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Stereospecific transduction of behavioral effects via diazepam-insensitive $GABA_A$ receptors

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Abstract

Previous studies reported a positive correlation between ligand affinities at diazepam-insensitive $GABA_A$ receptors and substitution for the discriminative stimulus effects of the benzodiazepine receptor antagonist, flumazenil, in pigeons. In the present experiments, bretazenil and Ro 14-5974 (ethyl-(*S*)-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]-pyrrolo-[2,1-*c*][1,4]benzodiazepine-1-carboxylate) partially substituted for, and blocked the discriminative stimulus effects of midazolam, congruent with their actions at diazepam-sensitive $GABA_A$ receptors in vitro. In addition, bretazenil and Ro 14-5974, but not their *R*-enantiomers, had high affinity for diazepam-insensitive receptors and fully substituted for the discriminative stimulus effects of flumazenil. The *R*-enantiomers of these compounds had low affinity ($K_i > 1$ μM) for diazepam-sensitive and diazepam-insensitive receptors, and did not share discriminative stimulus effects with flumazenil or midazolam. Ro 19-0528 (7-chloro-3-(3-cyclopropyl-1,2,4-oxadiazol-5-yl)-4,5-dihydro-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one), a structurally related compound with full agonist actions at diazepam-sensitive $GABA_A$ receptors, had high diazepam-insensitive receptor affinity ($K_i = 96$ nM) and partially substituted for the discriminative stimulus effects of flumazenil. These results are consistent with stereospecific mediation of the discriminative stimulus effects of flumazenil through high affinity binding to diazepam-insensitive receptors in pigeons.

Keywords: Benzodiazepine; $GABA_A$ receptor, diazepam-insensitive; Drug discrimination; Bretazenil; Flumazenil; (Receptor partial agonist); Ro 19-0528; GABA (γ-aminobutyric acid); (Pigeon)

1. Introduction

The γ-aminobutyric acid-A ($GABA_A$) receptor supramolecular complex includes allosteric modulatory sites that bind benzodiazepines as well as other structurally diverse molecules. A number of $GABA_A$ receptor subtypes have been identified on the basis of subunit composition (Lüddens and Wisden, 1991; Pritchett et al., 1989a,b), radioligand binding characteristics (Pritchett et al., 1989a; Wong et al., 1993a), and differential localization within the central nervous system (Young and Kuhar, 1980; Young et al., 1981; Edgar and Schwartz, 1990). For almost a decade, one of the defining characteristics of native $GABA_A$ receptors had been the ability to bind diazepam with high affinity. However, a number of additional $GABA_A$ receptors with characteristics consistent with α_6 subunit constitution (Lüddens et al., 1990) have been identified by labeling with [^{3}H]Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) (Sieghart et al., 1987) or ZG-63 (*tert*-butyl-8-chloro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) (Gu et al., 1993; Wong et al., 1993a), and unlike the majority of $GABA_A$ receptors, binding at a portion of these sites is not displaced by diazepam. These sites have been termed diazepam-insensitive $GABA_A$ receptors. The only ligands identified to date that bind to diazepam-insensitive receptors are not selective, and usually bind with higher affinity to diazepam-sensitive than to diazepam-insensitive $GABA_A$ receptors (Wong and Skolnick, 1992a; Wong et al., 1993b). Given the

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lack of selective diazepam-insensitive receptor ligands, identification of the pharmacological and behavioral effects transduced through diazepam-insensitive receptors has been difficult to establish.

The function of diazepam-insensitive binding sites in the modulation of $GABA_A$ receptor function has not yet been established because the efficacy of diazepam-insensitive receptor ligands to significantly modulate GABA binding is weak or absent when using the predictive GABA-shift assay in brain tissue (Turner et al., 1991; Uusi-Oukari, 1992; Wong and Skolnick, 1992a,b; Wong et al., 1993a). Although there is a recent report that the diazepam-insensitive receptor ligand, Ro 15-4513, can modulate GABA binding in a cell culture preparation expressing the α_6 subunit (Korpi and Seeburg, 1993), there is no functional in vitro assay in which to establish the efficacy of diazepam-insensitive receptor ligands in natural tissue. However, a behavioral assay has been proposed in which diazepam-insensitive receptor ligand binding affinity has been positively correlated with substitution for the discriminative stimulus effects of flumazenil in pigeons (Wong et al., 1993b).

Flumazenil (Ro 15-1788), the prototypic diazepam-sensitive $GABA_A$ receptor antagonist (Hunkeler et al., 1981), is devoid of behavioral activity across a wide dose range in mammals including man (Hunkeler et al., 1981; Bonetti et al., 1982; Brogden and Goa, 1988). When trained as a discriminative stimulus in rats, there have been reports of full substitution by receptor full agonists (De Vry and Slangen, 1985), partial substitution by a variety of compounds including the receptor inverse agonist DMCM (Woudenberg and Slangen, 1989), and full substitution by antagonists (Rowan and Lucki, 1992). In contrast to its apparent lack of specificity as a discriminative stimulus in rats, flumazenil can act as a potent and specific discriminative stimulus in pigeons, when considered in light of the diazepam-insensitive receptor subtype. In pigeons trained to discriminate flumazenil from vehicle, a number of compounds with affinity for diazepam-insensitive receptors fully substituted for flumazenil, despite functioning as partial agonists, antagonists, or partial inverse agonists at diazepam-sensitive $GABA_A$ receptors (Wong et al., 1993b). Conversely, compounds without affinity for diazepam-insensitive receptors did not substitute for flumazenil, despite being partial agonists, antagonists, or partial inverse agonists at diazepam-sensitive $GABA_A$ receptors. Providing evidence that the diazepam-insensitive $GABA_A$ receptor subtype may mediate a specific behavioral effect, Wong et al. (1993b) reported a significant correlation between the potency of a series of compounds to substitute for flumazenil in pigeons and their affinities at cerebellar diazepam-insensitive receptors, raising the possibility that a specific discriminative stimulus is mediated through binding at diazepam-insensitive receptors in the pigeon brain, and establishing a behavioral assay with which to evaluate the effects of diazepam-insensitive receptor ligands in this species.

