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Chronic Bretazenil Produces Tolerance to Chlordiazepoxide, Midazolam, and Abecarnil

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BRONSON, M. E. *Chronic bretazenil produces tolerance to chlordiazepoxide, midazolam, and abecarnil.* PHARMACOL BIOCHEM BEHAV 51(2/3) 481–490, 1995.—The purpose of the present study was to determine if chronic treatment with a nonsedative benzodiazepine partial agonist would confer tolerance to the rate-decreasing effects of other benzodiazepine ligands in a fixed-interval procedure in rats. A separate group of rats was treated chronically with the sedative benzodiazepine full agonist, chlordiazepoxide, for comparison. It was hypothesized that tolerance would develop rapidly to chlordiazepoxide due to loss of reinforcement density at rate-decreasing doses and that there would probably be cross-tolerance to other rate-decreasing benzodiazepine ligands such as midazolam and abecarnil. Because bretazenil does not produce rate decreases, however, it was not expected that tolerance would be found to chlordiazepoxide, midazolam, or abecarnil. After 8–12 weeks of chronic treatment with either chlordiazepoxide or bretazenil, however (final dose of benzodiazepine = 30 mg/kg/day), tolerance was found to the rate-decreasing effects of chlordiazepoxide, midazolam, and abecarnil in both groups. It is concluded that such tolerance was most likely due to a saturation of benzodiazepine receptors by this high-affinity partial agonist.

Benzodiazepines Chlordiazepoxide Bretazenil Midazolam Abecarnil Tolerance
Schedule-controlled behavior Fixed interval

IT IS WELL established that both tolerance and physical dependence develop upon chronic exposure to benzodiazepines. It appears that tolerance develops rapidly to the sedative and anticonvulsant effects of both short- and long-acting benzodiazepines; on the other hand, tolerance to anxiolytic and stimulant activity is less likely to occur (13). Because cellular tolerance is closely linked to the development of dependence (12), it has been suggested that tolerance may be a manifestation of changes occurring during drug dependence (8). Moreover, Chait and Balster (5) have postulated that tolerance to behavioral effects of a drug may be positively correlated with abuse potential of that drug.

Among the many procedures that have been used to examine tolerance and dependence, the assessment of schedule-controlled behavior is one procedure that is particularly sensitive to alterations in a drug effect as the result of tolerance or dependence. Schedule-controlled behavior has been used to examine tolerance to several different drug classes, including the opioids (11,18,26), barbiturates (18), psychomotor stimulants (5,28,32), and cannabinoids (2,16). In such procedures, animals are first trained to respond under a schedule of food presentation and then drug effects are measured as an alteration in the rate at which the animals respond for food. Dose-effect curves are obtained under two conditions: first following acute administration of the drug and then again during a regimen in which the drug is administered chronically. Rightward shifts in the dose-effect curve from the acute to the chronic conditions are used as an indicator of tolerance.

Although tolerance procedures such as these have not been used extensively to examine benzodiazepine tolerance, preliminary studies indicate that tolerance to benzodiazepines will develop under a schedule of food presentation. For example, tolerance to the rate-decreasing effects of benzodiazepines has been reported under both fixed-ratio and fixed-interval schedules of food presentation; however, tolerance to the rate-increasing effects of low doses of benzodiazepines has been reported under a fixed-interval schedule (38) but not under a fixed-ratio schedule (9). Similarly, whereas tolerance also does not develop to the locomotor stimulant effects of low doses of BDZs in mice (7), tolerance to other motor effects of the benzodiazepines has been reported (31). Thus, tolerance appears to depend not only on the effect being studied, but also on the experimental procedure used to measure the effect.

Another aspect of benzodiazepine tolerance that has received only limited investigation is that of cross-tolerance among compounds acting at the benzodiazepine receptor. Numerous compounds now exist that have a wide range of intrinsic activity at the benzodiazepine receptor, from full agonists

to partial agonists to inverse agonists (10). Bretazenil has been classified as a partial agonist, because although it has very high affinity for the benzodiazepine receptor and is a more potent anticonvulsant and anxiolytic than diazpam, sedative effects of bretazenil are seen only at very high doses (10). Bretazenil is also different from full benzodiazepine agonists in that it apparently does not produce physical dependence upon chronic treatment (15,21). The current study was therefore conducted to determine whether chronic treatment of rats with the nonsedative partial agonist, bretazenil, would confer tolerance to the rate-decreasing effects of full benzodiazepine agonists such as chlordiazepoxide and midazolam, and also to the partial agonist beta-carboline anxiolytic, abecarnil (29). A separate group of rats was treated chronically with the full benzodiazepine agonist, chlordiazepoxide. It was hypothesized that chronic treatment with chlordiazepoxide would result in rapid tolerance to the rate-decreasing effects of this drug because of a loss of reinforcement density (35). Bretazenil, on the other hand, because it does not result in loss of reinforcement density (i.e., has no rate-decreasing effects), should not confer tolerance to benzodiazepines that do have this effect.

