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ORIGINAL INVESTIGATION

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Discriminative stimulus effects of flumazenil in untreated and in diazepam-treated rhesus monkeys

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Abstract *Rationale:* Long-term use of benzodiazepine agonists can have adverse effects (e.g., development of dependence), thereby limiting their clinical usefulness. *Objectives:* The goal of the current study was to examine the discriminative stimulus effects of flumazenil in untreated and diazepam-treated monkeys to determine whether this type of procedure could be used to examine benzodiazepine dependence. *Methods:* Flumazenil (0.32 mg/kg s.c.) was established as a discriminative stimulus in eight monkeys receiving 5.6 mg/kg/day of diazepam (p.o.); four responded under a fixed ratio (FR)5 schedule of stimulus-shock termination (SST) and four responded under a FR5 schedule of food presentation. For comparison, 1.0 mg/kg flumazenil (s.c.) was established as a discriminative stimulus in four untreated monkeys responding under a FR5 schedule of SST. *Results:* Flumazenil dose-dependently increased responding on the flumazenil-appropriate lever in all monkeys. In diazepam-treated monkeys, Ro 15-4513, ethyl beta-carboline-3-carboxylate and bretazenil substituted for flumazenil with pentylenetetrazole substituting in some monkeys; other drugs failed to substitute for flumazenil. Acute administration of 10.0 mg/kg diazepam (s.c.) shifted the flumazenil dose–effect curve threefold to the right of the control dose–effect curve. Temporary suspension of diazepam treatment produced a time-related increase in flumazenil-lever responding that was reversed by diazepam. In untreated monkeys, midazolam substituted for flumazenil, with other drugs, including those with primary mechanisms of action at non-γ-aminobutyric $acid_A$ receptors, substituting in some monkeys. Ro 15-4513 did not substitute in any untreated monkey. *Conclusions:* The flumazenil discriminative stimulus appears to be pharmacologically selective in treated monkeys with only negative and low efficacy positive modulators substituting for flumazenil; in contrast, a variety of drugs substitute for flumazenil in untreated monkeys. This apparent difference in selectivity suggests that diazepam treatment modifies the flumazenil discriminative stimulus perhaps due to the development of dependence.

Key words Diazepam · Flumazenil · Benzodiazepine · Dependence · Drug discrimination · Rhesus monkey

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Introduction

Benzodiazepines have become the primary pharmacotherapy for anxiety and insomnia; unfortunately, benzodiazepines can also produce adverse effects that influence their clinical use (Woods and Winger 1995). For example, termination of long-term benzodiazepine treatment can result in the emergence of a withdrawal syndrome (Busto et al. 1986b). Another adverse effect of benzodiazepines is their abuse potential (Busto et al. 1986a). Although the incidence of benzodiazepine abuse is small compared with either the therapeutic use of benzodiazepines or the abuse of other classes of drugs, certain populations, such as opioid abusers (Barnas et al. 1992; San et al. 1993; Gutiérrez-Cebollada et al. 1994), are highly prone to benzodiazepine abuse and are susceptible to developing dependence.

Because dependence liability can limit the clinical use of benzodiazepines, a better understanding of this effect might improve treatment of anxiety and insomnia as well as decrease the severity of withdrawal. A number of studies have demonstrated the emergence of a withdrawal syndrome in nonhuman primates following either termination of benzodiazepine treatment (Yanagita and Takahashi 1973; Lukas and Griffiths 1982) or administration of a benzodiazepine antagonist (Lukas and Griffiths 1982, 1984). One procedure that has been used

to study opioid withdrawal involves treating subjects chronically with an agonist and training them to discriminate a pharmacological antagonist (Gellert and Holtzman 1979; Valentino et al. 1983). This procedure was also used to examine the discriminative stimulus effects of flumazenil in chlordiazepoxide (CDP)-treated subjects. In rats treated with 100 mg/kg/day CDP, termination of treatment produced responding on both the flumazenil- and saline-appropriate levers and re-administration of CDP resulted in predominantly saline-lever responding (Emmett-Oglesby and Rowan 1991), indicating that flumazenil can be established as a discriminative stimulus and suggesting that the stimulus might be related to withdrawal. However, in rhesus monkeys receiving a smaller dose of CDP (3.2 mg/kg/12 h) and discriminating flumazenil, suspension of CDP treatment did not result in flumazenil-appropriate responding (France and Gerak 1997); treatment conditions that have been shown to produce dependence under other conditions (Yanagita and Takahashi 1973) might be required to obtain flumazenil-lever responding when agonist treatment is terminated in rhesus monkeys.

A variety of γ-aminobutyric acid$_A$ ($GABA_A$) modulators have been established as discriminative stimuli, and, with some exceptions (e. g. Ator and Griffiths 1997), in untreated human as well as non-human primates (Spealman 1985; Oliveto et al. 1994; Ator and Griffiths 1997), the discriminative stimulus effects of positive $GABA_A$ modulators appear to be pharmacologically similar. Flumazenil also has been used as a discriminative stimulus in untreated subjects. Interestingly, doses of flumazenil that produce discriminative stimulus effects are not markedly different in untreated (De Vry and Slangen 1985b) and in CDP-treated rats (Emmett-Oglesby and Rowan 1991). One difference between untreated rats and those receiving CDP chronically is the apparent selectivity of the flumazenil stimulus for negative $GABA_A$ modulators in CDP-treated rats. For example, in CDP-treated rats, the negative $GABA_A$ modulator pentylenetetrazole (PTZ) substitutes for flumazenil and CDP does not substitute (Emmett-Oglesby and Rowan 1991); in untreated rats, both negative and positive $GABA_A$ modulators, including PTZ and CDP, produce flumazenil-lever responding (De Vry and Slangen 1985a). Thus, chronic CDP treatment might modify the pharmacological selectivity of the flumazenil discriminative stimulus.