There were several additional findings of Wong et al. (1993b) that specifically addressed the question of whether the discriminative stimulus effects of flumazenil were mediated through diazepam-insensitive or diazepam-sensitive receptors. First, a full agonist at diazepam-sensitive receptors, midazolam, did not substitute for the discriminative stimulus effects of flumazenil, and was ineffective in blocking the discriminative stimulus effects of flumazenil. Additionally, ZK 93,426 (ethyl-5-isopropoxy-4-methoxymethyl-β-carboline-3-carboxylate), a diazepam-sensitive receptor antagonist with affinity only at diazepam-sensitive receptors, did not substitute for, or block the discriminative stimulus effects of flumazenil. Further, the compounds that fully substituted for flumazenil had a range of intrinsic efficacies at diazepam-sensitive receptors, and shared only the characteristic of high affinity binding to diazepam-insensitive receptors. Finally, there was a significant correlation between binding affinities at diazepam-insensitive, but not diazepam sensitive receptors, and potency to substitute for the discriminative stimulus effects of flumazenil. Although Wong et al. (1993b) provided the above evidence to suggest that diazepam-insensitive receptors mediate the discriminative stimulus effects of flumazenil, additional confirmation of receptor mediation and the functional integrity of diazepam-insensitive binding sites would derive from the demonstration of stereospecificity of diazepam-insensitive receptor binding and the mediation of behavioral effects.

The present studies were therefore initiated to further examine the discriminative stimulus effects of diazepam-insensitive receptor ligands, and to specifically address several issues. The first purpose was to investigate whether enantiomeric specificity is necessary for transduction of behavioral effects of diazepam-insensitive receptor ligands in pigeons as well as for in vitro binding. The conformational requirements for diazepam-insensitive receptor binding in the rat were recently explored through the synthesis of enantiomers of pyrroloimidazobenzodiazepines. Fryer et al. (1994) reported that for diazepam-insensitive receptor binding in rat cerebellum, *S*-enantiomers bind with higher affinity than *R*-enantiomers by two orders of magnitude, similar to stereospecificity requirements for diazepam-sensitive receptor binding (Blount et al., 1983). However, behavioral effects of enantiomers at diazepam-insensitive receptor sites in pigeons, including discriminative stimulus effects, have not been evaluated to date. To that end, several imadazodiazepine compounds, including two pairs of enantiomers (see Fig. 1), were tested for diazepam-insensitive receptor

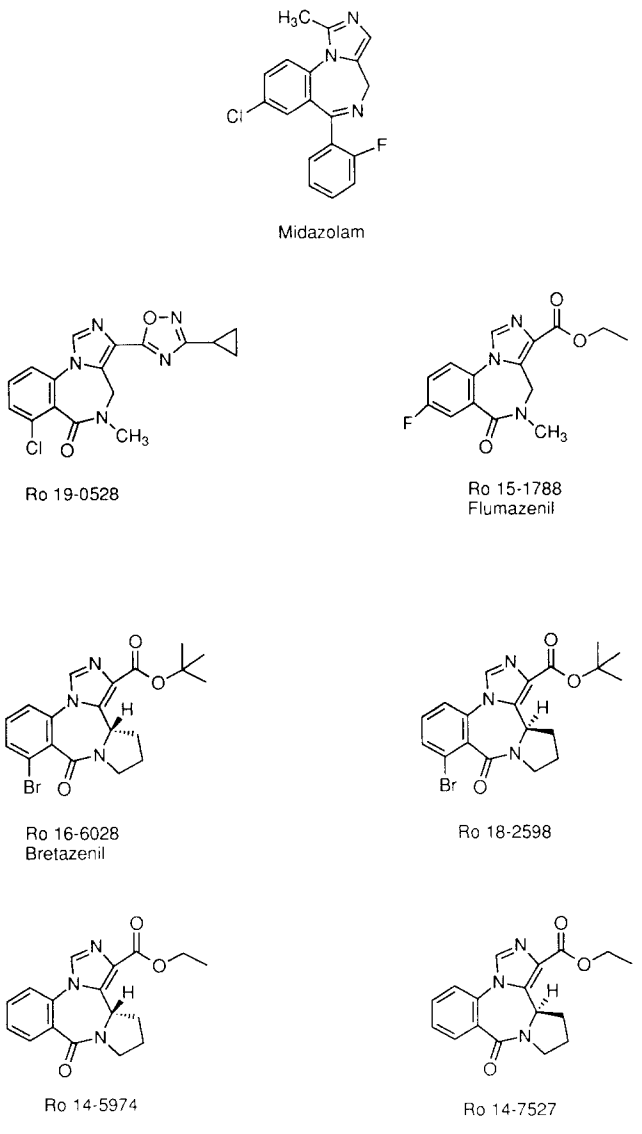

Fig. 1. Molecular structures.

binding affinity and ability to substitute for the discriminative stimulus effects of flumazenil. In addition, because compounds also have affinity for diazepam-sensitive $GABA_A$ receptors, compounds were assessed for classic, diazepam-sensitive receptor-mediated effects. Therefore, the abilities of compounds to substitute for, and to block discriminative stimulus effects of midazolam were determined. These results are required in order to establish that the effects of diazepam-sensitive receptor ligands in pigeons are consistent with effects in other species, and therefore cannot account for previously established unique behavioral effects of diazepam-insensitive receptor ligands in pigeons (Witkin and Barrett, 1985; Wong et al., 1993b) in contrast to rodents (Rowan and Lucki, 1992; De Vry and Slangen, 1985; Woudenberg and Slangen, 1989). Further, differences in the discriminative stimulus effects of compounds tested in midazolam-trained pigeons as compared to flumazenil-trained pigeons lend additional support for the dissociation of diazepam-sensitive and diazepam-insensitive receptor-mediated contributions to discriminative stimulus effects of these compounds.

