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Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity

(PD134308/PD135158)

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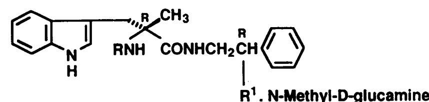
ABSTRACT **PD134308 and PD135158 are potent and selective antagonists at the cholecystokinin type B (CCK-B) receptors with IC_{50} values of 1.6 nM and 3.5 nM, respectively, in the radioligand binding assay and K_e values of 7.82 and 12.9 nM, respectively, in their blocking action on CCK responses in the rat lateral hypothalamic slice. PD134308 and PD135158 produced potent anxiolytic effects in the mouse black/white box test after either subcutaneous or oral administration. There was no evidence of the development of tolerance to the anxiolytic action of either PD134308 or PD135158 in mice treated twice daily for 7 days, nor was there any sign of withdrawal anxiogenesis after abrupt termination of this treatment. Both CCK-B antagonists were able to suppress the withdrawal anxiogenesis and produce an anxiolytic effect in mice previously made tolerant to diazepam. PD134308 and PD135158 produced potent anxiolytic effects in the rat elevated plus maze test and the rat social interaction test. The effects were comparable in magnitude to those seen with diazepam. However, unlike diazepam, PD134308 and PD135158 did not produce sedation. The CCK-B antagonists also showed powerful anxiolytic activity in the "marmoset human threat test." These results provide evidence of a selective role for CCK-B receptors in the control of anxiety. PD134308 and PD135158 are members of a class of anxiolytic agents that have a greatly improved profile compared with benzodiazepines or serotonin-related anxiolytics.**

Cholecystokinin (CCK), a member of the gastrin family, was first identified as a hormone in the gut with potent actions on secretions and gall bladder contractions (1). In 1975 the presence of gastrin-like immunoreactivity was demonstrated in brain (2). The majority of gastrin-like immunoreactivity in the brain is present as CCK-8, which exists as sulfated and desulfated forms (3, 4). Sulfated CCK is widely distributed and is present in neurons in the hippocampus, nucleus accumbens, caudate nucleus, cerebral cortex, and other brain regions (3, 5–8). In some neurons CCK coexists with other neurotransmitters—for example, dopamine, serotonin(5-HT), γ-aminobutyric acid (GABA), or other neuropeptides (4, 8, 9).

Evidence that CCK receptors might exist in more than one form was first produced by Innis and Snyder (10). Support for this was provided by the development of the benzodiazepine CCK antagonist L-364,718 (also known as MK-329 or devazepide), which was shown to be highly selective for peripheral versus brain CCK receptors (11). CCK/gastrin receptors are currently divided into three types; a gastrin receptor and two CCK receptors. CCK receptors are classified as CCK-A and CCK-B. The gastrin receptor is very similar to or identical with the CCK-B receptor. CCK-A receptors correspond to the receptors previously known as



PD No	R	R¹
134308	2-Adamantyloxycarbonyl-	$-NHCOCH_2CH_2CO_2H$
135158	1(S)-Endobornyloxycarbonyl-	$-NHCOCH_2CH_2CO_2H$

FIG. 1. Structure of PD134308 and PD135158. PD134308 is 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*-methyl-D-glucamine. PD135158 is 4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1.7.7-trimethyl-bicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[1*S*-1α.2β[*S**(*S**)]4α]}-butanoate *N*-methyl-D-glucamine (bicyclo system 1*S*-endo).

"peripheral CCK receptors." CCK-A receptors occur mainly in the periphery but also in some highly localized brain regions (12, 13). The majority of CCK receptors in the brain are, however, of the CCK-B subtype and these receptors are widely distributed throughout the brain (12). CCK-B receptors are characterized by a relatively high affinity for the agonists CCK-4 and pentagastrin and the antagonist L-365,260 and a low affinity for L-364,718 (11–13).