To determine whether the discriminative stimulus effects of flumazenil differ in untreated and benzodiazepine-treated monkeys, flumazenil was established as a discriminative stimulus in 12 monkeys. Eight monkeys received the largest dose of diazepam (5.6 mg/kg/day, p.o.) that did not produce marked sedation (unpublished observation); a similar dose has been shown to produce dependence in rhesus monkeys after 12 days of treatment (Gallager et al. 1986). Under some conditions, the effects of $GABA_A$ modulators are similar, regardless of the reinforcer that maintains responding (Spealman 1985); other studies have reported differential sensitivity to $GABA_A$ modulators depending on the reinforcer (e.g., food or shock; Barrett 1976; Ator 1979; Gerak and France 1997). In the current study, four monkeys responded for food and four responded to avoid shock. In order to compare the discriminative stimulus effects of flumazenil in benzodiazepine-treated and untreated monkeys, flumazenil was also established as a discriminative stimulus in four untreated monkeys.

Methods

Subjects

Eight juvenile male, three adult male and one adult female rhesus monkeys weighed between 4.5 kg and 13.0 kg. Monkeys were housed individually and maintained on a 14-h light/10-h dark schedule. Diet comprised primate chow (Harlan Teklad, High Protein Monkey Diet, Madison, Wis.), fresh fruit and peanuts; eight monkeys received food during experimental sessions. Sufficient quantities of food were provided either to maintain monkeys at 100% of their free-feeding weights (adults) or to allow for normal growth (juveniles). Two of the adult monkeys had restricted access to water in order to facilitate drinking of punch containing diazepam; in those monkeys, water was available 18 h per day. The other ten monkeys had free access to water. Of the eight monkeys receiving diazepam daily, seven had been treated chronically with CDP in previous experiments (unpublished observations), with three discriminating flumazenil from saline while receiving CDP daily (France and Gerak 1997); one monkey had received opioids and nonopioids in previous studies (Gerak et al. 1994). The last diazepam-treated monkey and the four untreated monkeys were experimentally naive prior to this study. Sessions were conducted 7 days per week.

Apparatus

Monkeys were seated in chairs that provided restraint at the neck. Chairs were placed in ventilated, sound-attenuating chambers equipped with three response levers, an array of stimulus lights and a cup to which food pellets could be delivered from a dispenser located outside of the chamber. For monkeys treated daily with diazepam, the center lever was retracted (inaccessible) throughout the experiment. For monkeys responding under a schedule of stimulus-shock termination (SST), feet were secured in shoes containing brass electrodes through which a brief electric shock (250 ms, 3 mA) could be delivered from a.c. shock generators located adjacent to chambers. An interface (MedAssociates, St. Albans, Vt.) connected the chambers to a microprocessor which controlled experimental events and collected data.

Procedure

Flumazenil discrimination in diazepam-treated monkeys responding under a schedule of SST

Four monkeys were treated daily with 5.6 mg/kg diazepam (p.o.) and discriminated 0.32 mg/kg flumazenil (s.c.) from vehicle while responding under a fixed ratio (FR)5 schedule of SST; diazepam was administered 3 h prior to sessions. Initially, sessions comprised a single 25-min cycle. The first 15 min of the cycle was a time-out period, during which the chamber was dark and responses had no programmed consequence; the last 10 min was a response period during which the schedule of SST was in effect. At the end of the time out, red stimulus lights were illuminated and shock was scheduled to occur every 10 s. Monkeys could extinguish stimulus lights and postpone the shock schedule for 30 s by emitting five responses (FR5) on the lever designated correct according to the injection administered during the first minute of the time out. For two monkeys, responding on the left lever postponed

shock following administration of flumazenil and responding on the right lever postponed shock following administration of vehicle; for the other two monkeys, the lever designations were reversed. Responding on the incorrect lever reset the response requirement on the correct lever. When monkeys satisfied the response requirement, a 30-s time out occurred, after which the chamber was again illuminated and the schedule of SST was in effect. Sessions ended after 10 min or ten shocks, whichever occurred first.

Monkeys were trained until the following criteria were satisfied for five consecutive or six of seven sessions: 80% or more of the total responses on the correct lever and fewer than five responses on the incorrect lever prior to completing the first response requirement. Test sessions were identical to training sessions except that responding on either lever postponed shock. Under test conditions, a dose–effect curve was determined for flumazenil; on separate occasions, various doses (0.0032–0.32 mg/kg) were administered during the first minute of the cycle. In addition, the time course of flumazenil was determined by administering the training dose (0.32 mg/kg) at various times (5–180 min) prior to the response period.

Once the flumazenil dose–effect and time–effect curves had been determined twice, sessions were changed to multiple 15-min cycles. The first 10 min of each cycle was a time out and the remaining 5 min comprised a response period during which the FR5 schedule of SST was in effect; the response period ended after 5 min or four shocks, whichever occurred first.

Testing criteria were identical to those described above and initially had to be satisfied during all cycles for five consecutive or six of seven sessions; these criteria were satisfied by three monkeys. The test compound was administered at the beginning of each cycle, with the cumulative dose increasing by 0.25 or 0.5 log units per injection, up to doses that either produced 80% or greater flumazenil-lever responding or decreased response rates sufficiently to result in the delivery of shock. After the flumazenil dose–effect curve was re-determined, substitution studies were conducted with the following drugs: the negative $GABA_A$ modulators Ro 15-4513, ethyl beta-carboline-3-carboxylate (β-CCE), and PTZ; the positive $GABA_A$ modulators triazolam, midazolam, pentobarbital and bretazenil; the *N*-methyl-D-aspartate (NMDA) antagonist ketamine; the κ-opioid agonist spiradoline; and cocaine. To determine whether a positive $GABA_A$ modulator modifies the effects of flumazenil, 10.0 mg/kg diazepam (s.c.) was administered 120 min after the normal daily dose of diazepam (p.o.) and 60 min prior to flumazenil.

