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Resolution of Two Biochemically and Pharmacologically Distinct Benzodiazepine Receptors

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KLEPNER, C. A., A. S. LIPPA, D. I. BENSON, M. C. SANO AND B. BEER. *Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors.* PHARMAC. BIOCHEM. BEHAV. 11(4) 457–462, 1979.—Brain-specific binding sites have been isolated on synaptosomal membrane fragments which recognize pharmacologically active benzodiazepines (BDZ's) and triazolopyridazines (TPZ's). While early evidence indicated the existence of a single homogeneous class of BDZ binding sites, more recent biological and pharmacological studies support the notion of BDZ receptor multiplicity. We now propose that two biochemically distinct BDZ receptors exist in brain which are responsible for the mediation of different pharmacological activities. Type I BDZ receptors display a high affinity for both BDZ's and TPZ's, are not coupled to GABA receptors or to chloride ionophores, and are the sites which mediate anxiolytic actions. Type II BDZ receptors display a high affinity for BDZ's, display a low affinity for TPZ's, are coupled to GABA receptors and/or chloride ionophores, and are the sites which mediate pharmacological effects other than anxiolytic activity.

Benzodiazepine receptors Triazolopyridazines GABA Chloride ionophores
Type I and Type II BDZ receptors

BRAIN-SPECIFIC benzodiazepine (BDZ) binding sites, which selectively recognize pharmacologically and clinically active BDZ's, have been isolated in all vertebrate species studied with the exception of the cartilagenous fish [3, 15, 19–21, 25]. ^{3}H-BDZ binding has been found to have a high affinity, to be saturable and stereo-specific, and to be sensitive to heat and proteolytic enzymes [15, 17–19, 26]. Brain regional distribution studies have revealed an uneven distribution of the binding sites with highest concentrations found in cortical regions and lowest concentrations found in pons-medulla and spinal cord [15, 19, 25]. A neuronal localization of the binding sites is supported by electrophysiological evidence [10] and by observation of a parallel decrease in binding sites with a degeneration of cerebellar Purkinje cells in nervous mice [12, 24, 27]. In general, biochemical criteria which are indicative of binding to specific receptor sites [6] are met by ^{3}H-BDZ binding.

Early evidence indicated that only a single homogeneous class of BDZ binding sites existed. Scatchard analyses of equilibrium binding in both rat and human brain yielded straight lines having no tendency to resolve into more than one component [15, 17, 19, 25, 26]. Hill analyses of displacement curves for 15 BDZ's having varying affinities for the receptor yielded Hill coefficients near unity [25], indicating a single binding site as well as the lack of cooperativity between binding sites. In mammalian brain, 14 BDZ's exhibited similar Ki values in both frontal cortex and cerebellum [18, 19, 25]. Finally, when membrane preparations were preincubated at 60°C in Tris·HCl buffer, the thermal inactivation of the sites was monophasic [28].

However, several lines of evidence have recently suggested the existence of multiple BDZ receptors. Recent studies have shown that after administration of diazepam at doses which produce significant activity in both anticonflict and anticonvulsant procedures (two pharmacological tests believed to be predictve of anxiolytic activity), only 10–20% of the total number of BDZ binding sites were occupied in the brain [10, 11, 24]. These data suggested that while a small percentage of BDZ binding sites may be involved in the mediation of anxiolytic effects, other binding sites may be involved in the mediation of other pharmacological effects (i.e., sedation, ataxia, etc.).

The discovery of a series of novel triazolopyridazines (TPZ's) [1] has added further pharmacological and biochemical support for the concept of multiple BDZ receptors. CL 218,872 (3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo [4,3-b]pyridazine), a member of this series, was the first non-BDZ reported to displace ^{3}H-BDZ from its brain-specific binding sites with a potency comparable to the BDZ's [9, 10, 28]. However, in contrast to the BDZ's, Hill analyses of dose response curves in ^{3}H-BDZ binding assays revealed Hill coefficients for CL 218,872 which significantly deviated from unity (0.5–0.7) [9,28]. Further, CL 218,872 was found to be as potent as diazepam in both anti-conflict and anti-convulsant studies in animals [9,10]. Unlike diazepam, CL 218,872 (administered at doses producing an-

xiolytic effects) did not produce the muscle incoordination nor the motor depression typically observed with BDZ's [9,10]. Comparisons of *in vivo* anxiolytic activity with *in vitro* potency in ^{3}H-BDZ binding for several TPZ's indicated that IC_2 values, as determined in the binding assay, correlated best with the pharmacological activity [1,9]. Again, these data suggested that only a small percentage of the BDZ binding sites were involved with the mediation of the anxiolytic effect.

