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Comparison of the behavioral effects of bretazenil and flumazenil in triazolam-dependent and non-dependent baboons

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Abstract

Behavioral effects of the benzodiazepine receptor partial agonist bretazenil were compared with those of the benzodiazepine receptor antagonist flumazenil under conditions in which three baboons received continuous intragastric (i.g.) infusion of vehicle and then continuous i.g. infusion of triazolam (1.0 mg/kg/day). In each condition, acute doses of flumazenil (0.01–3.2 mg/kg) and bretazenil (0.01–10.0 mg/kg) were administered every 2 weeks (beginning after 30 days of treatment in the triazolam-dependent condition). Food pellets were available during daily 20-h sessions. Following test injections, 60-min behavioral observations were conducted followed by a fine motor assessment. During chronic vehicle administration, neither drug produced changes in observed behaviors. Bretazenil increased pellets earned and time to complete the fine-motor task (10.0 mg/kg dose). During chronic triazolam dosing, both bretazenil and flumazenil precipitated benzodiazepine withdrawal syndromes, characterized by vomiting, tremors/jerks, and a decrease in pellets earned. Thus, bretazenil can function as an antagonist under conditions of benzodiazepine physical dependence.
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1. Introduction

A range of ligands bind to the benzodiazepine modulatory site on the gamma-aminobutyric acid $(GABA)_A$-benzodiazepine receptor and produce different pharmacological effects. Ligands that enhance $GABA_A$-stimulated chloride flux are called receptor agonists and function as anxiolytics. On the other end of the continuum are ligands that reduce $GABA_A$-stimulated chloride flux, have anxiogenic action, and are thus termed receptor inverse agonists. In the center of the continuum are ligands that prevent the binding of other ligands, but have little or no intrinsic effects and thus fit the classic definition of a receptor antagonist. Lying on the continuum between benzodiazepine receptor agonists and antagonists are ligands that potently bind the benzodiazepine site but have low to intermediate intrinsic efficacy and, as a result, have been called benzodiazepine receptor partial agonists (Haefely et al., 1990). The benzodiazepine receptor partial agonists have been of particular interest because preclinical studies have shown that they retained anxiolytic effects at doses lower than those that produced the undesirable effects of full benzodiazepine receptor agonists such as sedation and ataxia (Cooper et al., 1987; Haefely et al., 1990). Benzodiazepine receptor partial agonists also appear to be less likely to produce physiological dependence (Bronson, 1994; Jenck et al., 1992; Martin et al., 1995a; Moreau et al., 1991, 1990; Richards and Martin, 1998).

Bretazenil (Ro 16-6028) is a 1,4-benzodiazepine with a pharmacological profile characteristic of a benzodiazepine receptor partial agonist (Facklam et al., 1992a,b; Finn and Gee, 1993; Martin et al., 1988). Agonist-like effects have been reported in a number of procedures. For example, when compared to the full benzodiazepine receptor agonist

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diazepam, bretazenil potently produced anticonflict and anticonvulsant activity but only weak motor impairing activity and less ethanol potentiation (Facklam et al., 1992a; Martin et al., 1988). Similarly, in pigeons, bretazenil produced increases in punished responding that were comparable to or exceeded those observed following administration of full benzodiazepine receptor agonists (Kleven and Koek, 1999; Witkin et al., 1997, 2004). In drug discrimination studies in rats, bretazenil dose-dependently substituted for the full benzodiazepine receptor agonists midazolam (Acri et al., 1996; Ator, 1999; Sannerud and Ator, 1995) and chlordiazepoxide (Bronson and Chen, 1996), but only partially substituted for pentobarbital (Rowlett and Woolverton, 1998).

Bretazenil has also been reported to function as a competitive receptor antagonist, blocking the direct effects of full benzodiazepine receptor agonists in a manner similar to the benzodiazepine receptor antagonist flumazenil. When administered in combination with acute doses of a full benzodiazepine receptor agonist, bretazenil antagonized the effects of the agonist on motor behavior (Smith et al., 2004), shock-suppressed responding (Paronis and Bergman, 1999), defensive behaviors (Griebel et al., 1999), and response sequence acquisition and retention (Auta et al., 1995). Antagonism of the discriminative stimulus effects of full benzodiazepine receptor agonists has also been reported (e.g., Lelas et al., 1999). Thus, it appears that bretazenil can antagonize both anxiolytic and sedative/myorelaxant effects of a full benzodiazepine receptor agonist.

Despite demonstration that partial benzodiazepine receptor agonists antagonize the effects of full benzodiazepine receptor agonists under conditions of acute administration, it is unclear how they function under conditions of chronic benzodiazepine receptor agonist administration and physical dependence. Physical dependence following repeated administration of benzodiazepines has been well documented in both pre-clinical and clinical studies (for a review see Woods et al., 1992). In animals, administration of a competitive benzodiazepine receptor antagonist such as flumazenil produces a withdrawal syndrome that can include vomiting, anorexia, impairment of motor function, autonomic signs, and preconvulsive and convulsive phenomena (Woods et al., 1992). In addition, Sannerud et al. (1991) demonstrated that the agonist-like effects of midazolam were decreased while the inverse agonist-like effects of benzodiazepine receptor inverse agonist 3-carboethoxy-beta-carboline hydrochloride (β-CCE) were enhanced during chronic diazepam administration.

