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γ-Aminobutyric Acid Enhancement of CL 218,872 Affinity and Evidence of Benzodiazepine Receptor Heterogeneity

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SUMMARY

The inhibition of [^{3}H]flunitrazepam binding by a novel anxiolytic agent [CL 218,872; 3-methyl-6-[3-trifluoromethyl)phenyl]-1,2,4-triazolo-[4,3-b]pyridazine] was studied in membranes prepared from bovine retina and rat cerebral cortex, cerebellum, and kidney. The order of potency for the inhibition of [^{3}H]fluintrazepam binding by CL 218,872 in these tissues was cerebellum > retina ≅ cerebral cortex ≫ kidney. The slope factors (Hill coefficients) for CL 218,872 inhibition of [^{3}H]flunitrazepam were approximately 1.0 for kidney, 0.9 for cerebellum, and 0.7 for cerebral cortex and retina. In thoroughly washed membrane preparations from all of the central tissues, K_i values were significantly decreased an average of 60% in the presence of 100 μM γ-aminobutyric acid (GABA) ($p < 0.01$). With kidney membranes there was no apparent affect of GABA on the K_i of CL 218,872. (+)-Bicuculline (100 μM) could antagonize the effect of GABA on membranes from central tissues. Nonlinear least-squares regression analyses were used to reanalyze these data in terms of receptor models describing the interaction of a ligand with either one or two classes of independent binding sites. A two-site regression model resulted in a highly significant improvement in the fit of data obtained from retina, cerebral cortex, and cerebellum ($p < 0.01$), but not with data from kidney ($p > 0.05$). GABA was found to enhance the affinity of CL 218,872 for both of the sites without changing the proportion of sites. The results of these studies show that GABA enhances the affinity of CL 218,872 for the central benzodiazepine receptor(s) and that the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in bovine retina, rat cerebellum, and cerebral cortex may be explained by interactions with two classes of independent binding sites.

INTRODUCTION

CL 218,872[4] (see Fig. 1) is a prospective anxiolytic drug which can inhibit the binding of tritiated benzodiazepines to brain tissue (1–3). Another anxiolytic, zopiclone, is chemically unrelated to both the benzodiazepines and CL 218,872 and it, too, inhibits the binding of tritiated benzodiazepines to brain tissue (4). The evidence that pharmacologically related, but chemically dissimilar, compounds compete for a common binding site supports the hypothesis that the central benzodiazepine receptor is pharmacologically relevant and may be a physiological component of anxiety.

Unlike the benzodiazepines or zopiclone, the inhibition of [^{3}H]fluintrazepam binding by CL 218,872 does not obey the mass action law that governs simple molecular interactions (bimolecular association, monomolecular dissociation). The deviations of CL 218,872 from simple mass action law are visualized by log dose-inhibition curves that cover more than two orders of magnitude and Hill coefficients or slope factors that are less than 1 (1–3). Such deviations may be described by a number of molecular mechanisms, including the concepts of multiple classes of independent binding sites, transition states of a single binding site, negative cooperativity, and others (5). It was also observed that the atypical log dose-inhibition curves of CL 218,872 had a regional specificity. For example, CL 218,872 inhibition of [^{3}H]flunitrazepam binding to membranes prepared from the cerebral cortex gave slope factors less than 1, whereas with membranes from the cerebellum, slope factors close to 1 were found (1). These findings herald the possibility that unique

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[4] The abbreviations used are: CL 218,872, 3-methyl-6-[3-trifluoromethyl)phenyl]-1,2,4-triazolo-[4,3-b]pyridazine; GABA, γ-aminobutyric acid; PBS, phosphate-buffered saline; ANOV, analysis of variance.

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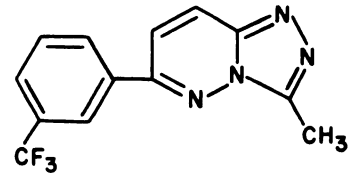
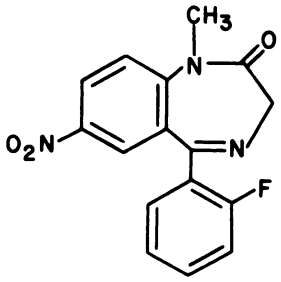


CL 218,872



flunitrazepam

FIG. 1. *Structures of CL 218,872 and of flunitrazepam*

aspects of the pharmacology of the benzodiazepines could reflect the particular molecular mechanism giving rise to these unorthodox inhibition curves. Therefore, the study of the interactions of CL 218,872 with benzodiazepine binding sites merits critical examination.