A second purpose of the present experiments was to examine the behavioral effects and diazepam-insensitive receptor binding affinity of a compound known to be a full receptor agonist at diazepam-sensitive sites. To date, most diazepam-insensitive receptor ligands that have been identified are partial modulators of GABA at diazepam-sensitive receptors (Korpi et al., 1992; Wong et al., 1993b; Wong and Skolnick, 1992a), with the exception of DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), a full inverse agonist at diazepam-sensitive receptors (Turner et al., 1991; Wong et al., 1993b). However, results of behavioral experiments with DMCM are equivocal because of proconvulsant effects (Wong et al., 1993b). Therefore, the full diazepam-sensitive receptor agonist, Ro 19-0528 (7-chloro-3-(3-cyclopropyl-1,2,4-oxadiazol-5-yl)-4,5-dihydro-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one) (Kyburz, 1986), was evaluated for diazepam-insensitive receptor binding affinity and behavioral effects based on its structural similarity to other diazepam-insensitive receptor ligands. Thus, Ro 19-0528 provides the first opportunity to examine discriminative stimulus effects of diazepam-insensitive receptor occupation in a diazepam-sensitive receptor full agonist.

2. Materials and methods

2.1. Binding assays

Methods for radioligand binding were similar to those described previously (Wong and Skolnick, 1992a; Wong et al., 1993b). Experimentally naive male pigeons of the same approximate age as those used in the behavioral experiments were maintained at approximately 90% of their free-feeding body weights. Pigeons were killed by decapitation, and brains were dissected on ice (Karten and Hodos, 1967). Cerebella were weighed and disrupted (Brinkmann Polytron, setting 7, 10 s) in either 30 or 60 volumes of 50 mM Tris-citrate buffer (pH 7.8). Homogenates were centrifuged at $20000 \times g$ for 30 min (4° C), resuspended in 60 volumes of buffer, and recentrifuged. This washing procedure was repeated a total of 5 times. Homogenates were frozen at −40° C and resuspended (Brinkmann Polytron setting 7, 5 s) immediately prior to use.

Total $GABA_A$ receptor (diazepam-sensitive + diazepam-insensitive receptor) binding assays were performed in a total volume of 0.5 ml consisting of 0.1 ml of tissue suspension (~ 50–100 μg protein), 0.05 ml

[^{3}H]Ro 15-4513 (specific activity 24.1 Ci/mmol, New England Nuclear, Boston, MA, USA, final concentrations ~ 2.42–2.53 nM), 0.05 ml of 0.2 M NaCl, and Tris-citrate buffer (pH 7.8) to volume. Nonspecific binding was determined in the presence of 10 μM flumazenil and typically represented less than 10% of total binding. For diazepam-insensitive receptor binding, 0.05 ml of buffer was replaced with 10 μM diazepam. Diazepam-sensitive receptor binding was determined by subtracting the concentration of diazepam-insensitive receptor sites from diazepam-sensitive + diazepam-insensitive receptor sites. Stock solutions of compounds (1 mM) in ethanol were diluted in buffer to yield the desired six drug concentrations for each experiment. Incubations (0–4° C) were initiated by the addition of tissue and terminated after 60 min by rapid filtration with two 5-ml washes of ice-cold Tris-citrate buffer through Whatman GF/B filters using a Brandel M-48R filtering manifold (Brandel Instruments, Gaithersburg, MD, USA). The radioactivity retained by the filter was measured in an LS 5801 liquid scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Protein content was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Assays were performed in duplicate and the values presented are the mean ± S.E.M. of three experiments. Data were analyzed with the GraphPad Inplot 4.0 iterative curve fitting program for competitive binding. All curves were fit with an R^2 value ≥ 0.92.

2.2. Subjects in behavioral tests

Subjects were male, white Carneaux pigeons (*Columba livia*, Bowman Gray University Breeders) maintained in excellent health at 80–90% of their free-feeding weights, and were similar to those which provided cerebella for radioligand binding assays. They were housed one per cage, with a 12 h light/dark cycle, with water and grit freely available. All animals had been used in previous experiments.

2.3. Materials

Midazolam maleate was dissolved in 0.9% NaCl and all other compounds were suspended with 1 drop Tween-80 per/5 ml distilled water. All compounds were administered intramuscularly in a volume of 1 ml/kg, 5 min prior to testing. Drugs, including bretazenil (Ro 16-6028) (*tert*-butyl-(*S*)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]-pyrrolo-[2,1-*c*][1,4]-benzodiazepine-1-carboxylate), Ro 18-2598 (*tert*-butyl-(*R*)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]-pyrrolo-[2,1-*c*][1,4]benzodiazepine-1-carboxylate), Ro 14-5974 (ethyl-(*S*)-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]-pyrrolo-[2,1-*c*][1,4]benzodiazepine-1-carboxylate), and Ro 14-7527 (ethyl-(*R*)-11,-12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]-pyrrolo-[2,1-*c*][1,4]benzodiazepine-1-carboxylate) were generously donated from Hoffmann-La Roche through Dr. Peter Sorter (Nutley, NJ, USA) and Dr. Willy Haefely (Basel, Switzerland).