The goal of the current study was to determine whether bretazenil would produce agonist-like effects or precipitate withdrawal (i.e., antagonism) in subjects that were maintained on a dose of triazolam (1.0 mg/kg/day) that was sufficient to produce physical dependence. Previous studies in baboons using similar procedures and doses have shown that 2–4 weeks of chronic dosing with 1.0 mg/kg triazolam produces physical dependence as evidenced by both flumazenil-precipitated and spontaneous withdrawal (Ator et al., 2000; Weerts and Griffiths, 1999). The present study compared the behavioral effects of bretazenil with those of the benzodiazepine receptor antagonist flumazenil under conditions in which baboons received chronic administration of vehicle (non-dependent condition) or triazolam (triazolam-dependent condition). To minimize the number of nonhuman primates exposed to physical dependence conditions, a single-subject design was used (Bordens and Abbott, 1996; Krishef, 1991; Sidman, 1960). The use of a single-subject design is further justified by the fact that maintaining intragastric catheters, long-term chronic drug dosing, and systematic observational procedures in large nonhuman primates are very labor intensive and costly (Ator and Griffiths, 2003). Thus, in the current study, each subject served as his own control, and bretazenil and flumazenil doses were manipulated as needed to fully characterize individual drug dose–effect curves. First, doses of bretazenil and flumazenil were administered during the non-dependent condition and food-maintained operant responding, a fine-motor task, and observed behaviors were examined to determine the direct effects of each drug alone. Similar doses of bretazenil and flumazenil were again administered after the baboons had been maintained on chronic triazolam for at least 30 days and the same behavioral measures were evaluated for evidence of a precipitated withdrawal syndrome or enhancement of agonist effects. Under carefully controlled experimental conditions, the demonstration of robust effects in three or four subjects is usually sufficient to show scientifically meaningful results using a single-subject design (Ator and Griffiths, 2003).

2. Methods

2.1. Subjects

Three adult male baboons (*Papio anubis*, obtained from Primate Imports, New York, NY) served as subjects. Baboons weighed 24.1– 36.3 kg at the beginning of the study. All three baboons (designated DI, ED, and GA) had previously served in studies of benzodiazepine/sedative dependence (Ator et al., 2000; Weerts and Griffiths, 1999) but had not received any experimental drugs for 4, 6 and 22 months, respectively, before the current experiment. As described below, the baboons had 20 h/day access to food pellets (1 g, banana-flavored, Bio-SERV, Inc., Frenchtown, NJ). Supplemental feeding of 1 or 2 pieces of fresh produce and a children's chewable multi-vitamin occurred at approximately 11 a.m. each day. Tap water was continuously available via an automatic watering system attached to the cage. The volume of water consumed was recorded at the same time each morning prior to the experimental session.

Intragastric (i.g.) catheters were surgically implanted into the stomach using methods based on Lukas et al. (1982). Before vehicle (distilled water) administration began, baboons showed normal dietary intake under the 20 h/day food access program (described below). Baboons were anesthetized with ketamine hydrochloride (HCl) (150–300 mg) every 2–3 weeks to permit weighing, physical

examinations, and cage washing. During the physical examination, routine catheter maintenance procedures were performed; the area around the catheter exit site was shaved, scrubbed with antiseptic solution, and treated with an antibiotic ointment. Ketamine anesthesia also occurred as needed to unblock the catheter or to repair catheter or tether-related equipment problems. Animal maintenance and research were conducted in accordance with the American Psychological Association ethical standards. The research protocol was approved the Institutional Animal Care and Use Committee.

2.2. Apparatus

Baboons were housed individually in standard primate cages equipped with an aluminum "intelligence panel," which was mounted on the rear wall. A more detailed description of the cage and panel has been presented previously (Weerts et al., 1998a). Briefly, the intelligence panel contained a Lindsley operandum (Gerbrands Corp., Arlington, MA), a colored jewel light, and a hopper for delivery of food pellets. Food pellets were delivered into the hopper using an electro-mechanical feeder. The i.g. catheters were protected by a customized vest and tether system that permitted the baboon free movement inside the cage (Lukas et al., 1982). The tether was connected to the infusion system via a customized liquid swivel (IITC Life Science, Woodland Hills, CA) mounted on top of the cage. Drug and vehicle suspensions or solutions were infused into the catheter using a peristaltic pump (Model 1201 or 1203, Harvard Apparatus, South Natick, MA). The pellet feeder, the water bottle, and the peristaltic pump were located on a grating above the cage. Room ceiling lights were brightly illuminated for 13 h/day (6:00 a.m.–7:00 p.m.) and dimly illuminated for the remainder of the day.

Experimental control and data collection were accomplished using personal computers with MED Associates Inc. (East Fairfield, VT) software and instrumentation. The behavioral observation data were collected using laptop personal computers and custom software programmed in BASIC. The fine-motor task used custom-made Plexiglas trays on which six 3-cm cups with 1-cm high rims were mounted. The fine-motor task was timed using a stopwatch and results were recorded using a pen and paper checklist.

2.3. Experimental conditions

2.3.1. Effects of bretazenil and flumazenil in non-dependent baboons

Chronic triazolam vehicle (1 g/l suspending agent) was continuously infused via the i.g. catheter. During chronic vehicle dosing, acute doses of bretazenil (0.01, 0.1, 1.0, 10.0 mg/kg) and flumazenil (0.01, 0.1, 0.32, 1.0, 3.2 mg/kg) were administered about every two weeks to assess their behavioral effects and to provide a comparison for corresponding administration during the triazolam-dependent condition. Doses of flumazenil or bretazenil were delivered as bolus i.g. injections 15 min before the beginning of pellet access (see below). A vehicle control test was conducted 3–4 days before each drug test. Doses were presented in different orders for each baboon. To determine if overt behavioral signs of receptor agonist effects (e.g., lip droop) or of a precipitated withdrawal syndrome occurred, 60-min observation sessions (described below) were conducted 5-min after the administration of bretazenil, flumazenil, or vehicle. Immediately following the observations, baboons were presented with the fine-motor task as described below.

2.3.2. Effects of bretazenil and flumazenil in triazolam-dependent baboons

Chronic triazolam (1.0 mg/kg/day) was continuously infused over a 24 h period via the i.g. catheter. After at least 30 days, the series of acute drug tests with flumazenil and bretazenil was repeated, and procedures were conducted as described above.