In addition to brain (6, 7), specific, saturable, high-affinity binding of tritiated benzodiazepines has been described for mammalian kidney (8, 9) and retina (10–14). If the unorthodox inhibition curves of CL 218,872 observed in cerebral cortex are important to the pharmacology of anxiolytics, then they should not exist in tissues which are not ostensibly related to the production of anxiety. To test this hypothesis we examined the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in membranes prepared from either bovine retina or rat kidney. Data were analyzed by conventional logit-log plots and by nonlinear least-squares regressions which permitted us to make objective choices between models describing ligand interactions with either one or two classes of independent binding sites.

Another interesting property of the central benzodiazepine receptor is the observation that GABA can increase the binding affinity of tritiated benzodiazepines (15, 16). This effect can be duplicated with other GABA receptor agonists and it can be blocked with the GABA antagonist (+)-bicuculline. With regard to CL 218,872, Klepner *et al.* (1) reported that GABA had no effect on the IC_{50} of CL 218,872 as measured by the inhibition of [^{3}H]flunitrazepam binding. These workers postulated that a subclass of benzodiazepine receptors is GABA-independent and that this subclass may mediate the anxiolytic effects of benzodiazepines. Since the possible involvement of GABAergic mechanisms is an important aspect of the pharmacology of the benzodiazepines, we reinvestigated the effect of GABA on the inhibition of [^{3}H]flunitrazepam binding by CL 218,872.

METHODS

Bovine eyes were obtained from a local slaughterhouse, and the retinae were removed and frozen at −20° until use. Kidneys, cerebral cortices, and cerebella were removed from decapitated rats on the day of the experiment. All tissues were homogenized in 39 volumes of buffer with a Brinkmann Polytron (Setting 6; two times, 30 sec each time). Following the initial homogenization, two methods of membrane preparation, utilizing different buffers, were employed. The first method (Method A), used in the experiments depicted in Figs. 2 and 3, involved the following. Tissues were homogenized in a 50 mM Tris-HCl buffer (pH 7.8 at 5°) and the homogenates were centrifuged at 49,000 × *g* for 15 min. The supernatants were discarded and the pellets were resuspended in the original volume of 50 mM Tris-HCl buffer. The membranes were washed once by repeating the centrifugation and resuspension. The second method of preparing membranes (Method B) was used for the experiments depicted in Tables 1 and 2. For the initial homogenization and all subsequent resuspensions, method B used 50 mM sodium-potassium phosphate buffer containing 100 mM NaCl, pH 7.4 (PBS). The tissues were homogenized as described above and the homogenates were centrifuged at 49,000 × *g* for 15 min. The supernatants were discarded and the pellets were resuspended in the original volume of PBS buffer. The membranes were washed three times by repeating the centrifugation and resuspension three more times. Following the final resuspension the membrane suspension was frozen for approximately 16 hr at

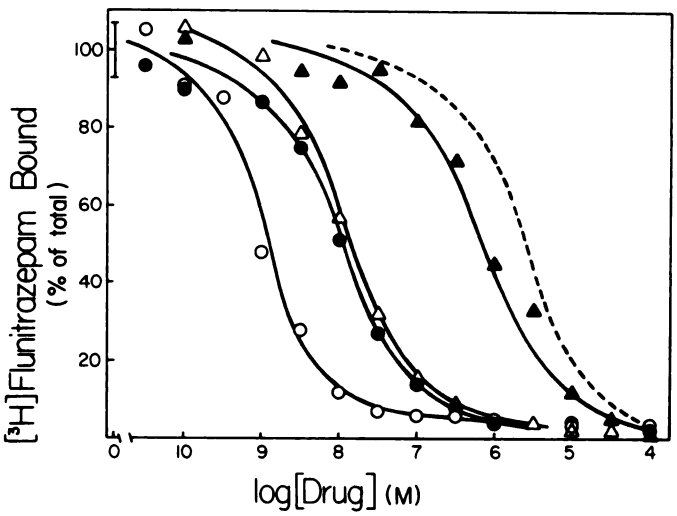


FIG. 2. *Inhibition of renal [^{3}H]flunitrazepam binding by Ro 5-4864 (○), flunitrazepam (●), diazepam (△), and CL 218,872 (▲)*