2.4. Flumazenil substitution

Pigeons were previously trained to discriminate 0.1 mg/kg flumazenil from vehicle as described in detail elsewhere (Wong et al., 1993b), in operant chambers equipped with two response keys, a feeder that provided mixed grain reinforcers, white noise, and a ventilating fan. Each response key was 2 cm in diameter and was transilluminated with red (right key) or white (left key) light. A minimum key peck force of 15 g was required to produce a relay click and record a response. When the 0.1 mg/kg training dose of flumazenil was administered, 30 consecutive responses on one key produced 4 s access to grain and responses on the other key reset the response requirement to 30. When vehicle was given, 30 consecutive responses on the opposite key were required for food delivery. The response key correlated with flumazenil was counterbalanced across pigeons. Food presentations were followed 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.

When responding occurred reliably on the injection-correlated key (at least 85% correct responses overall and before the first presentation of food), dose-response functions for test compounds were determined by substitution of the test compound for the training dose of flumazenil. During these test sessions, 30 consecutive responses on either response key produced food. Test sessions with the training dose of flumazenil or vehicle also occurred throughout the experiment. Test sessions were conducted no more than twice weekly, were separated by at least 2 days, and were conducted only if the pigeons met the 85% correct criterion on the previous experimental sessions.

2.5. Midazolam substitution

Compounds were also tested for their ability to substitute for midazolam, which has affinity only for diazepam-sensitive $GABA_A$ receptors. A separate group of pigeons had been trained to discriminate 1.0 mg/kg midazolam using the same methods as those described for flumazenil discrimination. After responding on the injection-correlated key was reliable as defined by the 85% correct criterion, dose-response functions for midazolam and other compounds were

Table 1
Ligand affinities for diazepam-sensitive and diazepam-insensitive $GABA_A$ receptors in pigeon cerebellum and behavioral effects

Compound	K_i (nM) (diazepam-sensitive receptors) [a]	K_i (nM) (diazepam-insensitive receptors) [a]	Enantiomer	Flumazenil substitution [b]	Midazolam substitution [b]	Midazolam blockade [c]
1. Ro 16-6028 (bretazenil) [d]	0.7 ± 0.1	14 ± 3	*S*	96.2% (3.0 mg/kg)	69.8% (0.3 mg/kg)	37.4% (0.3 mg/kg)
2. Ro 18-2598	$IC_{50} > 1000$	$IC_{50} > 1000$	*R*	2.8% (0.1 mg/kg)	1.8% (1.0 mg/kg)	99.6% (0.1 mg/kg)
3. Ro 14-5974 [e]	8.8 ± 2.6	427.0 ± 3	*S*	99.5% (1.0 mg/kg)	36.2% (0.3 mg/kg)	22.3% (1.0 mg/kg)
4. Ro 14-7527 [f]	$IC_{50} > 1000$	$IC_{50} > 1000$	*R*	0.9% (10.0 mg/kg)	0.1% (1.0 mg/kg)	99.3% (1.0 mg/kg)
5. Ro 19-0528	0.6 ± 0.03	95.8 ± 9.0	*S*	62.9% (0.3 mg/kg)	93.3% (1.0 mg/kg)	

[a] Affinities are means ± S.E.M. from three experiments. [b] Substitution data are shown as maximum percent drug-key responding in 4–8 pigeons, and the dose at which the response was obtained is shown in parentheses in mg/kg body weight. [c] Midazolam blockade results are shown as the minimum percent midazolam-key responding in 4–8 pigeons when the compounds were given in conjunction with the training dose of midazolam (1 mg/kg). The dose at which the response was obtained is shown in parentheses in mg/kg body weight. The training dose of midazolam produced 99.83 ± 5% midazolam-key responses when given alone. Midazolam blockade was not studied with Ro 19-0528 because it is a full agonist and fully substituted for midazolam. [d] Wong et al. (1993a,1993b report K_i (nM) values for this compound at diazepam-sensitive receptors = 0.6 ± 0.1 and diazepam-insensitive receptors = 12 ± 4 in pigeon cerebellum. [e] Fryer et al. (1994) report K_i (nM) values for this compound at diazepam-sensitive receptors = 0.9 ± 0 and diazepam-insensitive receptors = 45.1 ± 13 in rat cerebellum. [f] Fryer et al. (1994) report K_i (nM) at diazepam-sensitive receptors = 1667, and for diazepam-insensitive receptors, IC_{50} (nM) > 10000 in rat cerebellum.

determined by the substitution of test compounds for the training dose of midazolam, using methods described above.

2.6. Midazolam blockade

Compounds were tested for their ability to block the discriminative stimulus effects of midazolam in a third group of pigeons trained to discriminate 1.0 mg/kg midazolam from vehicle. Training methods were identical to those described above. Once responding occurred reliably on the injection-correlated key, dose-response functions for blockade of midazolam discrimination were determined. Test drugs were administered simultaneously with the training dose of midazolam, and tests were conducted no more than twice weekly.

2.7. Data analysis

The rate of responding and the overall percentage of responses occurring on the key correlated with injection of the training drug (flumazenil or midazolam) were the primary dependent measures. Each data point represents at least duplicate determinations in each animal, where availability of compound permitted. Data on the percentage of responses for individual animals were not used if response rates decreased below 15% of vehicle control values for that experiment. Dose-effect functions, derived from mean values across subjects, were analyzed using data from the linear portion of the curves using standard bioassay analysis of variance techniques for repeated measures (Finney, 1964; Snedecor and Cochran, 1980), followed by post-hoc Dunnett's tests.