2.4. Behavioral assessments

In both conditions described above, the effects of flumazenil and bretazenil were assessed using three measures: food-maintained operant responding, overt behavioral signs of receptor agonist or withdrawal effects, and a fine-motor task. These three measures have been reported to be sensitive to both benzodiazepine receptor agonist and antagonist-precipitated withdrawal effects (e.g., Ator et al., 2000; Weerts et al., 1998a).

2.4.1. Food-maintained operant behavior

During all conditions, the baboons had unlimited access to food pellets during daily 20-h sessions that began at approximately 9:00 a.m. Pellet availability was signaled by continuous illumination of the jewel light mounted above the Lindsley lever. Completion of 10 responses resulted in delivery of a 1-g food pellet and illumination of the food tray for 1 s (i.e., a fixed ratio (FR) 10 schedule of reinforcement was in effect). At this response requirement, pellet intake was sufficient to maintain normal body weights in the absence of any experimental manipulations and baboons ate all of the pellets delivered (i.e., pellets were not found on the floor or in the cage). Data were collected in 15-min intervals via the computer program.

2.4.2. Observations

Behaviors were recorded during 60-min observation periods according to methods described previously (Weerts et al., 1998a). Briefly, a trained observer sat in front of the baboon's cage and recorded the occurrence of 18 possible behavioral signs and 6 defined postures using a laptop computer. Data were collected in consecutive, 1-min intervals, and observers could type comments at any time. The signs and postures used are reliable indicators of benzodiazepine direct effects and benzodiazepine withdrawal effects in baboons (Ator et al., 2000; Lukas and Griffiths, 1982; Sannerud et al., 1991; Weerts et al., 1998a).

Baboons were habituated to the observers and observation procedures during practice sessions before the study began. The criterion for agreement on occurrence and nonoccurrence of all behaviors and postures between all possible pairs of observers was 90% or greater before experimental conditions were initiated. The overall inter-rater reliability for all behaviors and postures during the experiment was greater than 95%.

2.4.3. Fine-motor task

Fine-motor coordination was assessed each day using a food item retrieval task (Weerts et al., 1998a). One food item (raisin) was

placed in each of the six cups on the tray (described above) that was then presented at the front of the cage. A research technician presented the tray for 120 s or until all six raisins were retrieved, whichever occurred first. The technician completed a pen and paper checklist, which indicated the occurrence or non-occurrence of tremor and incoordination, the time (s) to retrieve all six raisins (or the maximum test time of 120 s if less than six raisins were retrieved), the number of raisins retrieved, the number of raisins dropped, and any comments about behaviors observed during the task. Before the study began, all baboons were given repeated trials on the task. Relevant behaviors (e.g., vomiting, diarrhea, tremors) that were observed outside of the observation periods were also recorded in the daily records by animal care technicians.

2.5. Evaluation of triazolam in blood

During the chronic conditions, 5–10 cc of venous blood was drawn while the baboons were anesthetized with ketamine for the regular examination described above. Blood was spun for 8 min at 3400 r.p.m. and plasma was pipetted into a tube. Total (free plus bound) plasma triazolam concentrations were determined by gas chromatography with electron capture detection using methods as described previously (von Moltke et al., 1996).

2.6. Drugs

Triazolam (Upjohn Pharmacia Corp., Kalamazoo, MI) was suspended in a vehicle of 1 g/l BIO-Serv Agent K (Frenchtown, NJ) and distilled water. Stock solutions (0.1 mg/ml) were mixed using a blender, and were kept in a brown glass bottle under refrigeration for up to 3 days. The daily dose for each baboon was mixed using the appropriate amount of triazolam stock and vehicle to yield a 450 ml volume, which was continuously infused (1.0 mg/kg/day) into the i.g. catheter. Levels of drug or vehicle infused were recorded at the same time each day (i.e., 8:30 a.m.), and then the drug bottle was refilled.

The acute doses of flumazenil and bretazenil (Hoffman-LaRoche, Basel Switzerland) were dissolved in 10 ml of polyethylene glycol to which 10 ml of sterile water was added per injection; the 20 ml dose was administered all at once via the i.g. catheter and was followed by a 5–10 ml injection of distilled water to flush out the line.

2.7. Data analysis

A single-subject design was used in which each subject served as its own control (Bordens and Abbott, 1996; Krishef, 1991; Sidman, 1960). As is typical for single-subject designs, multiple control tests (N=10–12 per baboon per condition) were conducted for each dosing condition (chronic vehicle and chronic triazolam) in each subject to provide a good baseline for comparison of effects of the test drugs. For each baboon, 95% confidence intervals were calculated from these control tests to determine normal range of frequencies for each behavior under each dosing condition. Specifically, two sets of z-scores were calculated for each behavior for each subject from observations conducted during 1) the chronic vehicle condition and 2) the chronic triazolam administration condition. Behavioral changes after administration of each acute dose of flumazenil or bretazenil were judged as *significantly* increased or decreased when values fell outside of the 95% confidence intervals determined by the z-scores for the appropriate condition(s). For behaviors predicted to increase, significance was concluded only if the observed frequency of that behavior exceeded the 95% confidence interval (i.e., $p<0.05$, using a one-tailed test). For all other behaviors for which the direction of effect was not predicted (e.g., pellets, locomotion), those that exceed the 95% confidence interval and fell in the top 2.5% or the bottom 2.5% (i.e., $p<0.025$, a two-tailed test) of values were considered significant. For the evaluation of acute effects of flumazenil and bretazenil alone, significant effects were determined based on the 95% confidence intervals determined for the chronic vehicle condition. For evaluation of the effects of flumazenil and bretazenil in the context of chronic triazolam, significant changes in withdrawal related behaviors were concluded only if behavioral frequencies were outside the range of frequencies for each dose of flumazenil and bretazenil administered alone (i.e., during chronic vehicle administration) as well as the 95% confidence intervals for the chronic vehicle condition and the 95% confidence intervals the chronic triazolam condition. For behaviors that never occurred under baseline conditions (e.g., seizure) any instance was considered significant. Thus, both time-related and triazolam-produced changes in behavior were accounted for in the analysis of precipitated withdrawal effects.