To assess whether discontinuation of diazepam treatment results in flumazenil-lever responding, monkeys drank drug-free punch 3 h prior to sessions comprising four cycles during which vehicle was administered. Vehicle was substituted for diazepam for three consecutive days; on the third day, vehicle was administered on the first cycle followed by increasing doses of diazepam (s.c.).

Flumazenil discrimination in diazepam-treated monkeys responding under a schedule of food presentation

Four monkeys were treated daily with 5.6 mg/kg diazepam (p.o.) and discriminated flumazenil (s.c.) from vehicle while responding under a FR5 schedule of food presentation. For three monkeys, the training dose of flumazenil was 0.32 mg/kg; for the fourth monkey, the training dose was 0.178 mg/kg, because larger doses disrupted responding in that monkey. The conditions used in this study were similar to those described above. Initially, monkeys responded under a single-cycle procedure with a 15-min time out followed by a 10-min response period, during which green lights were illuminated, and monkeys received a 300-mg banana-flavored pellet (Bio-Serv, Frenchtown, N.J.) after completing the FR requirement on the appropriate lever. Stimulus lights remained illuminated and the FR5 schedule was in effect until 10 min elapsed or the monkey received 50 pellets.

The testing criteria were identical to those described above; once satisfied, flumazenil dose–effect and time–effect curves were determined. The schedule was then changed to a multiple-cycle procedure with temporal parameters identical to those described above. Monkeys could receive up to ten food pellets per cycle; when the time required to receive the maximum number of food pellets was less than 5 min, the remainder of the response period was a time out.

Under the multiple-cycle procedure, only the three monkeys that discriminated 0.32 mg/kg of flumazenil satisfied the testing criteria during each cycle of five consecutive or six of seven sessions. Substitution studies were conducted using the same drugs and dosing conditions as described above.

Flumazenil discrimination in untreated monkeys responding under a schedule of SST

Four untreated monkeys responded under a FR5 schedule of SST and discriminated flumazenil from vehicle (s.c.). Initially, experimental sessions comprised a single 18-min cycle with the first 10 min being a time out. The next 2 min was a response period, during which a green light was illuminated above the center lever and a FR10 schedule of food presentation was in effect on the center lever; responding on other levers had no programmed consequence. The response period ended when monkeys received ten pellets or 2 min had elapsed; at that time, the green light was extinguished and the center lever was retracted. The 2-min response period was followed by a 1-min time out, then a 5-min response period; the beginning of the second response period was signaled by illumination of red lights, and monkeys discriminated between flumazenil and vehicle while responding under a FR5 schedule of SST. The session ended after 5 min or the delivery of ten shocks, whichever occurred first.

Initially, the training dose of flumazenil was 0.1 mg/kg. After monkeys had received this dose during 30 training sessions, with the total number of training sessions varying among subjects depending on the number of intervening vehicle training sessions, the dose was increased to 0.32 mg/kg, and, thereafter, the training dose was incremented by 0.25 log units after 30 sessions had been conducted with the same dose of flumazenil. After monkeys had received 1.0 mg/kg flumazenil for 30 sessions, stimulus control had been established in only two of the four monkeys. Because of limited solubility and availability of flumazenil, further increases in training dose were not possible; in order to improve the likelihood of obtaining stimulus control in all monkeys, the food component was removed from the schedule. The modified training sessions comprised a 10-min time out followed by a 5-min response period, during which the FR5 schedule of SST was in effect. All other conditions were identical to those described for the SST component of the multiple schedule. Under these conditions, all monkeys satisfied the testing criteria described for diazepam-treated monkeys. The flumazenil dose–effect curve was determined twice, while dose–effect curves for other drugs were determined once. A limited supply of bretazenil precluded evaluation of this compound in untreated monkeys.

Drugs

The vehicle for oral administration of diazepam was fruit punch (seven monkeys) or apple juice (one monkey) combined with Suspending agent K (Bio-Serv) so that a concentration of 1 g suspending agent per liter of punch was achieved. Tablets containing 10 mg diazepam (Zenith Laboratories, Inc., Northvale, N.J.) were crushed and mixed in a blender with the vehicle. The diazepam mixture was administered using a 12 G drinking needle attached to a 60 cc syringe. To obtain a dose of 5.6 mg/kg diazepam, a standard concentration of diazepam was prepared with different volumes administered among monkeys depending on body weights. The diazepam mixture was prepared immediately before administration.

The following drugs were administered s.c.: cocaine hydrochloride (Research Technology Branch, NIDA, Rockville, Md.),

flumazenil and bretazenil (F. Hoffmann LaRoche Ltd., Basel, Switzerland), ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, Iowa), midazolam hydrochloride (Roche Pharma, Inc., Manati, Puerto Rico), spiradoline mesylate (The Upjohn Co., Kalamazoo, Mich.), pentobarbital sodium (Sigma Chemicals, St. Louis, Mo.), Ro 15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine-3-carboxylate), PTZ, β-CCE, triazolam and diazepam (RBI, Natick, Mass.). Flumazenil, bretazenil and Ro 15-4513 were dissolved in a vehicle comprising 40% propylene glycol, 50% saline and 10% ethanol. Diazepam, triazolam and β-CCE were dissolved in a vehicle comprising 20% emulphor, 10% ethanol and 70% water. Cocaine, pentobarbital, PTZ and spiradoline were dissolved in sterile water. Commercially prepared solutions of ketamine and midazolam were diluted with water. For s.c. administration, drugs were typically administered in a volume of 0.1 ml/kg body weight. Doses are expressed in terms of the forms listed above.

