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Effect of the selective CCK_B receptor antagonist LY288513 on conditioned fear stress in rats

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Abstract

In order to investigate the involvement of cholecystokinin (CCK) in the regulation of anxiety, the effect of the selective non-peptide CCK_B receptor antagonist LY288513 ((4*S*,5*R*)-*N*-(4-bromophenyl)-3-oxo-4,5-diphenyl-1-pyrazolidinecarboxamide) on freezing behavior induced by conditioned fear stress was examined using a time-sampling procedure. Rats were individually subjected to 5 min of inescapable electric footshock in a shock chamber. Twenty-four hours after the footshock, the rats were again placed in the shock chamber and observed for 5 min without shocks: this procedure is termed conditioned fear stress. Subcutaneous administration of LY288513 30 min before footshock (0.3 mg/kg) and 30 min before conditioned fear stress (0.03–0.3 mg/kg) reduced conditioned freezing. This indicates that LY288513 blocked both the acquisition and expression of conditioned fear. The relatively selective non-peptide CCK_A receptor antagonist, lorglumide (D,L-4-(3,4-dichlorobenzoylamino)-5-(diphentylamino)-5-oxo-pentanoic acid), blocked the expression of conditioned fear, though only at a high dose (1.0 mg/kg). The peripheral non-peptide $CCK_{A/B}$ receptor antagonist, loxiglumide (D,L-4-(3,4-dichlorobenzoylamino)-5-(*N*-3-methoxypropyl-pentylamino)-5-oxo-pentanoic acid), failed to do so. These results suggest that brain CCK_B receptors are involved in the regulation of anxiety.

Keywords: CCK (cholecystokinin); CCK receptor antagonist; CCK_B receptor; Conditioned fear stress; Anxiety; LY288513 ((4*S*,5*R*)-*N*-(4-bromophenyl)-3-oxo-4,5-diphenyl-1-pyrazolidinecarboxamide)

1. Introduction

Recent studies have indicated a role for cholecystokinin (CCK) in anxiety. In experimental animals (Palmour et al., 1991, 1992; Singh et al., 1991a) and humans (De Montigny, 1989; Koszycki et al., 1993), the administration of CCK receptor agonists (CCK-4 (Trp-Met-Asp-Phe-NH_2) and CCK-8S (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) produces anxiogenic effects. Two subtypes of CCK receptors have been differentiated in rat, guinea pig, and human (Moran et al., 1986; Wank et al., 1994): CCK_A and CCK_B receptors. CCK_A receptors are found in some distinct regions of the central and peripheral nervous systems, and in pancreas, gallbladder and gastric mucosa. The CCK_B receptor is found throughout the central nervous systems, and in immune cells such as monocytes and T lymphocytes (Wank et al., 1994). In animal models of anxiety, such as the two-compartment test, the elevated plus-maze test, and the social interaction test, selective non-peptide CCK_B receptor antagonists have been reported to produce anxiolytic effects (Hughes et al., 1990; Ravard et al., 1990; Rataud et al., 1991; Singh et al., 1991b; Chopin and Briley, 1993). Results of few studies have suggested that the CCK_A receptor antagonist devazepide (formally MK-329, L-364,718; 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide), is also effective in animal models of anxiety (Hendrie and Dourish, 1990; Hughes et al., 1990), but that this anxiolytic effect of devazepide might be attributable to the CCK_B blocking effect of devazepide at high doses. Therefore, non-peptide CCK_B receptor antagonists have been developed as new anxiolytics. However, the mechanism of the anxiolytic effects of CCK_B receptor antagonists remains to be elucidated.

