Pharmacology Biochemistry and Behavior, Vol. 56, No. 1, pp. 1–7, 1997

0091-3057/97 $17.00 + .00

PII S0091-3057(96)00120-7

Blockade of Behavioral Effects of Bretazenil by Flumazenil and ZK 93,426 in Pigeons

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Received 18 September 1995; Accepted 9 February 1996

WITKIN, J. M., J. B. ACRI, S. GLEESON AND J. E. BARRETT. *Blockade of behavioral effects of bretazenil by flumazenil and ZK 93,426 in pigeons.* PHARMACOL BIOCHEM BEHAV **56**(1) 1–7, 1997.—Benzodiazepine receptor partial agonists manifest full efficacy in preclinical tests of anxiolytic drug action but do not fully reproduce the discriminative stimulus effects of benzodiazepine receptor full agonists in pigeons. The partial agonist, bretazenil, binds to both diazepam-sensitive and diazepam-insensitive $GABA_A$ receptors. Previous studies have suggested a role for each of these receptor populations in some behavioral effects of bretazenil in pigeons. A possible role for these receptor subtypes in the behavioral effects of bretazenil was further investigated through drug interaction studies with the benzodiazepine receptor antagonists, flumazenil and ZK 93,426. Whereas flumazenil binds with high affinity to both receptor isoforms, ZK 93,426 binds preferentially to diazepam-sensitive binding sites. Bretazenil markedly increased punished responding of pigeons without significantly affecting nonpunished responding. In pigeons discriminating the full benzodiazepine receptor agonist, midazolam, from saline, bretazenil produced only 60–75% maximal effect. Flumazenil and ZK 93,426 neither increased punished responding nor substituted for midazolam, but dose-dependently blocked the effects of bretazenil on punished responding. Flumazenil also dose-dependently blocked the effects of bretazenil in midazolam-discriminating pigeons, whereas ZK 93,426 only attenuated this effect. These results indicate that bretazenil's actions as a partial agonist at diazepam-sensitive benzodiazepine receptors mediate increases in punished responding and substitution for the discriminiative stimulus effects of midazolam in pigeons. The differences in the effects of flumazenil and ZK 93,426 on the discriminative stimulus effects of bretazenil suggest a potential contribution of diazepam-insensitive sites to this behavioral effect.

Bretazenil Flumazenil ZK 93,426 Midazolam Punished behavior Drug discrimination

BRETAZENIL is a benzodiazepine receptor partial agonist that produces many of the pharmacological effects of benzodiazepine receptor full agonists including clinical relief from anxiety (*cf.*, 9,25). Both preclinical (*cf.*, 24,26,39,45,50) and clinical (9,25,42) observations have indicated that bretazenil may be less sedating than full benzodiazepine receptor agonists. Bretazenil produced increases in punished responding of pigeons, a preclinical test of anxiolytic drug action, that were comparable in efficacy to that of full benzodiazepine receptor agonists (50). In contrast, bretazenil only partially mimicked the discriminative stimulus effects of the benzodiazepine receptor full agonist, midazolam, in this species (2,50). Because drug discrimination methods in animals have been widely used as a model of subjective effects (*cf.*, 27,32), these findings in pigeons suggest that, at anxiolytic doses, bretazenil may not fully reproduce the subjective effects of full benzodiazepine receptor agonists.

Although bretazenil is efficacious in preclinical anxiolytic tests (24,39,45), bretazenil also fully replicates the discriminative stimulus effects of full agonists in mammals (4,8,40, 43,46,49), but not in pigeons (2). Likewise, species differences in the effects of bretazenil have been observed in pigeons vs. rats trained to discriminate bretazenil from vehicle (1). In that experiment, midazolam was substituted for bretazenil in rats, but not in pigeons. The basis for the differences in the behavioral effects of bretazenil in pigeons vs. mammals is not known. However, the observation that bretazenil can fully mimic the discriminative stimulus effects of flumazenil in pigeons (52) and the discovery that this common behavioral action is mediated through a diazepam-insensitive $GABA_A$ receptor in pi-