Although CCK has been shown to excite central neurons (14, 15), the physiological and behavioral significance of CCK-B receptors and of the role of CCK in the central nervous system is not understood. Progress in elucidating the function of CCK in brain has been hampered by the lack of selective CCK-B antagonists with good bioavailability and brain penetrability. The CCK-B-selective benzodiazepine derivative L-365,260 was described by Lotti and Chang (16) but to our knowledge no details of any central nervous system actions have been reported (16).

We have developed methods for the synthesis of nonpeptide analogues of CCK (17, 18). In the present paper we describe the actions of two potent and highly selective CCK-B receptor antagonists (Fig. 1) developed by rational design using these methods. Details of the chemical synthesis will be described elsewhere. The use of our antagonists has allowed us to demonstrate an important role of CCK in brain function and an important therapeutic potential for CCK-B antagonists. PD134308 and PD135158 are potent and highly selective anxiolytic agents.

METHODS

Receptor Binding Studies. Membrane homogenates were prepared from mouse cerebral cortex and rat pancreas, essentially as described (11, 19). Final pellets were resuspended

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Abbreviations: CCK, cholecystokinin; ^{125}I-CCK, ^{125}I-labeled CCK; 5-HT, serotonin; GABA, γ-aminobutyric acid.
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in assay buffer [10 mM Hepes (pH 7.2) containing 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and bacitracin at 0.25 mg/ml] to 2 mg (cortex) and 0.5 mg (pancreas) of original wet weight per ml. Incubation assay conditions and analysis of binding data were as described (19) with the exception that the concentration of ^{125}I-labeled Bolton–Hunter reagent-labeled sulfated CCK-8 (^{125}I-CCK) used was 50 pM. ^{125}I-CCK binding to guinea pig dispersed gastric (fundic) glands was essentially as described (20) with the exception that an excess concentration of L-364,718 (3 nM) was added to the incubation assay buffer [15 mM Hepes (pH 7.4) containing 130 mM NaCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 3 mM NaH_2PO_4, 5 mM glucose, 4 mM glutamine, and 0.1% bovine serum albumin] to suppress any possible binding of the radioligand to CCK-A site that may occur in this preparation.

Electrophysiological Studies. Extracellular recordings from brain slices containing the ventromedial nucleus of the hypothalamus and data analysis were made as described (15).

Curve fitting. Best-fit curves for dose–response data were obtained by fitting a simple hyperbolic function. This provided estimates of maximum response and the ED_{50} for CCK.

Schild analysis. Agonist concentration–response curves in the absence and presence of increasing concentrations of CCK-B antagonists were determined and the method of Arunlakshana and Schild (21) was used to provide Schild plots of the data, obtain dissociation equilibrium constants (K_e), and verify the competitive nature of the inhibition. A second series of single antagonist concentration experiments was performed using a wide range of antagonist concentrations on different slice preparations and the equilibrium constant was calculated using the equation: (dose ratio − 1) = [antagonist]/equilibrium constant.

Anxiolytic Activity in the Mouse. To measure anxiolytic activity in mice, male albino mice (Bradford strain; 20–30 g) were used. They were housed in groups of 10 at constant temperature (21°C) and controlled lighting (dark period, 0700–1900). The mice were fed ad libitum on a standard laboratory chow. Behavioral testing was performed in a quiet darkened room illuminated with a red light, between the hours of 1300 and 1800. The test apparatus has been described elsewhere (22). Two-fifths of the box was painted black and illuminated with a red light. This section was partitioned from the rest of the box, which was painted white and brightly illuminated. The compartments were connected by an opening located at floor level in the center of the partition. The mice were placed into the center of the white brightly lit area and observed by remote video recording, and the following four behaviors were noted: (*i*) the number of exploratory rearings in the white and black sections, (*ii*) the number of line crossings in the white and black sections, (*iii*) the time spent in the white and the black areas, and (*iv*) the latency of the initial movement from the white to the black area. Mice were used once only in treatment groups of 5 or 10 with a pretreatment time of 40 min and a test length of 5 min. In the duration of action studies, animals were tested 2, 4, 8, 12, or 24 hr after dosing.

