Pharmacology Biochemistry & Behavior, Vol. 23, pp. 639–644, 1985. © Ankho International Inc. Printed in the U.S.A. 0091-3057/85 $3.00 + .00

CGS 8216 and CGS 9896, Novel Pyrazoloquinoline Benzodiazepine Ligands With Benzodiazepine Agonist and Antagonist Properties

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BOAST, C. A., E. W. SNOWHILL AND J. P. SIMKE. *CGS 8216 and CGS 9896, novel pyrazoloquinoline benzodiazepine ligands with benzodiazepine agonist and antagonist properties.* PHARMACOL BIOCHEM BEHAV 23(4) 639–644, 1985.—CGS 8216 and CGS 9896 are two recently described compounds which interact with benzodiazepine binding sites but have pharmacological, biochemical and behavioral characteristics which distinguish them from classical benzodiazepines. CGS 8216 shows properties of a weak inverse agonist, while CGS 9896 shows properties of a mixed agonist/antagonist. Experiments using quantitative autoradiography to determine benzodiazepine binding site interactions of these compounds in discrete anatomical areas are described. Results indicate that [3H]-CGS 8216 does not show any regional differentiation in binding characteristics in 7 brain areas studied. CGS 9896 preferentially inhibited [3H]-flunitrazepam from cerebellar sites compared to hippocampal dentate gyrus sites, but the magnitude of this effect was small. These data support the conclusion that CGS 9896 is acting preferentially at putative benzodiazepine type 1 sites and is consistent with the mixed agonist/antagonist profile of the compound.

CGS 8216 CGS 9896 Benzodiazepine Flunitrazepam Quantitative autoradiography Agonist/antagonist Dentate gyrus Cerebellum Hippocampus

CGS 9896 and CGS 8216 are representives of a class of pyrazoloquinoline compounds [31] which bind with high affinity to central benzodiazepine recognition sites [6]. The two compounds differ structurally by the presence of a chloro-substituent in the para position of the pendant phenyl group. This discrete chemical modification results in two compounds with quite different biochemical, pharmacological and behavioral properties. CGS 9896 has characteristics of a mixed agonist/antagonist [3,30] while CGS 8216 has characteristics of an inverse agonist. That is, CGS 9896 acts similar to diazepam (considered to be a benzodiazepine agonist) in some tests but reverses the actions of diazepam in other tests. CGS 8216 can reverse all of the actions of diazepam but also has effects of its own which are opposite to that of diazepam. This profile has been referred to as inverse agonism.

The efficacy of CGS 8216 in inhibiting [3H]-flunitrazepam binding was not altered by GABA [6], a property characteristic of other benzodiazepine antagonists [8,19]. In contrast, a slight reduction in the efficacy of CGS 8216 in inhibiting [3H]-flunitrazepam in the presence of GABA was reported by Wood *et al.* [30]. The magnitude of this GABA-shift was not considered to be of prime importance in distinguishing between benzodiazepine antagonists or inverse agonists. The latter report also indicated that CGS 8216 had no effect on the binding of [35S]-TBPS to the chloride channel, and did not alter [3H]-muscimol binding in the presence of etazolate. Together, these data are consistent with the classification of CGS 8216 as an inverse agonist [30].

CGS 8216 antagonizes the anticonvulsant, anxiolytic, muscle relaxant and sedative/hypnotic effects of diazepam [4]. Other data suggest that CGS 8216 has some intrinsic activity when administered alone [4, 9, 10, 15, 29]. The compound reduced by about 50% the ED50 for pentylenetetrazol (PTZ) to induce seizures [4]. Audiogenic seizures and electroshock seizures were also potentiated by CGS 8216 [15]. In a water lick conflict task, CGS 8216 demonstrated a proconflict effect [15]. Generalization of CGS 8216 to the PTZ-induced stimulus cue, which is considered to be related to endogenous anxiety in humans [18], has also been reported [15]. Social interaction time in animals exposed to a familiar environment was reduced by CGS 8216 [9,10], an effect not reversed by chlordiazepoxide or Ro15-1788. Avoidance of a dark chamber associated with the stimulus properties of CGS 8216 has also been reported [29]. In summary, CGS 8216 has neurochemical, behavioral and pharmacological properties indicating that it is an inverse agonist.