Additional biochemical studies with the BDZ binding sites have also suggested the existence of heterogeneous BDZ receptors. Thermal inactivation (at 60°C) of the BDZ binding sites in 50 mM sodium phosphate buffer, pH 7.5, revealed a biphasic degradation of the sites [28]. In studies utilizing P_2 synaptosomal membrane preparations which were thermally inactivated in sodium phosphate buffer for 30 min at 60°C (a treatment which eliminates approximately 50% of ^{3}H-BDZ binding), neither the Ki nor the Hill coefficient for diazepam differed from that observed in untreated membranes [28]. Unlike diazepam, the Ki for CL 218,872 more than doubled and the Hill coefficient approached unity in thermally inactivated membranes [28]. These data suggested that while diazepam had the same affinity for all binding sites, CL 218,872 appeared to have a higher affinity for the relatively heat-labile sites in comparison to the more heat-stable sites. Additionally, biphasic off-rates have been observed in the presence of large excesses of either flunitrazepam or diazepam [5].

A more detailed characterization of two biochemically and pharmacologically different BDZ receptors, designated as Type I and Type II, will be presented here. We suggest that the Type I receptor, having a high affinity for the TPZ's, is involved in the mediation of anxiolytic activity, while the Type II receptor is involved in the mediation of other pharmacological effects.

METHOD

Animals

Male, albino rats (100–200 g, Royalhart Farms, Wistar strain) were housed 4–6 per cage with food and water available ad lib.

Materials

The following substances were purchased from commercial sources: (N-methyl)-^{3}H-flunitrazepam, 84.3 Ci/mmol; flunitrazepam; clonazepam; GABA (γ-aminobutyric acid).

THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]-pyridin-3-ol) was provided by Dr. Anne Vibeke Christensen, H. Lundbeck and Co. A/S, Denmark.

CL 218,872 was synthesized at American Cyanamid Company, Pearl River, NY [1].

Preparation of P_2 Synaptosomal Membranes

After sacrificing by decapitation, whole frontal cortex was removed. The tissue was weighed and gently homogenized (Potter-Elvehjen, teflon-glass homogenizer) in 10 volumes of ice cold 0.32 M sucrose solution. Homogenates were centrifuged twice at 1000 g for 10 min at 4°C. The pellets were discarded and the supernatant solutions were re-centrifuged at 30,000 g for 20 min at 4°C. The resulting P_2 pellets were resuspended in 10 volumes of 10 mM Tris·HCl, pH 7.5, and stored in 2 ml aliquots at −20°C. At time of assay, frozen aliquots were thawed and resuspended in 80 volumes of test buffer, pH 7.5.

For brain regional studies, frontal cortex, cerebellar vermis and dorsal hippocampus were removed and prepared as described above. The freshly prepared P_2 pellets were resuspended in 50 mM Na_2HPO_4 buffer, pH 7.5 (frontal cortex and dorsal hippocampus, 80 volumes; cerebellar vermis, 60 volumes) and tested on that day.

^{3}H-Flunitrazepam Binding Assay

Three hundred μl of P_2 suspension (~0.2 mg tissue protein) was added to ice cold glass tubes containing test substance and ^{3}H-flunitrazepam (1.0 nM, final concentration) in a volume of 2.0 ml test buffer, pH 7.5. Samples, in duplicate, were incubated at 37°C for 15 min followed by an additional incubation at 0°C for 30 min (37°C incubation was omitted for brain regional studies). After incubation, the reaction was terminated by rapid filtration under vacuum through Whatman GF/C filters. Following two 5 ml washes with iced buffer, the filters were placed into scintillation vials, and radioactivity determined by conventional liquid scintillation counting.