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geons (2,56) may provide a foundation for understanding these effects. Thus, although partial agonism may account for some of the unique pharmacological actions of bretazenil and structurally related imidazobenzodiazepines (16,19,29,33,39), the molecular heterogeneity of $GABA_A$ receptors may also be responsible for the novel pharmacological character of this class of benzodiazepine receptor ligands (*cf.* 10,47). The heterooligomeric $GABA_A$ receptor appears to be encoded by genes for at least three subunit proteins (35,38,47). Pharmacological investigations of reconstituted $GABA_A$ receptors have demonstrated a dramatic ability of receptor structure to modify pharmacological function, both quantitatively and qualitatively (23,33,34,36,54).

In addition to their affinity for classic diazepam-sensitive benzodiazepine receptors, a number of partial agonists have high affinity for other isoforms of the $GABA_A$ receptor. Thus, many of the imidazobenzodiazepines, like bretazenil and Ro 17-1812, bind with nM affinity to diazepam-insensitive binding sites (55,56), which, at least in mammalian species, appear to be constituted by $\alpha 6$ or $\alpha 4$ in conjunction with $\beta 2$ and $\gamma 2$ subunits (36). In pigeons, these diazepam-insensitive receptor ligands fully mimic the discriminative stimulus effects of flumazenil (2,56).

One purpose of the present set of experiments was to demonstrate that bretazenil functions as a benzodiazepine receptor agonist on punished responding and in midazolam discriminations through the use of benzodiazepine receptor antagonists. Although, a host of in vivo observations have been consistent with the partial agonist nature of the effects of bretazenil (*cf.*, 2,3,50) there appears to be only one published report indicating that a behavioral effect of bretazenil can be blocked by benzodiazepine antagonists (14).

The second purpose of these experiments was to evaluate a potential role for diazepam-insensitive sites in the discriminative stimulus and antipunishment effects of the prototypic benzodiazepine receptor partial agonist, bretazenil. This goal was approached through drug-interaction experiments with the benzodiazepine antagonists flumazenil (28) and ZK 93,426 (31). Both antagonists have high affinity for diazepam-sensitive benzodiazepine receptors, but only flumazenil is nonselective and also binds with high affinity to diazepam-insensitive sites in pigeon brain membranes (56). The K_i for flumazenil binding to pigeon cerebellar membranes is 0.9 and 45 nM for diazepam-sensitive and diazepam-insensitive receptors, respectively. The K_i for ZK 93,426 is 2.4 vs. 574 nM for diazepam-sensitive and diazepam-insensitive receptors, respectively. The in vivo selectivites of these antagonists can also be addressed. Although both compounds can readily block behavioral effects of midazolam, ZK 93,426 did not block the discriminative stimulus effects of flumazenil, a behavior thought to be under the control of diazepam-insensitive $GABA_A$ receptors (56). Therefore, if diazepam-insensitive sites are involved in the behavioral effects of bretazenil, there should be a difference in the degree to which effects can be blocked by flumazenil as compared to ZK 93,426.

METHOD

Subjects

Adult, male, White Carneau pigeons (*Columba livia*) (Bowman Gray University Breeders, Winston-Salem, NC and Palmetto Pigeon Plant, Sumter, SC) were maintained in excellent health between 80–90% of their free-feeding body weights (18). The pigeons were used previously in comparative studies of a series of benzodiazepine receptor partial agonists under the same behavioral tests as used here (50). They were individually housed in a temperature- and humidity-controlled vivarium with a 12L : 12D cycle (lights on at 0700 h). Pigeons had continuous access to fresh water and oyster-shell grit. They were fed enough mixed grain after experimental sessions to maintain their body weights throughout the course of the experiment. Experiments were conducted during the light cycle, 5 days per week for behavioral studies.