METHOD

Subjects

Eighteen male Long-Evans hooded rats, approximately 1 year old at the start of the study, served as subjects. They were maintained at 80% of their free-feeding weight by supplemental feeding (Purina rat chow) immediately after each daily session, such that their final weights were 350–370 g. Rats were housed individually with unlimited access to water, in a colony maintained on a 12L : 12D cycle. All rats had been used in an acute benzodiazepine dependence study where they received acute doses of either chlordiazepoxide or bretazenil, followed by injections of flumazenil (4).

Apparatus

Experimental sessions were conducted in standard operant chambers 22 × 22 × 28 cm (Med-Associates Inc., East Fairfield, VT). Each chamber was equipped with three response levers located 8 cm above the floor and separated 1 cm from each other. Three 7-W white lights were mounted on the intelligence panel 5 cm above each response lever. A pan for food pellet delivery was located on the opposite wall. One 45-mg food pellet (BioServ) was delivered upon completion of the schedule requirements. All chambers were enclosed in sound-attenuating boxes equipped with white noise and an exhaust fan for ventilation. The reinforcement schedule and data collection were controlled by a microcomputer with Med-Associates Inc. interface and software. Sessions were conducted daily at the same time of day.

Behavioral Procedure

All of the rats had been trained to respond on one lever under a fixed- interval 1 min (FI 1′) schedule of food presentation with a limited hold of 10 s (LH 10″). In the FI component, the first response within 10 s after the 60-s interval produced a food pellet and started a 2-s timeout in which the chamber was dark and responses had no scheduled consequences. The timeout period started automatically without food presentation if a response was not made within 10 seconds after the 60-s interval had elapsed (i.e., limited hold). Training sessions lasted until 30 reinforcers had been earned. Cumulative dose–effect curves were determined for bretazenil (1, 10, 100 mg/kg), chlordiazepoxide (3.2, 10, 32 mg/kg), midazolam (3.2, 10, 32 mg/kg), and abecarnil (0.1, 1, 10 mg/kg) in that order, with at least 1 week separating dose–effect curves. During determination of dose–effect curves, a water injection was given 5 min prior to the first component of the session. Successive doses of drug were administered immediately after each session component. Each session component was approximately 5 min in duration (i.e., five reinforcers or five limited holds), and timeout after each injection was also 5 min. A 5-min timeout was chosen because prior studies in the same rats showed that chlordiazepoxide-induced rate decreases could be seen within this time period. Rats were returned to their home cages immediately following each injection. After completion of the dose–effect curves, rats were separated into three groups of six each according to chronic treatment with either bretazenil, chlordiazepoxide, or water. The initial daily dose of bretazenil and chlordiazepoxide was 10 mg/kg because this was the highest dose of chlordiazepoxide that did not significantly decrease responding. The daily

TABLE 1

SCHEDULING OF DOSE EFFECT CURVES

	Chronic Bretazenil (n = 6) Chronic Water (n = 3)	Chronic Chlordiazepoxide (n = 3*) Chronic Water (n = 1*)
Prechronic weeks 1–4	All rats—Acute dose–effect for bretazenil (week 1), chlordiazepoxide (week 2), midazolam (week 3), and abecarnil (week 4)	
Chronic treatment weeks 1–8	Acute dose–effect curves bretazenil every week	Acute dose–effect curves for chlordiazepoxide every week
Chronic treatment week 9	Acute dose–effect curve for chlordiazepoxide	Acute dose–effect curve for bretazenil
Chronic treatment weeks 10 11	All rats—acute dose–effect curves for midazolam (week 10) and abecarnil (week 11)	
Chronic treatment weeks 12 & 13	All rats—acute dose–effect curves for chlordiazepoxide (week 12) and bretazenil (week 13)	
Post chronic treatment weeks 2, 4, & 8	All rats—acute dose–effect curves for chlordiazepoxide	
Post-chronic treatment weeks 9, 15, & 17	All rats—acute dose–effect curves for bretazenil (pc-week 9), abecarnil (pc-week 15) and midazolam (pc-week 17)	

*The chronic chlordiazepoxide originally had an N of 6, and each chronic water group each had an N of 3, but due to deaths in the chronic chlordiazepoxide group and the chronic water group that had weekly redeterminations of the chlordiazepoxide dose–effect curve, data from these animals have been eliminated.

dose was administered 5 min prior to the session and behavioral testing occurred Monday–Saturday at 1000 h. Dose–effect curves were determined on Sundays at 1000 h. During chronic dosing, the daily dose was increased by 5 mg/kg at weekly intervals for a final daily dose of 30 mg/kg. A dose higher than 30 mg/kg was not used because this dose decreased responding by approximately 50%. Although bretazenil had no effect on responding, it was felt that the chronic dose for this drug should be the same as that for chlordiazepoxide. Table 1 shows the scheduling of dose–effect curves before, during, and after the chronic regimen. Tolerance in the chronic chlordiazepoxide group did not develop until week 8 (i.e., after 4 weeks at 30 mg/kg/day), as evidenced by a rightward shift in the dose–effect curve in the chronic chlordiazepoxide group. On week 9, all rats that had been receiving chronic bretazenil and those rats that had been receiving weekly bretazenil challenges now received a dose–effect challenge with chlordiazepoxide. Similarly, chronic chlordiazepoxide-treated rats and rats that had weekly dose–effect curve for chlordiazepoxide had a dose–effect curve for bretazenil on week 9. This was done to determine if tolerance to chlordiazepoxide had also developed in rats receiving chronic bretazenil, and also to determine whether the dose–effect curve for bretazenil would be the same in chronic chlordiazepoxide rats. On weeks 10 and 11, dose–effect curves for midazolam and abecarnil were redetermined in all rats. Chronic treatment was discontinued after additional dose–effect curves were redetermined for chlordiazepoxide and bretazenil, and all rats were

