1. Effect of CCK-4 and GB-115 on Total Locomotor Activity of Inbred BALB/c and C57Bl/6 Mice in the OF Test (*M*±*SEM*)

Group	BALB/c	C57Bl/6
Control	21.0±3.6	133.0±6.3
CCK-4	19.0±3.1	80.0±7.7*
CCK-4+GB-115	22.0±2.2	130.0±8.9
GB-115	81.0±7.9*	127.0±8.3

Note. *$p<0.01$ in comparison with the control (*ANOVA*; Dunnett's test). Each group consists of 9-11 animals.

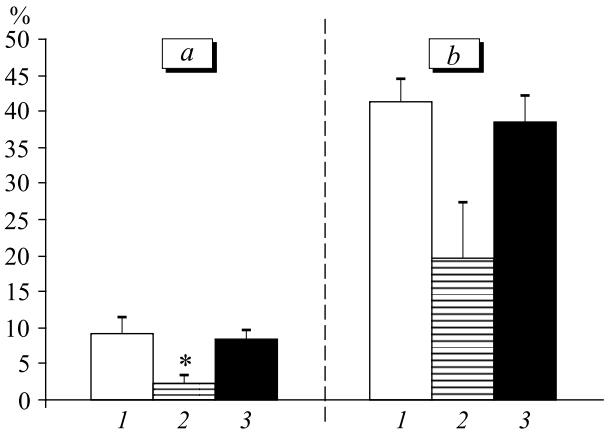

Fig. 1. Effect of GB-115 and CCK-4 on the behavior of outbred rats in EPM. Time spent in the open arms (*a*); number of entries into the open arms (*b*). Control (*1*); CCK-4 (4.0 μg/kg, *2*); CCK-4 (4.0 μg/kg)+GB-115 (0.05 mg/kg, *3*). **p*<0.05 in comparison with the control (nonparametric Mann–Whitney *U* test). Each group consists of 7-8 animals.

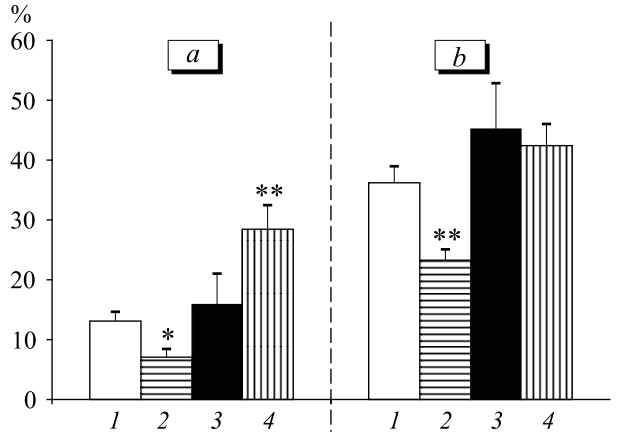

Fig. 2. Effect of GB-115 and GB-104 on the behavior of outbred rats in EPM. Time spent in the open arms (*a*); number of entries into the open arms (*b*). Control (*1*); GB-104 (0.2 mg/kg, *2*); GB-104 (0.2 mg/kg)+GB-115 (0.05 mg/kg, *3*); GB-115 (0.05 mg/kg, *4*). **p*<0.05 and ***p*<0.01 in comparison with the control (nonparametric Mann–Whitney *U* test). Each group consists of 7-9 animals.

genic effect was not observed after further treatment of animals with GB-115 in a dose of 0.05 mg/kg. Behavioral parameters of these animals did not differ from the control (Fig. 1).

Compound GB-104 decreased the time spent in the open arms ($p<0.05$), number of entries into the open arms ($p<0.01$), and locomotor activity of animals in EPM ($p<0.05$). These data illustrate a high level of animal's anxiety. GB-115 in a dose of 0.05 mg/kg prolonged the time spent in the open arms ($p<0.01$), but did not modulate locomotor activity of animals. These changes reflect the anxiolytic effect of GB-115 (Fig. 2). Activation of CCK receptors by GB-104 abolished the antianxiety effect of GB-115 in the EPM test, which is consistent with the results of experiments with a CCK receptor agonist CCK-4 (Fig. 1).

These data indicate that GB-115 produces a similar effect on the OF behavior of outbred rats (upon activation of CCK receptors) and inbred C57Bl/6 mice. Moreover, the anxiolytic effects of a dipeptide compound GB-115 depend on functional activity of CCK receptors.

Yohimbine did not affect the main behavioral parameters in high-anxiety BALB/c mice, which is consistent with the results of OF testing. These data illustrate an extremely high level of anxiety of BALB/c mice, which is typical of their genotype. GB-115 exhibited the anxiolytic properties. In this respect, GB-115 was less potent than diazepam ($p<0.05$ for the time spent in the open arms). Yohimbine did not modulate the anxiolytic effects of GB-115 (0.025 mg/kg intraperitoneally) and diazepam in BALB/c mice (Fig. 3, *a*).