The *dashed curve* represents the approximate location and shape for the inhibition of [^{3}H]flunitrazepam by six different benzodiazepines (clonazepam, flurazepam, lorazepam, nitrazepam, clobazam, and desmethyldiazepam). The data for the *dashed curve* and for the curves representing Ro 5-4864 and diazepam were obtained from experiments conducted similarly to the present one (9). The data for flunitrazepam and CL 218,872 were obtained from experiments which were conducted as described under Methods, using membranes prepared by Method A, and represent the mean of triplicate determinations from a typical experiment. An indication of the maximal variability of the data is given as the standard deviation of [^{3}H]flunitrazepam binding in the absence of inhibitor. The data have been corrected for nonspecific filter binding but not for nonspecific tissue binding. The mean ± standard error of the slope factors for CL 218,872 from three separate experiments was 0.99 ± 0.13; for flunitrazepam it was 0.97 ± 0.04.

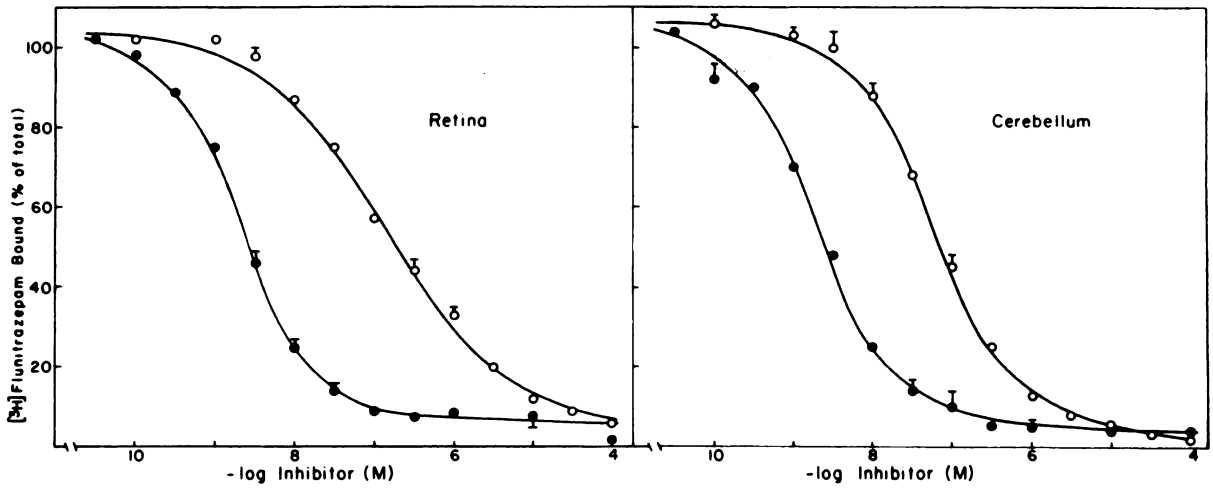


FIG. 3. *Inhibition of [^{3}H]flunitrazepam binding by unlabeled flunitrazepam (●) and by CL 218,872 (○) in bovine retina and rat cerebellum*
The inhibition of [^{3}H]flunitrazepam binding was studied as described under Methods, using membranes prepared by Method A. These data were corrected for filter binding and represent means ± standard errors from three separate experiments (*points* without *error bars* had a standard error of <1.0%). After correction for nonspecific tissue binding, logit-log analyses of these curves gave slope factors in cerebellum of 0.85 for CL 218,872 and 0.88 for flunitrazepam; in retina values of 0.66 and 1.06, respectively, were obtained.

TABLE 1

Parameters for inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in the presence and absence of 100 μM GABA[a]

Tissue[b]	No GABA		GABA	
	K_i	Slope factor	K_i[c]	Slope factor[d]
	nM		nM	
Cerebral cortex	179 ± 4	0.73 ± 0.02	76 ± 8	0.76 ± 0.01
Cerebellum	69 ± 2	0.87 ± 0.04	29 ± 2	0.88 ± 0.05
Retina	223 ± 43	0.64 ± 0.04	81 ± 4	0.67 ± 0.03

[a] The inhibition of [^{3}H]flunitrazepam binding was studied as described under Methods, using membranes prepared by Method B. K_i values and slope factors were calculated as described under Methods and represent the mean ± standard error of the mean of three separate experiments.