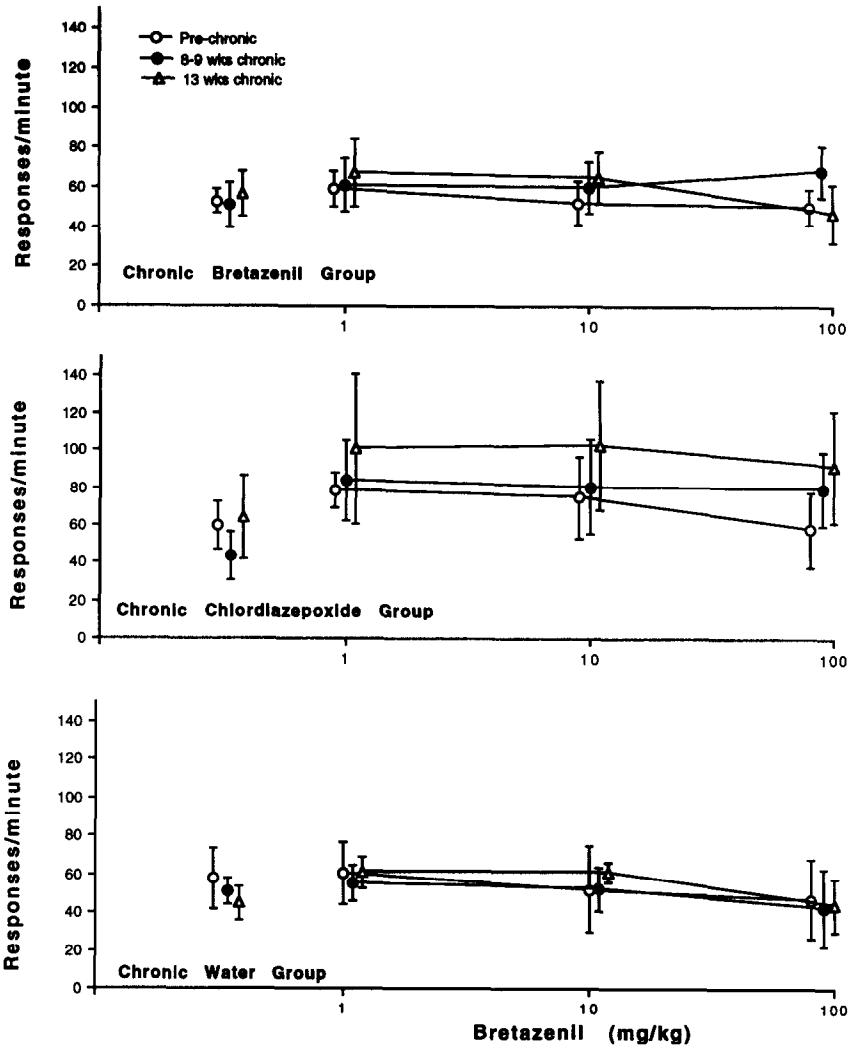

FIG. 1. Effects of cumulative bretazenil before (○), 8–9 weeks into the chronic regimen (●), and 13 weeks into the chronic regimen (△). Symbols represent the mean ± SE. The symbols to the left of the dose–effect curve represent the effect of a water injection during the first component of the cumulative dose–effect curve. The chronic bretazenil group is shown at the top, the chronic chlordiazepoxide group in the middle, and the chronic water group on the bottom. Due to three deaths in the chronic chlordiazepoxide group and two deaths in the chronic water group receiving weekly chlordiazepoxide, final was $n = 6$ in the chronic bretazenil group, $n = 3$ in the chronic chlordiazepoxide group, and $n = 4$ in the chronic water group.

run daily with no injections for a period of 2 weeks, after which the dose-effect curve for chlordiazepoxide was redetermined. Two weeks was chosen rather than 1 week, because some tolerance had developed in the chronic water group when dose-effect curves were determined at weekly intervals. Additional chlordiazepoxide dose-effect curves were redetermined 4 and 8 weeks after cessation of chronic treatment, and dose-effect curves for bretazenil, abecarnil, and midazolam were redetermined at 9-17 weeks after cessation of chronic treatment. An additional dose-effect curve for chlordiazepoxide was planned, but due to two additional deaths in the chronic chlordiazepoxide group (leaving an *n* of 1), this was not done (see Toxicity Analysis below).

Toxicity Analysis

During the chronic regimen, three rats in the chronic chlordiazepoxide group and two rats in the chronic water group that received chlordiazepoxide once a week died after developing severe bloating. Prior to bloating, all rats appeared to be healthy, as evidenced by shiny coats, healthy appetites, and the fact that rates of responding were fairly constant over a long period prior to bloating. When bloating developed, those rats were not tested, and all died within a period of 2 days. Data for these animals are not included in the Results section. Necropsy by the Auburn University School of Veterinary Medicine revealed intestinal obstruction and hepatic necrosis in these rats.

