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Ro 15-4513 Binding to $GABA_A$ Receptors: Subunit Composition Determines Ligand Efficacy

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WONG, G. AND P. SKOLNICK. *Ro 15-4513 binding to $GABA_A$ receptors: Subunit composition determines ligand efficacy.* PHARMACOL BIOCHEM BEHAV **42**(1) 107–110, 1992. – The bidirectional modulation of ligand binding to benzodiazepine receptors (BzR) by GABA (the "GABA shift") has been widely used to predict ligand efficacy. The present study examined the effects of GABA and muscimol on [^{3}H]Ro 15-4513 binding to "diazepam-insensitive" (DI) and "diazepam-sensitive" (DS) BzR. Neither GABA nor muscimol significantly altered [^{3}H]Ro 15-4513 binding to DI in cerebellum, while both compounds inhibit [^{3}H]Ro 15-4513 binding to cerebellar DS in a concentration-dependent fashion. The maximum reductions in [^{3}H]Ro 15-4513 binding to cerebral cortical and hippocampal membranes elicited by GABA were comparable to those obtained in cerebellar DS, but significantly less than obtained with the full inverse agonist [^{3}H]3-carbomethoxy-β-carboline. The qualitatively different effect of GABAmimetics on [^{3}H]Ro 15-4513 binding to DS and DI is not species specific since identical effects were obtained in rat and mouse brain. Based on previously established criteria, Ro 15-4513 can be classified as a "GABA-neutral" (antagonist) ligand at DI and "GABA negative" (inverse agonist) at other BzR. These findings suggest that $GABA_A$ receptor subunit composition determines not only ligand affinity but also ligand efficacy.

Benzodiazepine receptors Ro 15-4513 GABA Diazepam-sensitive receptors Diazepam-insensitive receptors

Ro 15-4513 is a high-affinity benzodiazepine receptor (BzR) ligand (11,14) that has been reported to antagonize various neurochemical, electrophysiological, and behavioral effects of ethanol (1,5,7,19). The blockade of these effects by specific BzR antagonists such as Ro 15-1788 suggests those actions of ethanol sensitive to Ro 15-4513 are mediated by the $GABA_A$ receptor complex. Nonetheless, the inverse agonist properties of Ro 15-4513 documented in behavioral, neurochemical, and electrophysiological [reviewed in (5,7,20)] measures, as well as the ability of other BzR inverse agonists (such as FG 7142) to mimick the actions of Ro 15-4513 as an alcohol antagonist in *some* paradigms [reviewed in (5,7,20)], have raised questions about the specificity of Ro 15-4513 as an alcohol antagonist.

When used as a photoaffinity ligand, [^{3}H]Ro 15-4513 binds to a population of cerebellar granule cells not labeled by [^{3}H]flunitrazepam (14). These sites, described in granule cell cultures and cerebellar homogenates (9,21), have been termed "diazepam insensitive" (DI) since a significant proportion of the [^{3}H]Ro 15-4513 bound to these cultures was not displaced by high (10 μM) concentrations of either diazepam or clonazepam. The remaining sites labeled by [^{3}H]Ro 15-4513 and displaced by diazepam were termed "diazepam sensitive" (DS) (9,21). A novel subunit (α_6) of the $GABA_A$ receptor complex localized to cerebellar granule cells has recently been cloned (8). Both the localization of this subunit and the pharmacological characteristics of a cell line expressing this α6 subunit as part of a reconstituted receptor strongly suggests it is an endogenous component of the native DI BzR. These findings have led to the proposal [(8); but see (4)] that the antagonism of alcohol-induced motor impairment by Ro 15-4513 is effected through DI.

Since the ability of GABA and related compounds to bidirectionally modulate ligand binding to BzR is widely used to predict BzR ligand efficacy in vivo (17), we investigated the effects of GABAmimetics on [^{3}H]Ro 15-4513 binding to DI and DS BzR.

We now report that the binding of [^{3}H]Ro 15-4513 to DI in adult rodent cerebellum is unaffected by either GABA or muscimol, but is reduced in a concentration-dependent fashion to DS in cerebellum, hippocampus, and cortex. Based on previously established criteria (17), Ro 15-4513 can be classified as a "GABA-neutral" (antagonist) ligand at DI and a "GABA-negative" (inverse agonist) ligand at other BzR. This

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qualitative difference in ligand behavior indicates that subunit composition of the $GABA_A$ receptor complex determines not only ligand affinity (6,8,12,17) but also ligand efficacy in native BzR.

METHOD

Adult, male Sprague-Dawley rats (Taconic Farms, Germantown, NY) and NIH Swiss mice (Harlan Sprague-Dawley, Frederick, MD) were used in these studies. Animals were killed by decapitation and brains placed in ice-cold 50 mM Tris-citrate buffer (pH 7.8). Brain regions were dissected, weighed, and disrupted (Brinkmann Polytron, setting 6, 10 s) in 60 volumes of 50 mM Tris-citrate buffer. Homogenates were centrifuged at 20,000 × *g* for 20 min (4°C), resuspended in 60 volumes of buffer, and recentrifuged. This "washing" procedure was repeated a total of six times.