In studies of the effects of CGS 9896 on components of the benzodiazepine complex, GABA, which caused a 2–3 fold increase in the binding affinity of flunitrazepam, only

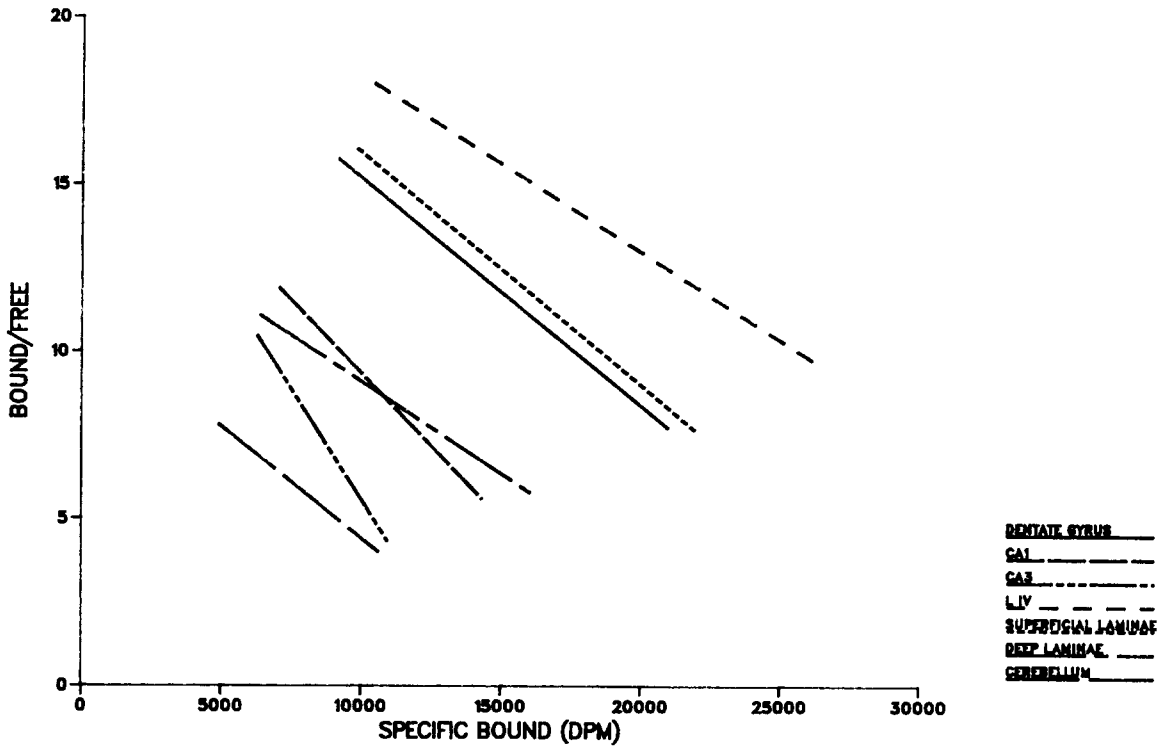

FIG. 1. Scatchard plots for [3H]-flunitrazepam binding in each of the seven brain regions indicated. There were no statistically significant differences among the Kd values (Table 1) (test of parallelism) ($r>0.86$ in all brain regions).

TABLE 1

Kd AND HILL VALUES FOR [3H]-FLUNITRAZEPAM* FOR EACH OF SEVEN RAT BRAIN REGIONS

Brain Region	Kd (nM)	Hill No.
CA3	4.52	0.92
Dentate Gyrus	8.73	0.93
CA1	6.85	0.96
Superficial Cortex	8.59	0.93
Deep Cortex	10.9	0.96
Lamina IV Cortex	11.3	0.94
Cerebellum	8.9	0.86

*Tissue sections on slides were incubated for 2.5 hr at 37°C in 170 mM Tris HCl (pH 7.4). [3H]-flunitrazepam (NEN, S.A. 76.4 Ci/mMole) was present over the concentration range of 1–20 nM. Non-specific binding was determined using 1 μM diazepam. There were no statistical differences (test of parallelism) among the Kd values. (N=4 or 5 for each brain region, the determinations are based on 7 concentrations).