3. Results

3.1. Radioligand binding

The affinities of two partial agonists and their *R*-enantiomers, and a full agonist at diazepam-sensitive receptor sites were determined at diazepam-sensitive and diazepam-insensitive benzodiazepine receptors in pigeon cerebellum (See Table 1). In accordance with previous characterization of requirements for binding at diazepam-sensitive receptor sites (Fryer et al., 1994; Blount et al., 1983), there was specific binding only by the *S*-enantiomers, bretazenil and Ro 14-5974, at both diazepam-sensitive and diazepam-insensitive receptor sites, with no measurable specific binding by the *R*-enantiomers, Ro 18-2598 and Ro 14-7527 ($IC_{50} > 1000$ nM). The benzodiazepine receptor full agonist, Ro 19-0528, bound with nanomolar affinity to both the diazepam-sensitive and diazepam-insensitive binding sites. All compounds with affinity for diazepam-insensitive receptors, including Ro 19-0528, had greater affinity for diazepam-sensitive receptors.

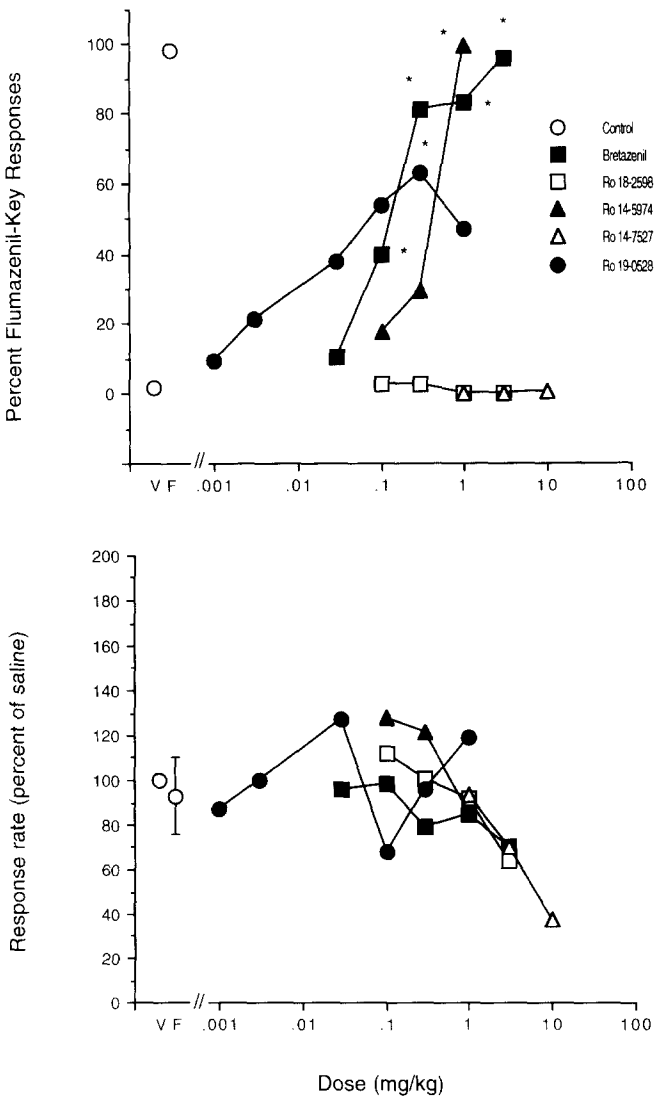

Fig. 2. Upper panel: The discriminative stimulus effects of bretazenil and Ro 14-5974 (filled symbols) and their inactive enantiomers Ro 18-2598 and Ro 14-7527 (corresponding open symbols) in pigeons trained to discriminate flumazenil from vehicle. Control values for the training dose of flumazenil (0.1 mg/kg) and vehicle are shown with open circles above F and V, respectively (means ± S.E.M.s). Filled circles indicate discriminative stimulus effects of the full receptor agonist Ro 19-0528. Asterisks signify points significantly different from vehicle control values. Lower panel: Effects of drugs on rates of responding. Rates were not significantly different from those of vehicle controls. Each point represents the mean of values derived from four pigeons.

3.2. Drug substitution experiments

Flumazenil substitution

Bretazenil and Ro 14-5974 dose-dependently substituted for the discriminative stimulus effects of flumazenil, and Ro 19-0528 partially substituted, reaching a maximum of 60% (Fig. 2). In contrast, *R*-enantiomers

of bretazenil and Ro 14-5974, Ro 18-2598 and Ro 14-7527, respectively, did not engender flumazenil-key responding. Dose-effect curves for bretazenil and Ro 14-5974 did not deviate from parallelism, and yielded similar maximum efficacy. In contrast, the substitution curve for Ro 19-0528 was not parallel to substitution curves for bretazenil or Ro 14-5974, and suggested lower efficacy by reaching a maximum of only 60%. The effects of test compounds on rates of responding were not statistically significant, although *R*-enantiomers Ro 18-2598 and Ro 14-7527 dose-dependently reduced response rates.