[b] Cerebral cortices and cerebella were obtained from rats; retinae were obtained from cows.

[c] K_i data were analyzed by a two-way ANOV with replications. The ANOV showed that there was a highly significant effect of 100 μM GABA on the K_i values for CL 218,872 inhibition of [^{3}H]flunitrazepam binding [$F(1,12) = 149.6$; $p < 0.01$]. The analysis also showed that there were significant differences in K_i values between tissues [$F(2,12) = 97.1$; $p < 0.01$] and that there were no interaction effects [$F(2,12) = 0.6$]. The Newman-Keuls multiple-range test was used to check for individual differences; the major findings were that, in every tissue, GABA caused a significant decrease in the K_i ($p < 0.01$). Additionally, it was found that the K_i values for cerebral cortex and retina were not significantly different from each other, in either the presence or absence of GABA ($p > 0.05$); however, K_i values for both cerebral cortex and retina were significantly different from the K_i values for cerebellum, in both the presence and absence of GABA ($p < 0.01$).

[d] The slope factor data were analyzed by a two-way ANOV with replications. The analysis showed that there was no significant effect of GABA on the slope factors [$F(1,12) = 0.7$; $p > 0.05$]; however, there was a significant difference in the slope factors between tissues [$F(2,12) = 20.6$; $p < 0.01$]. There were no interaction effects [$F(2,12) = 0.04$; $p > 0.05$]. The Newman-Keuls test showed that the slope factors for cerebellum were significantly different from the slope factors for cerebral cortex and retina ($p < 0.05$); however, the slope factors for cerebral cortex and retina were not significantly different from each other ($p > 0.05$).

−20°. Immediately prior to use, the suspension was thawed at room temperature and then centrifuged and resuspended as described above.

The binding of [^{3}H]flunitrazepam (83 Ci/mmole) was measured by filtration assay techniques. The final concentration of [^{3}H]flunitrazepam per assay was 1.0 nM. For membranes prepared by Method A, the final volume of the assays was brought to 2.0 ml using 50 mM sodium-potassium phosphate buffer, pH 7.4. For membranes prepared by Method B, the final volume was brought to 2.0 ml using the PBS buffer. Regardless of the method used to obtain membranes, the equivalent of 2.5 mg of original tissue was used per assay for the membranes prepared from kidney and retina; for cerebral cortex and

TABLE 2

Effects of (+)-bicuculline (BC) on inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in the presence and absence of GABA in membranes prepared from rat cerebral cortex[a]

Addition	K_i[b]	Slope factor[c]
	nM	
Control	183 ± 12	0.77 ± 0.05
100 μM GABA	90 ± 20	0.70 ± 0.01
100 μM BC	655 ± 95	0.80 ± 0.07
BC + GABA (100 μM each)	475 ± 118	0.86 ± 0.10

[a] The inhibition of [^{3}H]flunitrazepam binding was studied as described under Methods, using membranes prepared by isolation Method B. K_i values and slope factors were calculated as described under Methods and represent the mean ± standard error of the mean of three separate experiments.

[b] The K_i data were analyzed by a one-way ANOV, which showed that there were significant treatment effects [$F(3,8) = 20.7$; $p < 0.01$]. The Newman-Keuls test showed that the K_i obtained in the presence of GABA was different from that in all of the other groups ($p < 0.05$). In addition, the K_i values obtained in the presence of bicuculline and in the presence of bicuculline plus GABA were different from control ($p < 0.05$), but they were not different from each other ($p > 0.05$).

[c] The slope factor data were analyzed by a one-way ANOV, which showed no significant treatment effects [$F(3,8) = 1.71$; $p > 0.05$].

cerebellum, the equivalent of 1.25 mg of original tissue was used. In all of the inhibition experiments the assays were performed in triplicate using 12 concentrations of inhibitor. Total binding was determined in the absence of inhibitors. Binding to filters was measured in assays lacking both membranes and inhibitor. After the addition of membranes to the assays, an incubation period of 90 min at 0–5° followed. The incubation was terminated by filtration over Whatman GF/B glass fiber filters followed by three 5-ml rinses with ice-cold buffer. Filters were placed in 8 ml of scintillation solution, and 8–16 hr later the radioactivity was quantified by liquid scintillation spectroscopy.