[^{3}H]Ro 15-4513 binding was determined as previously described (10) with minor modifications. Saturation studies were performed in a a total volume of 0.5 ml consisting of 0.05 ml tissue (~100 μg protein), 0.05 ml [^{3}H]Ro 15-4513 (Sp. Act. 29 Ci/mmol; final concentrations 0.5–28 nM), and 0.4 ml Tris-citrate buffer (pH 7.8). Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. Other incubations were performed in a total volume of 1.0 ml consisting of: 0.1 ml tissue (~80–100 μg protein), 0.1 ml [^{3}H]Ro 15-4513 (final concentration ~2 nM), 0.1 ml GABA (final concentrations 1–100 μM) or muscimol (final concentrations 0.1–10 μM), 0.1 ml 2 M NaCl, and 0.6 ml Tris-citrate buffer (pH 7.8). [^{3}H]Ro 15-4513 binding displaced by Ro 15-1788 (10 μM) was defined as DS + DI binding. This typically represented 90–95% of [^{3}H]Ro 15-4513 binding with the remainder defined as nonspecific binding. [^{3}H]Ro 15-4513 binding displaced by Ro 15-1788 (10 μM) that was not displaced by diazepam (10 μM) was defined as DI binding. This value typically represented 30–40% of total binding in cerebellum, but was not detectable in other brain areas. The subtraction of [^{3}H]Ro 15-4513 binding to DI as described above from DS + DI defined ligand binding to DS. [^{3}H] 3-Carbomethoxy-β-carboline (βCCM) binding was assayed in a similar fashion except 0.1 ml [^{3}H]βCCM (Sp. Act. 80 Ci/mmol; final concentration ~2 nM) and 0.1 ml 5 M NaCl were used. Nonspecific binding was defined by Ro 15-1788 (10 μM) and typically represented less than 10% of total binding. 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). Protein content was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Assays were performed in duplicate unless otherwise specified. Radioligands were purchased from Du Pont-NEN (Boston, MA). Benzodiazepines were donated by Hoffmann-LaRoche (Nutley, NJ). GABA and muscimol were purchased from Sigma (St. Louis, MO). Other materials were from standard sources.

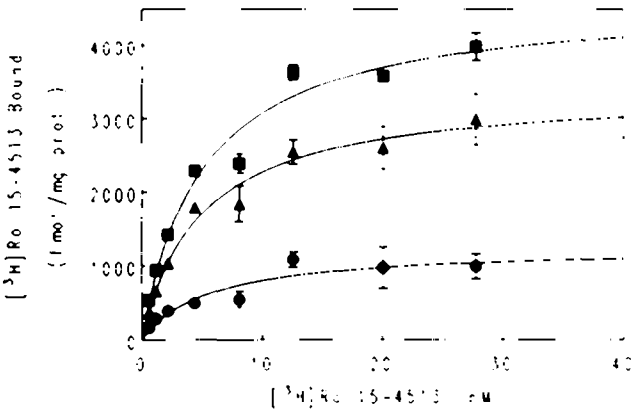

FIG. 1. Saturation analysis of [^{3}H]Ro 15-4513 binding to rat cerebellar (■) DS + DI, (▲) DS, and (●) DI BzR. Membranes were incubated with various concentrations of [^{3}H]Ro 15-4513 (0.5–28 nM). Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. DS + DI binding represents all specific binding sites determined. DI binding represents specific binding sites determined in the presence of 10 μM diazepam. DS sites were calculated by the subtraction of DI sites from DS + DI. Assays were performed in triplicate and data are the mean ± SEM (bars) values from three separate experiments. Iterative curve-fitting analysis (GraphPad Inplot 3.15) yielded K_d and B_{max} values of 5.1 ± 0.5 nM, 4577 ± 251 fmol/mg protein (DS + DI); 5.3 ± 1.2 nM, 3413 ± 513 fmol/mg protein (DS); 3.1 ± 0.1 nM, 1074 ± 156 fmol/mg protein (DI).

RESULTS

Analysis of [^{3}H]Ro 15-4513 binding to cerebellar membranes revealed large differences in the density of DS and DI BzR (Fig. 1), with B_{max} values approximately threefold higher at DS than at DI (3413 ± 512 vs. 1074 ± 156 fmol/mg cerebellar protein; $p < 0.02$). The K_d values of [^{3}H]Ro 15-4513 were not significantly different at DS and DI (5.3 ± 1.2 vs. 3.1 ± 0.1 nM; $p > 0.1$), respectively (Fig. 1).