Punished Responding

Keypeck responses of six pigeons were studied in standard operant chambers (18) described in detail previously (50). Briefly, a response key (Ralph Gerbrands, Arlington, MA) was located in the center of the front panel of the chamber, 23 cm above the floor. The key could be transilluminated with colored lamps and a force exceeding 0.15 N (15 g) produced the audible click of a feedback relay and was recorded as a response. A rectangular opening through which mixed grain could be presented was located below the response key. During the 4-s grain presentation, the food hopper was illuminated with white light. Electric shock (120 V AC, 60 Hz) could be delivered to stainless steel electrodes that were implanted bilaterally around the pubis bone and attached to a plug mounted on the back of a pigeon vest (5). The pigeon was connected to the shock source through a swivel-mounted cable in the ceiling of the chamber which provided free movement of the pigeon during experimental sessions. The impedance of the electrodes was monitored to ensure a constant level of current delivery across the experiment. Shock intensity was adjusted individually for each pigeon to suppress responding by at least 90% of nonpunished response levels (between 2 and 5 mA).

Keypecking was maintained under a multiple fixed-ratio 30 (food), fixed-ratio 30 (food + shock) schedule in which the keylight was either red or white during alternate 3-min components. Under this baseline, every 30th keypeck produced access to mixed grain in the presence of a white keylight. During the alternate schedule component in which the keylight was red, every 30th keypeck, in addition to food presentation, also produced a 200-ms electric shock. A 30-s timeout period, during which the keylights were extinguished and responses had no scheduled consequences, separated schedule components. Experimental sessions began with the component without shock and ended after 10 components of the multiple schedule. Drug experiments were conducted no more than 2 days a week and were separated from each other by at least 2 days.

Midazolam Discrimination

Pigeons were trained to discriminate 1 mg/kg midazolam from saline as described in detail elsewhere (2,50). The apparatus was a variant of that used in the experiments on punished responding described above, the major difference being the existence of three response keys equally distributed in a row across the front panel. In the present experiments, only the two side keys were used (full details in 56). Under the midazolam-saline discrimination, pigeons were injected (IM) with either 1 mg/kg midazolam or saline, 5 min prior to the illumination of the two response keys. When midazolam was administered, 30 consecutive responses on one key produced 4 s access to mixed grain. When saline was given, 30 consecutive responses on the opposite key were required for food delivery. The key associated with midazolam injections was randomized across subjects. Under both midazolam and saline conditions, a re-

sponse on the opposite key (non-injection-associated key) reset the response requirement on the injection-correlated key to 30. Food presentations were separated by 20-s time-out periods during which the chamber was dark and responding had no scheduled consequences. Experimental sessions lasted for 20 food presentations or 15 min whichever occurred first and were conducted 5 days per week.

Evaluation of effects of bretazenil and drug interactions were investigated only if a pigeon responded with an accuracy of at least 85% correct responses across the experimental session and before the first presentation of food in the preceeding baseline training sessions for both midazolam and saline. In these experiments bretazenil was studied either alone (+ vehicle) or in combination with an antagonist in test sessions in which the 30th consecutive response on either the left or right key produced food. Test sessions with 1 mg/kg midazolam or saline were also conducted regularly during dose-effect determinations to establish control values against which to compare the effects of test compounds.

Drugs

ZK 93,426 (ethyl-5-isopropoxy-4-methoxymethyl-β-carboline-3-carboxylate) was donated by Schering AG (Berlin, Germany). Bretazenil and flumazenil were donated by Hoffmann-La Roche (Basel, Switzerland and Nutley, NJ, USA). Compounds were suspended in water and Tween 80 (1 drop/5 ml) by light heating and sonication. Drugs were administered IM (pectoral muscle) in a volume of 1 ml/kg, 5 min prior to testing. Drug antagonism studies were conducted against the effects of 0.3 mg/kg bretazenil. This dose was selected since it produced maximal effects in both behavioral assays (50).