For the data analysis, the triplicates were averaged and the average for the filter binding was subtracted from the average for the total binding and from the averages for the binding occurring at each inhibitor concentration. These data were graphed (as in Figs. 2 and 3) and the level of nonspecific tissue binding was taken as the amount of binding occurring at maximal displacement. The nonspecific tissue binding was then subtracted from the total binding and from the binding occurring at each inhibitor concentration to give specific binding. Slope factors were calculated from a least-squares linear regression of log $\{B_i + (B_e - B_i)\}$ on log $[I]$, where $[I]$ is the concentration of inhibitor in moles per liter, B_e is the amount of specific [^{3}H]flunitrazepam bound at equilibrium (in the absence of inhibitor), and B_i is the amount of specific [^{3}H]flunitrazepam bound at a given concentration of inhibitor. This analysis is analogous to the Hill equation; however, since the present analysis uses indirect binding data, the term slope factor is used instead of Hill coefficient (17). The actual values used in the linear regression included all of the data between the asymptotes for the maximal and minimal specific binding as judged from the inhibition curves. For a given inhibitor, the apparent equilibrium dissociation constant (K_i) was calculated from its IC_{50} using the following formula: $K_i = IC_{50} \div (1 + [L]/K_d)$, where $[L]$ is the free concentration of radiolabeled ligand and K_d is the apparent equilibrium dissociation constant of the labeled ligand (18). In all experiments the total amount of [^{3}H]flunitrazepam bound (in the absence of inhibitor) was generally much less than 5% of the total quantity of [^{3}H]flunitrazepam added; thus the total concentration of [^{3}H]flunitrazepam could be used as an approximation for the free concentration of [^{3}H]flunitrazepam. K_d values for [^{3}H]flunitrazepam were determined in concurrent saturation studies using previously described methods (19).

The data in Tables 1 and 2 were analyzed either by a one-way or two-way ANOV (20). Tests of significance were made at either $p = 0.05$ or $p = 0.01$. If significant treatment effects were found, individual differences were determined using the Newman-Keuls multiple range test. If significant interaction effects were found in the two-way ANOVs, the effects of a particular factor were determined for either the rows or the columns, depending upon which factor was of the greatest interest. All of the data are expressed as the mean ± the standard error of the mean. The variance of K_i values tends to be proportional to their means, and in the present experiments this was found to be true (Bartlett's test, $p < 0.05$); thus for the statistical analyses, K_i data were logarithmically transformed (20).

The statistics presented in Tables 3 and 4 were obtained from nonlinear least-squares regression analyses using a computerized iterative procedure that was written in BASIC for an Apple II microcomputer. Our program corresponds to the program of Horowitz and Homer (21) with the modifications described by Peck and Barrett (22). The performance of our program was compared with NONLIN (23) and was found to give comparable values for the weighted residual sum of squares and for the parameter estimates; comparable values for the standard errors of the parameter estimates were not obtained and thus are not included in the Tables 3 and 4. The nonlinear regression models that were used consisted of a one-parameter equation[5] and a three-parameter equation[6] which described the interaction of a ligand with either one or two classes of independent binding sites, respectively (24). Mean data from sets of experiments were successively fit to each of the regression models and the residuals were weighted with the reciprocal of the variance. Examination of the weighted residual sum of squares and an F-test were used to evaluate the two models (25).

[^{3}H]Flunitrazepam was purchased from the New England Nuclear Corporation (Boston, Mass). CL 218,872 was obtained from ICI Americas, Inc. (Wilmington, Del). With the exception of clobazam, unlabeled benzodiazepines were gifts from Hoffman-La Roche, Inc. (Nutley, N. J.); clobazam was a gift from Hoechst-Roussel, Inc. (Somerville, N. J.). GABA and (+)-bicuculline were purchased from the Sigma Chemical Company (St. Louis, Mo.). Other chemicals were purchased from commercial sources.

RESULTS

Figure 2 shows the inhibition of [^{3}H]flunitrazepam binding to renal membranes by CL 218,872 and by a number of benzodiazepines. Ro 5-4864, flunitrazepam and diazepam are rather potent, with IC_{50} values in the nanomolar range: logit-log analyses of these curves reflect simple molecular interactions (slope factors ~1). The other benzodiazepines inhibit [^{3}H]flunitrazepam binding with IC_{50} values in the range of 1–6 μM. The non-benzodiazepine anxiolytic, CL 218,872, inhibits [^{3}H]flunitrazepam with a potency comparable to that of the majority of the benzodiazepines tested. In addition, the shape of the displacement curve of CL 218,872 is similar to that of the other benzodiazepines, and its slope factor is approximately 1.