TABLE 2

Kd AND HILL VALUES FOR [3H]-CGS 8216* FOR EACH OF SEVEN RAT BRAIN REGIONS

Brain Region	Kd (nM)	Hill No.
Dentate Gyrus	0.13	0.96
CA1	0.15	1.0
CA3	0.25	0.94
Lamina IV Ctx	0.14	0.91
Superficial Cortex	0.12	0.75
Deep Cortex	0.10	1.1
Cerebellum	0.11	1.0

*Tissue sections on slides were incubated for 3 hr at 37°C in 170 mM Tris HCl (pH 7.4). [3H]-CGS 8216 (CIBA-Geigy, Horsham, S.A. 20.1 Ci/mMole) was present over the concentration range of 0.3–1 nM. Non-specific binding was determined using 1 μM diazepam. There were no statistical differences (test of parallelism) among the Kd values (N=4 or 5 for each brain region, the determinations are based on 7 concentrations).

increased the affinity of CGS 9896 by 1–1.5 fold [22,30]. [35S]-TBPS binding to the chloride channel and etazolate stimulated [3H]-muscimol binding to GABA sites were only stimulated to 50% of the level obtained with classical benzodiazepine agonists [30]. By themselves these data suggest a partial agonist profile for CGS 9896. In experiments utilizing photolabelled membranes, CGS 9896 did not decrease flunitrazepam affinity, thereby resembling antagonist compounds [5,22]. Taken together these findings suggest that CGS 9896 posesses neurochemical properties shared by other compounds regarded as mixed agonist/antagonists [30]. Additional behavioral evidence supports this conclusion.

Behaviorally, CGS 9896 possesses partial benzodiazepine agonist activity in certain anticonvulsant assays. For example, CGS 9896 protected rats against convulsions induced by PTZ [31], prevented seizures induced with bicuculline in mice [12] and also prevents audiogenic seizures in DBA/2