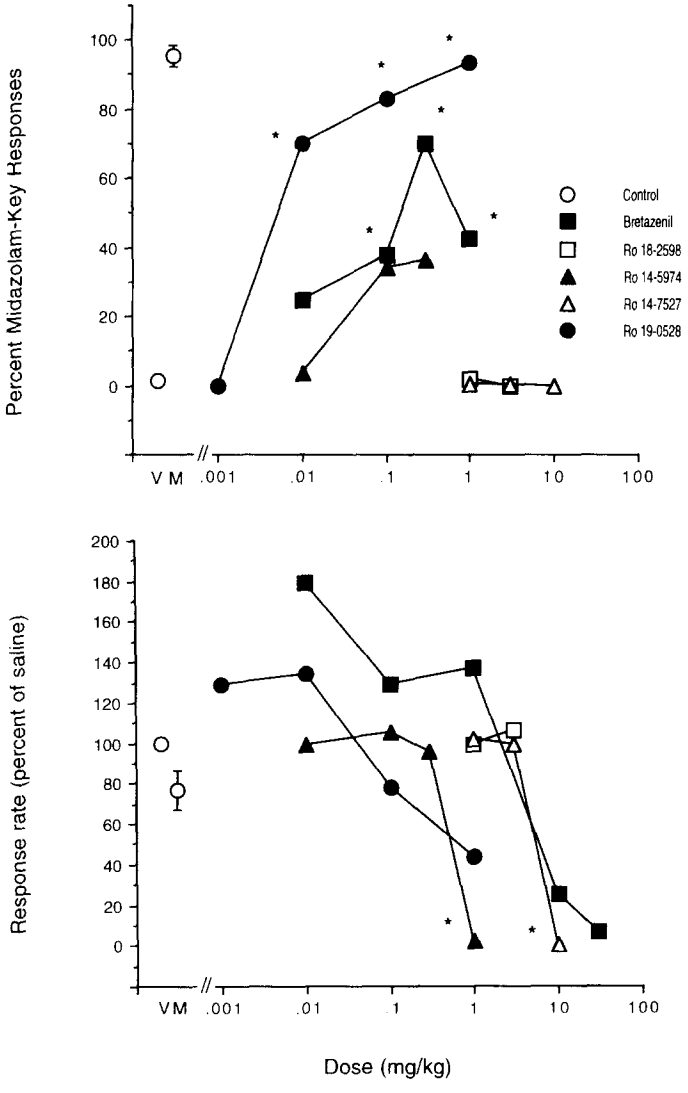

Fig. 3. Upper panel: The discriminative stimulus effects of bretazenil and Ro 14-5974 (filled symbols) and their inactive enantiomers Ro 18-2598 and Ro 14-7527 (corresponding open symbols) on midazolam discrimination. Filled circles indicate discriminative stimulus effects of the full receptor agonist Ro 19-0528. Control values for the training dose of midazolam (1.0 mg/kg) and vehicle are shown with open circles above M and V, respectively (means ± S.E.M.s). Asterisks signify points significantly different from vehicle control. Lower panel: Effects of drugs on rates of responding. Rates were significantly different from those of vehicle controls only for 1.0 mg/kg Ro 14-5974 and its enantiomer, Ro 14-7527 at 10 mg/kg. Each point represents the mean of values derived from four pigeons.

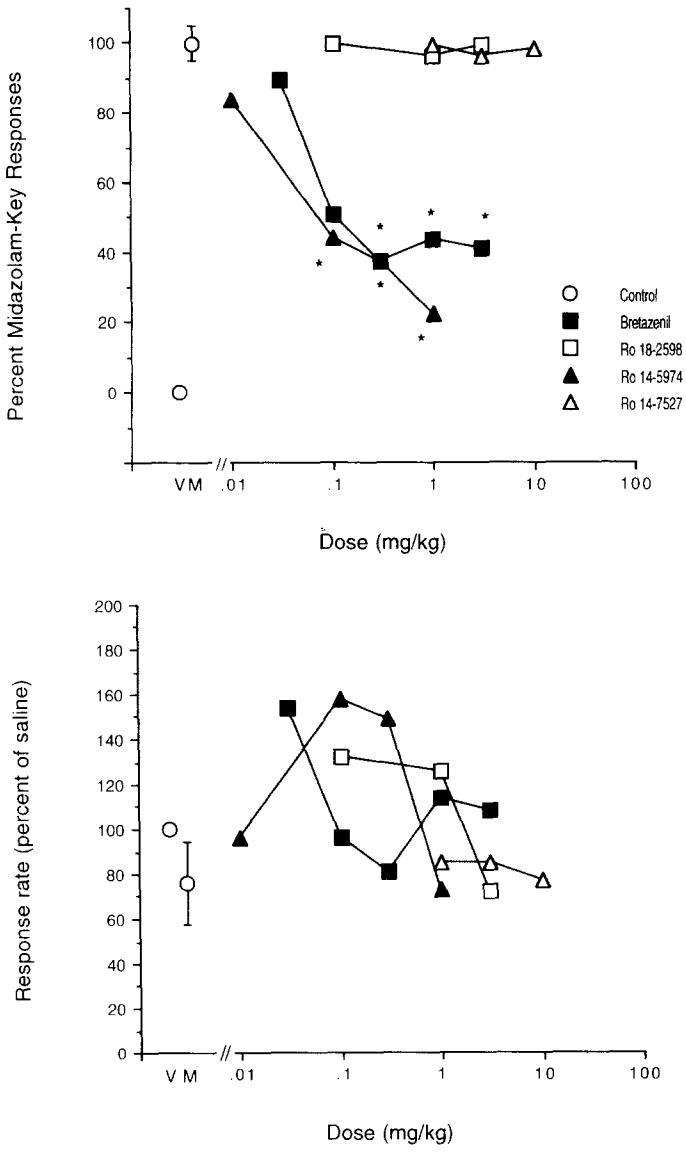

Fig. 4. Upper panel: Blockade of midazolam discriminative stimulus effects by receptor partial agonists (filled symbols) and their enantiomers (corresponding open symbols) in pigeons trained to discriminate midazolam from vehicle. Control values for the training dose of midazolam (1.0 mg/kg) and vehicle are shown with open circles above M and V, respectively (means ± S.E.M.s). Asterisks signify points significantly different from midazolam control, indicating blockade of discriminative stimulus effects of midazolam. Lower panel: Effects of drugs in combination with 1.0 mg/kg midazolam on rates of responding. Each point represents the mean of values derived from eight pigeons.

Midazolam substitution

The diazepam-sensitive receptor full agonist, Ro 19-0528, substituted fully for the discriminative stimulus effects of midazolam, while both bretazenil and Ro 14-5974, partial agonists at diazepam-sensitive receptors, substituted only partially for midazolam (Fig. 3).