Specific binding was defined as the total binding minus non-specific binding (as measured in the presence of 1 μM clonazepam, 3–5% of total binding). Protein was determined by the method of Lowry [13]. All statistical analyses were performed utilizing the student's *t*-test, two-tailed.

RESULTS AND DISCUSSION

Brain Regional Distribution Studies

Early studies revealed that while BDZ binding sites are unevenly distributed in the brain with highest concentrations found in cortical regions [15, 19, 25], the potencies of several BDZ's to displace ^{3}H-BDZ from its binding sites in cortex and cerebellum were found to be equal [15, 19, 25]. However, more recent studies indicate that this is not the case for CL 218,872 in either the ^{3}H-diazepam [22] or ^{3}H-flunitrazepam (see below) binding assays.

IC_{50} values derived from concentration-response curves utilizing CL 218,872 to displace bound ^{3}H-flunitrazepam differ significantly ($p<0.001$) in three brain regions examined (Table 1). CL 218,872 was found to be most potent in cerebellar vermis (IC_{50}=37 nM), less potent in frontal cortex (IC_{50}=142 nM), and least potent in dorsal hippocampus (IC_{50}=330 nM). Of interest is the finding that while Hill coefficients in both frontal cortex and dorsal hippocampus deviate from unity, Hill coefficients in cerebellar vermis approach unity (Table 1, Fig. 1). Since the Hill coefficient approaches unity in the cerebellar region, we have speculated that this membrane preparation is enriched in a single homogeneous type of receptor (which will be designated as Type I), while frontal cortex and dorsal hippocampus contain a heterogeneous population of receptors (Type I and Type II). Because of the extremely high potency of CL 218,872 in the cerebellar region in comparison to the potency of CL 218,872 in the other two regions, we believe that CL 218,872 has a much higher affinity for Type I receptors than for Type II receptors.

In contrast, flunitrazepam was found to be equipotent in all three brain regions having Hill coefficients near unity (Table 1). These data indicate that BDZ's display an equally high affinity for both Type I and Type II receptors.

TABLE 1

POTENCY AND HILL COEFFICIENTS OF CL 218,872 AND FLUNITRAZEPAM IN FRONTAL CORTEX, DORSAL HIPPOCAMPUS AND CEREBELLAR VERMIS

	CL 218,872			Flunitrzepam		
Brain Region	Hill Coefficient	IC_{50} (nM)	r	Hill Coefficient	IC_{50} (nM)	r
Cerebellar Vermis	0.9 ± 0.05	37.4 ± 2.1	.997 ± .003	0.9 ± 0.07	2.54 ± 0.96	.992 ± .006
Frontal Cortex	0.7 ± 0.02	142 ± 37	.994 ± .003	1.0 ± 0.05	2.90 ± 0.29	.998 ± .001
Dorsal Hippocampus	0.6 ± 0.03	330 ± 65	.990 ± .010	1.0 ± 0.05	2.32 ± 0.52	.993 ± .011

IC_{50}'s and Hill coefficients were determined for CL 218,872 and flunitrazepam in ^{3}H-flunitrazepam binding assay in three brain regions in rat. The binding assay proceeded as described in the text using fresh P_2 synaptosomal membranes. Data are representative of the mean ± S.D. of 3 individual experiments performed on separate days.

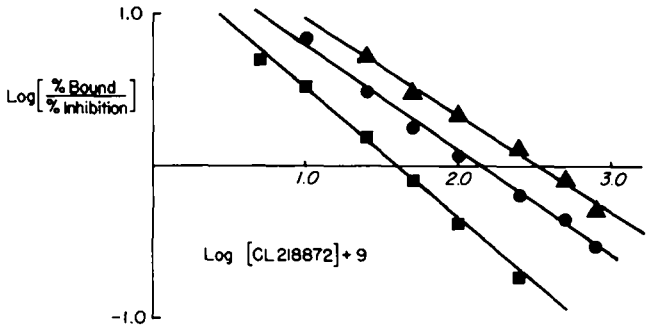

FIG. 1. Hill plots of dose response curves determined in ^{3}H-flunitrazepam binding for CL 218,872 in three rat brain regions: cerebellum (■), frontal cortex (●) and dorsal hippocampus (▲). The assay proceeded as described in the text. Each point is representative of the mean of three separate experiments, performed in duplicate, on separate days.