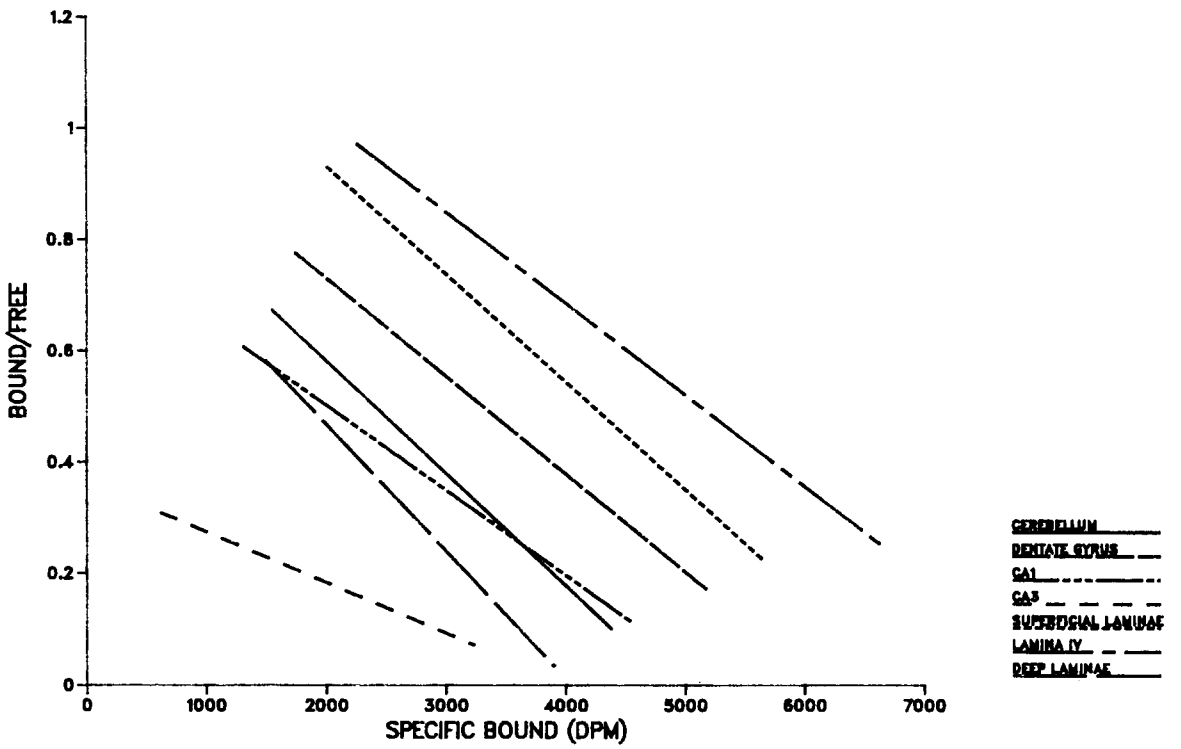

FIG. 2. Scatchard plots for [3H]-CGS 8216 binding in each of the seven brain regions indicated. There were no statistically significant differences among the Kd values (Table 2) (test of parallelism) ($r>0.91$ in all brain regions).

mice [3]. CGS 9896 was not effective in preventing tonus induced by a maximal transcorneal electroshock, whereas diazepam was moderately active in this test [3].

CGS 9896 has full agonist activity in a variety of models predictive of anxiolytic efficacy. For example, the compound was as effective as diazepam [31] in the Cook/Davidson [7] conflict model and Patel *et al.* [21] reported that the compound increased responding in a lick suppression paradigm. In a drug discrimination paradigm, CGS 9896 antagonized the stimulus properties of PTZ [1,26]. In another non-conflict paradigm, CGS 9896 was found to selectively increase the latency to terminate intracranial electrical stimulation [3]. This finding was interpreted to indicate a reduction in the accumulation of aversive properties associated with long-duration hypothalamic stimulation. Other known anxiolytics which shared this effect were diazepam, chlordiazepoxide and CL 218872 [14].

In contrast to these effects, CGS 9896 appears to lack muscle relaxation and/or sedative/hypnotic activity. For example, rotorod performance was not impaired at doses up to 300 mg/kg PO of CGS 9896, whereas diazepam exhibited an ED50 value of 5–10 mg/kg PO in this test [3]. Gee and Yamamura [12] reported the absence of ataxia or sedation at effective anticonvulsant doses of CGS 9896. Also, in several tests (e.g., Cook/Davidson, drug discrimination, shuttle box) designed to characterize anxiolytic activity, there were no reductions in normal response rates [1].

Pharmacological studies of interactions between CGS 9896 and diazepam or alcohol have added complexity to the profile of CGS 9896. Specifically, the question of whether CGS 9896 would modify the motor impairing properties of diazepam and alcohol, was assessed using rotorod measures [3]. CGS 9896 antagonized the rotorod-impairing properties of diazepam. This effect was probably not due to a direct muscle activation effect of CGS 9896, because the rotorod impairing effects of ethanol, in contrast, were potentiated by CGS 9896, although at a dose that was six fold higher than that required by diazepam to produce the same degree of ethanol potentiation. Further studies also examined the interaction of CGS 9896 with diazepam against electroconvulsive shock-induced seizures. A relatively high dose of CGS 9896 (100 mg/kg PO), ineffective alone against electroshock induced seizures, reduced the protective effect of diazepam in this test [3]. This effect is possibly the result of reduced muscle relaxation as seen in the diazepam interaction studies using rotorod. The consistent finding in these studies is that CGS 9896 has some benzodiazepine antagonist properties.