Drugs

Chlordiazepoxide and midazolam hydrochlorides (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.9% saline. Bretazenil (kindly supplied by Hoffmann-La Roche Inc., Basel, Switzerland and Nutley, NJ) and abecarnil (a gift of Schering AG, Berlin, Germany) were suspended in distilled water with 1-2 drops of Tween 80 per 10 ml. All doses are expressed as the salt, and all injections were IP.

Data Analysis

Data were analyzed by a two-way subject by component repeated-measures analysis of variance (ANOVA). When

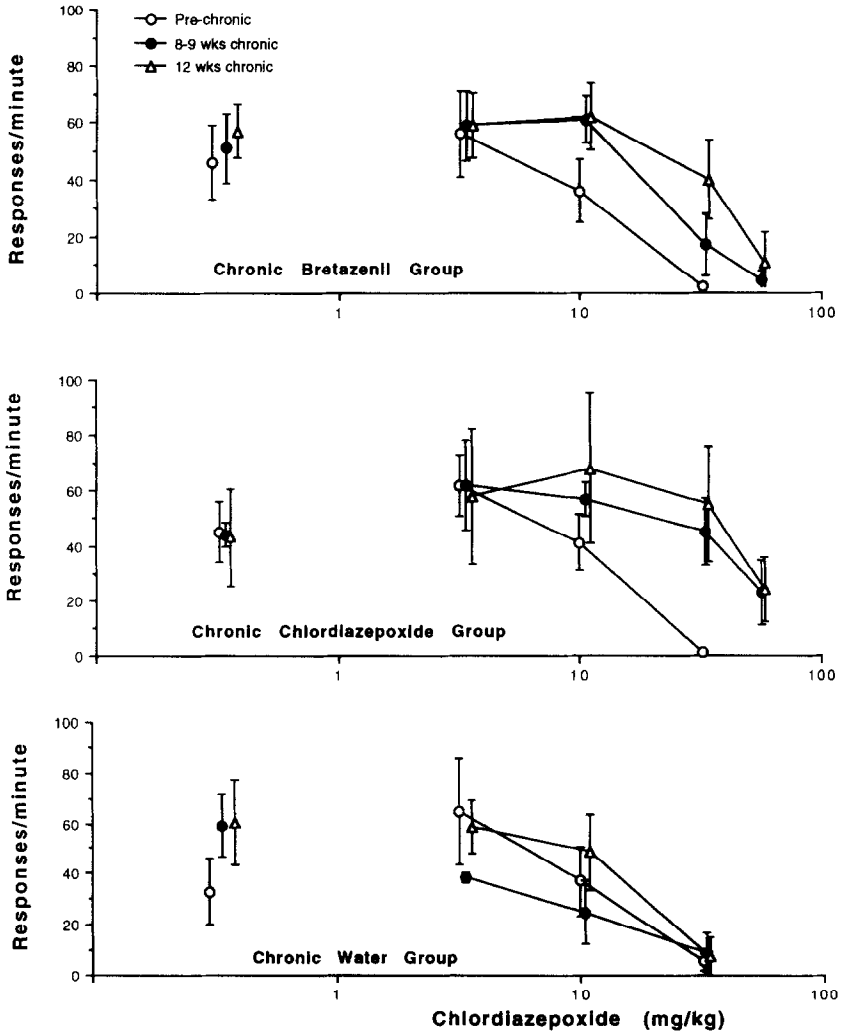

FIG. 2. Effects of cumulative chlordiazepoxide before (○), 8-9 weeks into the chronic regimen (●), and 12 weeks into the chronic regimen (△). Other details are the same as in Fig. 1.

there was a significant component effect, post hoc comparisons were done with the Bonferroni test to determine where the significance lay. Significance level was set at $p < 0.05$.

RESULTS

In Fig. 1 it can be seen that bretazenil alone either had no effect or produced a slight increase in rate of responding before chronic treatment with bretazenil, chlordiazepoxide, or water, even at cumulative doses as high as 100 mg/kg. After chronic treatment with chlordiazepoxide, however, bretazenil significantly increased responding as determined by repeated-measures ANOVA, $F(3, 2) = 10.417$, $p = 0.0003$, and post hoc analysis revealed that this increase in responding was apparent at both 9 and 13 weeks but not before the chronic regimen ($p < 0.05$). In Fig. 2, however, it can be seen that chronic treatment for 9–13 weeks with a final dose of 30 mg/kg/day bretazenil resulted in tolerance to the rate-decreasing effects of chlordiazepoxide, as evidenced by a rightward shift in the chlordiazepoxide dose-effect curve. Chronic treatment for 8 weeks with a final dose of 30 mg/kg/day chlordiazepoxide also produced tolerance to the effects of chlordiazepoxide, as evidenced by a shift to the right in the chlordiazepoxide dose-effect curve of greater than ¼ log. In this group, a dose of 56 mg/kg only decreased rates approximately 50%, whereas before the chronic regimen there was no responding at the 32-mg/kg dose. A slight degree of tolerance to chlordiazepoxide also developed in the chronic water group, as evidenced by an upward shift in the dose-effect curve.