Anxiolytic Activity in the Rat. Hooded Lister rats (Bradford University; 275–325 g) were used in both the elevated plus maze test and the social interaction test. Animals were transferred to the experimental room at least 1 hr prior to testing. In the elevated plus maze test, the apparatus used was as described (23). The 10-min testing period commenced when each animal was placed onto the central square of the maze. The time spent by each rat in the end section of the open arms of the maze was then determined over the entire test period. Drugs were injected subcutaneously or intraperitoneally before the animals were placed in the maze. The social interaction test was carried out as described by Jones *et al.* (24). The behavior of the rats was observed over a 10-min period by remote video recording. The following two behaviors were noted: (*i*) social interaction between the rats was determined by timing, sniffing of the partner, crawling under or climbing over the partner, genital investigation of partner, and following of partner; and (*ii*) exploratory locomotion was measured as the number of crossings of the lines marked on the floor of the test box. Twenty naive animals were used in drug-treated pairs in treatment groups of 6 (i.e., 12 animals). Animals were injected intraperitoneally or subcutaneously with test compound 40 min before being placed in the test box. Data were analyzed using a single factor analysis of variance followed by Dunnett's *t* test.

Anxiolytic Activity in the Marmoset. The behavior of single sex pairs of adult marmosets (290–390 g), bred at Bradford University, was assessed over a 2-min period, during which the number of "postures" exhibited by each animal was recorded using an electronic keyboard connected to a microcomputer (23). These postures were defined as body movements directed toward the threatening stimulus (i.e., a human observer standing in close proximity to the home cage) and include tail postures (elevation of the tail to expose the genital region), slit stares (confronting the observer with flattened ear tufts and partial eye closure), scent marking of the cage surfaces, and arching of the back with associated piloerection. The amount of time that the animals spent at the front of the cage was also determined. Test substances were administered subcutaneously 40 min prior to testing. Data were analyzed using a paired *t* test.

RESULTS

PD134308 and PD135158 produced a concentration-dependent inhibition of ^{125}I-CCK binding to CCK-B sites in mouse cortex with IC_{50} values of 1.7 nM and 2.8 nM, respectively (Table 1). In contrast the IC_{50} values of PD134308 and PD135158 for the ^{125}I-CCK-labeled CCK-A sites in rat pancreas were 2.7 μM and 1.2 μM, respectively, which results in an approximate 1600- and 400-fold selectivity, respectively, for the CCK-B receptor.

The IC_{50} values for various compounds are as follows: sulfated CCK-8 (IC_{50} = 0.24 ± 0.05 nM) > PD134308 (1.4 ± 0.2 nM) = L-365,260 (1.7 ± 0.2 nM) > PD135158 (3.4 ± 0.7 nM) = gastrin (3.7 ± 1.4 nM). All inhibited specific ^{125}I-CCK binding to the gastrin receptor in dispersed guinea pig gastric glands (n = 5 experiments) with almost identical affinities to those obtained at the CCK-B receptor in mouse cortex.

In contrast, PD134308 and PD135158 had negligible affinity for $GABA_A$; benzodiazepine; substance P; neurotensin; κ, μ, and δ opioid; bradykinin; and 5-HT_3 binding sites (IC_{50} > 10 μM).