Our previous studies have shown that conditioned fear stress (exposure to an environment previously paired with

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footshock), an animal model of anxiety, increases both dopamine and 5-hydroxytryptamine (5-HT) metabolism in the medial prefrontal cortex, and induces freezing behavior, a fear- or anxiety-related behavior (Inoue et al., 1993, 1994). In addition, other investigators have reported that conditioned fear stress increases brain noradrenaline metabolism (Tsuda et al., 1986) and dopamine metabolism (Herman et al., 1982). Since conditioned fear stress involves no physical stimuli at the time of testing and does not use appetitive or consummatory behaviors, it is regarded as a purely psychological stress, and therefore appropriate for studying the relationship between neurochemical changes and anxiety. Conditioned fear stress-induced freezing has been reported to be attenuated by several classes of anxiolytics, such as benzodiazepines, 5-HT_{1A} receptor agonists and selective 5-HT reuptake inhibitors (Fanselow and Helmstetter, 1988; Hashimoto et al., 1995; Inoue et al., 1995). These findings indicate that conditioned fear stress is closely associated with monoamine neurotransmision, and that conditioned fear stress-induced freezing is attenuated by various anxiolytics, especially 5-HT-related drugs. However, the effects of CCK_B receptor antagonists on conditioned fear stress have so far not been reported upon.

The present study examined the effects of three types of non-peptide CCK receptor antagonists, LY288513, lorglumide and loxiglumide, on conditioned fear stress-induced freezing behavior in rats. LY288513 is highly selective for CCK_B receptors (Rasmussen et al., 1993), lorglumide is relatively selective for CCK_A receptors (Woodruff and Hughes, 1991), and loxiglumide is a peripheral $CCK_{A/B}$ receptor antagonist (Kaken Co. and Tokyo Tanabe Co., 1990).

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (the Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 250–300 g, were used. The rats were housed four per cage, and kept on a 12 h light-dark cycle, in a temperature-controlled environment, with free access to food and water. All experiments were performed between 08:00 and 13:00 h.

2.2. Drugs

The following drugs were used: LY288513 ((4*S*,5*R*)-*N*-(4-bromophenyl)-3-oxo-4,5-diphenyl-1-pyrazolidinecarboxamide) (Eli Lilly and Co., IN, USA); lorglumide sodium salt (D,L-4-(3,4-dichlorobenzoylamino)-5-(diphentylamino)-5-oxo-pentanoic acid sodium salt) (RBI, Natick); loxiglumide (D,L-4-(3,4-dichlorobenzoylamino)-5-(*N*-3-methoxypropyl-pentylamino)-5-oxo-pentanoic acid) (Tokyo Tanabe Co., Tokyo, Japan). LY288513 and loxiglumide were suspended in 0.5% sodium carboxymethyl cellulose, and lorglumide was dissolved in saline. All three drugs were injected subcutaneously (s.c.) in a volume of 1 ml/kg.

2.3. Conditioned fear

The rats were individually subjected to 5 min of inescapable electric footshock [2.5 mA of scrambled shock, on a variable interval schedule, with a mean intershock interval of 60 s (35–85 s) and a shock duration of 30 s] in a chamber with a grid floor ($19 \times 22 \times 20$ cm, Medical Agent Co., Kyoto). Twenty-four hours after the footshock, the rats were again placed in the shock chamber (this time without shocks), and observed for 5 min. Drugs, or the vehicle, were administered either 30 min before footshock, 5 min after footshock, or 30 min before placing the rats in the shock chamber again. During the 5-min observation period, freezing behavior was recorded using a time-sampling procedure (Fanselow, 1980). Every 10 s, the behavior that the animal was currently engaged in was classified as either freezing or activity. Freezing was defined as the lack of any observable movement of the body and the vibrissae, except movements related to respiration. The percentage scores for freezing were calculated for a 5-min observation period. These procedures had been approved by the Hokkaido University School of Medicine Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine.

2.4. Pain

The effects of LY288513 on footshock-induced pain were examined. Four behaviors, vocalization, limb withdrawal of the forepaw, limb withdrawal of the hindpaw, and jumping, were used as indices of nociception (Carter, 1991). Thirty minutes after drug injection (LY288513 0.3 mg/kg, s.c.), the rats were placed individually in the shock chamber which was to be used for the conditioned fear. After a 5-min adaptation period, the rats were subjected to scrambled electric footshocks presented in ascending order, ranging from 0.2 mA to 3.0 mA in 0.2-mA steps. Each shock was of 10-s duration, and spaced at 40-s intervals. The responses of the rats to each shock were observed, and the minimal intensities of electric footshocks at which each of the four behaviors appeared were determined.