Since pharmacological studies have shown that CL 218,872 exhibits anxiolytic activity relatively devoid of sedative or ataxic effects, it is proposed that drug actions at Type I receptors may mediate anxiolytic activity, while Type II receptors may be involved in the mediation of other pharmacological effects.

GABA Involvement with BDZ Receptors

Studies investigating the ability of substances to protect BDZ receptors against thermal degradation at 60°C led to the discovery of both GABA-associated and GABA-independent BDZ receptors in rat brain [2,8]. It was found that both GABA and muscimol (a GABA-mimetic) could protect one type of BDZ binding sites, but had no thermal protective effects on another type of BDZ binding sites. Protection of the binding sites as a function of GABA concentration yielded flat dose response curves with maximum protection corresponding to approximately 30% of the total number of BDZ binding sites. Hill and Eadie-Hofstee analyses of these data indicated the presence of two GABA-associated BDZ binding sites with significantly ($p<0.05$) different affinity constants of 8 and 40 μM [2,8].

In contrast to GABA and muscimol, two GABA-mimetics, isoguvacine (1,2,3,4-tetrahydro-4-pyridinecarboxylic acid) and THIP were unable to protect the BDZ binding sites against thermal inactivation at 60°C [2,8]. In addition, isoguvacine and THIP differed from GABA and muscimol in the standard ^{3}H-BDZ binding assay. While GABA and muscimol enhanced ^{3}H-flunitrazepam binding, isoguvacine and THIP selectively inhibited limited populations of binding sites. The inhibition of binding produced by either isoguvacine or THIP was found to be completely reversible in the presence of GABA or muscimol. Isoguvacine consistently inhibited a smaller population (40%) of the binding sites than did THIP (60%). Combinations of isoguvacine and THIP did not inhibit binding more than THIP alone, which indicated that while THIP acted on both GABA-associated receptors, isoguvacine acted on only one of the GABA-associated receptors [2,8].

Further biochemical studies have now revealed that in the presence of THIP both the affinity of ^{3}H-flunitrazepam for the BDZ binding sites and the maximum number of binding sites were decreased (Fig. 2). When 100 μM THIP was added to P_2 synaptosomal membranes (rat whole cortex) and incubated in varying concentrations of ^{3}H-flunitrazepam, the Kd was significantly ($p<0.05$) increased from 0.74 ± 0.09 nM to 1.73 ± 0.66 nM (mean ± S.D., n=4), while the B_{max} was significantly ($p<0.005$) decreased from 1.68 ± 0.14 to 1.06 ± 0.21 pmoles/mg protein (mean ± S.D., n=4). We interpret these data to suggest that THIP may partially overlap onto adjacent BDZ receptors when occupying GABA receptors causing a B_{max} decrease and additionally causing a conformational change in the binding sites resulting in a reduction of their affinity for flunitrazepam.

Since approximately 40% of the total number of ^{3}H-flunitrazepam binding sites were found to be GABA-independent (not inhibited by THIP or not protected by GABA against thermal inactivation), it became essential to investigate the effects of GABA on the inhibition of ^{3}H-BDZ binding produced by CL 218,872. It was found that 1 mM GABA did not alter the inhibition of ^{3}H-flunitrazepam binding produced by CL 218,872. Neither IC_{50} values nor Hill coefficient values for CL 218,872 changed significantly ($p>0.05$) in the presence of GABA (Table 2). In contrast, this concentration of GABA was found to completely reverse the inhibition of ^{3}H-flunitrazepam binding produced by THIP or isoguvacine [2,8]. From these data it is concluded that CL 218,872 activity is not mediated through GABA receptors. This conclusion is supported by pharmacological data in which BDZ's, but not CL 218,872, protect against the convulsions produced by GABA antagonists [9,10].