Table 1. Inhibition of specific ^{125}I-CCK binding to mouse cortex (CCK-B) and rat pancreas (CCK-A) membranes by various compounds

Ligand	IC_{50}, nM: Mouse cortex CCK-B	IC_{50}, nM: Rat pancreas CCK-A	Pancreas/cortex ratio
Agonist			
CCK-8S	0.27 ± 0.03 (3)	0.12 ± 0.01 (4)	0.44
Pentagastrin	0.85 ± 0.13 (4)	605 ± 55 (4)	712
CCK-8US	2.6 ± 0.5 (4)	59 ± 16 (4)	23
Gastrin	5.1 ± 0.5 (4)	845 ± 78 (4)	166
CCK-4	2.6 ± 0.5 (3)	5330 (2)	2050
Antagonists			
PD134308	1.7 ± 0.2 (7)	2717 ± 1037 (6)	1598
PD135158	2.8 ± 0.4 (6)	1232 ± 223 (5)	440
L-365,260	5.2 ± 0.2 (4)	240 ± 48 (4)	46
L-364,718	31.7 ± 5.0 (4)	0.19 ± 0.01 (3)	0.006
CR1409	>1000 (4)	7.1 ± 0.5 (3)	<0.007

Each value represents the mean ± SEM for the number of experiments listed in parentheses and performed in triplicate.

In comparison, of the peptide ligands investigated, only the sulfated form of CCK-8 (IC_{50} = 0.3 nM) was found to have significantly higher affinity, and only CCK-4 [binding ratio CCK-A/CCK-B sites (A/B) = 2050] had higher selectivity, than PD134308 or PD135158 for the ^{125}I-CCK-labeled CCK-B sites (Table 1). The nonpeptide benzodiazepine analogue L-365,260 also bound with lower affinity (IC_{50} = 5.2 nM) and selectivity (A/B = 46) relative to PD134308 or PD135158 (Table 1).

The high affinity (IC_{50} = 0.2 nM) and 170-fold selectivity of L-364,718 for the CCK-A receptor (Table 1) was consistent with previous observations (11).

Electrophysiological Studies. Extracellular recordings were made from single neurons in the ventromedial hypothalamus of rat brain slices. As described (25), CCK-8 added to the perfusing artificial cerebrospinal fluid caused a concentration-dependent increase in spontaneous action potential firing. It has been shown (15) that the CCK-B receptor mediates this response. PD134308 blocked the increase in firing rate produced by CCK-8 (Fig. 2 *a* and *b*) in a concentration-dependent fashion (Fig. 2*c*) and Schild analysis revealed the block to be competitive (Fig. 2*d*) with a K_e of 7.87 nM (n = 4). In single antagonist concentration studies (concentration range, 10–1000 nM), the mean equilibrium constant was calculated to be 2.1 ± 1.4 nM (n = 10). Similar results were obtained when the experiments were repeated using PD135158. The K_e from multiple-concentration studies was 12.9 nM (n = 4), in good agreement with that obtained from single antagonist concentration experiments (18.6 ± 8.3 nM, n = 4). The specificity of the blocking action of PD134308 was obtained by testing the response to two other CCK-B ligands, pentagastrin and caerulein (1.0–1000 nM), and the response of carbachol (0.1–10 μM) in the presence of the antagonist. The response to pentagastrin and caerulein was abolished by PD134308 (300 nM) whereas the response to 1 μM carbachol remained intact (n = 5).

In three experiments, we recorded the CCK-8 (300 nM) response intracellularly from a ventromedial hypothalamus neuron. When PD134308 was applied (100–1000 nM), there was no change in resting potential or input resistance of the neuron but the CCK response was reduced or blocked in the presence of the antagonist.

Behavioral Tests. PD134308 and PD135158 were remarkably effective as anxiolytics in the mouse black/white box. The minimum anxiolytic dose of PD134308 was 100 ng/kg subcutaneously and produced an increase in exploratory behavior (rears and line crossings) in the white section of the box with a concomitant decrease in exploratory behavior in the black section. The compounds also decreased the percentage of time spent in the black section and increased the initial latency for the animals to cross from the white to the black section. The range of doses over which PD134308 and PD135158 were active was very large with no fall-off in activity at doses up to 30 mg/kg (Fig. 3). PD134308 was similarly effective as an anxiolytic after oral administration (Fig. 3) in mice.

