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HIGH AFFINITY INHIBITION OF [^{3}H]-FLUNITRAZEPAM BINDING TO BRAIN BENZODIAZEPINE RECEPTORS BY CGS 9896, A NOVEL PYRAZOLOQUINOLINE

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Inhibition of specific [^{3}H]-Flunitrazepam([^{3}H]-FLU) in rat cerebral cortex by the novel pyrazoloquinoline, CGS 9896, was examined. CGS 9896 inhibited [^{3}H]-FLU binding with high affinity (K_I=0.035nM). Hill slope data derived from inhibition curves suggested CGS 9896 adheres to classic mass action behavior. CGS 9896 also exhibits typical competitive-type inhibition of [^{3}H]-FLU binding.

INTRODUCTION

The observation that certain non-benzodiazepine compounds will label brain benzodiazepine receptors with high affinity has provided new perspectives in the characterization of benzodiazepine receptors. CL 218872, a member of the triazolopyridazine series of compounds, is an effective inhibitor of benzodiazepine receptors binding (1,2). Studies with CL 218872 yielded some of the initial evidence for benzodiazepine receptor heterogeneity (3). Furthermore, CL 218872 was found to be as potent as diazepam in both anti-conflict and anti-metrazol studies in animals (4). The alkyl beta-carboline carboxylate derivatives have been shown to be potent antagonist of the effects of the benzodiazepines in vivo and in vitro (5,6). In binding studies, [^{3}H]-propyl beta-carboline-3-carboxylate possessed the ability to discriminate benzodiazepine receptor

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subtypes providing further evidence of receptor heterogeneity. Recently, [^{3}H]-CGS 8216 (2-phenylpyrazolo [4,3-c]quinolin-3(5H)-one) a novel compound of the pyrazoloquinoline class has been reported to label brain benzodiazepine receptors with very high affinity but did not discriminate between benzodiazepine receptor subtypes (7). CGS 8216 was also reported to possess no benzodiazepine-like activity in pharmacological tests but was found to be a potent antagonist of the actions of diazepam (8). In this report, the inhibition of specific [^{3}H]-FLU in rat cerebral cortex by CGS 9896 (2-(4-Chlorophenyl)-2,5 dihydropyrazolo[4,3-c]quinoline-3(3H)-one), a structural analogue of CGS 8216, is examined.

METHODS

Whole brains from male Sprague-Dawley rats (250-300g) were rapidly removed following sacrifice. The cerebral cortex was dissected over ice and homogenized in 100 volumes of ice-cold 50mM Na/K phosphate buffer, pH 7.4. These homogenates were subsequently washed 3 times in the same buffer (100 vol) by centrifugation at 48,000xg for 10 minutes. Following the third wash, the pellets were resuspended in a final concentration of 5 mg original wet weight/ml buffer. In the inhibition studies using CL218872 and flunitrazepam, a final tissue concentration of 20 mg original wet weight/ml buffer was used.

The specific binding of [^{3}H]-FLU (83.6 Ci/mmol, New England Nuclear Corp.) was determined by a filtration technique. All assays were performed in triplicate by adding 100 μl of cortical homogenate to glass tubes containing [^{3}H]-FLU and various concentrations of inhibitor (CGS 9896, CL 218872 or flunitrazepam) with the final incubation volume being 2 ml. Specific binding of [^{3}H]-FLU was defined as the difference between the amount of [^{3}H]-FLU bound in the presence and absence of 1.0 μM clonazepam. All tubes were incubated for 90 minutes in an ice bath (0-4^{o}C) following the addition of the tissue homogenate. The incubations were terminated by vacuum filtration through Whatman GF/B glass fiber filters followed by 3 washes with 5 ml of ice-cold buffer. Filters were placed in 8 ml of scintillation fluid. Filter-bound radioactivity was measured by liquid scintillation spectrophotometry 6-8 hours later with an efficiency of 46%.

The IC_{50} and Hill slope values were derived from the inhibition data by least squares linear regression of $\log(B_{max}/B - 1)$ vs log [I] plots where I is the concentration of inhibitor in moles/liter, B_{max} is the amount of specific [^{3}H]-FLU bound at equilibrium in the absence of inhibitor and B is the amount of specific [^{3}H]-FLU bound at a given concentration of inhibitor. K_I values were determined from the equation $K_I=(IC_{50}/(1+[L]/K_D))$ where L is the free ligand concentration and K_D is the independently determined apparent dissocation constant.