Data analyses

Control response rates were determined individually for each subject. For single-cycle procedures or flumazenil training sessions, control rates represent the average of ten consecutive training sessions, during which either vehicle or the training dose of flumazenil was administered and subjects satisfied the testing criteria. For multiple-cycle procedures, vehicle control rates were determined by averaging the rates for all cycles within a session during which only vehicle or "sham" injections were administered, then averaging these means across ten sessions; for response rates to be included in the overall mean, monkeys had to satisfy the testing criteria during all cycles within a session. A paired *t*-test was used to analyze differences in the control response rates among conditions ($P<0.05$). The percentage of responses emitted on the flumazenil lever (% DR) is plotted as a function of dose or time; drugs that produced 80% or greater responding on the flumazenil-appropriate lever were considered to have substituted for flumazenil. Response rates for each component are expressed as a percentage of vehicle control rates and are plotted as a function of dose or time. When response rates decreased to less than 25% of control for an individual monkey, the discrimination data were not included in analyses. Because results of substitution studies were indistinguishable between the two groups of diazepam-treated monkeys, some of the data from these two groups were combined with each dose–effect curve representing data from six monkeys (three from each group). When dose–effect curves were determined twice, the data for individual subjects were averaged first; subsequently, the mean (±SEM) among subjects was calculated with the SEM representing the variance among subjects. Estimates of the dose required to produce 50% flumazenil-lever responding (ED_{50}) were determined by linear regression when three appropriate data points were available, otherwise by interpolation.

Results

Stimulus control with flumazenil was considered adequate for testing after: 104.5 (range 82–121) sessions in the diazepam-treated monkeys responding under the schedule of SST; 146.5 (range 136–167) sessions in the diazepam-treated monkeys responding under the schedule of food presentation; and 226.8 (range 143–282) sessions in the untreated monkeys responding under the schedule of SST. In three diazepam-treated monkeys responding under the schedule of SST, an additional 20.0 (range 12–29) training sessions were required to re-establish stimulus control under the multiple-cycle procedure. Three diazepam-treated monkeys responding under the schedule of food presentation needed an average of 40.3 (range 10–81) sessions to re-establish stimulus control; there was no significant difference in the number of sessions-to-criteria between the two groups.

There were no significant differences between the group mean response rates obtained during vehicle training sessions and those obtained during flumazenil training sessions for monkeys responding under the schedule of SST; however, response rates obtained during flumazenil training cycles were significantly lower than rates obtained during vehicle training cycles in diazepam-treated monkeys responding to receive food under the multiple-cycle procedure (Table 1). Although response rates in the untreated monkeys tended to be higher under both training conditions than in monkeys treated with diazepam, there was no significant difference in mean response rate among the groups.

In all groups, flumazenil dose-dependently increased responding on the flumazenil lever with 0.1 mg/kg producing more than 80% flumazenil-lever responding in diazepam-treated monkeys responding for food, and doses larger than 0.1 mg/kg producing more than 80% flumazenil-lever responding in the other groups (upper panel, Fig. 1). The ED_{50} values for flumazenil were not different among the three groups: 0.040±0.009 mg/kg in diazepam-treated monkeys responding under a schedule of SST, 0.042±0.006 mg/kg in diazepam-treated monkeys responding under a schedule of food presentation, and 0.083±0.028 mg/kg in untreated monkeys. Flumaze-

Table 1 Control response rates (responses/s) obtained during vehicle or flumazenil training sessions in monkeys discriminating flumazenil

Group	Vehicle Single cycles	Vehicle Multiple cycles	Flumazenil[a] Single cycles	Flumazenil Multiple cycles
Diazepam-treated SST	1.47±0.33[b]	1.52±0.46	1.25±0.19	1.26±0.22
Diazepam-treated food	1.46±0.29	1.33±0.16	1.14±0.06	0.79±0.13*
Untreated SST	1.69±0.28	n.s.	1.61±0.08	n.s.

*$P<0.05$ for response rates obtained during flumazenil training sessions compared with response rates obtained during multiple-cycle vehicle training sessions

[a] The training dose of flumazenil was 0.32 mg/kg for diazepam-treated monkeys and 1.0 mg/kg for untreated monkeys

[b] Mean response rates (±SEM) obtained during ten single-cycle or ten multiple-cycle training sessions, with each multiple-cycle session comprising at least four cycles during which vehicle was administered. Values represent the average response rates for three (diazepam-treated) or four (untreated) monkeys

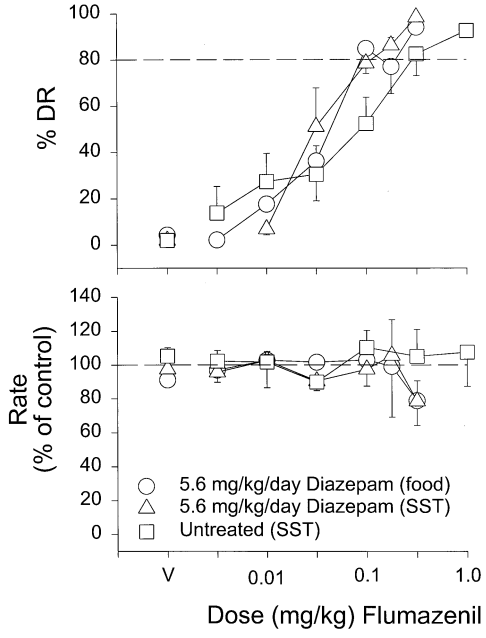

Fig. 1 Discriminative stimulus effects of flumazenil in diazepam-treated and untreated monkeys. One group of monkeys (*n*=3) received 5.6 mg/kg/day diazepam (p.o.) and discriminated 0.32 mg/kg of flumazenil s.c. while responding under a fixed ratio (FR)5 schedule of food presentation (○), and a second group of monkeys (*n*=4) received the same daily dose of diazepam and discriminated the same dose of flumazenil while responding under a FR5 schedule of SST (△). The third group (*n*=4) did not receive diazepam and discriminated 1.0 mg/kg flumazenil while responding under a FR5 schedule of SST (□). Dose-effect curves were determined using a single-cycle procedure with each curve determined twice in each monkey. *Abscissae*: dose expressed as milligrams per kilogram body weight; point above *V* represent the effects of vehicle. *Ordinates*: percentage of total responding (±SEM) that occurred on the flumazenil-appropriate lever (% DR, *upper panel*) and response rate expressed as a percentage of control rates (±SEM) (*lower panel*)