2.5. Motor activity

Motor activity was measured using an apparatus with an infrared sensor that detects thermal radiation from animals, as described by Ohmori et al. (1994). The horizontal movements of the rats were digitized and fed into a computer every 10 min. Locomotion predominantly contributed to the count, but other non-specific body move-

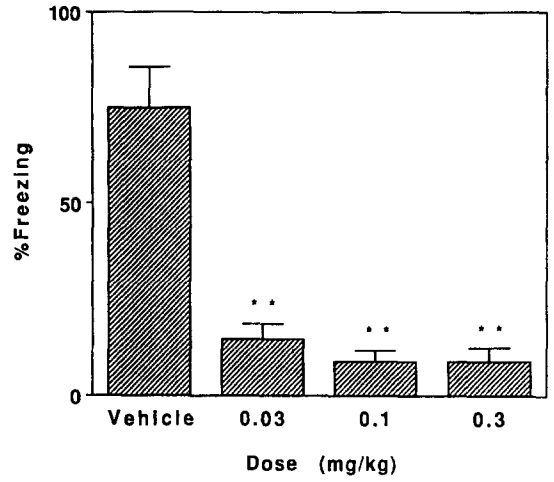

Fig. 1. Effect of LY288513 on the acquisition of conditioned freezing. LY288513 was administered to rats 30 min before footshock. The mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. $n = 8$ rats/group. ** $P < 0.01$ compared to vehicle controls.

ments could also contribute to the count when these movements had substantial horizontal components. The rats were housed individually for 2 days prior to testing. Two hours after the home cage of the rat had been placed under an infrared sensor, 0.3 mg/kg of LY288513 (s.c.) or 1.0 mg/kg of lorglumide (s.c.), which significantly inhibited freezing in the experiment on conditioned fear was injected. The rats' motor activity in the home cages was recorded for 120 min after the injection.

2.6. Data analysis

All the data are presented as the means ± S.E.M. of the individual values for the rats from each group. The statistical significance of differences between the two groups was tested using an unpaired t-test (two-tailed). Multiple group comparisons were made using a one-way analysis of variance (ANOVA) followed by Duncan's test.

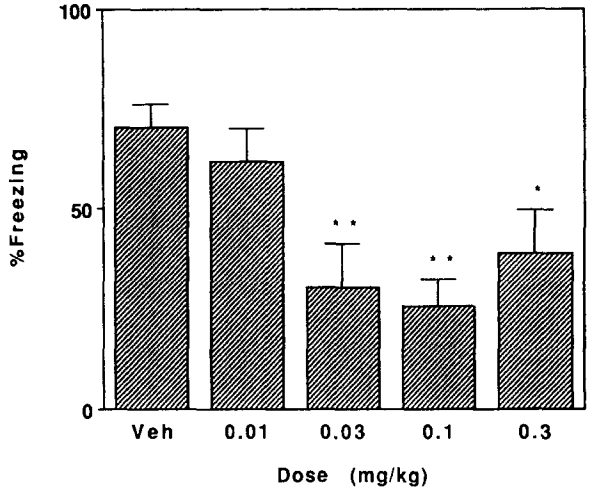

Fig. 3. Effect of LY288513 on the expression of conditioned freezing. LY288513 was administered to rats 30 min before conditioned fear stress. The mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. The numbers of rats/group (n) for each experiment were: vehicle, $n = 16$; LY288513, $n = 8$–12. * $P < 0.05$; ** $P < 0.01$ compared to vehicle controls.

3. Results

3.1. LY288513

Figs. 1–3 display the effects of LY288513 on the duration of conditioned fear stress-induced freezing behavior. Administration of LY288513 (0.03–0.3 mg/kg) 30 min before footshock significantly reduced the acquisition of conditioned freezing [$F(3,28) = 28.114$, $P < 0.01$] (Fig.