Data Analysis

Rates of responding during the punishment experiments were expressed as percentage changes from baseline levels which included vehicle and non-vehicle control sessions which did not differ from one another. The rate of responding and the overall percentage of responses on the response key correlated with midazolam injections were the primary dependent measures in the drug discrimination experiments. Duplicate determinations of each drug combination were generally collected. Data on the percentage of midazolam-key responses were not used if response rates were decreased below 15% of vehicle control values. Dose-effect functions, were analyzed using data from the linear portion of the curves using standard bioassay analysis of variance techniques (20,48) for repeated measures. Differences in drug effects from control values in the behavioral experiments were determined with Dunnett's test for multiple comparisons.

RESULTS

Punished Responding

Baseline rates of punished responding (0.14 ± 0.02 responses/s) were about 4% of nonpunished response levels (3.8 ± 0.30 responses/s). Bretazenil (0.3 mg/kg) increased rates of punished responding ~3000-fold (Fig. 1 and 2, filled symbol above C in upper panels) without significantly affecting nonpunished response rates (Fig. 1 and 2, filled symbol above C in lower panels). When given alone, flumazenil did not increase punished responding (Fig. 1, open symbols) but dose-dependently blocked the effects of 0.3 mg/kg bretazenil (Fig. 1, top panel, filled symbols). Significant attenuation of the effects of bretazenil was achieved by doses of flumazenil from 0.3–3 mg/kg. The ED_{50} for this effect was 0.18 mg/kg (95% confidence limits = 0.09–0.28). Although flumazenil did not significantly reduce rates of nonpunished responding, there was a significant difference in the effect of 0.3 mg/kg flumazenil alone and 0.3 mg/kg flumazenil in the presence of bretazenil (bottom panel).

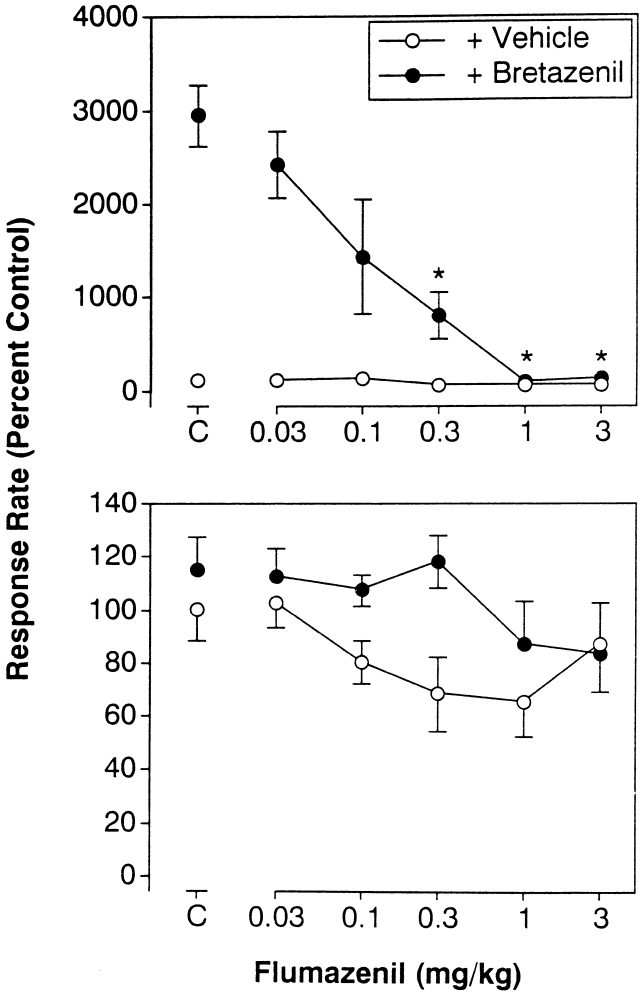

FIG. 1. Effects of flumazenil alone (○) and in combination with 0.3 mg/kg bretazenil (●) on punished (top panel) and nonpunished responding (bottom panel). Each point represents mean ± SEM effects in 4–6 pigeons. $p < 0.05$ compared to effects of 0.3 mg/kg bretazenil alone by Dunnett's test for multiple comparisons.