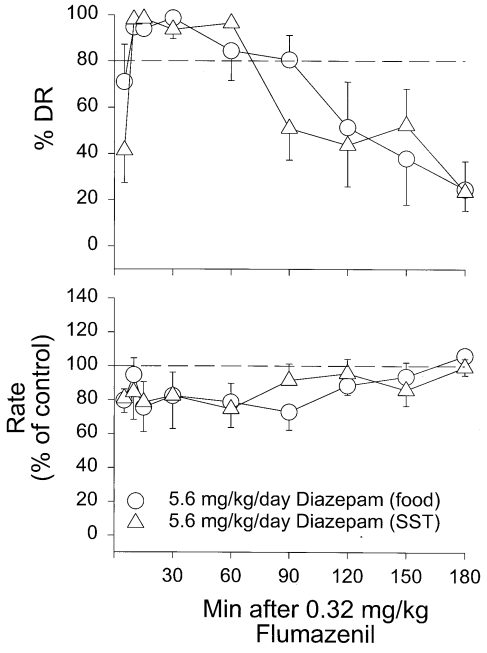

Fig. 2 Time course for the discriminative stimulus effects of the training dose (0.32 mg/kg) of flumazenil in the two groups of diazepam-treated monkeys. *Abscissae*: time after injection of flumazenil in minutes. See Fig. 1 for other details

nil did not markedly alter response rates (lower panel, Fig. 1).

Diazepam-treated monkeys emitted some responses on the flumazenil lever 5 min after administration of the training dose (0.32 mg/kg) of flumazenil with more than 80% flumazenil-lever responding occurring from 10 min to 60 min after administration (upper panel, Fig. 2). After 60 min, responding on the flumazenil lever decreased with monkeys responding predominantly on the vehicle lever 180 min after receiving flumazenil. Response rates were not markedly decreased (lower panel, Fig. 2).

Once the flumazenil dose–effect and time–effect curves were completed in diazepam-treated monkeys, the procedure was changed to multiple cycles. Flumazenil was slightly less potent with the cumulative-dosing procedure than with the single-cycle procedure. In diazepam-treated monkeys responding under the SST schedule, the mean ED_{50} obtained with cumulative dosing (0.099±0.043 mg/kg) was twofold larger than the ED_{50} obtained with single cycles; in diazepam-treated monkeys responding under the food schedule, the mean ED_{50} (0.062±0.01 mg/kg) was 1.5-fold larger than the ED_{50} obtained with single cycles. Periodic re-determination of the flumazenil dose–effect curve indicated that there was no change in the potency of flumazenil in the 2 years since stimulus control was established.

Substitution studies were conducted with drugs that are known to have actions at the $GABA_A$ receptor complex as well as with drugs whose primary mechanisms of action are not at this receptor complex. Drugs that substituted for flumazenil in all diazepam-treated monkeys included the negative $GABA_A$ modulators Ro 15-4513 and β-CCE, and the positive modulator bretazenil (upper panel, Fig. 3); there was no difference in substitution between the two groups. The rank order potency of these compounds was flumazenil = Ro 15-4513 (ED_{50}=0.10±0.01) > bretazenil (ED_{50}=0.19±0.06) > β-CCE (ED_{50}=0.58±0.18). None of the four drugs markedly decreased response rates (lower panel, Fig. 3). In contrast, the $GABA_A$ antagonist PTZ substituted for flumazenil in three of the six diazepam-treated monkeys (upper panel, Fig. 3); of the three monkeys responding more than 80% on the flumazenil-appropriate lever, only one responded under the schedule of food presentation. Up to a dose of 32.0 mg/kg, PTZ did not decrease response rates in any subject; larger doses were not studied to avoid toxicity.

Drugs from other pharmacological classes failed to substitute for flumazenil in any diazepam-treated monkey (data not shown). Ketamine failed to substitute for flumazenil up to the dose that eliminated responding (3.2 mg/kg; *n*=6), although 1.0 mg/kg produced some (<50%) flumazenil-lever responding in three monkeys. Up to

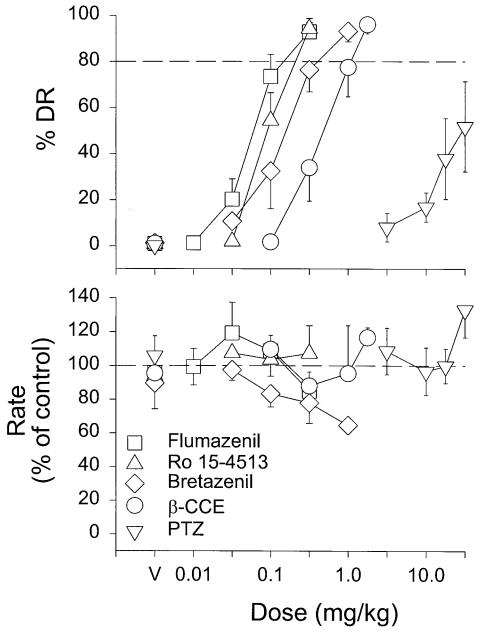

Fig. 3 Discriminative stimulus and rate-decreasing effects of γ-aminobutyric acid$_A$ (GABA$_A$) modulators that substituted for flumazenil in diazepam-treated monkeys. Data obtained in monkeys responding under both the schedule of food presentation and stimulus-shock termination (SST) were averaged to generate the dose–effect curves shown. The flumazenil dose–effect curve was determined twice in six monkeys, while those for Ro 15-4513 and ethyl beta-carboline-3-carboxylate (β-CCE) were determined once in six monkeys and that for bretazenil was determined once in five monkeys; for all curves, three of the monkeys responded under the schedule of SST. See Fig. 1 for other details