Thus, both neurochemical and behavioral data indicate that CGS 9896 is a mixed agonist/antagonist at benzodiazepine binding sites. An expectation that follows from this conclusion is that CGS 9896 might demonstrate differential interactions with putative subtypes of benzodiazepine receptors. Previous reports have described cerebellar benzodiazepine binding sites as primarily one subtype (BZ1) and forebrain sites as a mixture of subtypes (BZ1 and BZ2) [13,16]. Petrack *et al.* [22] reported that CGS 9896 did not show any regional differences in its ability to inhibit [3H]-flunitrazepam binding. Gee and Yamamura [12] reported similar findings but cautioned that, due to a Hill coefficient less than one for dorsal hippocampal binding, it was possible that CGS 9896 showed differential interaction at subtypes of benzodiazepine binding sites. In a forebrain homogenate both type 1 and type 2 benzodiazepine sites are present. More discrete anatomical analysis can be provided by using

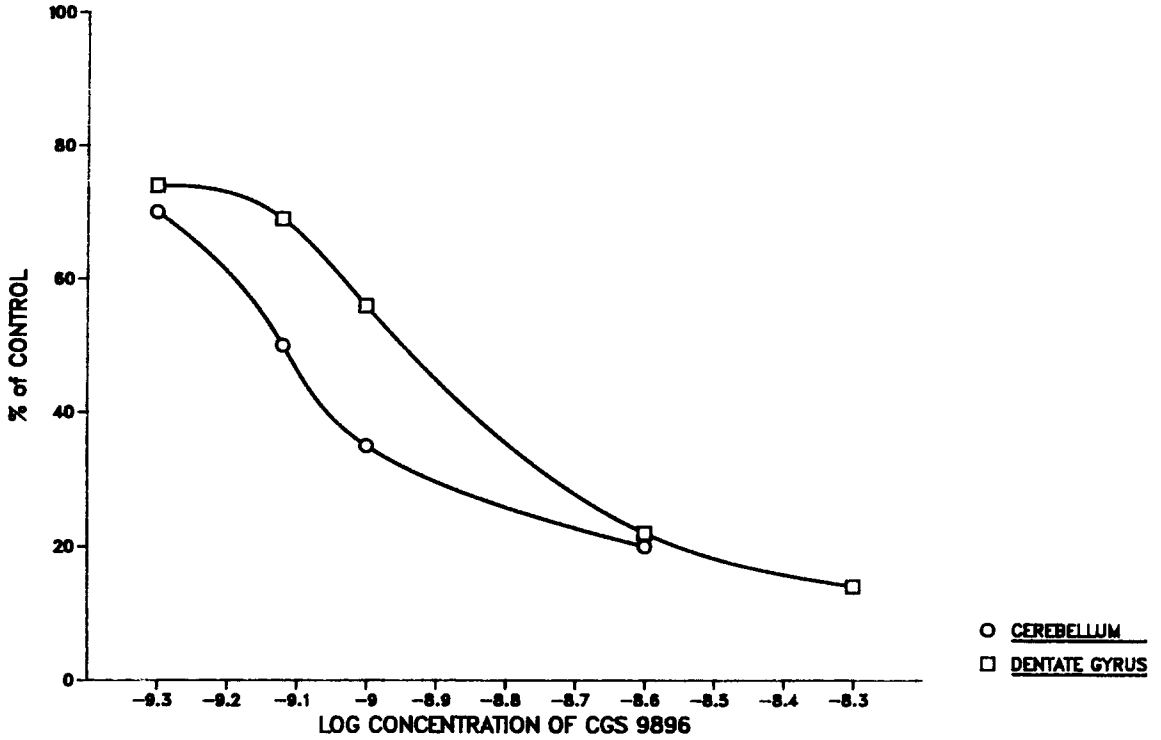

FIG. 3. Inhibition of [3H]-flunitrazepam binding as a function of the concentration (log) of CGS 9896 in cerebellum and dentate gyrus. The relative potency of CGS 9896 in the two regions was 0.69 (95% fiducial limits=0.55–0.87), indicating a preference for cerebellar sites.

TABLE 3

IC50 VALUES FOR CSG 9896 INHIBITION OF [3H]-FLUNITRAZEPAM IN DENTATE GYRUS AND CEREBELLUM

Brain Region	IC50 (nM)
Dentate Gyrus	1.18
Cerebellar	0.79

*Tissue sections on slides were incubated for 2.5 hr at 37°C in 170 mM Tric HCl (pH 7.4), with 5 nM [3H]-flunitrazepam and various concentrations of CGS 9896 over the range of 10^{-10} to 10^{-8}. Non-specific binding was determined using 1 μM diazepam. The IC50 values were statistically different (relative potency=0.69, 95% fiducial limits=0.55–0.87).

quantitative autoradiographic techniques [17,28]. The following experiments were designed to elucidate the selective benzodiazepine binding site interactions of CGS 9896 and CGS 8216 in various brain regions.