As with flumazenil, ZK 93,426 alone did not increase punished (Fig. 2, open symbols in top panel) or nonpunished responding (Fig. 2, open symbols in bottom panel). Nonetheless, ZK 93,426 attenuated the increases in punished responding produced by 0.3 mg/kg bretazenil in a dose-dependent manner, with significant blockade observed at 3 and 10 mg/kg (filled symbols in top panel). The ED_{50} for ZK 93,426 was 1.9 mg/kg (0.9–3.0).

Midazolam Discrimination

Under the midazolam discrimination, administration of 1 mg/kg midazolam produced predominately midazolam-appropriate keypeck responses (94 ± 2.1%), whereas saline injec-

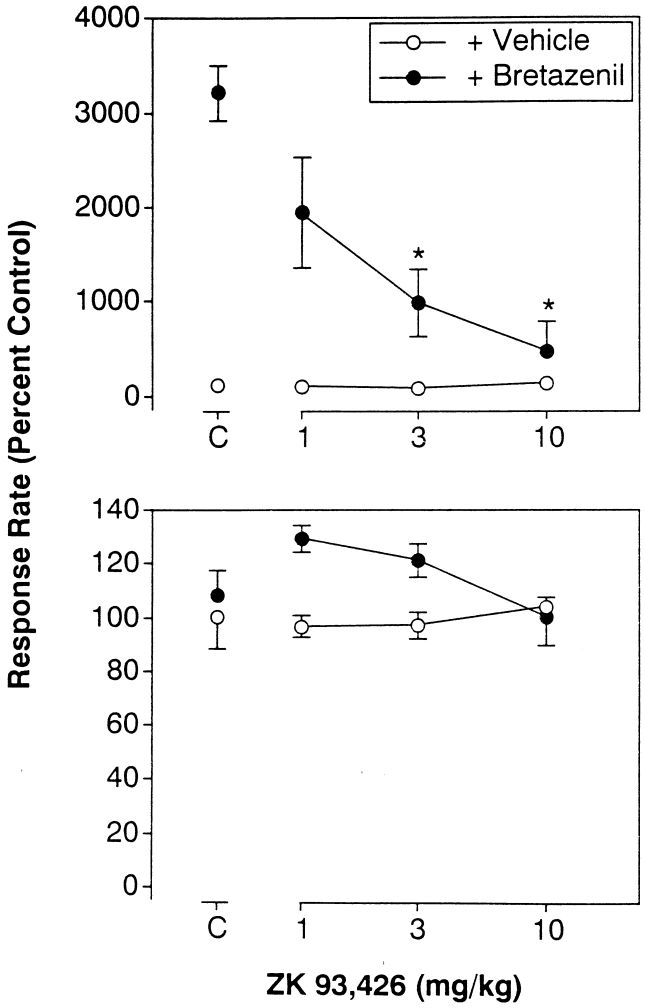

FIG. 2. Effects of ZK 93,426 alone (○) and in combination with 0.3 mg/kg bretazenil (●) on punished (top panel) and nonpunished responding (bottom panel). Each point represents mean ± SEM effects in 4–6 pigeons. $p < 0.05$ compared to effects of 0.3 mg/kg bretazenil alone by Dunnett's test for multiple comparisons.

