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Gastrointestinal Effects of Diazepam-withdrawal are Linked to Activation of Central Cholecystokinin-ergic Pathways in Rats

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Abstract—The influence of flumazenil-precipitated diazepam withdrawal on intestinal myoelectric activity and colonic transit was evaluated, in diazepam-dependent rats. Administered intraperitoneally, flumazenil (15 mg kg^{-1}) induced a strong stimulation of the duodenal spiking activity lasting 197 ± 20 min, and accelerated colonic transit corresponding to a significantly ($P < 0.05$) increased value of the geometric centre (3.52 ± 0.23 vs 2.44 ± 0.1 for the control). Both devazepide and L365260 administered intracerebroventricularly at a dose of 10 μg kg^{-1} abolished the flumazenil-induced withdrawal effect on the duodenum, whereas at a lower dose (1 μg kg^{-1}) only L365260 was able to antagonize this effect. In the same way, devazepide, loxiglumide and L365260 suppressed the effect of precipitated withdrawal on colonic transit when administered intracerebroventricularly at a dose of 10 μg kg^{-1}, whereas similar blockade was obtained at a dose of 5 μg kg^{-1} with L365260, and 10 ng kg^{-1} with PD135-158. It is concluded that in rats precipitated diazepam-withdrawal altered intestinal motility and colonic transit and that these effects are mediated by central release of cholecystokinin (CCK) or activation of CCK-ergic neurons.

Benzodiazepines are widely used in therapeutic management of anxiety, insomnia, muscle rigidity and convulsions. Like other sedative hypnotics, long-term treatment induces tolerance and dependence (Kalant et al 1971; Owen & Tyrer 1983). Numerous reports indicate withdrawal symptoms in chronically treated patients upon abrupt cessation of treatment, leading to excessive anxiety, tremor, muscle cramps, appetite and weight loss, and gastrointestinal distress (Pevnick et al 1987; Winokur et al 1980). Anxiety-related changes, vomiting and diarrhoea were also noted as symptoms of precipitated withdrawal by benzodiazepine-receptor antagonists in several animal species (Cumin et al 1982; McNicholas et al 1983; Lukas & Griffiths 1984; Giorgi et al 1988; Wilson & Gallager 1988). Recently, Martinez et al (1992a) have shown that benzodiazepine withdrawal in diazepam-dependent rats induces alterations of the intestinal and caecal myoelectric activity with an acceleration of gastrointestinal transit.

Administered either acutely or chronically, benzodiazepines such as flurazepam, lorazepam or diazepam could selectively antagonize the cholecystokinin $(CCK)_8$-induced activation of rat hippocampal pyramidal neurons (Bradwejn & De Montigny 1984; Bouthillier & De Montigny 1988). On the other hand, it was reported that cessation of long term-diazepam treatment produces changes of CCK binding in rat brain, corresponding to an increased number of sulphated [^{3}H]CCK_8 binding sites in several brain areas (Harro et al 1990a). Furthermore, CCK_B-receptor antagonists are able to suppress the withdrawal anxiogenesis and to produce an anxiolytic-like effect in mice previously made tolerant to diazepam (Hughes et al 1990).

Chlordiazepoxide, diazepam and medazepam have been shown to inhibit nerve-mediated response of guinea-pig ileal longitudinal muscle to CCK_8 (Meldrum et al 1986) and also contractile response of strips from guinea-pig gallbladder (Kubota et al 1986). Moreover, in fasted dogs, acute systemic administration of diazepam is followed by a long-lasting change in intestinal motor profile (Fargeas et al 1984), a pattern resembling that observed postprandially, or after systemic infusion of CCK_8 (Weisbrodt et al 1974).

Consequently, this work was performed to evaluate the effect of withdrawal, precipitated by a central benzodiazepine-receptor antagonist, flumazenil, on intestinal motility and colonic transit in diazepam-dependent rats, and to determine the relationship with CCK_8 by central administration of selective antagonists of CCK_A- and CCK_B-receptor subtypes.