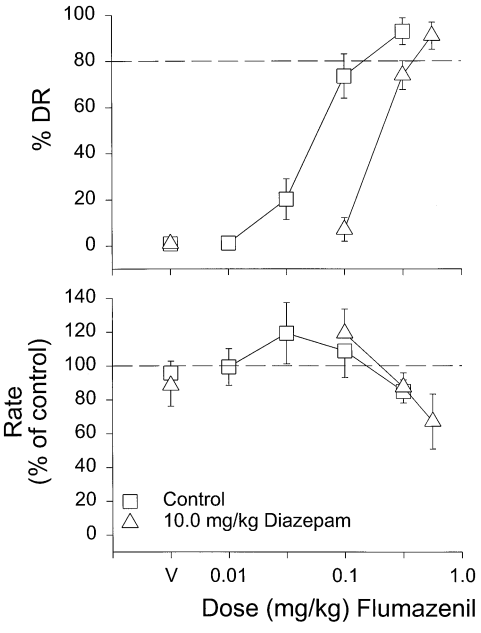

Fig. 4 Discriminative stimulus and rate-decreasing effects of flumazenil in diazepam-treated monkeys that received a dose of 10.0 mg/kg diazepam (s.c.) 45 min before the start of the session and 60 min before administration of the first dose of flumazenil. The dose–effect curve for flumazenil in the presence of the supplemental dose of diazepam was determined once in five monkeys. See Fig. 3 for other details

doses that eliminated responding, neither the κ-agonist spiradoline (0.01–0.1 mg/kg; *n*=6) nor the positive GABA$_A$ modulator pentobarbital (3.2–32.0 mg/kg; *n*=5) produced more than 10% flumazenil-lever responding. Finally, monkeys responded predominantly on the vehicle lever following administration of triazolam (0.032–1.0 mg/kg; *n*=6), midazolam (1.0–5.6 mg/kg; *n*=5) or cocaine (0.32–3.2 mg/kg; *n*=6).

When administered acutely prior to the session (in monkeys that had received 5.6 mg/kg of diazepam p.o. 3 h earlier), diazepam attenuated the discriminative stimulus effects of flumazenil. Monkeys responded predominantly on the vehicle lever at control rates 45 min after administration of a dose of 10.0 mg/kg of diazepam (Δ above V, Fig. 4). However, the flumazenil dose–effect curve was shifted threefold to the right following diazepam administration (ED$_{50}$=0.23±0.03; Δ, upper panel, Fig. 4).

Diazepam treatment was suspended in four monkeys (two in each group) for 3 days, and monkeys drank vehicle 3 h prior to sessions. There was a time-related increase in responding on the flumazenil lever, with 76% of the total responses occurring on the flumazenil lever on day 3 (Fig. 5). Increasing doses of diazepam (s.c.), which were administered after the vehicle cycle on day 3, reversed flumazenil-lever responding with monkeys responding predominantly on the vehicle lever after receiving 1.0 mg/kg diazepam (upper panel, Fig. 5). Response rates were decreased to less than 65% of control in one monkey responding under the food schedule and in one monkey responding under the SST schedule; this effect was partially reversed by diazepam (lower panel, Fig. 5).

Substitution studies were conducted in four untreated monkeys discriminating flumazenil while responding under the single-cycle procedure. Midazolam produced more than 80% flumazenil-lever responding in all monkeys at doses that did not decrease response rates (diamonds, Fig. 6). Spiradoline, triazolam, pentobarbital and β-CCE substituted for flumazenil in some monkeys (upper right panel, Fig. 6), whereas ketamine, cocaine and Ro 15-4513 failed to substitute in any subject (data not shown). Spiradoline (∇, lower right panel, Fig. 6), triazolam (Δ, lower right panel, Fig. 6) and ketamine (3.2 mg/kg; data not shown) were studied up to doses that markedly reduced response rates in all subjects. The largest doses of pentobarbital (lower right panel, Fig. 6) and cocaine (data not shown) decreased response rates in some subjects. Ro 15-4513 (data not shown) and β-CCE (○, lower right panel, Fig. 6) did not modify response rates in any subject. Solubility or toxicity of these compounds precluded administration of larger doses.

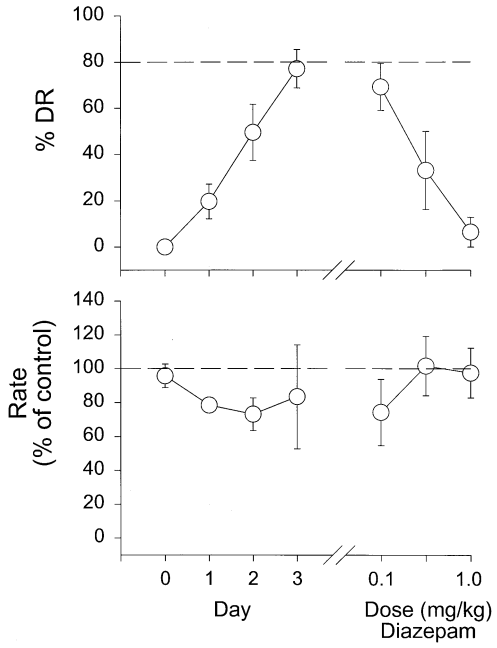

Fig. 5 Discriminative stimulus and rate-decreasing effects during a period of diazepam deprivation. On day 0, monkeys consumed the normal dose of diazepam 3 h prior to the session. On days 1, 2 and 3, monkeys consumed vehicle 3 h prior to the session. Experimental sessions conducted on days 0, 1 and 2 comprised four cycles during which only vehicle was administered. The experimental session conducted on day 3 comprised a vehicle cycle followed by cycles during which diazepam was administered (s.c.) using a cumulative-dosing procedure. Data from four monkeys are included in the figure. *Abscissae*: the *left portion* represents the days since the last administration of diazepam and the *right portion* represents increasing doses of diazepam administered cumulatively 3 days after the last daily dose of diazepam. See Fig. 1 for other details