In Figs. 3 and 4, it can be seen that chronic bretazenil or chronic chlordiazepoxide also produced profound tolerance to midazolam and abecarnil, with doses as high as 56 mg/kg of these drugs having very little effect of rate of responding. There was also tolerance to both drugs in the chronic water group, although to a greater extent with abecarnil. When the abecarnil dose-effect curve was redetermined 15 weeks after cessation of chronic treatment, some tolerance to the rate-decreasing effects of abecarnil was still apparent in the chronic chlordiazepoxide group but not in the other groups. When the midazolam dose-effect curve was redetermined 17 weeks after

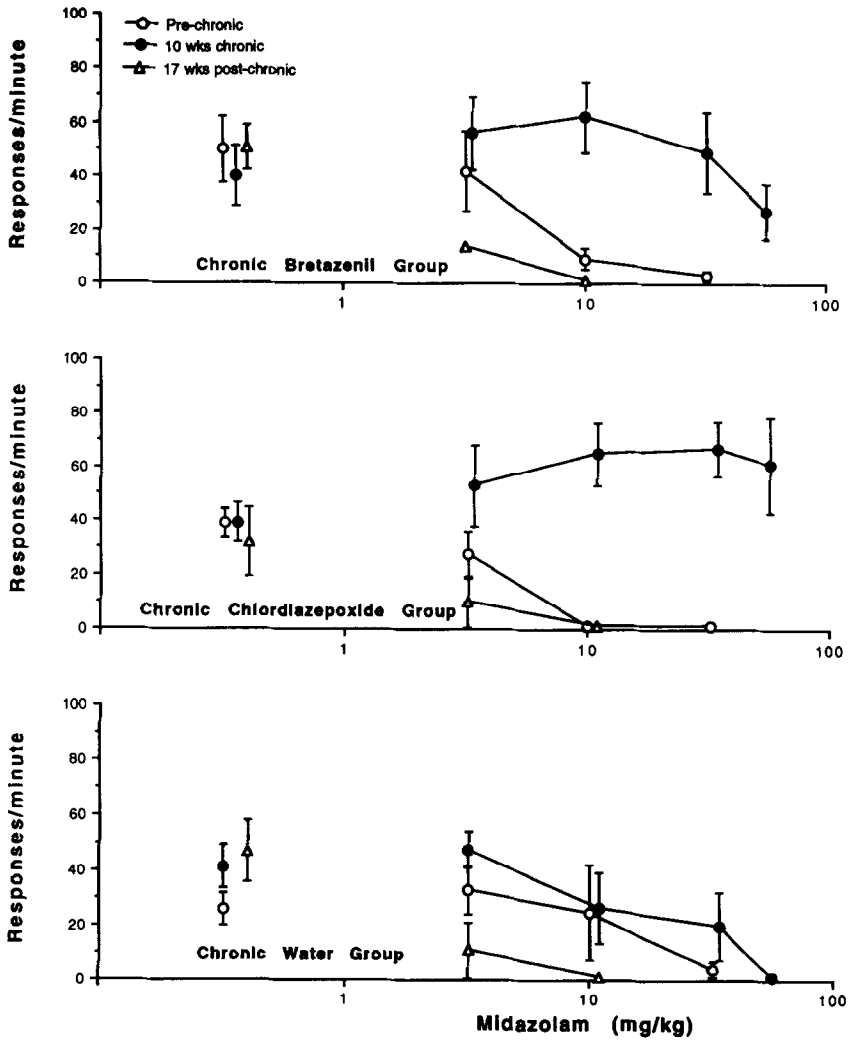

FIG. 3. Effects of cumulative midazolam before (○), 10 weeks into the chronic regimen (●), and 17 weeks after cessation of the chronic regimen (△). Other details are the same as in Fig. 1.