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Materials and Methods

Induction of dependence and withdrawal

Two groups of male Wistar rats, 200–250 g, individually housed and fed with pelleted rat diet (AO4, UAR, France), were used for these experiments. Animals were made benzodiazepine-dependent by intraperitoneal (i.p.) administration of diazepam (Roche, Paris, France) diluted in dimethylsulphoxide (DMSO) at a dose of 15 mg kg^{-1} day^{-1}. Diazepam was given three times daily at 0900, 1500 and 2100 h for seven consecutive days. Vehicle-treated rats received DMSO in the same conditions as diazepam-treated rats. Twelve hours after the last injection of diazepam, the abstinence syndrome was triggered by intraperitoneal administration of flumazenil (15 mg kg^{-1}), a central benzodiazepine-receptor antagonist, a gift from Hoffman La Roche (Paris, France). Vehicle-treated rats received 0·3 mL DMSO.

Studies on intestinal myoelectrical activity

Animal preparation. Forty-eight rats were prepared for long-term electromyographic recordings (Ruckebusch & Fioramonti 1975). Briefly, under 100 mg kg^{-1} intraperitoneal

ketamine (Imalgene-1000, Rhône-Mérieux, Lyon, France) anaesthesia, nichrome wire electrodes (80 μm diam.) were implanted in the wall of the proximal and distal duodenum (3 and 6 cm from the pylorus). The insulated electrode wires (80 cm in length) were exteriorized on the back of the neck and protected by a glass tube attached to the skin. Rats were also fitted with permanent polyethylene catheters (PE 10) inserted into the lateral ventricles of the brain (2 mm from the saggital suture and 1 mm from the coronal suture).

Myoelectric recordings and analysis. Electromyographic recordings were started five days after surgery. The spiking activity was amplified by an electroencephalograph machine (Minihuit, Alvar, France), summed every 20 s by an integrator circuit and automatically plotted on a potentiometric recorder with a paper speed of 5 cm h^{-1} (Latour 1973). This integrated record permitted a clear determination of the different patterns of intestinal activity and the identification of the migrating myoelectric complexes (MMCs), characterizing the fasted pattern. Intestinal myoelectric activity was analysed over a daily 10-h period, from 0900 to 1900 h. Modifications appearing after precipitated withdrawal were appraised by measuring the duration of motor changes, i.e. disruption of MMCs.

Procedure. Of the 48 animals used for these experiments, six were vehicle-treated rats and 42 were treated with diazepam (15 mg kg^{-1} day^{-1}, i.p.). Thirty diazepam-treated rats were divided into five groups receiving intracerebroventricularly (i.c.v.), 10 min before flumazenil, either devazepide, a CCK_A-receptor antagonist or L365260, a CCK_B-receptor antagonist (gifts from MSD, West Point, PA) at doses of 1 or 10 μg kg^{-1}, or their vehicle (DMSO 3 μL). Twelve diazepam-treated rats were divided into two groups receiving i.c.v., 10 min before DMSO (flumazenil vehicle) devazepide or L365260 at a dose of 10 μg kg^{-1}.

Studies on colonic transit

Animal preparation. Colonic transit was evaluated according to a previously described technique (Williams et al 1988). Sixty-six male Wistar rats, 250–300 g, were equipped, under 100 mg kg^{-1} ketamine anaesthesia, with a permanent polyethylene catheter implanted into the lumen of the proximal colon. The catheters were brought subcutaneously to the scapular region of the back, where they were externalized and sutured to the skin. Rats were also fitted with permanent polyethylene catheters inserted into the lateral ventricles of the brain. Five minutes before receiving flumazenil or its vehicle, a solution of 1 mCi $Na^{51}CrO_4$, dissolved in 0·1 mL 0·9% NaCl was injected in the colonic catheter. The radioactive chromium served as a nonabsorbable marker for measuring the quantitative movement of contents along the lumen of the colon. Twenty minutes after the administration of the radioactive marker, the animals were killed by cervical dislocation and the colon was removed and divided, without spillage of contents, into five equal segments by use of a premeasured template. Each segment was placed into an individual vial and counted for γ-emissions for 5 min in a gamma-counter (MR 252 C. Kontron, Basel, Switzerland).