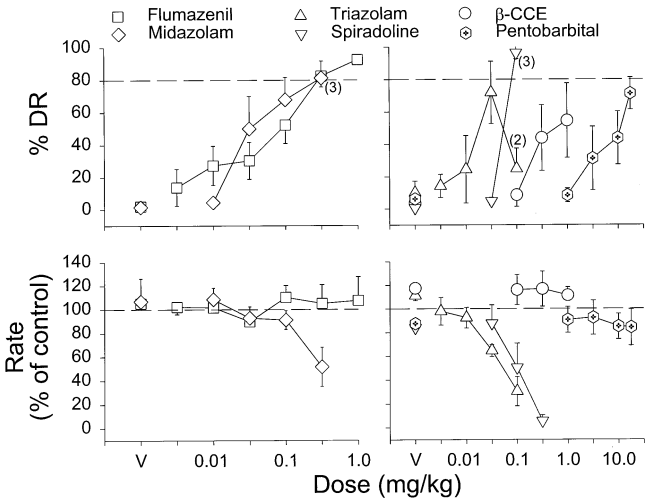

Fig. 6 Discriminative stimulus and rate-decreasing effects of drugs that substituted for flumazenil in untreated monkeys. Midazolam, which substituted for flumazenil in four monkeys, is shown in the *left panels* and drugs that substituted for flumazenil in one to three monkeys are shown in the *right panels*. The flumazenil dose–effect curve determined twice in four monkeys with the remaining dose–effect curves were determined once in four monkeys. *Numbers in parentheses* indicate that response rates were less than 25% of control for at least one monkey and, therefore, the discrimination data obtained with that dose for that monkey were excluded from analyses; the number of monkeys included in the data point is shown in *parenthesis*. See Fig. 1 for other details

Discussion

Drug discrimination procedures have been used extensively to characterize abused drugs from many pharmacological classes (Kamien et al. 1993), and these procedures have also been adapted to study opioid dependence and withdrawal (Gellert and Holtzman 1979; France and Woods 1989). Despite the demonstrated utility of these procedures, there has been little effort devoted to evaluation of dependence on and withdrawal from other drugs using drug discrimination. The goal of the current study was to apply this discrimination procedure to another pharmacological class, the $GABA_A$ modulators, in order to determine the feasibility of using this procedure to examine benzodiazepine dependence and withdrawal.

One important feature of any drug discrimination procedure is pharmacological selectivity; if flumazenil-lever responding is related to benzodiazepine withdrawal, then the only drugs that should substitute for flumazenil in diazepam-treated monkeys are those that attenuate the effects of diazepam under other conditions, and thus would precipitate withdrawal in diazepam-treated subjects. The negative $GABA_A$ modulators Ro 15-4513 and β-CCE substituted for flumazenil in diazepam-treated monkeys, and doses that produced more than 80% flumazenil-lever responding were similar to doses of each that attenuated the ventilatory-depressant effects of diazepam (Gerak et al. 1998b). Like flumazenil, Ro 15-4513 and β-CCE have high affinity for benzodiazepine receptors (Braestrup et al. 1980; Möhler et al. 1984) and antagonize positive $GABA_A$ modulators under other conditions (Herling and Shannon 1982; Rees and Balster 1988; Sannerud et al. 1991). Another negative $GABA_A$ modulator, PTZ, substituted for flumazenil in some diazepam-treated monkeys, and it is possible that larger doses of PTZ would have substituted for flumazenil in other monkeys; however, larger doses could not be studied safely. PTZ acts directly at GABA receptors on the $GABA_A$-receptor complex and some effects of PTZ are similar to those produced by drugs acting at benzodiazepine receptors. For example, PTZ substitutes for flumazenil in CDP-treated rats (Emmett-Oglesby and Rowan 1991) and flumazenil substitutes for PTZ in diazepam-treated (Emmett-Oglesby et al. 1983) and not in untreated rats (Rowan and Lucki 1992). Thus, the flumazenil discriminative stimulus appears to be pharmacologically selective for negative $GABA_A$ modulators. The pharmacological selectivity of flumazenil is further demonstrated by the failure of drugs from other classes to substitute for flumazenil in diazepam-treated monkeys.

Positive $GABA_A$ modulators with less efficacy than diazepam can attenuate the effects of diazepam under

some conditions; such low efficacy positive modulators should also precipitate withdrawal and substitute for flumazenil in diazepam-treated subjects. Drugs with efficacy equal to or greater than diazepam should neither attenuate diazepam nor substitute for flumazenil. The positive $GABA_A$ modulators triazolam and midazolam did not produce flumazenil-lever responding up to doses 30-fold larger than those required to decrease ventilation in monkeys (Gerak et al. 1998a). Rate-decreasing effects of these positive modulators further indicated that active doses were studied in diazepam-treated monkeys. For example, in untreated monkeys, 0.1 mg/kg of triazolam markedly decreased responding, whereas in diazepam-treated monkeys 1.0 mg/kg of triazolam did not have rate-decreasing effects. In contrast, the low efficacy agonist bretazenil (Martin et al. 1988) substituted for flumazenil in all diazepam-treated monkeys. These data support the notion that bretazenil has less efficacy than diazepam, triazolam or midazolam and further indicate the pharmacological selectivity of the flumazenil discriminative stimulus.

Pharmacological selectivity of the flumazenil discriminative stimulus does not provide direct evidence for a relationship between this stimulus and benzodiazepine withdrawal. One requirement for relating flumazenil-lever responding to withdrawal is to show that effects obtained with an antagonist are mimicked by discontinuation of chronic treatment. Temporary suspension of diazepam treatment resulted in a time-related switch in responding from the vehicle lever to the flumazenil lever with flumazenil-lever responding reversed by subsequent administration of diazepam. The fact that comparable effects were obtained following administration of flumazenil and discontinuation of diazepam treatment might reflect the development of dependence.