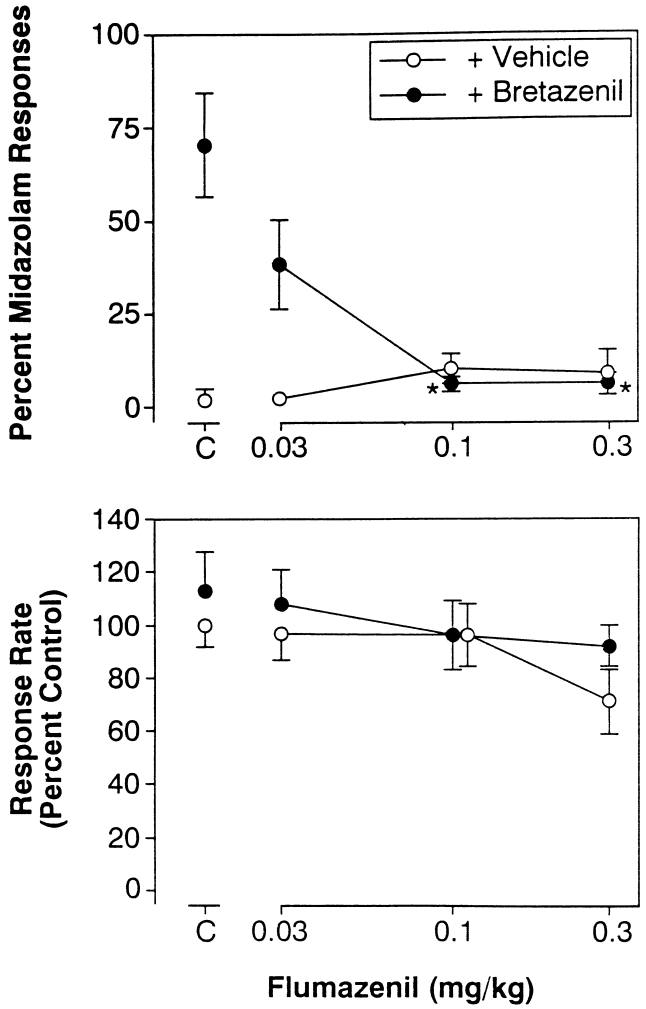

FIG. 3. Effects of flumazenil alone (○) and in combination with 0.3 mg/kg bretazenil (●) in pigeons trained to discriminate 1 mg/kg midazolam from saline. Effects on the percentage of midazolam-appropriate responses (top panel) and on rates of responding under the midazolam-saline discrimination (bottom panel) are shown. Control rates of responding after saline and 1 mg/kg midazolam were 2.1 ± 0.6 and 1.4 ± 0.5 responses/s, respectively. Each point represents the mean ± SEM in four pigeons except at the higher doses of some of the compounds where responding was reduced in individual animals to below 15% of saline control values; at these doses data from at least three pigeons are shown. $p < 0.05$ compared to effects of 0.3 mg/kg bretazenil alone by Dunnett's test for multiple comparisons.

tions engendered only 6.1 ± 1.4% midazolam-appropriate responses. Bretazenil (0.3 mg/kg) set the occasion for either ~75% (Fig. 3) or ~60% (Fig. 4) responses on the midazolam-associated response key (Fig. 3 and 4, filled symbol above C in upper panel), but did not significantly affect rates of responding (Fig. 3 and 4, filled symbol above C in lower panel). Flumazenil did not engender midazolam-appropriate responding (Fig. 3, open symbols in top panel) or alter response rates (Fig. 3, open symbols in bottom panel). Nonetheless, flumazenil completely prevented the effects of bretazenil (filled symbols in top panel) with an ED_{50} of 0.03 mg/kg (0.005–0.13).

ZK 93,426 also did not produce significant midazolam-appropriate responding (Fig. 4, open symbols in top panel) or affect response rates (Fig. 4, open symbols in bottom panel). ZK 93,426 produced an attenuation of the discriminative stimulus effects of bretazenil under the midazolam discrimination (filled symbols, top panel). However, none of the effects ZK 93,426 achieved statistical significance. The ED_{50} for this effect of ZK 93,426 was 4.6 mg/kg (3.1–6.4)