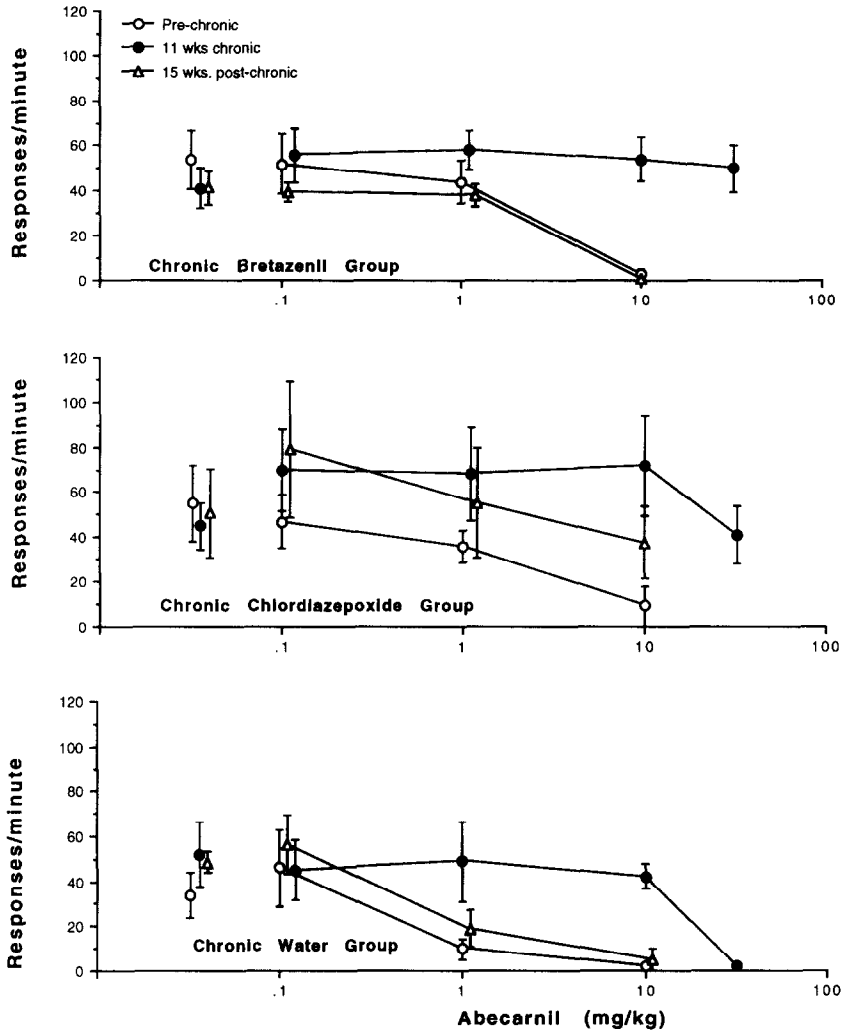

FIG. 4. Effects of cumulative abecarnil before (○), 11 weeks into the chronic regimen (●), and 15 weeks after cessation of the chronic regimen (△). Other details are the same as in Fig. 1.

cessation of chronic treatment, tolerance was not evident in any group. In fact, rats in all groups were more sensitive to the rate-decreasing effects of midazolam at this time.

Figure 5 shows that after discontinuation of the chronic regimen, profound tolerance to chlordiazepoxide in the chronic chlordiazepoxide group (but not in the other groups) was still evident at the end of 2 weeks. Furthermore, baseline rates of responding were increased more than 100% in this group and only returned to prechronic baseline levels at the highest (cumulative 56 mg/kg) dose of chlordiazepoxide. These effects were long lasting, as baseline responding was still elevated 8 weeks after cessation of the chronic regimen, and some degree of tolerance was still in evidence. In fact, responding in the chronic chlordiazepoxide group did not return to prechronic levels until 15 weeks after cessation of chronic treatment, when the final abecarnil dose-effect curve was redetermined.

Figure 6 shows that the effects of bretazenil 13 weeks after cessation of the chronic regimen were somewhat similar to prechronic levels. It is noteworthy, however, that the highest dose of bretazenil now decreased responding in the chronic bretazenil and chronic water groups, but not in the chronic chlordiazepoxide rats. In the chronic chlordiazepoxide group, 10 and 100 mg/kg bretazenil decreased responding relative to postchronic baseline, but did not decrease rates to below the prechronic baseline.

Chlordiazepoxide was more toxic than bretazenil in that no animals in the chronic bretazenil or the chronic water group that had weekly redeterminations of the bretazenil dose-effect curve died. In contrast, during the chronic regimen, three rats in the chronic chlordiazepoxide group and two rats in the chronic water group that received chlordiazepoxide once a week died after developing severe bloating. Two additional rats in the chronic chlordiazepoxide group died with similar symptoms during the postchronic period. Necropsy by the Auburn University School of Veterinary Medicine revealed intestinal obstruction and hepatic necrosis in these rats.