Colonic transit was evaluated by calculating the geometric center (GC) according to the following equation (Williams et al 1988):

$$GC = \Sigma((\text{Counts per segment}) \times (\text{segment number})) \times (\text{total count})$$

GC ranges from values of 1 to 5, such that a value of 1 indicates that colonic transit was maximally inhibited, whereas a value of 5 indicates that transit was maximal.

Procedure. Six animals were vehicle-treated; all others (n = 60) received diazepam (15 mg kg^{-1} day^{-1}, i.p.) and flumazenil (15 mg kg^{-1}, i.p.). Diazepam-treated rats were divided into ten groups receiving i.c.v., 10 min before flumazenil, devazepide or L365260 at doses of 1, 5 and 10 μg kg^{-1}, loxiglumide (CCK_A-receptor antagonist, Rotta, Milan, Italy) at doses of 1 and 10 μg kg^{-1} or PD135-158 (CCK_B-receptor antagonist, gift from Parke-Davis, Cambridge, UK) at a dose of 10 ng kg^{-1}, or their vehicle (DMSO 3 μL).

Statistics

Values were expressed as means ± s.e.m. and compared using analysis of variance followed by unpaired Student's *t*-test when appropriate. Differences were considered significant for $P < 0.05$.

Results

Intestinal myoelectric activity

Control studies. Rats fed only during the night presented a typical alternation of intestinal motility pattern. During the night period, they exhibited an irregular spiking activity of the duodenum, characteristic of the fed state. During daytime, rats were fasted, and exhibited an electrical activity of the duodenum characterized by the cyclic occurrence of migrating myoelectric complexes (MMC). Each MMC consisted of an irregular spiking activity (phase II) lasting 6–8 min, followed by a short period (4–5 min) of intense and regular spiking activity (phase III), separated by a 3–5-min quiescent period (phase I). These MMCs occurred firstly on the proximal duodenum and were propagated aborally at a mean rate of 3·5 ± 0·6 cm min^{-1} (n = 6).

Antagonism of precipitated diazepam-withdrawal by devazepide and L365260. Rats treated chronically with diazepam (15 mg kg^{-1} day^{-1}, i.p.) presented signs of withdrawal on the duodenum, after flumazenil administration. There was a significant and progressive decrease of the daily number of MMCs, whereas vehicle-treated rats exhibited a normal duodenal-fed-like pattern.

The administration of flumazenil (15 mg kg^{-1}, i.p.) to diazepam-treated rats immediately induced a strong stimulation of duodenal spike activity lasting 197 ± 20 min, characterized by a continuous irregular spiking activity resembling that observed after a meal (Fig. 1).

Ten minutes before flumazenil, both devazepide and L365260 at a dose of 10 μg kg^{-1} (i.c.v.) significantly reduced the flumazenil-induced withdrawal effect, whereas vehicle injected in the same conditions, had no effect on withdrawal disturbances (Table 1). Similarly L365260 administered at one-tenth the dose (1 μg kg^{-1}, i.c.v.) still reduced withdrawal

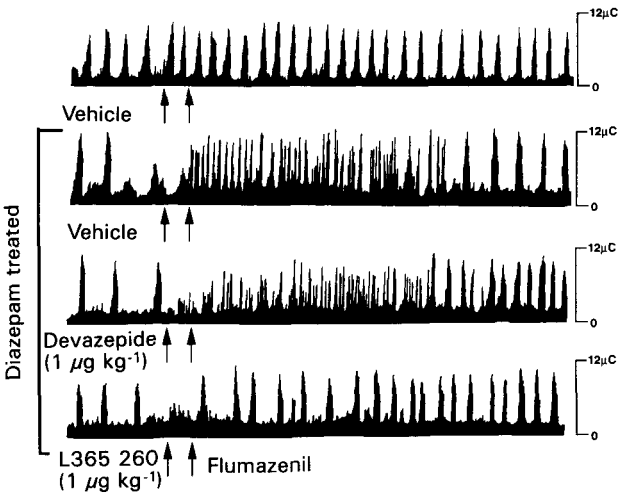

FIG. 1. Influence of intracerebroventricular (i.c.v.) administration of devazepide and L365260 on the duration of duodenal myoelectric alterations induced by flumazenil-precipitated withdrawal in 7-day diazepam-treated rats (integrated records). L365260 but not devazepide at a dose of 1 $\mu g\,kg^{-1}$ (i.c.v.) blocked the withdrawal effect.