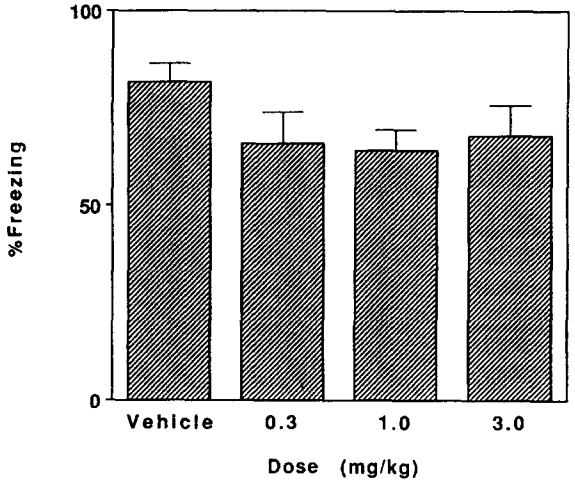

Fig. 2. Effect of LY288513 on conditioned freezing. LY288513 was administered to rats 5 min after footshock. The mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. $n = 8$ rats/group.

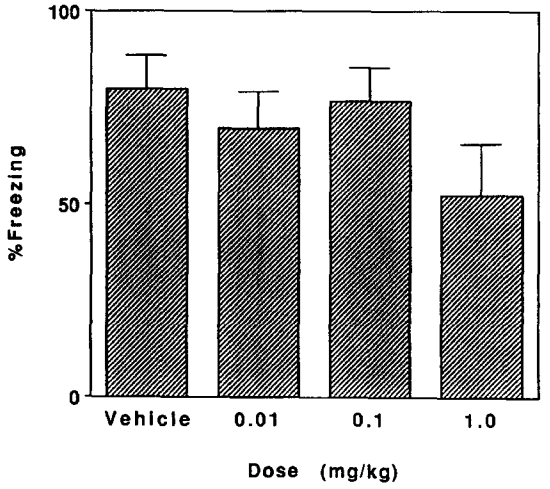

Fig. 4. Effect of lorglumide on the acquisition of conditioned freezing. Lorglumide was administered 30 min before footshock. The mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. $n = 8$ rats/group.

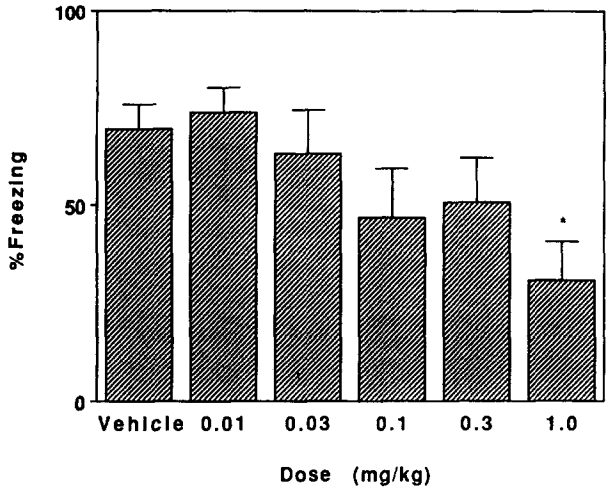

Fig. 5. Effect of lorglumide on the expression of conditioned freezing. Lorglumide was administered 30 min before conditioned fear stress. The mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. The numbers of rats/group (n) for each experiment were: vehicle, $n = 16$; lorglumide, $n = 8$. * $P < 0.05$ compared to vehicle controls.

1). The possibility that effects of LY288513, administered 30 min before footshock, had persisted up to the time of testing and directly affected the expression of conditioned freezing could be eliminated, because the administration of LY288513 5 min after footshock failed to reduce freezing behavior at doses of 0.3–3.0 mg/kg [$F(3,28) = 1.423$, $P = 0.26$] (Fig. 2). This indicates that LY288513 blocked the acquisition of conditioned fear. Administration of LY288513 (0.03–0.3 mg/kg) 30 min before placing the rats in the shock chamber again without shocks, that is, conditioned fear stress, significantly reduced freezing behavior [$F(4,55) = 5.976$, $P < 0.05$] (Fig. 3). Thus,