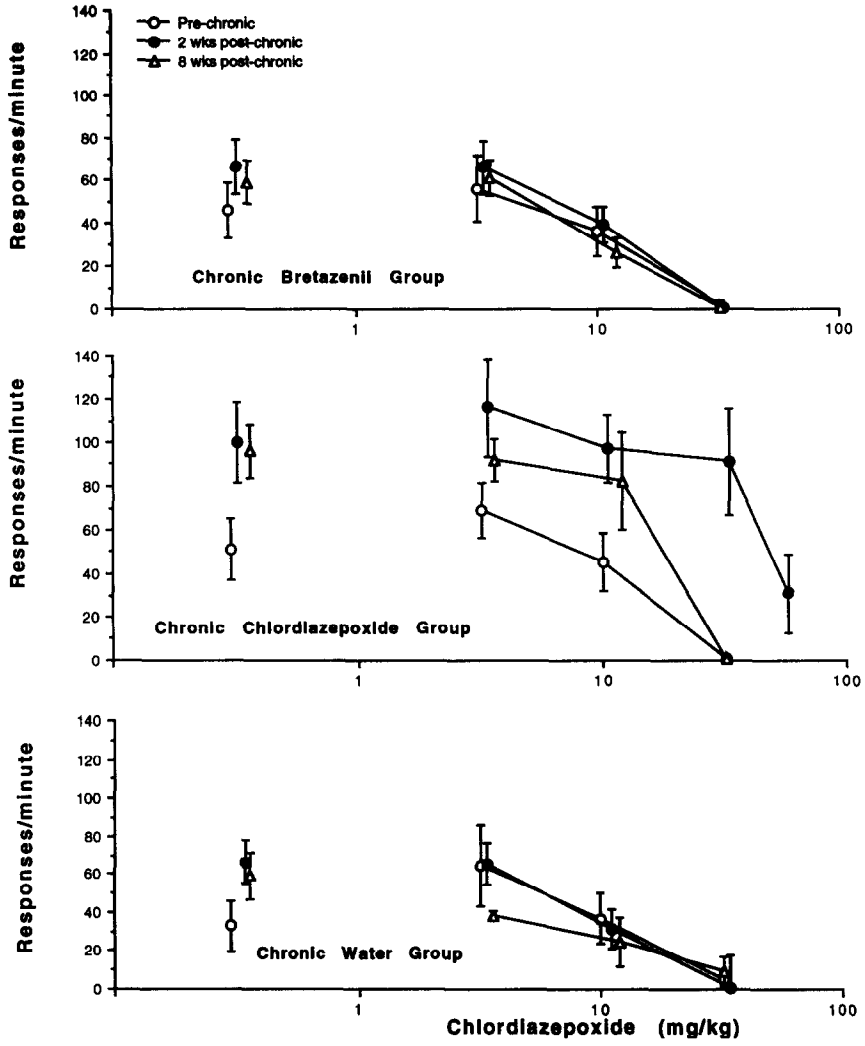

FIG. 5. Effects of cumulative chlordiazepoxide before (○), 2 weeks after cessation of the chronic regimen (●), and 8 weeks after cessation of the chronic regimen (△). Other details are the same as in Fig. 1.

DISCUSSION

In the current study, chronic treatment with either a sedative benzodiazepine, chlordiazepoxide, or a nonsedative benzodiazepine, bretazenil, resulted in tolerance to the rate-decreasing effects of chlordiazepoxide, midazolam, and the beta-carboline anxiolytic, abecarnil. Interestingly, some degree of tolerance was also noted in the chronic water group, a group that received only weekly redeterminations of dose-effect curves for either chlordiazepoxide or bretazenil, or on two occasions midazolam and abecarnil. In the group that was treated chronically with chlordiazepoxide, tolerance to chlordiazepoxide was very long lasting, with the dose-effect curve only approaching prechronic values 8 weeks after cessation of chronic treatment. Tolerance to abecarnil was also evident as long as 15 weeks after cessation of chronic treatment in the chlordiazepoxide group. These results are similar to those obtained by Margules and Stein (14) and Sanger and Zivkovic (25) where tolerance was still apparent weeks after cessation of chronic oxazepam and midazolam, respectively. In contrast to the chronic chlordiazepoxide group, tolerance to chlordiazepoxide was essentially gone 2 weeks after cessation of chronic treatment in both the chronic water and chronic bretazenil groups. These results are similar to those of File (6), who found that tolerance to the sedative effects of lorazepam disappeared within 48 h of the last dose. Taken together, the results of the various studies indicate that some benzodiazepines produce long-lasting tolerance whereas others do not.

Because chronic injections were administered immediately prior to the daily sessions, tolerance may have been due to behavioral factors (i.e., response-contingent tolerance where the animal compensates for lack of reinforcement caused by the drug) (27). This seems unlikely, however, in that chronic bretazenil, a partial agonist that did not affect responding, resulted in tolerance to three rate-decreasing anxiolytics: chlordiazepoxide, midazolam, and abecarnil. Furthermore, tolerance to the discriminative stimulus effects of chlordiazepoxide has been obtained under circumstances that were not response contingent (i.e., while training was suspended) (3).