effects, whereas devazepide, at the same dosage had no effect on precipitated-withdrawal motor disturbances (Fig. 1, Table 2). Injected alone in vehicle-treated rats, neither L365260 nor devazepide at these doses affected the fasted pattern of duodenal myoelectric activity. Moreover, devazepide and L365260 had no effect in diazepam-treated rats given flumazenil-vehicle when injected at a dose of 10 $\mu g\,kg^{-1}$ (Fig. 2).

Colonic transit

Effect of precipitated diazepam-withdrawal on colonic transit and antagonism by administration of devazepide and L365260. The administration of flumazenil (15 $mg\,kg^{-1}$, i.p.) after seven days of diazepam treatment produced an acceleration of colonic transit characterized by a significant ($P < 0.05$) increase of the colonic geometric centre displaced distally (Table 2).

Both devazepide and L365260, administered at a dose of 10 $\mu g\,kg^{-1}$ (i.c.v.) antagonized the increase of colonic transit induced by precipitated diazepam-withdrawal. At a dose of

Table 1. Effect of intracerebroventricular administration of devazepide and L365260 on the duration of duodenal MMC disruption induced by peripheral administration of flumazenil in diazepam-dependent rats. Flumazenil induced a duodenal MMC disruption of 197 ± 20 min.

Treatment	Dose	Duration of MMC disruption (min)
Control		10 ± 2
Diazepam-dependent rats + flumazenil (15 $mg\,kg^{-1}$, i.p.)		
Vehicle	3 μL (i.c.v.)	197 ± 20†
Devazepide	1 $\mu g\,kg^{-1}$	161 ± 18†
	10 $\mu g\,kg^{-1}$	42 ± 9*
L365260	1 $\mu g\,kg^{-1}$	38 ± 10*
	10 $\mu g\,kg^{-1}$	34 ± 10*

†$P < 0.05$ compared with control, *$P < 0.05$ compared with vehicle (n = 6).

Table 2. Effect of precipitated diazepam-withdrawal on colonic transit (geometric centre of ^{51}Cr) and antagonism of this effect by intracerebroventricular administration of devazepide and L365260.

Treatment	Dose	Geometric centre (arbitrary units)
Control		2·4 ± 0·1
Diazepam-treated rats + flumazenil (15 $mg\,kg^{-1}$, i.p.)		
Vehicle	3 μL (i.c.v.)	3·52 ± 0·2†
Devazepide	1 $\mu g\,kg^{-1}$	2·8 ± 0·3
	5 $\mu g\,kg^{-1}$	2·9 ± 0·2
	10 $\mu g\,kg^{-1}$	2 ± 0·3*
L365260	1 $\mu g\,kg^{-1}$	2·8 ± 0·4
	5 $\mu g\,kg^{-1}$	2·4 ± 0·1*
	10 $\mu g\,kg^{-1}$	1·9 ± 0·2*

†$P < 0.05$ compared with control, *$P < 0.05$ compared with vehicle.

5 $\mu g\,kg^{-1}$ (i.c.v.), only L365260 still antagonized the withdrawal effect, whereas devazepide at this dosage had no significant effect. Both CCK-receptor antagonists had no effect at one-fifth of the dose.

Administered i.c.v. at a dose of 10 $\mu g\,kg^{-1}$ loxiglumide antagonized ($P < 0.05$) the increase of colonic transit induced by flumazenil-precipitated withdrawal, whereas at a dose of 1 $\mu g\,kg^{-1}$ it had no effect (Table 3). PD135-158, at the dose of 10 $ng\,kg^{-1}$ (i.c.v.) significantly reduced ($P < 0.05$) the withdrawal effect.