RESULTS

The results of the inhibition experiments demonstrate that under our conditions CGS 9896 is a potent inhibitor of [^{3}H]-FLU binding. The K_I values (Table 1) show CGS 9896 being over 30 fold more potent than flunitrazepam and almost 4000 times more potent than CL 218872 in inhibiting the specific binding of [^{3}H]-FLU in rat cerebral cortex. In addition, CGS 9896 at a 10 pM final concentration exhibits typical competitive type inhibition of specific [^{3}H]-FLU binding in the cerebral cortex (data not shown). Inhibition of specific radiolabeled benzodiazepine binding by nonlabeled benzodiazepines are typically characterized by competition curves with Hill slopes of one suggesting adherence to classic mass action behavior. CGS 9896 appears to follow similar behavior since the Hill slope obtained for this compound did not differ significantly from the Hill slope for flunitrazepam inhibition of [^{3}H]-FLU binding. In contrast, the Hill slope for CL 218872 showed significant deviation from unity in agreement with previous reports (3).

DISCUSSION

The most significant finding from this study was CGS 9896 appeared to be one of the most potent inhibitors of specific [^{3}H]-FLU binding in rat cerebral cortex. This novel pyrazoloquinoline may interact competitively with [^{3}H]-FLU for benzodiazepine receptor sites. Hill slopes for CGS 9896 inhibition of [^{3}H]-FLU binding were not significantly different from one which may be interpreted as evidence for classic mass action behavior. Recent studies using radiolabeled CL 218872 and propyl beta-carboline-3-carboxylate have provided strong evidence in support of benzodiazepine receptor heterogeneity (3,6). Thus, CGS 9896 does not appear to discriminate between receptor subtypes in rat cerebral cortex.

Table 1: Inhibition of [^{3}H]-Flunitrazepam binding in rat cerebral cortex by CGS 9896, CL 218872 and Flunitrazepam[a]

INHIBITOR	K_I (nM)	HILL SLOPE
CGS 9896 (4)	0.035 ± 0.006*	0.85 ± 0.06
CL 218872 (5)	136.8 ± 22.8*	0.67 ± 0.01*
Flunitrazepam (5)	1.03 ± 0.04	0.92 ± .03

[a]A final concentration of 2 nM [^{3}H]-FLU was used in the CGS 9896 inhibition studies. 0.5 nM [^{3}H]-FLU was used in when the inhibitors, were CL 218872 or flunitrazepam. All values expressed as the mean ± SEM. The numbers in ( ) following each inhibitor represents the number of determinations.

*Significantly different from flunitrazepam at $P < 0.001$ by Student's t-test.

The data presented in this study did not yield information on whether CGS 9896 is a pharmacological antagonist similar to CGS 8216 or a benzodiazepine-agonist type compound. A recent proposal by Ehlert et al, 1981 suggested that the effects of gamma-aminobutyric acid (GABA) on ligand binding may distinguish antagonist from agonists of the benzodiazepine receptor (9). GABA would enhance the potency of agonist-type compounds, but not antagonist. Several studies have provided evidence in support of this hypothesis, receptor antagonists such as RO 15-1788; certain alkyl beta-carboline-3-carboxylates and CGS 8216 did not show GABA enhancement while the potency of agonists such as CL 218872 and other benzoidazepines with agonist-type activity were enhanced by GABA (9,10). Preliminary studies with CGS 9896 indicate that GABA increased the potency of this pyrazoloquinoline suggesting it may be a pharmacological agonist (11). Further evidence for agonist-like activity came from the observation that CGS 9896 protected mice against bicuculline induced seizures at doses that did not produce ataxia or sedation (11).

In summary, CGS 9896 is a very potent inhibitor of [^{3}H]-FLU binding in rat cerebral cortex. Thus, this pyrazoloquinoline may be

of potential value as a high affinity ligand for the further study of benzodiazepine receptors. CGS 9896 may also possess some very interesting pharmacological effects which may prove to be of therapeutic value in the treatment of anxiety and seizure disorders.

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