Behaviors considered to be indicative of benzodiazepine withdrawal were summarized as a composite withdrawal score for each subject (Weerts et al., 1998a). In deriving the withdrawal score, 1 point was assigned in each of nine categories of behaviors or postures; the maximum score was 9. Only behaviors that exceeded 95% confidence intervals (as described above) were assigned points for withdrawal scores. The nine categories of behavioral and postural differences used to determine the withdrawal score were as follows: 1) pellets per day decreased; 2) time to complete the fine-motor task increased; 3) the number of 1-min intervals increased in which eyes were closed or in which lying down, head-lower-than-torso posture and/or a withdrawn chin-on-chest posture were observed; 4) locomotion increased or decreased; 5) self-directed behaviors (nose rub, nose wipe, scratch, and wet dog shake) increased; 6) aggressive behaviors (threat, bruxism, or yawn) increased; 7) tremors (limb/body), jerks, and/or rigidly braced posture increased; 8) vomit and/or retch increased; 9) seizures occurred.

3. Results

3.1. Triazolam in blood

During chronic i.g. dosing with 1.0 mg/kg/day triazolam, the level of triazolam in plasma was: 80.0 ng/ml for baboon GA, 31.2 ng/ml for DI, and 77.1 ng/ml for ED.

3.2. Food-maintained responding

During chronic administration of vehicle, the mean (±1 S.E.M.) number of pellets delivered per day was 182.1 (±14.2) for baboon DI, 190.7 (±8.75) for GA, and 344.8 (±25.6) for ED. Compared to the non-dependent condition, chronic triazolam produced a significant increase in pellets per day in all 3

baboons. Specifically, the mean (±1 S.E.M.) number of pellets delivered per day was 292.5 (±14.8) for baboon DI, 545.8 (±33.2) for GA and 572.8 (±41.2) for ED. Flumazenil and bretazenil produced effects primarily in the first 2 h of the 20 h of pellet availability, when the rate of pellet-maintained responding was also the highest; therefore only that data was used for additional analyses.

In the non-dependent condition (Fig. 1, unfilled circles), there was a tendency for bretazenil to dose-dependently increase the number of pellets earned. In two baboons (ED and GA), bretazenil significantly increased the number of pellets earned in the first 2 h at 1.0 mg/kg. At 10 mg/kg, there was a decrease relative to the 1.0 mg/kg dose of bretazenil in these two baboons, although pellets remained significantly higher when compared to vehicle for baboon GA. Flumazenil did not alter pellets earned in two of three baboons but reduced pellets in baboon ED in a dose-related manner.

In the triazolam-dependent condition (Fig. 1, filled circles), the number of pellets earned was decreased by bretazenil and this effect was an increasing function of dose in two baboons (DI and GA) and an inverted U-shaped function of dose in the third (ED). In all baboons, there was a clear differentiation between the effect of bretazenil on the number of pellets during the non-dependent and triazolam-dependent conditions. Flumazenil produced significant decreases in the number of pellets earned in all three baboons during the triazolam-dependent condition. In fact, food-maintained responding was completely suppressed during the first 2 h of pellet availability after most or all flumazenil doses in all three baboons. The decrease in pellets earned was dose-related in 2 of 3 baboons. Pellets were completely suppressed at all doses in the 3rd baboon (GA). Complete suppression was also obtained at 0.32, 1.0 and 3.2 mg/kg in baboon ED and 0.1, 0.32, 1.0, and 3.2 mg/kg in baboon DI.

3.3. Fine-motor task

During chronic administration of vehicle, the mean (±1 S.E.M.) time (s) to complete the fine-motor task was 5.2 (±0.3) for baboon DI (N=12), 5.7 (±0.6) for GA (N=10), and 7.3 (±0.3) for ED (N=12). Chronic triazolam produced a significant increase in time to complete the task in all 3 baboons, compared to chronic vehicle (see Fig. 2, data points above "V"). Specifically, the mean (±1 S.E.M.) time in seconds to complete the task was 13.3 (±0.9) for baboon DI (N=12), 17.8 (±2.4) for GA (N=11) and 15.8 (±1.6) for ED (N=12). As shown by the S.E.M., triazolam also produced an increase in the variability in the time taken to complete the task relative to the non-dependent condition.

In the non-dependent condition (Fig. 2, unfilled circles), bretazenil significantly increased the time to complete the fine-motor task in a dose-related manner in 2 of 3 baboons (ED and DI), with the peak effect observed at the highest dose (10 mg/kg). In contrast, flumazenil generally did not increase the time to complete the task, except for one occasion (baboon ED at 0.1 mg/kg).

During the triazolam-dependent condition (Fig. 2, filled circles), the highest dose of bretazenil (10 mg/kg) significantly affected completion of the task; time (s) to retrieve all six food items was greater compared to vehicle in all three baboons; in some cases, the task was not completed within 120-s (DI and GA). Lower doses of bretazenil did not have a consistent effect across baboons. The highest dose of flumazenil (3.2 mg/kg) produced effects similar to bretazenil. All three baboons failed to complete the task within 120-