TABLE 2

EFFECT OF 1 mM GABA ON IC_{50} AND HILL COEFFICIENT OF CL 218,872 IN ^{3}H-FLUNITRAZEPAM BINDING

Substance	IC_{50} (nM)	Hill Coefficient	r
CL 218,872	257 ± 21	0.6 ± 0.06	0.998
CL 218,872 +1 mM GABA	221 ± 16	0.6 ± 0.04	0.996

The assay method proceeded as described in the text (rat whole cortex, 25 mM Tris·HCl, pH 7.5). Data are representative of the mean ± S.D. of 2 experiments performed on different days. No significant differences were observed with regard to the IC_{50}'s or Hill coefficients ($p>0.05$).

Relationship Between Chloride Ionophores and BDZ Receptors

Several investigators have studied the effects of both cations and anions on ^{3}H-BDZ binding. Macherer and Kochman [14] have reported that divalent cations (as their chloride salts) produced significant enhancement of specific ^{3}H-diazepam binding to synaptosomal membranes from whole rat brain by increasing the affinity of the binding sites. Further, they reported that monovalent cations produced only slight enhancement of binding while monovalent anions (as sodium salts) produced either slight enhancement or inhibition of binding. Costa *et al.* [4] have reported that those anions which have the ability to penetrate post-synaptic membranes of cat motoneurones enhance ^{3}H-diazepam binding. In particular, they investigated the effects of iodide and concluded that the halides increased the affinity of the binding sites for ^{3}H-diazepam, but did not significantly change the B_{max}. Our present studies indicated that for at least one monovalent anion in the Eccles series [7], chloride, this was not the case.

Scatchard analysis of specific ^{3}H-flunitrazepam binding in varying concentrations of chloride ion revealed a significant change in the total number of binding sites (B_{max}), but had no effect on the affinity constant (Kd) (Fig. 3). B_{max} values (*p* moles/mg protein, mean ± S.D.) for 1 mM, 10 mM and 50 mM chloride were found to be 0.45 ± 0.20, 1.23 ± 0.05 and 1.61 ± 0.08, respectively. Statistical analysis produced values of $p<0.001$ and $p<0.01$ for comparisons between 1 mM and 10 mM chloride and 10 mM and 50 mM chloride, respectively. In contrast, Kd values (nM, mean ± S.D.) were calculated to be 0.88 ± 0.07, 0.89 ± 0.10 and 0.83 ± 0.19 for 1 mM, 10 mM and 50 mM chloride, respectively. Statistical analysis of these data revealed no significant differences ($p>0.05$).

Several investigators have speculated that the BDZ receptor may be associated with a chloride ion channel which is closely linked to a GABA-recognition site. Evidence for this hypothesis stemmed from their data demonstrating that enhancement of ^{3}H-diazepam binding was markedly and synergistically increased by the presence of halides in the incubation medium [4,16]. In support of this theory, our studies indicated the inhibition of ^{3}H-flunitrazepam binding by THIP is enhanced in the presence of chloride. Concentration-response curves performed simultaneously in 25 mM Tris·HCl buffer, pH 7.5, and in 50 mM Na_2HPO_4 buffer, pH 7.5 (Fig. 4), reveal that while a maximum of 60% of the binding sites can be inhibited in the