DISCUSSION

Bretazenil produced marked increases in punished responding of pigeons. Increases in punished responding have also been reported in mammalian species (24,39,44,45). Despite the efficacy of bretazenil in these tests, bretazenil only partially substituted for the discriminative stimulus effects of midazolam in pigeons, as observed earlier (2,50). These latter results contrast with the full substitution of bretazenil in rats discriminating full benzodiazepine agonists including midazolam (8,40,43,46). Two, structurally distinct benzodiazepine receptor antagonists, flumazenil and ZK 93,426, dose-dependently attenuated the antipunishment and the discriminative stimulus effects of bretazenil. These observations are the first

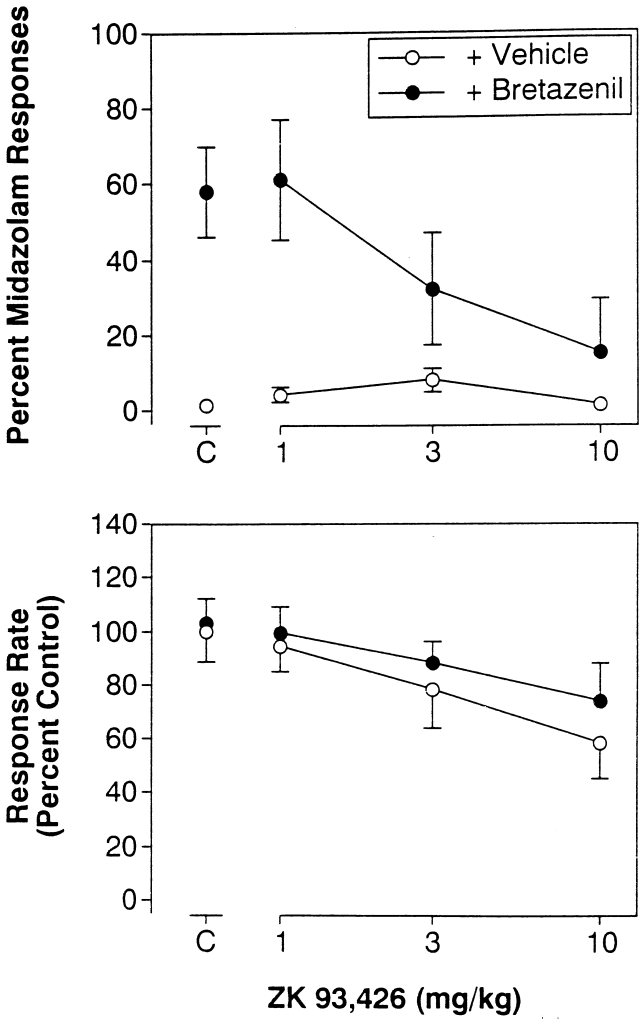

FIG. 4. Effects of ZK 93,426 alone (○) and in combination with 0.3 mg/kg bretazenil (●) in pigeons trained to discriminate 1 mg/kg midazolam from saline. Effects on the percentage of midazolam-appropriate responses (top panel) and on rates of responding under the midazolam-saline discrimination (bottom panel) are shown. Control rates of responding after saline and 1 mg/kg midazolam were 2.4 ± 0.6 and 1.5 ± 0.5 responses/s, respectively. Each point generally represents the mean ± SEM in four pigeons except at the higher doses of some of the compounds where responding was reduced in individual animals to below 15% of saline control values; at these doses data from at least three pigeons are shown.

report of blockade of these effects of bretazenil and are in agreement with a previous report where another behavioral effect of bretazenil (conditioned place preference) was blocked by flumazenil (14). Importantly, the antagonism data indicate that these behavioral effects of bretazenil are due to their agonist actions at benzodiazepine receptors.