In a separate series of experiments, mice were treated twice daily with diazepam to induce dependence. This dependence was characterized by a withdrawal anxiogenesis, which could be detected in the mouse black/white test box 24 hr after cessation of treatment. The results obtained in animals treated twice daily for 7 days with diazepam at 2.5 mg/kg and then withdrawn for 24 hr are shown in Fig. 4. The withdrawn animals show a marked reduction in exploratory behavior in the white section of the box and a concomitant increase in exploratory behavior in the black section compared with the untreated controls. These mice also spend significantly more time in the black section of the box than controls and had a reduced latency to enter the black section for the first time. In these animals undergoing diazepam withdrawal, PD134308 produced a clear anxiolytic response (Fig. 4).

When PD134308 was administered on a chronic basis (twice daily dosing for 7 days), no signs of tolerance were observed. Unlike diazepam, PD134308 produced no withdrawal anxiogenesis 24 hr after cessation of treatment but clear signs of anxiolytic activity were still evident (Fig. 4).

PD134308 displayed potent and selective anxiolytic activity in two additional rodent anxiolytic tests. In the rat elevated plus maze, PD134308 was tested at doses of 0.01 mg/kg and 1 mg/kg. Both doses produced a near maximal anxiolytic effect (Fig. 5) with no signs of sedation. The lowest dose produced a near maximal anxiolytic effect in this model.