Figure 3 shows the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 and by unlabeled flunitrazepam in bovine retina and rat cerebellum. Compared with the

[5] $B_i = 100 \div (1 + [I]_i/IC_{50})$, where B_i is the specific binding of [^{3}H]flunitrazepam at a given concentration of inhibitor ($[I]_i$) and IC_{50} is the concentration of inhibitor giving 50% of the specific binding in the absence of inhibitor.

[6] $B_i = \{B_{max_H} \div (1 + [I]_i/IC_{50_H})\} + \{(100 - B_{max_H}) \div (1 + [I]_i/IC_{50_L})\}$, where B_{max_H} is the percentage of high-affinity binding site and IC_{50_H} and IC_{50_L} are the IC_{50} values of the high- and low-affinity sites, respectively.

other curves, the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in bovine retina is more shallow, which is reflected by the value of its slope factor (0.66). The slope factor for CL 218,872 in the retina differs markedly from the slope factor for flunitrazepam in this tissue (1.06). In cerebellum the slope factors for CL 218,872 and flunitrazepam are nearly the same (~0.9).

Table 1 shows the results of a study of the effects of GABA on the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in several tissues. In all of these tissues the presence of 100 μM GABA causes a dramatic decrease in the K_i of CL 218,872. This decrease in K_i is remarkably uniform, averaging about 60%. A comparison of K_i values between tissues, in both the presence and absence of GABA, shows a corresponding pattern; i.e., the K_i values for retina and cerebral cortex are not significantly different from each other, but both are larger than the K_i for CL 218,872 in cerebellum. The presence of chloride ion was not essential for the GABA enhancement of CL 218,872 inhibition of [^{3}H]flunitrazepam binding. (Instead of using the PBS buffer, membranes were prepared with, and binding assays were conducted in, a 50 mM sodium-potassium phosphate buffer; in the presence of 100 μM GABA a 57% decrease in K_i was obtained.)

TABLE 3

Comparison of one-site versus two-site models for inhibition of [^{3}H]flunitrazepam binding by CL 218,872[a]

Tissue	Model[b]	Parameters[c]			WSS[d]
		IC_{50_H}	B_{max_H}	IC_{50_L}	
		nM	%	nM	
Cerebral cortex	1	174	100		235.2
	2	35	58	1,000	12.1[e]
Cerebellum	1	58	100		33.7
	2	43	95	6,600	0.4[e]
Retina	1	235	100		615.9
	2	34	61	1,600	8.4[e]
Kidney	1	2,600	100		125.1
	2	2,700	99	0.22	113.3

[a] Data for cerebral cortex, cerebellum, and retina were obtained from the experiments described in Table 1 that were conducted in the presence of 100 μM GABA. Kidney data were obtained from the experiments described in Fig. 1. For each tissue, values for percentage inhibition of [^{3}H]flunitrazepam binding for each of the 12 inhibitor concentrations from 3 experiments were averaged and were analyzed by the nonlinear least-squares regression technique described under Methods.

[b] Model 1 corresponds to a regression model (see Footnote 5, under Methods) describing ligand binding to one class of high-affinity sites; only IC_{50_H} is solved for in this model, B_{max_H} being defined as 100%. Model 2 corresponds to a regression model (see Footnote 6, under Methods) describing ligand binding to two classes of independent sites. Three parameters are solved for in this model; the total number of sites is defined as 100%, and the percentage of low-affinity sites is the difference between 100 and B_{max_H}.

[c] IC_{50_H} and IC_{50_L} are the concentrations of CL 218,872 needed to cause 50% inhibition of [^{3}H]flunitrazepam binding to the high- and low-affinity benzodiazepine binding sites, respectively. B_{max_H} is the density of high-affinity binding sites as a percentage of the total number of sites.

[d] WSS = the weighted residual sum of squares (a measure of the variability between the fitted regression and the actual data).

[e] Significant improvement over Model 1 [$F(2,9) > 10.1$; $p < 0.01$].