Neither of the benzodiazepine antagonists studied, flumazenil or ZK 93,426, increased punished responding nor mimicked the discriminative stimulus effects of midazolam when given alone. Although these compounds have also been reported to lack substitution for the discriminative stimulus effects of full and partial agonists in other studies (1,3,15,21) and have been shown to be devoid of activity in anxiolytic drug screens in pigeons (6) as well as mammals (7,51), other data has demonstrated partial agonist effects of these compounds. Thus, the absence of a partial agonist profile for flumazenil and ZK 93,426 observed here contrasts with the weak anticonvulsant effect reported for these compounds (22,30,31). Flumazenil has also demonstrated occasional, small antipunishment effects (17,37,53), and agonist-like effects in some drug discrimination studies in rats (11–13,41). The lack of benzodiazepine receptor agonist effects of flumazenil (e.g., increases in punished responding of pigeons) may be related, in part, to the tendency of flumazenil to decrease schedule-controlled responding in this species (present study and 51).

Previous observations have indicated a partial agonist pharmacological profile for bretazenil in pigeons. For example, the weak intrinsic efficacy of bretazenil in pigeon brain (as assessed by GABA shift) (52) and the ability of bretazenil to attenuate the discriminative stimulus effects of the full agonist, midazolam (2), are both consistent with partial agonism. However, a number of benzodiazepine receptor partial agonists, including bretazenil and Ro 17-1812, bind with high affinity to an isoform of the $GABA_A$ receptor for which diazepam and midazolam do not bind (2,55,56). These diazepam-insensitive sites potentially contribute to the pharmacological profile of these drugs. Because flumazenil binds with high affinity to both diazepam-sensitive and diazepam-insensitive sites, whereas ZK 93,426 has high affinity only for diazepam-sensitive sites (*cf.*, 56), these antagonists were used as tools to investigate the contribution of these receptor subtypes to the pharmacological effects of bretazenil. Both antagonists produced a dose-dependent blockade of the discriminative stimulus effects of bretazenil, although this effect did not reach statistical significance for ZK 93,426. The lack of significant blockade by ZK 93,426 and the 100-fold greater potency of flumazenil over ZK 93,426 to block the discriminative stimulus effects of bretazenil could be used to argue that the ability of bretazenil to partially substitute for midazolam is at least partially under the control of diazpam-insensitive $GABA_A$ receptors. While this arguement is appealing for present purposes, it is at odds with the fact that the discriminative stimulus of midazolam are mediated by diazepam-sensitive receptors (*cf.*, 15) and that midazolam does not bind to to diazepam-insensitive sites (56). Nonetheless, bretazenil has high affinity for diazepam-insensitive receptors and actions at this site have specific discriminative stimulus effects (56). Therefore, some as yet unspecified dynamic interplay may occur between actions at diazepam-sensitive and diazepam-insensitive $GABA_A$ receptors. In contrast, the full blockade of the effects of bretazenil on punished responding by both antagonists, suggests that diazepam-sensitive benzodiazepine receptors primarily underlie this effect of bretazenil.

The high affinity binding of bretazenil for these sites also appears to regulate other behavioral effects of this compound. Bretazenil and other ligands of diazepam-insensitive $GABA_A$ receptors fully reproduce the discriminative stimulus effects of the GABA-neutral antagonist (52), flumazenil (2,56). Both direct and correlative data from these studies has linked this common behavioral effect to diazepam-insensitive receptors. In addition, diazepam-insensitive binding may be involved in the discriminative stimulus effects of bretazenil in pigeons trained explicitly to discriminate bretazenil from vehicle (1). It is also possible that this receptor subtype is involved in the reduced side effect profile of bretazenil, as diazepam-insensitive receptors are largely located in the cerebellum (*cf.*, 34,56) and have been implicated in the motor effects of benzodiazepines (34). The discovery of a selective antagonist for

diazepam-insensitive $GABA_A$ receptors will be essential to a more refined analysis of this and related problems.

ACKNOWLEDGEMENTS

We thank Drs. Nancy Ator and Dai Stephens for their comments on an initial draft of this manuscript. The expert technical assistance of Michael Chider is gratefully acknowledged. We thank W. Haefely (Hoffmann-La Roche, Basel, Switzerland), P. Sorter (Hoffmann-La Roche, Nutley, NJ, USA), and D. N. Stephens (Schering AG, Berlin, Germany) for their help in obtaining the drugs used in this experiment. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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