Discussion

Our study highlights the fact that precipitated diazepam-withdrawal induced duodenal myoelectric disturbances which are prevented by previous treatment with CCK antagonists. Our findings are in agreement with previous work, showing that precipitated diazepam withdrawal induces intestinal disturbances, characterized by MMC disruption replaced by a strong irregular spiking activity (Martinez et al 1992a). This type of activity is often described as associated with increased intestinal flow of digesta and observed in various experimentally-induced diarrhoea states. The fact that precipitated diazepam-withdrawal in rats induces alterations in motor activity is also in accordance with digestive symptoms such as nausea, vomiting and diarrhoea, observed in particular after abrupt cessation of chronic treatment with benzodiazepines (Schauben et al 1992).

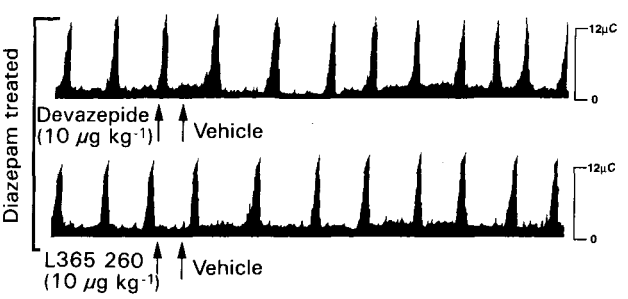

FIG. 2. Influence of intracerebroventricular administration of devazepide and L365260 on fasted pattern in diazepam-treated rats not given flumazenil but its vehicle.

Table 3. Effect of precipitated diazepam-withdrawal on colonic transit (geometric centre of ^{51}Cr) and antagonism of this effect by intracerebroventricular administration of loxiglumide and PD 135-158.

Treatment	Dose	Geometric centre (arbitrary units)
Control		2·4 ± 0·1
Diazepam-treated rats + flumazenil (15 mg kg^{-1} i.p.)		
Vehicle	3 μL i.c.v.	3·3 ± 0·3†
Loxiglumide	1 μg kg^{-1}	2·7 ± 0·3
	10 μg kg^{-1}	2·4 ± 0·2*
PD 135-158	10 ng kg^{-1}	2·6 ± 0·2*

†$P < 0.05$ compared with control, *$P < 0.05$ compared with vehicle.

Our results also indicate an accelerated colonic transit following flumazenil administration. This is in agreement with a previous study showing that caecal frequency was doubled during the first hour after benzodiazepine-withdrawal (Martinez et al 1992a). Flumazenil has been often used to accelerate the time course of withdrawal (Gonsalves & Gallager 1985), and Martinez et al (1992b) have demonstrated that behavioural withdrawal syndromes, such as increased startle response, precipitated by flumazenil, an antagonist of central benzodiazepine receptors, were more pronounced compared with that of PK 11-195, an antagonist at the peripheral benzodiazepine receptor.

Our results show that both duodenal and colonic disturbances induced by precipitated withdrawal are antagonized by central administration of devazepide, L365260, loxiglumide and PD135-158. We can hypothesize that the motor alterations induced by benzodiazepine withdrawal are linked to the CNS release of CCK or activation of CCK-ergic neurons. Indeed devazepide has a greater than 150-fold selectivity for CCK_A vs CCK_B receptors whereas L365260 has 50-fold selectivity for CCK_B vs CCK_A (Hughes et al 1990). Although they have low potency in displacing [^{3}H]flunitrazepam, these two CCK-receptor antagonists are benzodiazepine derivatives which might interact with benzodiazepine receptors (Chang et al 1986; Chang & Lotti 1989). The fact that loxiglumide and PD135-158 (non-benzodiazepine derivatives) also significantly reduced withdrawal effect on colonic transit strongly supports the hypothesis that CCK receptors are involved in this mechanism.