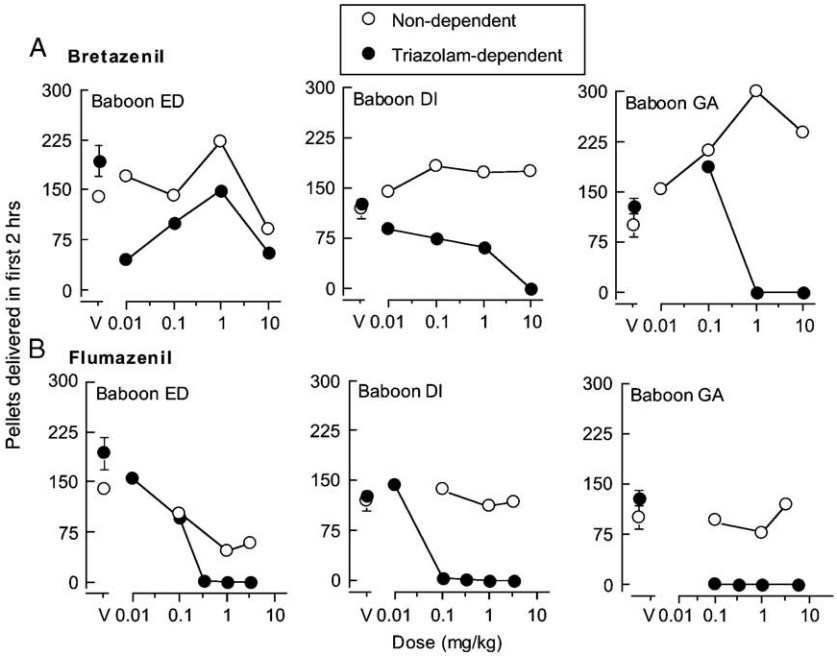

Fig. 1. Effects of acute administration of A. bretazenil and B. flumazenil on the number of pellets delivered during the first 2-h of pellet availability during daily 20-h sessions. Each drug was tested under conditions in which baboons were maintained on chronic vehicle (open circles) and chronic triazolam (filled circles). Data shown are for three individual baboons (ED, DI and GA). Points above "V" are the mean (±1 S.E.M.) number of pellets earned after acute bretazenil/flumazenil vehicle administration during each condition. In some cases, the data point encompasses the error bar. Doses of bretazenil and flumazenil administered during each condition are shown on the x-axes.

s. This effect was also obtained at 1.0 mg/kg for ED and 0.32 mg/kg for GA. Other doses did not reliably affect time to complete the task.

3.4. Behavioral observations

Figs. 3 and 4 show the effects of administration of flumazenil and bretazenil, respectively, on behaviors relevant to a benzodiazepine withdrawal syndrome as summarized by withdrawal scores. When administered during the non-dependent condition, flumazenil and bretazenil did not increase signs of withdrawal as compared to vehicle (data not shown). As shown in Fig. 3, administration of the lowest dose of bretazenil (0.01 mg/kg) during chronic triazolam did not produce any signs of precipitated withdrawal in any baboon. At 0.1 mg/kg bretazenil, withdrawal scores ranged from 0 to 1. Following administration of 1.0 mg/kg bretazenil withdrawal scores were 1, 4 and 6, which included vomiting in two baboons (GA and DI). The highest dose of bretazenil (10.0 mg/kg) produced withdrawal scores of 6 out of a possible maximum of 9 in all three baboons. Withdrawal-related behaviors included decreases in the number of pellets earned and increases in the time taken to complete the fine-motor task, withdrawal postures, self-directed behaviors, aggressive behaviors, tremors, and vomiting.

As shown in Fig. 4, administration of the lowest dose of flumazenil (0.01 mg/kg) did not increase signs of withdrawal. At 0.1 mg/kg flumazenil, there were marked individual differences in precipitated withdrawal as evidenced by withdrawal scores of 1–5. While one baboon (GA) showed a decrease in the number of pellets earned in the first two hours, the other 2 baboons showed tremors/jerks (ED and DI) and vomiting (DI), as well as a decrease in the number of pellets earned. Withdrawal scores ranged from 4–7 for doses of 0.32, 1.0, and 3.2 mg/kg flumazenil with one exception (a score of 3 for DI at 1.0 mg/kg). Tremors/jerks were observed in 2 or 3 baboons at doses of 0.1–3.2 mg/kg, and all three baboons showed vomiting at doses of 1.0 and 3.2 mg/kg flumazenil.

Figs. 5 and 6 show the dose-dependent increase in frequency of tremors and vomiting, respectively. Although flumazenil-precipitated withdrawal scores were not dose-related above 0.32 mg/kg, the intensity of flumazenil and bretazenil precipitated withdrawal was generally dose related. Neither bretazenil nor flumazenil produced vomiting in the non-dependent baboons (Fig. 5, unfilled circles). In the triazolam-dependent condition (Fig. 5, filled circles), the frequency of vomiting increased in all three baboons following both bretazenil and flumazenil administration, and frequency was directly related to dose. A similar dose-related increase in tremor frequency was observed following bretazenil administration to triazolam-dependent baboons (Fig. 6a, filled circles). Flumazenil also produced tremors in all three baboons in the triazolam-dependent condition, but this was a direct function of dose only in baboon ED (Fig. 6b, filled circles). Bretazenil and flumazenil did not produce tremors in the non-dependent condition. (Fig. 6, unfilled circles).

4. Discussion

In the present study, evidence of a precipitated withdrawal syndrome was observed following administration of the benzodiazepine receptor partial agonist bretazenil to baboons that were chronically administered triazolam. The characteristics and the intensity of the bretazenil-precipitated withdrawal were similar to flumazenil-precipitated withdrawal under the same conditions. Precipitated withdrawal was evident across three different behavioral measures;