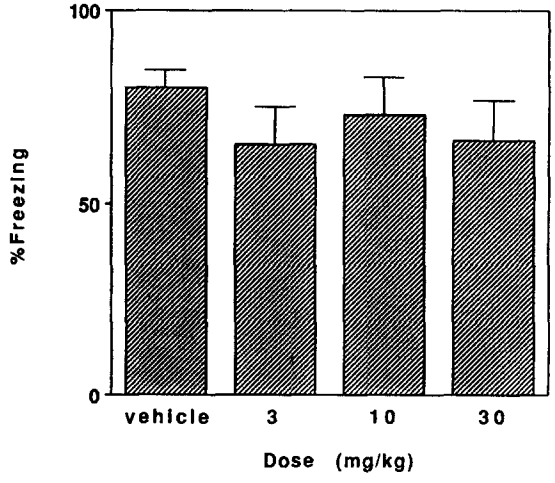

Fig. 6. Effect of loxiglumide on the expression of conditioned freezing. Loxiglumide was administered 30 min before conditioned fear stress. The mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. $n = 8$ rats/group.

Table 1
Effect of LY288513 on pain-related behavior induced by footshock. The mean thresholds ± S.E.M. of electric footshocks at which pain-related behaviors first appeared 30 min after injection of LY288513 or the vehicle are given

Behavior	Shock threshold (mA)		
	Vehicle ($n = 16$)	LY288513 ($n = 16$)	
Vocalization	1.47 ± 0.03	1.55 ± 0.05	N.S.
Limb withdrawal of the forepaw	1.17 ± 0.06	1.13 ± 0.08	N.S.
Limb withdrawal of the hindpaw	1.61 ± 0.08	1.72 ± 0.11	N.S.
Jumping	2.15 ± 0.05	2.21 ± 0.08	N.S.

N.S.: not significantly different

LY288513 blocked, not only the acquisition, but also the expression of conditioned freezing.

3.2. Lorglumide

Figs. 4 and 5 display the effects of lorglumide (0.01–1.0 mg/kg) on the duration of conditioned fear stress-induced freezing behavior. The administration of lorglumide 30 min before footshock failed to cause a significant change in freezing behavior [$F(3,28) = 1.402$, $P = 0.26$] (Fig. 4); but its administration 30 min before conditioned fear stress significantly reduced freezing behavior, though only at a dose of 1.0 mg/kg [$F(5,50) = 2.838$, $P < 0.05$] (Fig. 5). This suggests that lorglumide blocked the expression, but not the acquisition of conditioned fear.

3.3. Loxiglumide

Fig. 6 displays the effect of loxiglumide (3–30 mg/kg) on the duration of conditioned fear stress-induced freezing behavior. The administration of loxiglumide 30 min before

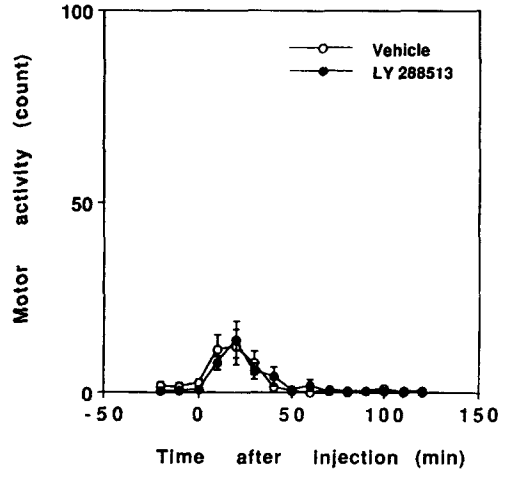

Fig. 7. Effect of LY288513 on motor activity. Vehicle and LY288513 (0.3 mg/kg) were administered at time 0. Each point represents the mean ± S.E.M. for $n = 12$ rats/group.

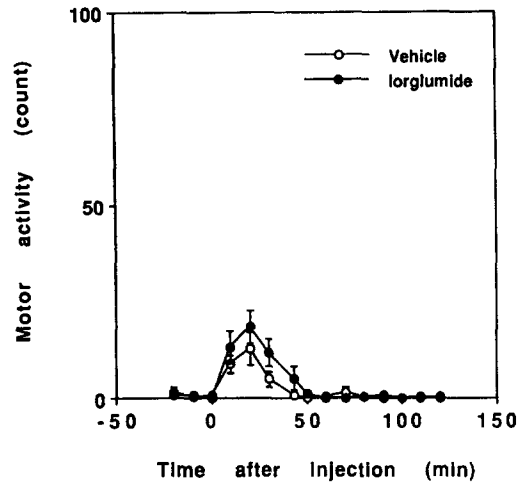

Fig. 8. Effect of lorglumide on motor activity. Vehicle and lorglumide (1.0 mg/kg) were administered at time 0. Each point represents the mean ± S.E.M. for $n = 12$ rats/group.

the conditioned fear stress failed to cause a significant change in freezing behavior [$F(3,28) = 0.570$, $P = 0.64$].