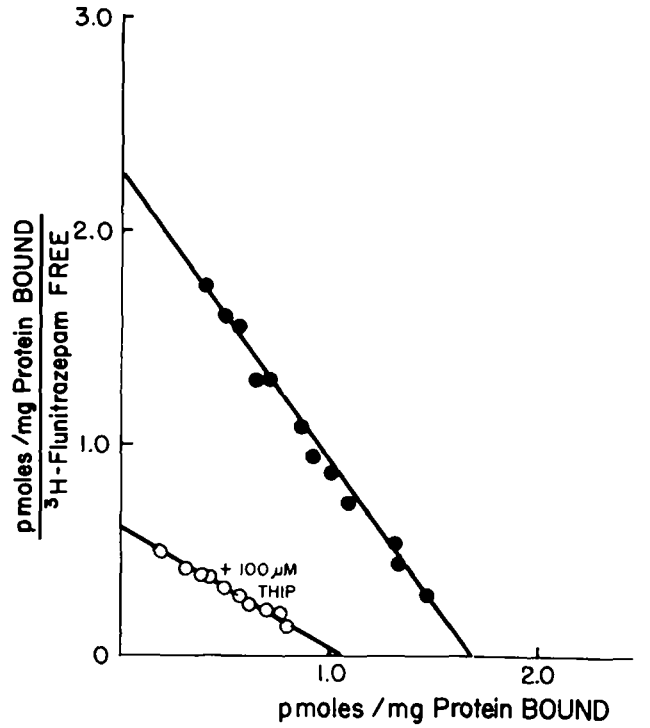

FIG. 2. Scatchard analyses of ^{3}H-flunitrazepam binding in 25 mM Tris·HCl, pH 7.5, with (○) and without (●) 100 μM THIP. The assay proceeded as described in the text (rat whole cortex). Each point on the figure represents the mean value of 4 individual experiments.

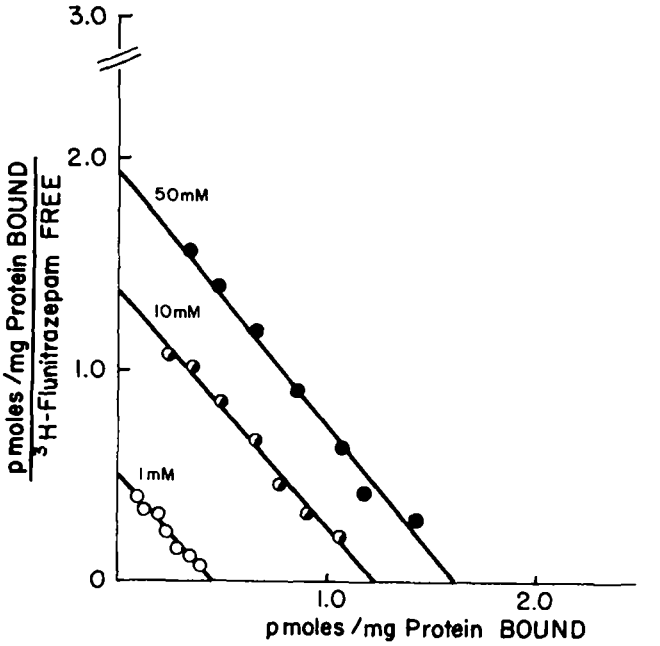

FIG. 3. Scatchard analyses of ^{3}H-flunitrazepam binding (rat whole cortex) in the presence of 1 mM (○), 10 mM (◑) and 50 mM (●) chloride. The ion was tested as its Tris·salt, pH 7.5. The assay method proceeded as described in the text. Each point is the mean of three individual experiments. Kd values did not significantly change ($p>0.05$) with chloride concentration. However, B_{max} values were significantly increased with increasing chloride concentration ($p<0.001$ and $p<0.01$ for comparisons between 1 mM and 10 mM chloride and 10 mM and 50 mM chloride, respectively).

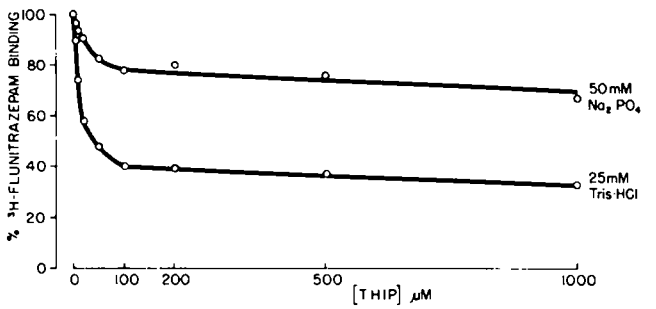

FIG. 4. Dose response of THIP in ^{3}H-flunitrazepam binding (rat whole cortex) in 50 mM Na_2HPO_4 buffer and in 25 mM Tris·HCl buffer, both pH 7.5. Assay method proceeded as described in the text. This figure is representative of one experiment which was repeated one additional time with similar results. As can be seen, the potency of THIP with respect to its ability to inhibit binding was found to be ion-dependent. While a maximum of 60% of the total number of binding sites were inhibited by THIP in 25 mM Tris·HCl, only 20% of the binding sites were blocked by THIP in 50 mM Na_2HPO_4 buffer.

presence of chloride, only 20% of the binding sites can be inhibited in an incubation medium devoid of chloride.