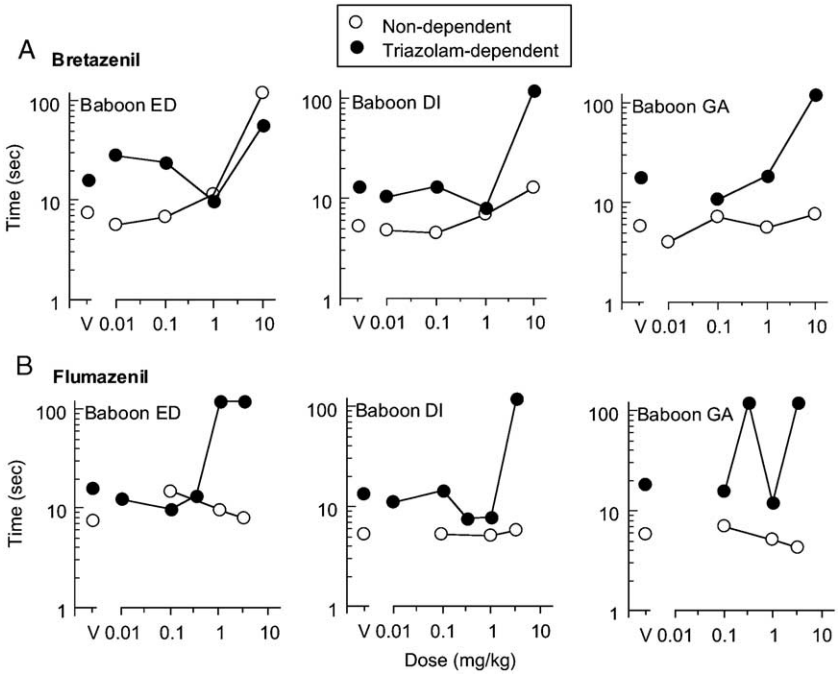

Fig. 2. Effects of acute administration of A. bretazenil and B. flumazenil on the time to complete the fine motor task in three baboons. Data points show the time (s) to retrieve six food items (raisins) from a tray presented at the front of the cage. The maximum time allowed for retrieving all six raisins was 120 s. The data points over "V" represent the mean (±1 S.E.M.) for tasks conducted during each condition. For other details, see Fig. 1.

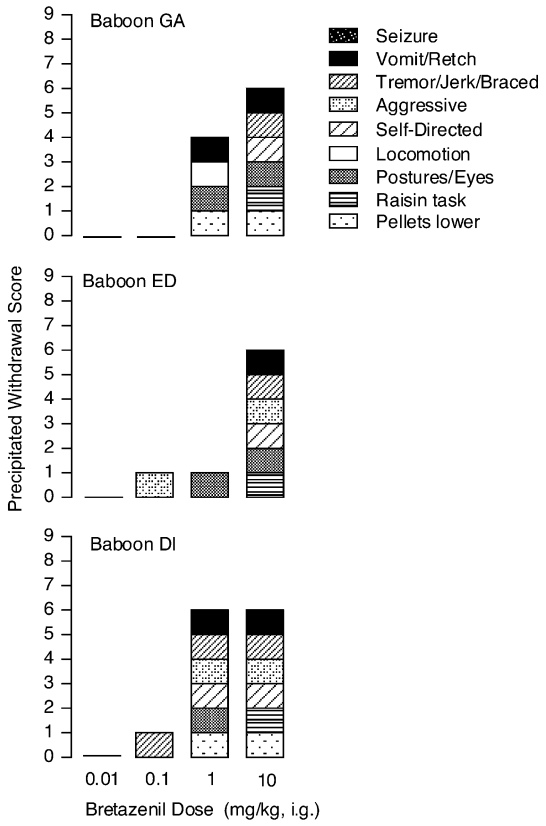

Fig. 3. Bretazenil-precipitated withdrawal scores during chronic triazolam administration. Sixty-min observations and the fine motor task were conducted after i.g. administration of vehicle, 0.01, 0.1, 1.0, and 10.0 mg/kg bretazenil. The maximum total possible score that could be obtained was 9. Points were assigned as follows: 1 point for each of 7 categories of behavior recorded during observations that differed significantly ($p<0.05$) when compared to controls; 1 point for increased duration or not completing the fine motor task (within 120 s) conducted at the end of the observational session; and 1 point if the number of pellets delivered in the first 2 h of the 20-h session was decreased.

namely, the number of pellets earned, time taken to complete a fine-motor task, and occurrence of behaviors characteristic of a benzodiazepine withdrawal syndrome in baboons (Lukas and Griffiths, 1982). The present results indicate that the benzodiazepine receptor partial agonist bretazenil can function as an antagonist under conditions of chronic benzodiazepine administration and, like flumazenil, reveal physical dependence.

Flumazenil-precipitated withdrawal from benzodiazepine receptor agonists has been both well documented and well characterized in baboons (Ator et al., 2000; Kaminski et al., 2003; Lamb and Griffiths, 1984, 1985, 1987; Lukas and Griffiths, 1982, 1984; Sannerud et al., 1991; Weerts et al., 1998a,b). To facilitate comparison of results across studies Yanagita and Takahashi (1970) developed a system to classify signs of withdrawal as "mild," "intermediate," or "severe." This system has been used to grade withdrawal from benzodiazepine receptor agonists qualitatively (Ator et al., 2000; Kaminski et al., 2003; Weerts et al., 1998a). Applied to the present data, withdrawal would be classified as mild if any of the following were observed: decreases in pellets delivered per day, and increases in aggression, self-directed behaviors, and postures that may be associated with abdominal discomfort and nausea (e.g., head-lower-than-torso posture). The presence of either vomiting (or retching) or jerks/tremors (limb or body) results in an intermediate classification regardless of whether signs of mild withdrawal were also observed. The occurrence of seizures, not observed in the present study, would have resulted in classification as a severe withdrawal syndrome.