METHOD

Tissue

Brains were extracted from five male Sprague-Dawley rats (Charles River, Wilmington, MA) following transcardial perfusion with phosphate buffered saline. The brains were quickly frozen onto microtome chucks. Sections 20 microns thick were taken from the forebrain (region with dorsal hippocampus) and the cerebellum. They were thaw mounted onto chromalum-gelatin coated microscope slides. Sections were selected such that anatomically adjacent sections were available for total and non-specific binding conditions for a given concentration of radioactive ligand (saturation studies), or unlabelled drug (inhibition studies). The sections were stored frozen (−20°C) for at least 24 hours before use.

Incubations

Optimal equilibrium and wash time conditions were determined [24] for [3H]-flunitrazepam (Flu) and for [3H]-CGS 8216 using modifications of previous methods [6, 28, 32]. Sections were incubated for 2.5 hours in the [3H]-Flu assay, and for 3 hours in the [3H]-CGS 8216 assay, both at 37°C in 170 mM Tris-HCL (pH 7.4). Following this incubation the slides were transferred to fresh buffer at 4°C for 4 minutes, rinsed briefly in cold distilled water and placed on a slide warmer to dry. For determination of regional affinities, [3H]-Flu (New England Nuclear, S.A. 76.4 Ci/mMole) was present over the concentration range of 1–20 nM, and [^{3}H]-CGS 8216 (CIBA-Geigy, Horsham, S.A. 20.1 Ci/mMole) was present over the concentration range of 0.3–1 nM. For subsequent inhibition studies [3H]-Flu was present at 5 nM, and CGS 9896 was present over the concentration range of 10^{-10} to 10^{-8} M. Non-specific binding was determined in all experiments by adding 1 μM diazepam to the incubation buffer.

Autoradiography/Densitometry

Incubated slides were apposed to LKB Ultrofilm, along with a set of standards prepared by sectioning frozen brain paste containing known amounts of [3H]-deoxyglucose.

After exposure times of 8–10 days for [3H]-Flu, or 4–6 weeks for [3H]-CGS 8216, the films were developed using Kodak D-19 (5 min, 20°C) and Rapid Fix (5 min). The tissue images were then quantitated with an EYE COM III (Spatial Data, Goleta, CA) image analysis system [25].

Binding was quantitated in detail for three cerebral cortical layers (superficial, lamina IV, and deep), three regions of hippocampus (CA1, CA3, and dentate gyrus), and the molecular layer of the cerebellar cortex. Statistical analysis of the data was performed using linear regression analysis and tests of parallelism [11]. This was done for each of three experiments examining (1) the regional affinity of [3H]-Flu binding, (2) the regional affinity of [3H]-CGS 8216 binding, and (3) the regional inhibition of [3H]-Flu binding by CGS 9896. In addition, an estimate of the relative potency was determined in the inhibition experiment.

RESULTS

Regional Affinity of [3H]-Flu Binding

Affinity constants (Kd) for [3H]-Flu binding were determined for each of the seven brain regions of interest (Table 1). These values ranged from 4.5 nM in the CA3 region of the hippocampus, to 11.3 nM in lamina IV of the cerebral cortex. These values are consistent with the range of Kd values that have been reported in the literature for tissue homogenates [20,27]. No statistically significant differences were found among these values, $F(6,196)=0.44$, $p>0.5$. Scatchard plots [2] for all brain regions are shown in Fig. 1.

Hill coefficients were also calculated for each brain region (Table 1). These values ranged from 0.86 for cerebellum to 0.96 for CA1 and laminae V–VI. These differences were not considered significant nor different from 1.0. This is consistent with previous findings [20,27].