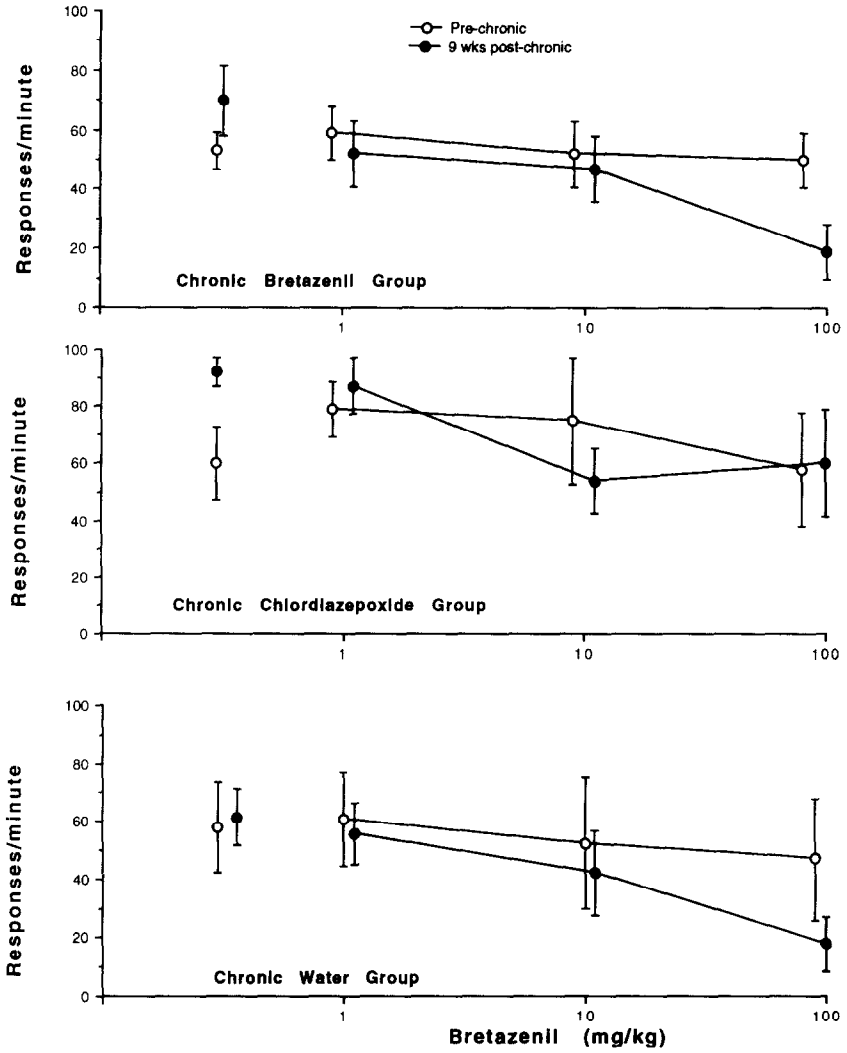

FIG. 6. Effects of cumulative bretazenil before (○) and 9 weeks after cessation of the chronic regimen (●). Other details are the same as in Fig. 1.

The greatly increased responding seen on termination of chronic chlordiazepoxide may represent a manifestation of withdrawal, especially in view of the fact that the increased responding was reversed by chlordiazepoxide. These results are similar to those obtained by McMillan and Leander (17), who found that removal of chlordiazepoxide from the drinking water of chronically treated rats resulted in increased unpunished fixed interval responding in rats. Furthermore, withdrawal signs such as twitches, tremor, piloerection, etc., have been reported up to 2 weeks after the last dose of chlordiazepoxide (24), although in the current study no overt signs of withdrawal were noted. In humans, withdrawal signs and symptoms may persist up to 4 weeks after termination of nitrazepam, another sedative benzodiazepine (22,23). These withdrawal symptoms may be correlated with the increase in norepinephrine and dopamine reported during benzodiazepine withdrawal in rats (1), although no known studies to date have measured levels of these neurotransmitters 2 weeks after benzodiazepine continuation. The fact that bretazenil discontinuation did not produce similar increases in rates of responding in the current study suggests that chronic bretazenil treatment does not result in physical dependence, a finding reported by this investigator and others (4,15,21). Increases in responding were also seen in the chronic chlordiazepoxide group after administration of bretazenil at both 9 and 13 weeks of chronic treatment. Bretazenil has been reported to produce a mild abstinence syndrome in diazepam-dependent monkeys (10), and the increase in responding seen in the current study in chlordiazepoxide-dependent rats may also represent a mild form of bretazenil-elicited chlordiazepoxide withdrawal.

Tolerance to the rate-decreasing effects of chlordiazepoxide, midazolam and abecarnil seen after chronic treatment with bretazenil, a drug that by itself did not affect rate of responding, was unexpected. The daily dose of bretazenil used in this study, 30 mg/kg/day, is a very high dose for this particular drug. In view of studies done in mice, where benzodiazepine receptors were saturated at much lower doses (19), it is likely that the benzodiazepine receptors in the chronic bretazenil treatment group were also saturated in the present study. If benzodiazepine receptors were indeed saturated by breta-

zenil, then it is quite likely that adaptive changes resulted in tolerance. It is possible, for example, that diazepam binding inhibitor (DBI), a negative allosteric regulator of $GABA_A$, increased during the period of chronic drug exposure, effectively attenuating the rate-altering effects of the sedative benzodiazepines. Indeed, it has been shown that DBI is increased in the cerebellum and cortex of rats made tolerant to diazepam (20).

In the present study, chlordiazepoxide was found to be more toxic than bretazenil, at least in this group of "middle-aged" rats. Adverse reactions to chlordiazepoxide have been reported in the 1994 Physicians' Desk Reference, and these include constipation and hepatic dysfunction. Both of these symptoms were found in the chronic and weekly chlordiazepoxide groups. A total of seven rats died following abdominal distention, and necropsy revealed intestinal obstruction and hepatic necrosis. To the author's knowledge, these findings have not been reported in other tolerance studies. This discrepancy may be due to the fact that rats in the current study were over 1 year of age and had already been exposed to a wide range of doses of bretazenil, chlordiazepoxide, and flumazenil. In rats of the same age and with the same drug history, but receiving either chronic or weekly bretazenil, however, these symptoms were not present, and these rats continued to perfom until the age of approximately 2.5 years. Death in these groups was attributed to "old age," as no overt physical abnormalities were found on necropsy.

In summary, it appears that chronic exposure to bretazenil leads to tolerance to the rate-decreasing effects of other drugs that act on the benzodiazepine receptor, even though bretazenil itself does not produce decreases in rate of responding. This tolerance cannot be response contingent because bretazenil does not affect reinforcement density in the fixed interval procedure, even at very high doses. Futher studies will be necessary to determine what is occurring at the biological level.

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