Both bretazenil and flumazenil produced a dose-dependent intermediate intensity precipitated withdrawal syndrome in the baboons maintained on 1.0 mg/kg/day triazolam. The present study is the first demonstration of a bretazenil-precipitated withdrawal syndrome comparable to that precipitated by flumazenil. In a study in which rats were administered triazolam (3 mg/kg) via implanted osmotic pumps for 11 days, all doses of flumazenil (3.0–30.0 mg/kg) decreased response rates in the triazolam-treated rats, consistent with precipitated withdrawal, where as bretazenil (0.1–10.0 mg/kg) did not (Cohen and Sanger, 1994). Using a similar procedure, Martin et al. (1995b) compared precipitated withdrawal from diazepam (30 mg/kg, p.o. once daily for 11 days) in squirrel monkeys following administration

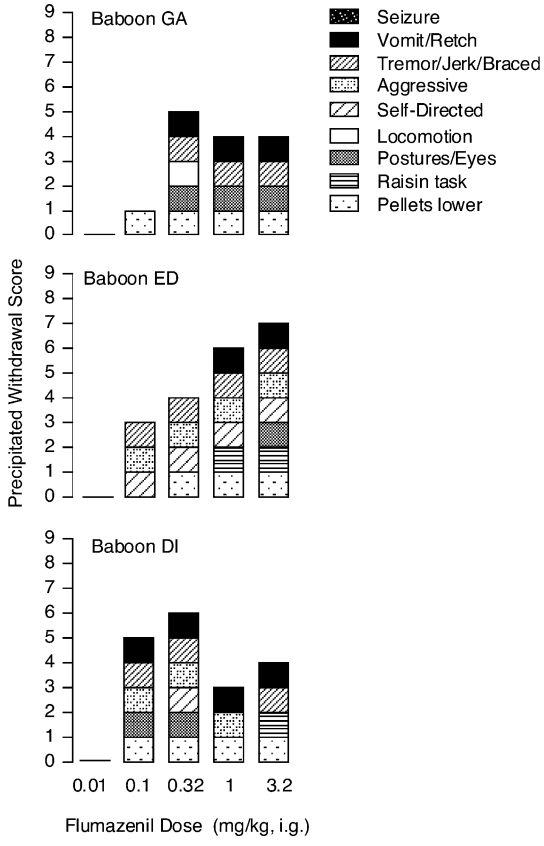

Fig. 4. Flumazenil-precipitated withdrawal scores during chronic triazolam administration. Sixty-min observations and the fine motor task were conducted after i.g. administration of vehicle, 0.01, 0.1, 0.32, 1.0, and 3.2 mg/kg flumazenil. For other details, see Fig. 3.

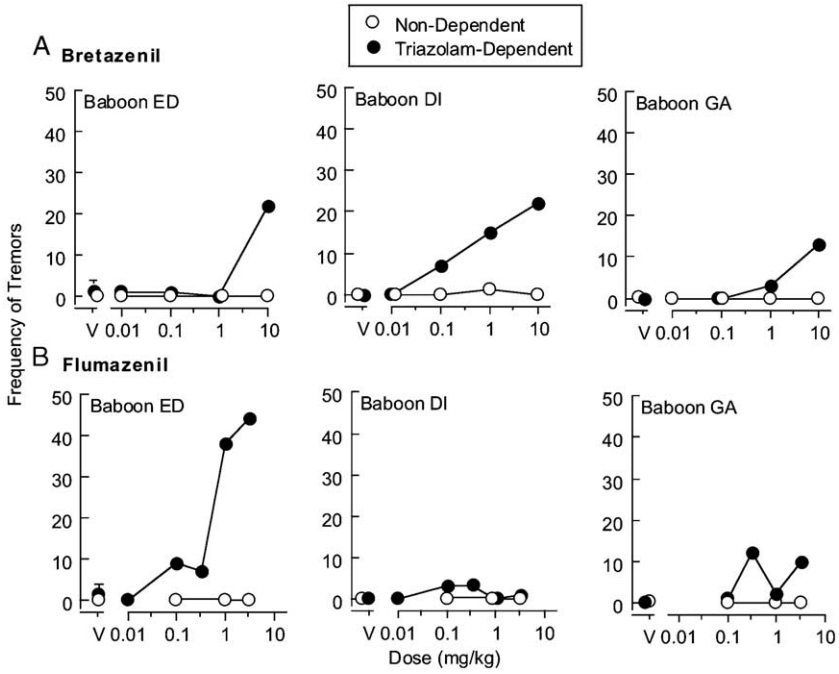

Fig. 5. Frequency of vomiting after acute i.g. administration of A. bretazenil and B. flumazenil during chronic administration of vehicle and chronic administration of triazolam. Points above "V" are the mean (±1 S.E.M.) vomiting during acute i.g. vehicle administration during each condition. Doses of bretazenil and flumazenil administered during each condition are shown on the *x*-axes.

flumazenil (0.03–3.0 mg/kg) or bretazenil (0.1–3.0 mg/kg, i.v.). The monkeys received the i.v. injection at 5, 24, and 48 h after the last diazepam dose. When administered to the diazepam-maintained monkeys, flumazenil induced tremor at all doses in all monkeys, vomiting at the two higher doses in all but one monkey, and short-duration convulsions in 4 of the 9 monkeys tested at all three doses. Bretazenil produced tremors in all monkeys at the two higher doses, but vomiting in only one in each group at the highest and lowest doses, and a convulsion in only one at the middle dose. The authors found the intensity of tremors and convulsions to be greater with flumazenil as well. An important point of their study