Another approach for determining whether diazepam treatment modifies the flumazenil stimulus is to compare the effects of flumazenil in untreated monkeys with those observed in diazepam-treated monkeys. Untreated rats and pigeons (De Vry and Slangen 1985b; Rowan and Lucki 1992; Wong et al. 1993) have been trained to discriminate flumazenil, and, in the current study, stimulus control was established four untreated monkeys. One difference between untreated and diazepam-treated monkeys appears to be the pharmacological selectivity of the flumazenil stimulus; for some drugs, substitution data in untreated monkeys were not consistent among the subjects. For example, the positive $GABA_A$ modulator midazolam substituted for flumazenil in all monkeys and other positive modulators, triazolam and pentobarbital, substituted in some monkeys. The negative $GABA_A$ modulator β-CCE, as well as a drug whose primary mechanism of action is not related to the $GABA_A$ receptor complex (spiradoline), also produced flumazenil-lever responding in some untreated monkeys. Although there were some drugs that failed to substitute in any monkey, including a negative $GABA_A$ modulator (Ro 15-4513), the fact that drugs with different mechanisms of action occasioned flumazenil-lever responding demonstrates that the flumazenil stimulus in untreated monkeys is not selective for negative $GABA_A$ modulators.

The apparent lack of pharmacological selectivity in the untreated monkeys is consistent with other studies that have examined flumazenil in untreated subjects. In pigeons and rats, some positive and negative $GABA_A$ modulators substitute for flumazenil (De Vry and Slangen 1985a; Rowan and Lucki 1992; Wong et al. 1993). It is possible that the stimulus effects of flumazenil in untreated subjects are mediated by more than one subtype of benzodiazepine receptor, including diazepam-insensitive receptors (Wong et al. 1993). Although the affinity of flumazenil is 50-fold greater at diazepam-sensitive receptors than diazepam-insensitive receptors, flumazenil has relatively high affinity at diazepam-insensitive receptors (K_i=45±5 nM; Wong et al. 1993). In contrast, diazepam as well as other benzodiazepines have low affinity for these receptors, which could account for the apparent lack of selectivity in untreated monkeys; however, the only drug that substituted in all untreated monkeys was midazolam, a drug with very low affinity for the diazepam-insensitive receptor (K_i>10000 nM; Wong et al. 1993). Little is known about endogenous compounds that act at benzodiazepine receptors and how the actions of endogenous substances might modify $GABA_A$ receptors; these interactions could have profound effects on the nature of the flumazenil stimulus in untreated animals.

One consistent feature among the wide variety of protocols that have been used to establish drugs as discriminative stimuli (Jarbe 1989) is that pharmacologically-specific stimulus effects can be identified and characterized (Overton 1991), i.e., the discriminative stimulus effects of drugs from one pharmacological class are different from those of drugs from other pharmacological classes. The positive $GABA_A$ modulator midazolam has been established as a discriminative stimulus in several species using either single- or multiple-cycle procedures (Spealman 1985; Evans and Johanson 1989; Lelas et al. 1999). Positive $GABA_A$ modulators reliably substitute for midazolam and drugs from other pharmacological classes do not substitute, indicating that the pharmacological selectivity of the midazolam discriminative stimulus is maintained despite differences in species, procedure and mode of administration. Thus, it is unlikely that procedural differences alone can account for the dramatic differences in selectivity of the flumazenil stimulus between untreated and diazepam-treated monkeys. An alternative explanation for these differences is that chronic diazepam treatment qualitatively modifies the discriminative stimulus effects of flumazenil.

Daily administration of diazepam can produce dependence in rhesus monkeys (Gallager et al. 1986); in the current study, withdrawal signs were not evident following administration of flumazenil or when monkeys were deprived of diazepam for 3 days, although flumazenil-lever responding was obtained under both conditions.

These initial studies were designed to test the feasibility of studying benzodiazepine dependence and withdrawal using drug discrimination procedures, and these results support the notion that, under the appropriate set of conditions, this procedure might be useful for study benzodiazepine withdrawal. However, it is also possible that the discriminative stimulus effects of flumazenil are unrelated to benzodiazepine withdrawal; instead, monkeys might be discriminating the presence or absence of diazepam administered 3 h prior to the session, with the vehicle lever associated with the presence of diazepam and the flumazenil lever associated with the absence of diazepam. Additional studies are warranted to investigate the relationship between flumazenil-lever responding and withdrawal.

Repeated administration of flumazenil has been shown to alter its potency in benzodiazepine-treated subjects (Lamb and Griffiths 1985; Gallager et al. 1986); however, the flumazenil discriminative stimulus was maintained for more than 2 years in diazepam-treated monkeys, with no indication that the stimulus was changing, and this consistency in effect over time has also been demonstrated in other studies (Emmett-Oglesby and Rowan 1991; France and Gerak 1997; Gerak and France 1997). Under some conditions, the effects of $GABA_A$ modulators vary depending on the reinforcer that maintains responding (Barrett 1976; Ator 1979; Gerak and France 1997); in the current study, the discriminative stimulus effects of flumazenil did not appear to vary depending on the reinforcer used. Moreover, the discriminative stimulus effects of flumazenil did not appear to vary among monkeys despite differences in experimental histories. With one exception (France and Gerak 1997), this is the only demonstration of stimulus control with an antagonist in a non-human primate treated daily with a positive $GABA_A$ modulator, and, given the consistency of the flumazenil stimulus in diazepam-treated monkeys and the differences in the flumazenil stimulus between treated and untreated monkeys, these results will provide the basis for future studies of the dependence potential of benzodiazepines and other $GABA_A$ modulators.

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