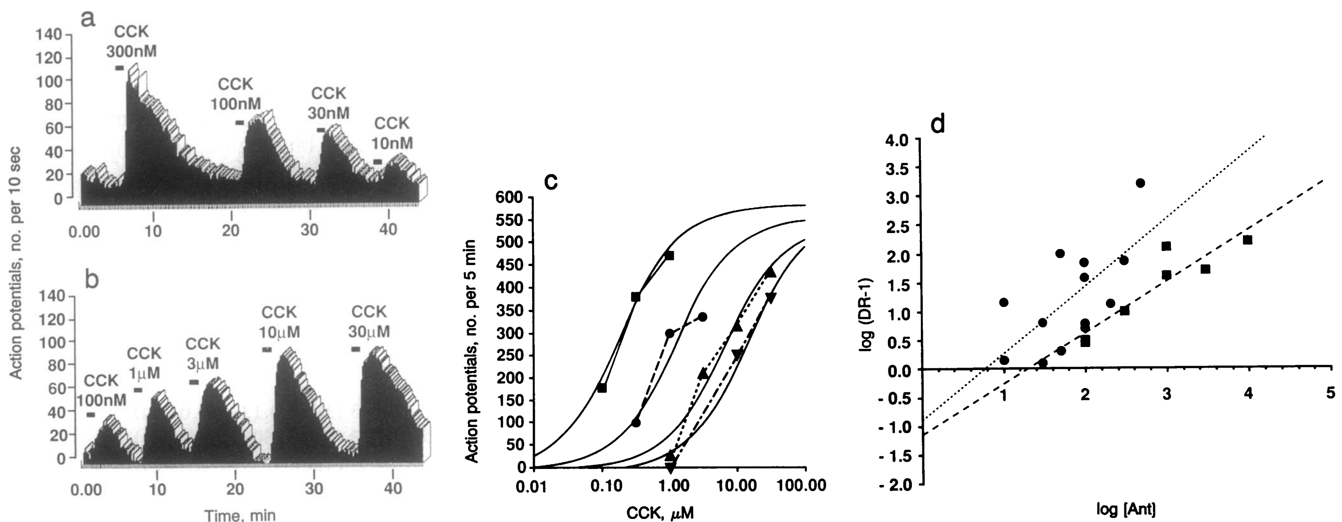

FIG. 2. (*a* and *b*) Ratemeter histograms of spontaneous action potential firing with time, recorded from a VMH neuron. Ordinate is number of action potentials in successive 10-sec intervals. (*a*) One-minute applications of CCK (indicated by the solid bar) produced a concentration-dependent increase in firing rate. (*b*) Same neuron 10 min after the application of PD134308 (100 nM) showing that the compound produced a potent antagonism of the CCK response. The dose–response curves in *c* are from a separate experiment on another neuron that was exposed to 30 (●), 100 (▲), and 200 (▼) nM PD134308, illustrating the concentration-dependent nature of the effect of the drug. ■, Control. (*d*) All data from multiple dose experiments for PD134308 (●) and PD135158 (■) (n = 4 for each compound) are plotted in the form of a Schild plot [log(dose ratio − 1 (DR − 1) versus log(antagonist concentration)]. The best-fit lines give slopes of 1.14 and 0.87, respectively, and K_e values of 7.87 nM for PD134308 and 12.9 nM for PD135158. Ant, antagonist. Dotted line, $1.14 \times X - 0.896$; dashed line, $0.87 \times X - 1.11$.

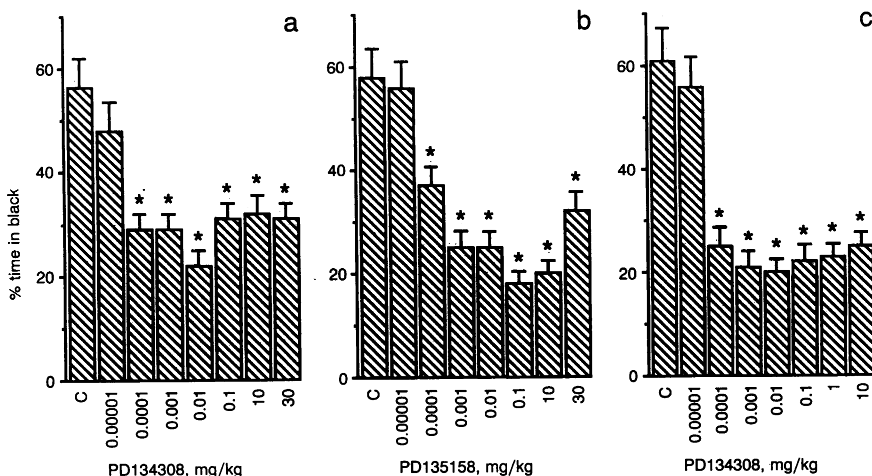

FIG. 3. Anxiolytic effect of PD134308 and PD135158 in the mouse black/white test box. PD134308 was given subcutaneously (*a*) or orally (*c*) in the doses indicated. PD135158 was given subcutaneously (*b*). c, Control. *, $P < 0.001$.

PD134308 was very effective in the rat social interaction test at doses of 1 μg/kg or 100 μg/kg. As in the mouse, there was no evidence of any sedative effect in the two rat tests.

In the marmoset human threat test in which PD134308 was tested, the response of vehicle-treated marmosets to a human threat was to spend only 20% of the total testing time forward and demonstrating 16 postures in the 2-min testing period. PD134308 (0.001 mg/kg subcutaneously) failed to significantly ($P > 0.05$) modify either of these parameters. However, higher doses of 0.01 and 1 mg/kg subcutaneously significantly ($P < 0.05$) increased the time the animals spent forward to 44% and 55%, respectively. This was accompanied, at the highest dose tested (1 mg/kg subcutaneously) by a significant ($P < 0.05$) decrease in the number of postures exhibited by the animals. Locomotor activity, assessed as the number of jumps between perches, was not significantly modified by treatment with PD134308.