TABLE 4

Two-site analysis of the effects of (+)-bicuculline (BC) on inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in the presence and absence of GABA in membranes prepared from rat cerebral cortex[a]

Addition	Parameters[b]		
	IC_{50_H}	B_{max_H}	IC_{50_L}
	nM	%	nM
Control	101	68	3,500
100 μM GABA	29	51	1,200
100 μM BC	402	75	16,000
BC + GABA (100 μM each)	380	67	7,900

[a] Data from the experiments described in Table 2 were analyzed by the nonlinear least-squares regression techniques described under Methods and in Table 3. Analyses revealed that, for each set of data, a two-site model gave an improved fit over a one-site model ($p < 0.01$).

[b] IC_{50_H} and IC_{50_L} are the concentrations of CL 218,872 needed to cause 50% inhibition of [^{3}H]flunitrazepam binding to the high- and low-affinity benzodiazepine binding sites, respectively. B_{max_H} is the density of high-affinity binding sites as a percentage of the total number of sites; the total number of sites is defined as 100% (see also Footnote 6, under Methods).

In a single experiment, using membranes prepared from rat kidney, GABA did not enhance the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 (IC_{50} values: control, 2.5 μM; plus 100 μM GABA, 4.6 μM). The procedures used to prepare membranes for experiments concerned with the effects of GABA (Method B) seemed to damage the kidney membranes as judged by a large increase in nonspecific binding and from an increase in the variability of replicates. Experiments with kidney-derived membranes were not pursued any further.

The data in Table 1 show that GABA did not significantly affect the slope factors for CL 218,872 in any tissue. Additionally, the slope factors for CL 218,872 in cerebral cortex and retina are not different from each other, but they are significantly different from the slope factor obtained in cerebellum. These data also show that the slope factor for CL 218,872 in cerebellum may not actually equal 1, which corroborates data in Fig. 3 and other published results (1).

Table 2 shows the effects of the GABA antagonist, (+)-bicuculline, on the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 in the presence and absence of GABA in membranes prepared from rat cerebral cortex. These data confirm the decrease in K_i due to GABA that is observed in Table 1. They also show that, when GABA and (+)-bicuculline are present in equimolar quantities, the normally observed decrease in K_i is reversed; i.e., there is a highly significant 160% increase in the K_i of CL 218,872. In the presence of 100 μM (+)-bicuculline alone, there is a remarkable 258% increase in the K_i of CL 218,872. This compares with the 106% increase in the K_d of [^{3}H]flunitrazepam that is obtained in simultaneously determined saturation isotherms (data not shown). The slope factors for CL 218,872 that are obtained with the control and with 100 μM GABA compare favorably with those shown in Table 1. Slope factors obtained in the presence of (+)-bicuculline and (+)-bicuculline plus GABA are slightly increased over the control; however, the increase is not statistically significant.

The results of nonlinear least-squares regression analyses are shown in Table 3 for models describing ligand interactions with either one or two classes of independent binding sites. Examination of the weighted residual sum of squares show that, for all of the central tissues, fitting the data to the two-site model results in a significant decrease in the residual variation as compared with the one-site model. In kidney membranes, however, fitting the data to the two-site model is not associated with a significant decrease in the weighted residual sum of squares. In addition, the two-site regression model for kidney membranes gives an estimate for IC_{50_L} that is unreasonable. Similarly, when the two-site model was used for regressions with data obtained from the inhibition of [^{3}H]flunitrazepam binding by unlabeled flunitrazepam, there was either no improvement over a one-site model, or else irrational parameter estimates were obtained (data not shown). Table 3 shows that the two-site model gives estimates of IC_{50_H} that are of a similar magnitude regardless of the tissue; the same is true for IC_{50_L}. These data also show that the proportion of high- and low-affinity sites differs greatly between cerebral cortex and retina on the one hand and cerebellum on the other.

Table 4 summarizes the results of nonlinear least-squares regression analyses on the data obtained from the experiments described in Table 2. Comparing the results obtained in the presence of 100 μM GABA with the results of the two-site analyses shown in Table 3 reveals that the estimates of the IC_{50} values of the high- and low-affinity sites are of the same magnitude. Additionally, Table 4 shows that GABA and/or (+)-bicuculline affect the magnitude of the IC_{50} values for both the high- and low-affinity sites. In the presence of 100 μM GABA there was a 71% decrease in the magnitude of IC_{50_H} and a 66% decrease in IC_{50_L}. Similarly, in the presence of 100 μM (+)-bicuculline the IC_{50} of the high-affinity site increased 298% and for the low-affinity site the IC_{50} increased by 357%. Regardless of the addition, the proportion of high-affinity sites was not consistently affected.