3.4. Pain

Table 1 shows the effects of LY288513 (0.3 mg/kg) on pain-related behaviors induced by footshock. The minimal intensities of electric footshocks at which pain-related behaviors appeared, that is the pain thresholds, in animals treated with LY288513 were not different from those of the controls.

3.5. Motor activity

Motor activity was not significantly affected at the effective doses of LY288513 (0.3 mg/kg) or lorglumide (1.0 mg/kg) in the conditioned fear test (Figs. 7 and 8).

4. Discussion

In the present study, the selective non-peptide CCK_B receptor antagonist, LY288513, blocked the expression of conditioned freezing at doses of 0.03, 0.1, and 0.3 mg/kg. Since administration of LY288513 did not affect motor activity, the blocking effect of LY288513 on the expression of conditioned freezing appears to be independent of any effects on motor activity at the dose required to significantly reduce freezing. Clinically effective anxiolytics, such as benzodiazepines, $5\text{-}HT_{1A}$ receptor agonists, and selective 5-HT reuptake inhibitors have also been reported to reduce the expression of conditioned freezing (Fanselow and Helmstetter, 1988; Inoue et al., 1995). This supports the proposition that the blocking effect of LY288513 on the expression of conditioned freezing reflects an anxiolytic effect.

Several studies have indicated anxiolytic effects of CCK_B receptor antagonists in other animal models of anxiety. Previous studies have shown that the selective non-peptide CCK_B receptor antagonist, L-365,260 (3*R*(+)-*N*-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl-*N*1-(3-methyl-phenyl)urea), displayed anxiolytic activity in the elevated plus-maze test in both rats and mice, and in the mouse two-compartment test at the doses of 0.001–0.1 mg/kg (Ravard et al., 1990; Rataud et al., 1991; Chopin and Briley, 1993).

Hughes et al. (1990) reported that another selective non-peptide CCK_B receptor antagonist, CI-988 (PD-134308) (4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1.^{3,7}]-dec-2-yloxy)-carbonyl]-amino]-propyl]-amino]-1-phenylethyl]-amino}-4-oxo-[*R*-(*R**,*R**)]-butanoate-*N*-metyl-D-glucamine), produced anxiolytic effects in the rat elevated plus-maze test and in the rat social interaction test at doses of 0.01–0.001 mg/kg. Thus, the results of these four studies presented here, indicate that the minimum effective doses of L-365,260 and CI-988 in animal models of anxiety are about 0.001 mg/kg to 0.01 mg/kg. In the present experiments, the minimum effective dose of LY288513 that blocked the expression of conditioned freezing was 0.03 mg/kg. The effective doses of these CCK_B receptor antagonists in animal models of anxiety correlate with in vitro affinities (IC_{50}) of these drugs for CCK_B receptors: L-365,260, 5.2 nM; CI-988, 1.7 nM; LY288513, 16 nM (Hughes et al., 1990; Rasmussen et al., 1993).

The mechanism for the effects of LY288513 on conditioned freezing is unclear. Previous studies have shown that conditioned fear stress increases dopamine, noradrenaline, and 5-HT metabolism in rat brain, and suggested that these brain monoaminergic systems (especially 5-HT) might be associated with the anxiety shown in conditioned fear stress (Herman et al., 1982; Tsuda et al., 1986; Inoue et al., 1993, 1994, 1995). CCK is known to be co-localized with 5-HT in neurons of rat dorsal raphe (Van der Kooy et al., 1981). The anxiolytic effects of CCK_B receptor antagonists may be mediated by interaction with 5-HT systems.