Regional Affinity of [3H]-CGS 8216 Binding

Affinity constants for [3H]-CGS 8216 binding ranged from 0.10 nM in the CA3 region of the hippocampus, to 0.25 nM in the deep layers of the cerebral cortex (Table 2). This range of values is consistent with previously published data using tissue homogenate preparations [6]. There was no anatomic variation in these Kd values as they were not statistically different, $F(6,196)=1.76$, $p>0.1$. Scatchard plots for these brain regions are shown in Fig. 2.

The Hill coefficients for these brain regions (Table 2) ranged from 0.75 in the superficial layers of the cerebral cortex to 1.1 in the deep layers of the cerebral cortex. These Hill coefficients were not considered different from 1.0.

Regional Inhibition of [3H]-Flu Binding by CGS 9896

The mean percent binding of [3H]-Flu at each of several concentrations of CGS 9896 in the dentate gyrus and in the molecular layer of the cerebellum is shown in Fig. 3. The IC50 values for each of these brain regions are presented in Table 3. These IC50 values differed statistically but this difference was small (<2 fold). The analysis of relative potency indicated that CGS 9896 was significantly more potent in displacing cerebellar [3H]-Flu binding as compared with that in the dentate gyrus (relative potency=0.69, 95% fiducial limits=0.55–0.87).

DISCUSSION

In the present inhibition experiments, using CGS 9896 to inhibit [3H]-flunitrazepam binding, the IC50 data indicated slight differences in the regional inhibition properties of CGS 9896. A preference for cerebellar over dentate gyrus sites was suggested. This finding is in agreement with recent data from homogenate experiments [23] indicating a threefold preference for cerebellar sites over hippocampal sites by CGS 9896. The highly discrete anatomical analysis afforded by quantitative autoradiography is sufficient to explain why these small regional differences were not seen in an earlier homogenate study (22). Since it was confirmed in the first set of quantitative autoradiographic studies reported here, that [3H]-Flu did not show any regional differences in binding characteristics across the seven brain regions examined, then these differences in the IC50 values for CGS 9896 would be reflected in inhibitor constants (Ki).

The difference in affinity of CGS 9896 for dentate gyrus and cerebellar cortex suggests that this compound may show a differential interaction with subtypes of benzodiazepine binding sites. Cerebellar cortex has been reported to contain primarily type 1 sites, while the highest relative concentration of type 2 sites is in the dentate gyrus of the hippocampus. Thus, CGS 9896 shows some preference for type 1 benzodiazepine sites. The physiological significance of these receptor subtypes is currently unknown.

These data are consistent with the findings indicating that CGS 9896 has mixed benzodiazepine agonist and antagonist effects [3,30]. That is, the compound could act as an agonist at one binding site subtype and as an antagonist at another subtype. There is no information available, however, to indicate at which binding site subtypes the compound acts as an agonist and at which as an antagonist. Studies of receptor activation of second messengers or of specific electrophysiological changes would help clarify this issue.

The magnitude of the difference in the observed regional IC50 values between cerebellar cortex and dentate gyrus is small. This may be due in part to the use of a ligand that does not show subtype selectivity. The small difference seen in the present experiments may be magnified if subtype selective radioligands were available.

In the present experiments using [3H]-CGS 8216, no regional differences in affinity were found despite the discrete anatomical assessment by quantitative autoradiography. The compound had a higher affinity than [3H]-flunitrazepam in all brain regions. These data are consistent with previous reports using homogenate techniques [6]. The similarity of affinity for [3H]-CGS 8216 across subtypes of benzodiazepine binding sites is also consistent with the ability of this compound to antagonize all of the actions of diazepam [4].

In summary, CGS 8216 acts as a weak inverse agonist and does not show any regional preferences for subtypes of benzodiazepine binding sites. This is consistent with the ability of this compound to reverse all of the effects of diazepam. CGS 9896 shows a slight preference for cerebellar type 1 benzodiazepine binding sites but the magnitude of this preference is small. This is consistent with its neurochemical and behavioral profile which suggests mixed agonist and antagonist actions at benzodiazepine binding sites.

ACKNOWLEDGEMENTS

The authors would like to thank Nancy Hall for statistical contributions and Drs. Jeff Liebman, Michael Williams and Paul Wood for comments on an earlier version of the manuscript.

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