DISCUSSION

Considerable effort has been expended over the last 15 years on experiments designed to elucidate the role of CCK in the brain. A major group of CCK-containing neurons is associated with the dopaminergic neurons in the mesolimbic system and medial substantia nigra (26–28) and this has led to speculation that CCK may act as a modulator of dopaminergic function. There is some evidence that CCK acts as a satiety signal and CCK receptors in the hypothalamus may be involved in the control of feeding behavior (29–31). CCK has also been implicated in analgesia after the finding that CCK itself can have analgesic actions (32, 33) and the more recent discovery that CCK antagonists, such as proglumide and devazepide (L-364,718), can potentiate morphine analgesia albeit over a narrow dose range (34). The widespread distribution of CCK receptors in brain suggests other functions of this peptide. One of the major obstacles in research on central CCK has been the absence of good selective antagonists of CCK-B receptors, the receptor subtype that is predominant in brain.

In the present study we have described and characterized PD134308 and PD135158, two potent and highly selective CCK-B antagonists. PD134308 and PD135158 are between 400 and 1600 times selective for CCK-B versus CCK-A receptors with both ligands binding with nanomolar affinity to ^{125}I-CCK-labeled CCK-B receptors in mouse brain. Furthermore, PD134308 is inactive or very weakly active in displacement of binding of the appropriate radioligands to $GABA_A$; benzodiazepine; substance P; neurotensin; bradykinin; 5-HT_3; or κ, μ, or δ opioid binding sites. In this respect PD134308 and PD135158 are by far the most selective CCK-B antagonists yet developed.

A functional block of brain CCK-B receptors by PD134308 and PD135158 was demonstrated in electrophysiological studies in rat brain slices; these experiments also enabled us to further demonstrate the selectivity of the compound for CCK-B versus CCK-A receptors. Thus in slices of rat ventromedial hypothalamus where we have previously shown that the excitatory effects of CCK-8 are mediated through CCK-B receptors (15), PD134308 and PD135158 were potent antagonists of CCK responses and the K_e for PD134308 (7.9 nM) was in good agreement with the K_i value obtained in binding studies. Other experiments showed that the same compounds were very

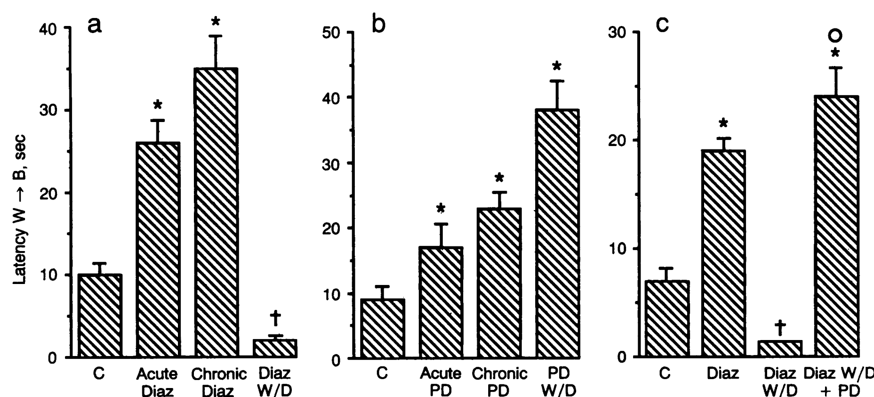

FIG. 4. Assessment of anxiolytic and anxiogenic effect of chronic treatment of diazepam or PD134308 in the mouse black/white test box. W, white box; B, black box; C, control. (*a*) Acute or chronic treatment with diazepam (Diaz) had an anxiolytic effect. After chronic diazepam treatment and withdrawal (W/D) there was a rebound anxiogenic response. (*b*) Acute or chronic treatment with PD134308 (PD) had an anxiolytic effect that was maintained after withdrawal. (*c*) PD134308 blocked the rebound anxiogenic effect after withdrawal from chronic diazepam. ★, $P < 0.001$ (anxiolysis); †, $P < 0.001$ (anxiogenesis); ○, $P < 0.001$ (reversal anxiogenesis).