Alternatively, the effects of LY288513 may be mediated by γ-aminobutyric acid (GABA)/benzodiazepine systems. CCK is co-localized with GABA in neurons of rat cerebral cortex. Yaksh et al. (1987) reported that in vivo release of CCK-8-like immunoreactivity from rat cerebral cortex was reduced by administration of GABA into the cortical superfusate or intraperitoneal injection of diazepam. Both CCK_B receptor antagonists and benzodiazepine can block withdrawal symptoms from the chronic use of benzodiazepines in animals (Hughes et al., 1990). Chopin and Briley (1993) reported that the benzodiazepine receptor antagonist, flumazenil (Ro 15-1788) (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo(1,5*a*)(1,4)-benzodiazepine-3-carboxylate), blocks the anxiolytic effects of selective CCK_B receptor antagonists in the elevated plus-maze test.

Obviously, additional neurochemical and behavioral experiments are needed to further investigate the mechanism of the anxiolytic effects of CCK_B receptor antagonists.

LY288513 blocked not only the expression, but also the acquisition, of conditioned freezing. LY288513 administration (0.03-0.3 mg/kg) 30 min before footshock significantly reduced conditioned freezing. The possibility that the effect of LY288513, administered 30 min before footshock, persisted at the time of testing and directly affected the expression of conditioned freezing can be eliminated, because the administration of LY288513 5 min after footshock failed to reduce conditioned freezing. Furthermore, the present results demonstrate that LY288513 did not affect the threshold of footshock-induced pain. This finding is consistent with the results of a previous study showing that the selective CCK_B receptor antagonist, L-365,260, had no effect on pain threshold (Dourish et al., 1990). This indicates that the effect of LY288513 on the acquisition of conditioned fear is not due to the effects of LY288513 on nociception.

The CCK_A receptor antagonist, lorglumide, blocked the expression of conditioned freezing only at a dose of 1 mg/kg. Since lorglumide (1 mg/kg) did not affect motor activity, the blocking effect of lorglumide on the expression of conditioned freezing is not attributable to drug-induced hyperactivity. This result suggests that lorglumide may have an anxiolytic effect. However, there is no general agreement on the anxiolytic effects of CCK_A receptor antagonists. Results of three studies have suggested that the CCK_A receptor antagonist, devazepide, was anxiolytic in the two-compartment test and the elevated plus-maze test (Hendrie and Dourish, 1990; Ravard et al., 1990; Chopin and Briley, 1993); on the other hand results of two other studies showed no anxiolytic effects of devazepide (Rataud et al., 1991; Singh et al., 1991b). In an in vitro binding study, while LY288513 was a highly selective CCK_B receptor antagonist with a negligible affinity for CCK_A receptors (Rasmussen et al., 1993), lorglumide had only 70-fold selectivity for CCK_A receptors as against CCK_B receptors (Woodruff and Hughes, 1991). In addition, it should be noted that, as a CCK_B antagonist, LY288513 is 30 times more potent than lorglumide (IC_{50}: LY288513, 16 nM; lorglumide, 500 nM). In the present study, the minimum effective dose of LY288513 in the inhibition of conditioned fear stress-induced freezing was 0.03 mg/kg, and that of lorglumide, 1.0 mg/kg. Thus, LY288513 is 30 times more potent to reduce freezing than is lorglumide. Taken together, the effect of lorglumide on conditioned freezing may be explained by the CCK_B blocking effect of this compound.

Loxiglumide was regarded as a peripheral CCK receptor antagonist because [^{14}C]loxiglumide showed very low brain retention 15 min after intravenous injection in the rat (Kaken Co. and Tokyo Tanabe Co., 1990). In the present study, loxiglumide had no siginificant effect on the expression of conditioned freezing. This suggests that the peripheral CCK_B receptors are not related to the inhibitory effects of LY288513 on the expression of conditioned freezing.

In conclusion, the selective non-peptide CCK_B receptor antagonist, LY288513, inhibited both the acquisition and expression of conditioned freezing, suggesting an anxiolytic effect for LY288513. These results suggest that brain CCK_B receptors are involved in the regulation of anxiety, and that CCK_B receptor antagonists may represent a novel class of anxiolytic drugs.

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