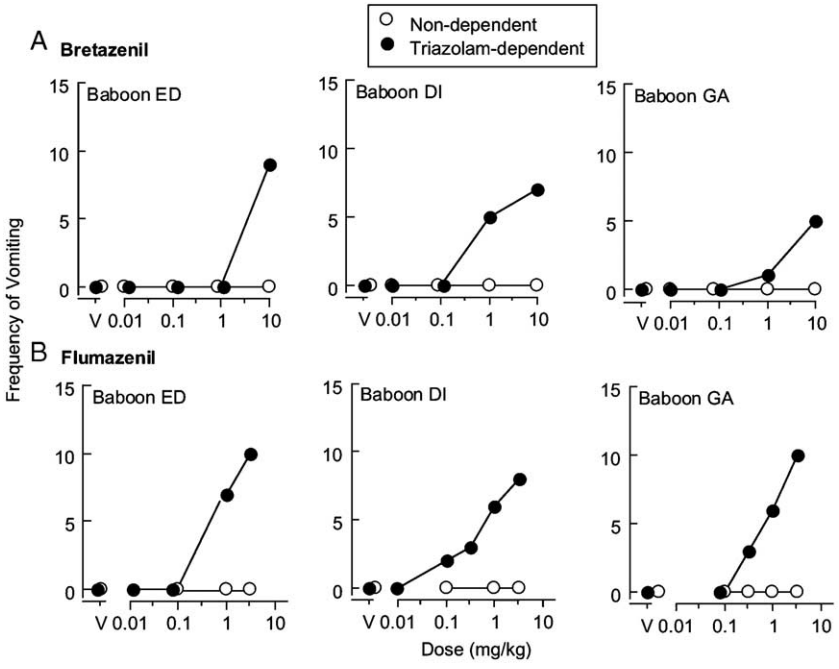

Fig. 6. Frequency of tremors after acute i.g. administration of A. bretazenil and B. flumazenil during chronic administration of vehicle and chronic administration of triazolam. Points above "V" are the mean (±1 S.E.M.) of tremors after acute i.g. vehicle administration during each condition. Doses of bretazenil and flumazenil administered during each condition are shown on the *x*-axes.

was that the doses of flumazenil and bretazenil used had been selected because they were equivalent in reversing hypnotic effects of flunitrazepam in squirrel monkeys.

There are a number of differences between those two studies and the present study, including the species, method and duration of exposure to the full benzodiazepine receptor agonist, route of administration of the bretazenil and flumazenil, and the procedure for the precipitated withdrawal tests. In the procedures used by Cohen and Sanger (1994) and by Martin et al. (1995a,b), the precipitated withdrawal tests were conducted after chronic administration of the benzodiazepine receptor agonist ended. In the present study, the precipitated withdrawal tests occurred during the ongoing administration of the receptor agonist. Also, given the almost 30-fold difference in the weights of the two monkey species between Martin et al. and the present study, the total amount of bretazenil in the present study may have been effectively higher than in Martin et al. despite the equivalence of the high doses (for review see Dews, 1976).

Several recent studies suggest that partial receptor agonists can have both agonist-like and antagonist-like effects that are dependent upon the conditions under which they are studied. In baboons and rats trained to discriminate lorazepam from no drug (Ator and Griffiths, 1999), the imidazoquinoxaline U-78875, which acts as a benzodiazepine receptor partial agonist in vitro, increased the discriminative potency of lorazepam in animals that generalized to it and antagonized the discriminative stimulus effects of lorazepam in those that did not. Tang and Franklin (1991) reported similar effects for U-78875 when diazepam was the training drug. Smith et al. (2004) reported that bretazenil decreased motor performance in a rotorod test under conditions of high behavioral demand (e.g., faster speeds), but did not alter motor performance under conditions of low demand (low speeds). Bretazenil did, however, antagonize the effects of diazepam and clonazepam under conditions in which it did not disrupt performance when administered alone (i.e., lower speed conditions). Paronis and Bergman (1999) reported that both bretazenil and flumazenil antagonized the rate-decreasing effects of midazolam in a shock termination procedure. However, in a shock-suppressed responding procedure, both bretazenil and the full benzodiazepine receptor agonist midazolam increased suppressed responding; and the effects of both drugs were antagonized by flumazenil.

In the current study, agonist-like effects were observed when bretazenil was administered. Acute administration of bretazenil increased food-maintained responding, which is characteristic of benzodiazepine receptor agonists. For example, increases in food-maintained responding were observed during triazolam administration in the current study and also previously in baboons (Ator et al., 2000; Weerts and Griffiths, 1999). Increases in food intake have also been reported following bretazenil administration in mice (Witkin et al., 2004), rats (Clifton and Cooper, 1996; Yerbury and Cooper, 1987), and squirrel monkeys (Weerts et al., 1998c). Benzodiazepine receptor agonist effects of bretazenil were also detected during the fine motor task, which provides a measure of motor impairment and incoordination. Bretazenil increased the time taken to complete the task in 2 of 3 baboons. Similar increases in this measure were observed during chronic triazolam administration in the current study, and after administration of other benzodiazepine receptor agonists to baboons in other studies (e.g., zaleplon and triazolam, Ator et al., 2000). A number of studies have reported that bretazenil produced less motor impairment, muscle relaxation, and sedation than full benzodiazepine receptor agonists (Fack-lam et al., 1992a; Martin et al., 1988; Yerbury and Cooper, 1987), although signs of sedation and/or myorelaxation were observed at high doses (Martin et al., 1988). In the present study, bretazenil did not produce overt signs of sedation (e.g., eyes closed, lying down) or muscle relaxation (most prominently shown in the baboon by lip droop) at the doses tested. Flumazenil can exert some agonist-like and inverse agonist-like effects under certain conditions (File and Pellow, 1986; Neave et al., 2000; Weerts et al., 1993; Witkin and Barrett, 1985). In the current study, however, flumazenil did not increase food-maintained responding, time taken to complete the fine-motor task, and no signs of sedation were evident during the behavioral observations. Thus, given that increases in these behaviors appear to be due to positive modulation of GABA, the current data are consistent with in vitro research showing that flumazenil has less intrinsic efficacy than bretazenil.

These data are consistent with previous studies, which have shown that benzodiazepine physical dependence may produce functional shifts in the behavioral effects of ligands that bind at the benzodiazepine receptor site (Sannerud et al., 1991). Sannerud et al. (1991) demonstrated that the agonist-like effects of midazolam were decreased, whereas the inverse agonist-like effects of β-CCE were enhanced by chronic diazepam administration (Sannerud et al., 1991). Similarly, in the present study, whether bretazenil functioned as a weak benzodiazepine receptor agonist or as a competitive antagonist was dependent upon whether it was administered alone or during a state of benzodiazepine physical dependence.

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