DISCUSSION

Several important aspects of the interaction of CL 218,872 with benzodiazepine binding sites may be deduced from the results of this study. First, as demonstrated by the inhibition of [^{3}H]flunitrazepam binding, CL 218,872 interacts with both the peripheral-type benzodiazepine binding site and the central ones. Second, the affinity of CL 218,872 for central benzodiazepine receptors is markedly increased in the presence of GABA. This increase in affinity is of a similar magnitude for retina, cerebral cortex, and cerebellum and can be antagonized with (+)-bicuculline. Additionally, (+)-bicuculline, in the absence of exogenously added GABA, significantly decreases the affinity of CL 218,872 in extensively washed membranes of cerebral cortex. Finally, slope factors for CL 218,872 are resistant to change.

As demonstrated in this study and in others (1–3), CL 218,872 inhibits the binding of [^{3}H]flunitrazepam and [^{3}H]diazepam to cerebral membranes. The present findings also show that CL 218,872 inhibits the renal binding of [^{3}H]flunitrazepam in a manner similar to that of many benzodiazepines. There are fundamental differences between the central benzodiazepine binding sites and those of the periphery: the differences include different orders of potency for a number of drugs, and, for the peripheral binding site, a lack of correlation between binding and pharmacological activity and the absence of a GABA effect. Given these differences one would not expect *a priori* similarities in the binding characteristics of two chemically dissimilar compounds on a fundamentally different type of binding site. However, similarities with respect to both potency and slope factor were observed between a number of benzodiazepines and CL 218,872 on the inhibition of [^{3}H]flunitrazepam to membranes prepared from the rat kidney. These findings suggest that there may be similarities between the molecular interactions that govern binding to the renal benzodiazepine binding site and those that govern binding to the central benzodiazepine receptor.

For tissues from the central nervous system the results of the present investigation are consistent with the idea that there are two classes of benzodiazepine binding sites. The best evidence for this conclusion is provided by the results of the nonlinear least-squares regression analyses, which consistently showed that a two-site regression model gave an improved fit of the data over that of a one-site model. These analyses can also explain the apparent differences in the K_i and slope factor values that are observed between cerebellum and cerebral cortex when the CL 218,872 inhibition data are analyzed by a conventional logit-log technique. For example, the larger K_i obtained by logit-log analysis for CL 218,872 inhibition of [^{3}H]flunitrazepam binding in cerebral cortex as opposed to cerebellum may be a reflection of the greater porportion of low-affinity sites in the cerebral cortex. The observation that the respective IC_{50} values for the high- and low-affinity sites are of a similar magnitude for both the cerebral cortex and cerebellum suggests that the benzodiazepine binding sites of these tissues are similar and that it is only the ratio of high- to low-affinity sites which differs between these tissues. An interesting consideration in this regard is the development of retina and cerebral cortex as compared with cerebellum. Both retina and cerebral cortex are derived from the telencephalon, whereas cerebellum develops from the metencephalon: perhaps the distribution of alleged central benzodiazepine receptors is determined by the embryological origin of the tissue.

Another concept which is often used to explain results of the present kind is that of negative cooperativity. Although this concept has a precise molecular meaning within the context of certain well-studied enzymatic systems, it embraces a number of molecular models when used to explain equilibrium binding data (5). Included in these models are the concepts of multiple classes of independent binding sites, slowly convertible transition states of a single receptor, coupling between ligand binding and receptor aggregation, and others. The present experimental design cannot resolve among these models; however, studies of the thermal inactivation (3) and of the solubilization (26, 27) of benzodiazepine binding sites suggest the existence of multiple classes of sites.

In summary, the parameters of the inhibition of [^{3}H] flunitrazepam binding by CL 218,872 have been investigated in several tissues and under different conditions. There are two fundamental types of benzodiazepine binding sites: those represented by membranes prepared from retina, cerebral cortex, and cerebellum (so-called central benzodiazepine receptors) and those represented by membranes prepared from kidney (termed peripheral benzodiazepine binding sites). Of the former, the parameters of the inhibition of [^{3}H]flunitrazepam binding by CL 218,872 show that there are apparent differences between membranes prepared from cerebral cortex and retina as compared with membranes prepared from cerebellum. One possible explanation of these results is that there are two classes of central benzodiazepine receptors which are independent and of differing affinities. If this is true, then both classes are modulated by GABA and the relative proportion of the two classes varies between tissues.

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