Of the receptor partial agonists, only bretazenil engendered levels of responding on the midazolam key that were significantly different from drug vehicle. Linear portions of the dose-effect curves were compared in pairs and did not differ significantly from parallelism. Bretazenil and Ro 14-5974 were both less efficacious than Ro 19-0528 in substitution for midazolam, but did not differ from each other in efficacy. In contrast to bretazenil and Ro 14-5974, the *R*-enantiomers, Ro 18-2598 and Ro 14-7527, did not engender midazolam-key responding. Rates of responding were significantly decreased by 1 mg/kg Ro 14-5974 and 10 mg/kg Ro 14-7527.

3.3. Midazolam blockade experiments

Bretazenil and Ro 14-5974, which partially substituted for midazolam, also partially blocked the discriminative stimulus effects of midazolam at doses that did not significantly reduce response rates. Analysis of the linear portions of dose-effect curves indicated no deviation from parallelism and suggested similar efficacy (Fig. 4). The *R*-enantiomers, Ro 18-2598 and Ro 14-7527, had no effect on the discriminative stimulus effects of midazolam or on response rates.

4. Discussion

The present experiments support the hypothesis that compounds binding at the diazepam-insensitive $GABA_A$ receptor produce discriminative stimuli by establishing stereospecificity as a requirement for both binding and the transduction of discriminative stimulus effects. Consistent with Wong et al. (1993b), compounds with affinity at the diazepam-insensitive $GABA_A$ receptor substituted for flumazenil in pigeons trained to discriminate flumazenil from vehicle. Moreover, *R*-enantiomers of these compounds that did not bind to diazepam-insensitive $GABA_A$ receptors did not substitute for flumazenil. While the selectivity of rat diazepam-insensitive sites for the *S*-enantiomers of pyrroloimidazobenzodiazepines has recently been established (Fryer et al., 1994), the selectivity demonstrated by the lack of binding and behavioral effects of the *R*-enantiomers lends further support for the relationship between binding and discriminative stimulus effects of diazepam-insensitive receptor ligands in pigeons.

Bretazenil and Ro 14-5974, diazepam-sensitive receptor partial agonists with nanomolar affinities at diazepam-insensitive receptors, fully substituted for the discriminative stimulus effects of flumazenil, an action previously linked to diazepam-insensitive receptor binding (Wong et al., 1993b). The substitution dose-effect curves did not deviate from parallelism and revealed the same efficacy. However, Ro 19-0528, a diazepam-sensitive receptor full agonist, only partially substituted for flumazenil and yielded a flumazenil substitution dose-effect curve that was not parallel to curves of the other ligands, and that displayed lower efficacy. These results differ from substitution patterns of other diazepam-insensitive receptor ligands (Fig. 2; Wong et al., 1993b), and suggest that the discriminative stimulus produced by Ro 19-0528 may be dissimilar to that produced by other diazepam-insensitive receptor ligands. Whether such differences are qualitative or quantitative remains to be determined. Similarly, differences also have been suggested by studies with DMCM, a receptor full inverse agonist at most diazepam-sensitive sites, with high affinity at diazepam-insensitive receptors (Wong et al., 1993b). When the convulsant effects of DMCM were blocked by ZK 93,426 (a selective diazepam-sensitive receptor antagonist), DMCM still failed to substitute for the discriminative stimulus effects of flumazenil (unpublished observations). Taken together with the results from Ro 19-0528, these results suggest that diazepam-insensitive receptor affinity may not always be the sole determinant of a compound's ability to fully substitute for flumazenil, in light of full agonist (Ro 19-0528) or full inverse agonist (DMCM) actions at diazepam-sensitive receptors.

In contrast to flumazenil substitution, dose-effect curves for substitution of the discriminative stimulus effects of midazolam by Ro 19-0528, bretazenil, and Ro 14-5974 did not deviate from parallelism, despite differences in efficacy to substitute. The diazepam-sensitive receptor full agonist, Ro 19-0528, fully substituted for the discriminative stimulus effects of midazolam, consistent with previous findings for other full agonists (Garcha et al., 1985; Woudenberg and Slangen, 1989; Evans and Johanson, 1989), and the receptor partial agonists bretazenil and Ro 14-5974 resulted in partial substitution, consistent with receptor actions as full and partial agonists in GABA-shift assays in vitro (Kyburz, 1986; Haefely, 1990). A number of previous reports have indicated that compounds with receptor full agonist actions in vitro will substitute for other receptor full agonists in drug discrimination experiments (Garcha et al., 1985; Woudenberg and Slangen, 1989; Evans and Johanson, 1989). Additionally, compounds with receptor partial agonist actions have been reported to result in full substitution in rats (Andrews and Stephens, 1991), unless animals are trained to discriminate higher doses of receptor full agonists (Tang and Franklin, 1991). However, results of the present experiments are consistent with other studies in which receptor partial agonists only partially substitute for the discriminative stimulus effects of midazolam in pigeons (Gleeson et al., 1991). Consistent with their lack of affinity for the diazepam-sensitive recep-

tor, the *R*-enantiomers, Ro 18-2598 and Ro 14-7527, did not substitute for the discriminative stimulus effects of midazolam.