When similar studies were performed utilizing CL 218,872 as the displacing agent, it was found that the presence or absence of chloride in the incubation medium does not affect the potency nor the Hill coefficient of CL 218,872 (Table 3). These data indicate that while GABA-associated or Type II receptors may be coupled to a chloride ionophore, Type I receptors most likely are not.

Electrophysiological evidence for the existence of BDZ receptors coupled with chloride ionophores and BDZ receptors independent of chloride ionophores has been reported [10]. Activation of BDZ receptors located on cerebellar Purkinje cells by microiontophoretic application of flurazepam was found to inhibit the overall firing rate of these cells. Concurrent microiontophoretic application of picrotoxin antagonized the depressant actions of flurazepam in approximately 50% of the cells tested. Since pircotoxin antagonized the depressant effects of GABA on those same cells in which it failed to affect flurazepam, it was speculated that one type of BDZ receptors depressed cerebellar activity through a picrotoxin-sensitive (possibly chloride mediated conductance) mechanism, while another type of BDZ receptors did not utilize this mechanism.

CONCLUSIONS

While early studies suggested the existence of a single homogeneous class of ^{3}H-BDZ binding sites [15, 17–19, 25, 26], the present results, in agreement with other recent studies [2, 5, 8–11, 22, 28], provide evidence for the existence of two biochemically and pharmacologically distinct subpopulations of BDZ receptors. Both TPZ's and BDZ's competitively displace ^{3}H-BDZ binding at a high affinity site (Type I) [22]. These Type I receptors appear to be GABA-independent and chloride-independent (present results). Additionally, Type I receptors are more heat labile at 60°C in 50 mM sodium phosphate buffer [28] and exhibit a relatively faster dissociation rate in comparison to Type II receptors [5]. In contrast, TPZ's non-competitively displace ^{3}H-BDZ binding from a low affinity site (Type II) [22]. Type II receptors are additionally characterized by their associations with GABA receptors and chloride conductance mechanisms [2, 4, 8, 10, 16]. The relative amounts of Type I and Type II receptors differ in various brain regions ([22] present results).

TABLE 3

EFFECT OF IONS ON THE POTENCY OF CL 218,872 TO INHIBIT SPECIFIC ^{3}H-FLUNITRAZEPAM BINDING IN RAT WHOLE CORTEX

Buffer	IC_{50} (nM)	Hill Coefficient	r
25 mM Tris·HCl	155 ± 30	0.5 ± 0.02	0.998
50 mM Na_2HPO_4	131 ± 60	0.6 ± 0.04	0.998

Dose response curves for Cl 218,872 were performed in either 25 mM Tris·HCl, pH 7.5, or 50 mM Na_2HPO_4, pH 7.5, in ^{3}H-flunitrazepam binding assay (described in text). Data presented are the mean ± S.D. of 6 individual experiments performed on separate days.

A large body of evidence now suggests that pharmacological actions on the GABA-independent Type I receptors may endow a drug with anxiolytic properties, while actions on GABA-associated Type II receptors may produce sedation and ataxia. In accordance with this, CL 218,872, having a ten-fold higher affinity for Type I receptors, produces anxiolytic actions in animals at less than one-tenth the doses at which it produces ataxia and protects against the convulsions produced by GABA antagonists [9,10]. Furthermore, GABA antagonists reverse the ataxia produced by BDZ's in doses which do not alter the anxiolytic effects of these drugs (see Reference [10] for review). Since BDZ's have similar affinities for both Type I and Type II receptors, it is not surprising that these drugs produce sedation and ataxia in the same dose range at which they produce anxiolytic effects.

One question which still remains to be answered is whether Type I and Type II receptors are actually two different binding sites for which the BDZ's have equal affinities. These two binding sites certainly appear to differ with respect to their biochemical, pharmacological and functional properties. The brain regional independence of Type I and Type II receptors support such a hypothesis. Alternatively, Type I and Type II receptors may actually be the same binding sites and their different properties may be attributable to their close association with other functional units such as GABA receptors and/or chloride ionophores.

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