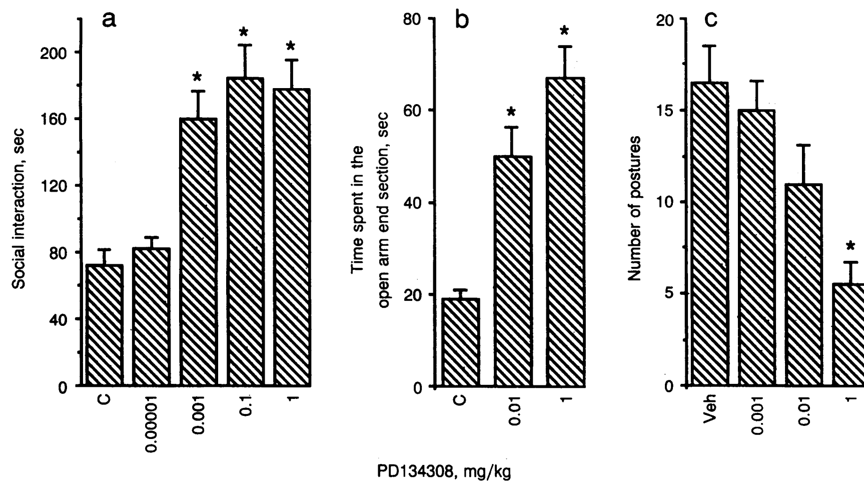

FIG. 5. Anxiolytic effect of PD134308 in the rat social interaction test (*a*), the rat elevated plus maze test (*b*), and the marmoset human threat test (*c*). PD134308 was given subcutaneously in the doses indicated. Veh, vehicle. *, $P < 0.001$.

weak CCK antagonists in the dorsal raphe slice, where the receptors are of the CCK-A type (unpublished data).

PD134308 and PD135158 have provided us with a research tool to investigate the role of CCK in brain. We, therefore, carried out a series of behavioral studies with these compounds. The distribution of CCK-B receptors in brain and the colocalization of CCK with GABA and 5-HT suggests that one function of CCK might be to serve a modulatory role on other transmitter systems. Thus it was decided first to investigate PD134308 and PD135158 in animal models of anxiety. These experiments provided evidence for a potent and highly selective anxiolytic action of PD134308 and PD135158.

In the mouse black/white box, PD134308 and PD135158 were remarkably effective anxiolytics with a minimum effective dose of 0.001 mg/kg. A maximum and highly efficacious anxiolytic effect was obtained at doses up to 30 mg/kg with no signs of sedation. A similar profile of anxiolytic effect was shown in the rat elevated plus maze test and in the rat social interaction test. In the mouse and rat models, PD134308 was equally as potent after oral administration as when given subcutaneously showing that the compound is highly bioavailable when absorbed orally. The marmoset human threat test is a useful primate model highly predictive of anxiolytic activity in man. PD134308 was active in this test with an ED_{50} very similar to that seen in the rat models.

Our findings raise several interesting points. Whether CCK-B antagonists exert their anxiolytic actions through similar or separate neural substrates to the benzodiazepine and 5-HT_3-related anxiolytic ligands should be determined. Irrespective of the neural substrates we would expect a neuropeptide antagonist such as PD134308 or PD135158 to be more selective and free of side effects compared with benzodiazepine agonists. Moreover, we hypothesize that CCK neurons related to anxiety may only be activated in abnormal (anxiogenic circumstances) and thus CCK-B antagonists will only modify abnormal behavior. Indeed PD134308 and PD135158 appear to be remarkably free of overt behavioral effects. In all three species used, there were no signs of sedation or ataxia detected even at the highest doses given. In a separate series of experiments, no sedation was seen in either rats or mice when the compound was given at up to 500 mg/kg (unpublished data). Thus the compound shows far superior anxiolytic profile to either benzodiazepines or 5-HT_3 antagonists in these models, the former being highly sedative and the latter showing a bell-shaped dose–response curve with activity in a relatively narrow dose range.

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