Both diazepam-sensitive receptor partial agonists (bretazenil and Ro 14-5974) and their *R*-enantiomers (Ro 18-2598 and Ro 14-7527, respectively) were also tested for their abilities to block the discriminative stimulus effects of midazolam. According to receptor theory (cf. Koek and Woods, 1989), agonists with low intrinsic efficacy should partially antagonize the discriminative stimulus effects of full agonists. In the present experiments, both receptor partial agonists, when co-administered with the training dose of midazolam, partially blocked the discriminative stimulus effects of midazolam, results that are consistent with actions of partial agonists at diazepam-sensitive $GABA_A$ receptors (Tang and Franklin, 1991). The degree of blockade resembled the degree of substitution. In contrast, the *R*-enantiomers of bretazenil and Ro 14-5974 did not bind to diazepam-sensitive receptors and did not either substitute for, or block the discriminative stimulus effects of midazolam. Despite the inability of these compounds to substitute for, or to block effects of midazolam, a dose 10 mg/kg Ro 14-7527 significantly reduced response rates relative to vehicle control in the midazolam substitution experiments, and both *R*-enantiomers dose-dependently (but non-significantly) decreased response rates for flumazenil substitution. These response rate reductions indicate that Ro 18-2598 and Ro 14-7527 are physiologically active within the dose range studied.

Unlike bretazenil and Ro 14-5974, where the flumazenil substitution dose-effect curves were parallel and revealed similar maximum effects, the flumazenil substitution dose-effect curve for Ro 19-0528 was less steep, and reached a maximum of only 63%, despite high affinity diazepam-insensitive receptor binding. This discrepancy invites speculation as to potential differences in the mechanisms mediating these behavioral effects. One possibility is that Ro 19-0528 acts as a partial agonist at diazepam-insensitive receptors. Although binding data previously failed to discriminate among diazepam-insensitive receptor ligands on the basis of GABA-shift ratios (Wong et al., 1993a; Turner et al., 1991), recent evidence from studies using cell lines expressing only the α_6 subunit have suggested that a range of intrinsic efficacies may be possible (Korpi and Seeburg, 1993). Further, under non-standard assay conditions with incubations at 37° C, differing results also have been reported (Uusi-Oukari, 1992). Whether discrepancies in the intrinsic efficacies of diazepam-insensitive receptor ligands to modulate GABA through diazepam-insensitive receptors are a function of assay conditions, different receptor compositions, or other factors, the results of Korpi and Seeburg (1993) and Uusi-Oukari (1992) suggest that diazepam-insensitive receptors are GABA-linked and ligands can potentially have a range of efficacies at these receptors. Behavioral results with Ro 19-0528 are consistent with the possibility of a range of efficacies associated with diazepam-insensitive receptor binding.

Another possibility that may explain the present results with Ro 19-0528 is that there are as yet undetermined interactions between diazepam-sensitive and diazepam-insensitive receptor sites in vivo. This explanation is consistent with findings that rat strains lacking the diazepam-insensitive receptor subtype are more sensitive to the impairment of postural reflexes by diazepam (Hellevuo et al., 1989), and reports that in transfected cells, diazepam-sensitive and diazepam-insensitive receptors can be expressed in the same neurons (Korpi and Lüddens, 1993). Finally, this explanation is consistent with the present and previous findings that a full agonist (Ro 19-0528) and a full inverse agonist (DMCM) at diazepam-sensitive receptors do not fully substitute for flumazenil, despite affinity for the diazepam-insensitive receptor. However, whether diazepam-insensitive receptor binding or ligand intrinsic efficacy has any direct or indirect effect on diazepam-sensitive receptor-mediated behaviors, or vice versa, remains to be demonstrated.

While substitution results in the present experiments could be interpreted in light of weak partial agonist actions of flumazenil at diazepam-sensitive receptors, consistent with results obtained in rats (De Vry and Slangen, 1985; Woudenberg and Slangen, 1989), the present findings are also consistent with results obtained in pigeons, in which the discriminative stimulus effects of flumazenil in this species were not similar to those of a diazepam-sensitive receptor partial agonist, but were related to affinity for diazepam-insensitive receptors (Wong et al., 1993b). The present findings, indicating a stereospecificity requirement for both binding and flumazenil substitution, support the hypothesis that diazepam-insensitive receptors mediate the discriminative stimulus effects of flumazenil only when these results are coupled with the previously reported findings that in pigeons, only ligands that bind to diazepam-insensitive receptors substitute for flumazenil, despite a range of intrinsic efficacies at diazepam-sensitive receptors. Conversely, ligands with a range of intrinsic efficacies at diazepam-sensitive receptors that do not have diazepam-insensitive receptor affinity, such as midazolam, chlordiazepoxide, ZK 93,426, or FG 7142, did not substitute for, or block the discriminative stimulus effects of flumazenil in pigeons (Wong et al., 1993a,b). Finally, Wong et al. (1993b) reported that the potencies of compounds to substitute for flumazenil were correlated with affinity for diazepam-insensitive, but not diazepam-sensitive receptors. Despite this evidence that diazepam-insensitive receptors mediate the discriminative stimulus effects of

flumazenil in pigeons, the fact that the diazepam-insensitive receptor ligand, DMCM, and in the present study, Ro 19-0528, did not fully substitute for flumazenil suggests that diazepam-insensitive receptor affinity (as measured using [^{3}H]Ro 15-4513) is not the sole determinant of the ability of a compound to substitute for the discriminative stimulus effects flumazenil.

In conclusion, the present experiments support the stereospecificity requirement for diazepam-insensitive receptor binding and transduction of discriminative stimulus effects in pigeons. Consistent with radioligand binding studies in rats (Fryer et al., 1994), only the *S*-enantiomers were active in binding and behavioral assays for diazepam-insensitive and diazepam-sensitive receptor ligands. Stereospecificity requirements for diazepam-insensitive receptors are consistent with those previously established for diazepam-sensitive receptor binding (Kyburz, 1986; Blount et al., 1983). Finally, the results of the present experiments are consistent with the major finding of Wong and colleagues (Wong et al., 1993b), that occupation of diazepam-insensitive $GABA_A$ receptors is associated with transduction of the discriminative stimulus effects of flumazenil in pigeons. Further clarification of the functional role of diazepam-insensitive receptors must await the availability of selective ligands for this site.

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