By contrast, administration of yohimbine to stress-resistant C57Bl/6 mice was followed by a decrease in the time spent in the open arms (4.4±0.7 *vs*. 21.6±2.9 sec in the control; $p<0.001$) and reduction of locomotor activity (2.0±0.4 *vs*. 6.9±0.5 in the control; $p<0.001$). These changes are typical of the anxiogenic reaction. GB-115 did not affect the behavior of C57Bl/6 mice, which is consistent with the data on pharmacological activity of this dipeptide and its primary influence on animals with a passive phenotype of the emotional-and-stress response. Diazepam in a dose of 1.0 mg/kg decreased the total locomotor activity of animals, but did not have the anxiolytic effect. It was probably related to the sedative effect of benzodiazepine derivatives in C57Bl/6 mice [11]. GB-115 did not prevent the development of yohimbine-induced anxiety in C57Bl/6 mice (by the time spent in the open arms; Fig. 3, *b*).

Previous studies showed that inbred BALB/c mice with a genetically determines passive response to emotional stress prefer the closed/safe arms of the maze and exhibit low level of total locomotor activity in the OF test (as compared to stress-resistant C57Bl/6 mice) [2,3]. The dependence of anxiogenic activity of CCK-4 on the baseline level of anxiety is confirmed by published data [15]. As differentiated from Sprague-Dawley rats, PVG rats demonstrated a prolonged freezing response during the predator presentation. This response was reduced after treatment with a CCK_2 receptor antagonist LY225910. By contrast, the tetrapeptide CCK-4 (CCK_2 receptor antagonist) had the anxiogenic effect only in stress-resistant Sprague Dawley rats. The antagonists of central CCK receptors were low effective in these animals [15]. Moreover, a correlation was found between low exploratory activity of animals in the EPM test and high activity of the central CCK-ergic system [13].

A large body of evidence exists that various types of stress (immobilization; social defeat; and treatment with anxiogenic agents, including diethyl ether and

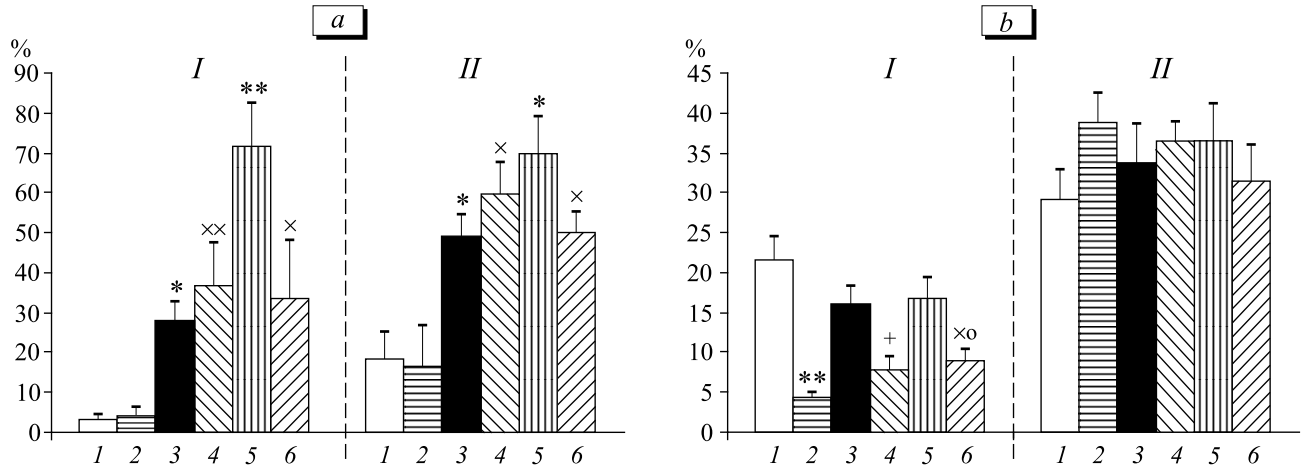

Fig. 3. Effect of GB-115 and diazepam on the yohimbine-induced stress response of BALB/c (*a*) and C57Bl/6 mice (*b*) in EPM. Time spent in the open arms (*I*); number of entries into the open arms (*II*). Control (*1*); yohimbine (5.0 mg/kg, *2*); GB-115 (0.025 mg/kg, *3*); yohimbine (5.0 mg/kg)+GB-115 (0.025 mg/kg, *4*); diazepam (1.0 mg/kg, *5*); yohimbine (5.0 mg/kg)+diazepam (1.0 mg/kg, *6*). **p*<0.01 and ***p*<0.001 compared to the control; ^{x}p<0.05 and ^{xx}p<0.01 compared to the yohimbine group; ^{+}p<0.05 compared to the GB-115 group; ^{o}p<0.05 compared to the diazepam group (nonparametric Mann–Whitney *U* test). Each group consists of 6-8 animals.

yohimbine) cause an increase in CCK content in the extracellular space of the frontal cortex [6]. This state is characterized by the increased expression of CCK_2 receptors in the structures responsible for emotional behavior (cortex, hippocampus, and hypothalamus) [14]. CCK_2 receptor blockade probably decreases the manifestations of anxiety/fear.

There are contradictory data on the efficiency of CCK receptor antagonists [8,9]. We revealed that the dipeptide GB-115 possessing a CCK-negative activity and phenotype-specific properties produces the anxiolytic effect. This selectivity of pharmacological activity is not a unique property of CCK_2 receptor antagonists. It is also typical of various derivatives of endogenous regulatory peptides, including Selank (synthesized from tuftsin [4], an endogenous dipeptide cycloprolylglycine) [3].

The existence of functional antagonism between anxiogenic agents (CCK-4 and GB-104) and GB-115 indicates that the anxiolytic effects of this dipeptide depend on its interaction with CCK receptors.

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