At low doses, only L365260 and PD135-158 block the withdrawal-induced disturbances of duodenal and colonic motility. However, according to their relative selectivity for CCK_A-(devazepide, loxiglumide) and CCK_B-(L365260, PD135-158) receptor subtypes, and according to the fact that CCK_B receptor antagonists are 100 times more potent than CCK_A-receptor antagonists, we cannot determine which CCK-receptor subtype is involved in this mechanism.

CCK and benzodiazepine receptors are co-localized in different parts of the brain. Indeed, co-localization of GABA (γ-amino butyric acid) and CCK_8 in rat cortical and hippocampal neurons has been observed (Somogyi et al 1984). Moreover, benzodiazepine and CCK_8 can interfere with each other; benzodiazepines antagonize the satiety action of CCK_8 in the CNS (Kubota et al 1986) and CCK antagonists can potentiate some effects of benzodiazepines. For example, lorglumide potentiates the protective effect of diazepam against seizures induced by pentetrazole (Penari et al 1986).

Many studies have shown that a single administration of benzodiazepine or GABA depresses central CCK transmission. Bradweijn & De Montigny (1984) have demonstrated that benzodiazepines selectively antagonize the CCK_8-induced activation of rat hippocampal pyramidal neurons. Moreover, the activation of the GABA-receptor/chloride-channel complex has been reported to inhibit the release of CCK. Indeed, GABA added to cerebroventricular superfusate diminishes the release of CCK-like immunoreactivity in perfusate of cerebral cortex (Yaksh et al 1987) and spinal cord (Benoliel et al 1992). Likewise, diazepam significantly depresses the CCK-evoked release by hippocampal neurons (Yaksh et al 1983). It has been demonstrated that long-term benzodiazepine treatment reduces neuronal responsiveness to cholecystokinin (Bouthillier & De Montigny 1988). The lack of this neuropeptide at the CCK_8-receptor level during diazepam treatment, could possibly induce an increased number of available binding sites. Consequently, it could be thought that repeated diazepam administration induces a decrease of the CCK release or a reduction of neuronal responsiveness to CCK, leading to CCK accumulation in neurons. Concurrently, the number of CCK-binding sites could increase during dependency, and then withdrawal may be associated with an overflow of CCK release, leading to gastrointestinal disturbances. The fact that devazepide and L365260 had no effect in diazepam-dependent rats not given flumazenil supports the hypothesis that CCK_8 and CCK receptors are involved, especially during withdrawal states, but not during dependency.

The increase of CCK_8-binding sites observed in rat brain during benzodiazepine treatment (Yaksh et al 1987) and the abrupt CCK_8 release associated with withdrawal may also be responsible for the anxiogenesis. Consistent with this hypothesis, systemic administration of caerulein results in a significant decrease in the exploratory activity of mice and this effect is blocked by proglumide, a CCK_8 antagonist, indicating the participation of CCK_8 receptors (Harro et al 1990b). Moreover, the capacity of L365260, a CCK_B-receptor antagonist, to block duodenal and colonic disturbances induced by diazepam-withdrawal, is in good agreement with a previous study which shows the ability of CI-988, a selective CCK_B-receptor antagonist, to block behavioural benzodiazepine withdrawal effects (Singh et al 1992a). Many studies support the findings that CCK_8 plays a major role in anxiogenesis, and CCK_B-receptor antagonists exhibit potent anxiolytic activity in several animal models. For example, CI-988 and PD 135-158, two CCK_B antagonists, produce anxiolytic effects in the mouse black/white box test (Hughes et al 1990) and the elevated X-maze test (Costall et al 1991). CI-988 (PD134308) increases responses of squirrel monkeys (Powell & Barret 1991) and produces an anxiolytic-like action in three different rodent tests of anxiety (Singh et al 1992b). L365260 reverses the natural aversion of mice for the open arms of an elevated-plus maze (Rataud et al 1991).

Finally, our findings indicate that gut disturbances

observed after abrupt benzodiazepine withdrawal are probably linked to the central release of CCK_8, acting on central CCK receptors; this released CCK_8 may also be responsible for excessive anxiety observed during benzodiazepine withdrawal.

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