GABA produced a concentration-dependent (1–100 μM) inhibition of [^{3}H]Ro 15-4513 binding to rat cerebellar membranes (Table 1). Significant differences ($p < 0.001$, Kruskal-Wallis) were observed in the maximum inhibition of ligand binding elicited by 100 μM GABA to DS + DI, DS, and DI, as well as between DS + DI and DS ($p < 0.05$, Mann-Whitney). The maximum inhibition (Δ_{max}) of [^{3}H]Ro 15-4513 binding to DS was ~57% greater than that observed to DS + DI (23.5 ± 4.4% vs. 15.0 ± 3.1%). In contrast, [^{3}H]Ro 15-4513 binding to DI was unaffected by GABA (Table 1). While significantly more potent than GABA (Table 1), the maximum inhibition of [^{3}H]Ro 15-4513 binding to DS, DI, and DS + DI obtained with muscimol (0.1–10 μM) was nearly identical to the values obtained with GABA (Table 1).

[^{3}H]Ro 15-4513 binding to DS, DI, and DS + DI was also examined in mouse cerebellar membranes to determine whether the qualitative difference in the effect of GABA on ligand binding to DS and DI was species specific. As was observed in the rat, [^{3}H]Ro 15-4513 binding to DI was unaffected by muscimol but was inhibited in a concentration-dependent manner at DS. While significant differences in the potency of GABA and muscimol, as well as the potency of muscimol between species were observed, the efficacy of GABA and muscimol to inhibit [^{3}H]Ro 15-4513 binding to DS was similar (Table 1).

The effects of GABA and muscimol on [^{3}H]βCCM binding to mouse cerebellar membranes were also examined. [^{3}H]βCCM binding was inhibited to the same extent by 10 μM diazepam or Ro 15-1788 (Fig. 2). Significant differences in the efficacy of GABA or muscimol to inhibit [^{3}H]βCCM binding were not observed. However, [^{3}H]Ro 15-4513 binding to cerebellar DS was inhibited by muscimol to a significantly lesser extent than [^{3}H]βCCM binding (24.1 ± 1.4% vs. 48.4 ± 7.0%, $p < 0.05$, Student-Newman-Keuls).

DI were not detected in either mouse cerebral cortex or

TABLE 1

EFFECTS OF GABA AND MUSCIMOL ON [^{3}H]Ro 15-4513 BINDING TO DS AND DI BzRs

	Rat				Mouse	
	GABA		Muscimol		Muscimol	
	EC_{50} (μM)	Δ_{max}	EC_{50} (μM)	Δ_{max}	EC_{50} (μM)	Δ_{max}
DS	7.8 ± 0.4	−23.5 ± 4.4	0.6 ± 0.2	−26.4 ± 1.6	7.4 ± 1.2	−24.1 ± 1.6
DS + DI	6.0 ± 1.6	−15.0 ± 3.1	0.7 ± 0.1	−15.0 ± 0.6	7.9 ± 0.9	−18.6 ± 1.1
DI	–	2.0 ± 3.8	–	−3.0 ± 4.3	–	4.0 ± 3.5

Radioligand binding to cerebellar membranes was determined as described in the Methods Section. Δ_{max} is expressed as % inhibition of basal radioligand binding. The Δ_{max} for DI binding was determined at 100 μM GABA or 10 μM muscimol. A statistically significant difference was found among DS, DI, and DS + DI Δ_{max} values ($p < .001$, Kruskal-Wallis). No significant differences were found between the effects of GABA and muscimol (rats) or rat and mouse (muscimol). Assays were performed in duplicate and values presented are the mean ± SEM of three experiments. Basal binding of [^{3}H]Ro 15-4513 (2 nM) was 927 ± 87 in rats and 789 ± 71 fmol/mg protein in mice, respectively.

hippocampus (Fig. 3). Moreover, in these tissues GABA inhibited [^{3}H]Ro 15-4513 binding with both potency and efficacy values not significantly different from those obtained at cerebellar DS (Fig. 3).

DISCUSSION

Ro 15-4513 binds with high affinity to a population of DI BzR previously described in cultured cerebellar granule cells and membranes (9,21). At least three different subunits (α,β,γ) expressed and assembled on cell membranes are required to form receptors with native $GABA_A$ receptor pharmacology [reviewed in (15)]. Depending upon the subunits expressed, multiple $GABA_A$ conformations are possible. A novel $GABA_A$ receptor subunit (α_6) (8) exhibits a pharmacological profile resembling native DI (i.e., with the exception of Ro 15-4513, structurally diverse BzR ligands such as diazepam, CL 218,872 and βCCM have affinities > two orders of magnitude lower than at other BzR) when expressed with β_2 and γ_2 subunits of adult rodent cerebellum (8). Moreover, like DI, α_6 appears confined to cerebellar granule cells in adult rodent cerebellum (8,9). The unique pharmacological profile of DI indicates the cerebellum could be a useful model to examine the role of native subunit composition in determining BzR ligand efficacy (15) since several lines of evidence indicate the Type I BzR originally described in cerebellum (6,18) corresponds to an α_1-containing heterooligomer (12).

Ro 15-4513 behaves as a GABA-neutral ligand at DI (Table 1) but as a GABA-negative ligand at DS in cerebellum, as well as BzRs in cortex and hippocampus (Fig. 3). The apparent qualitative difference in ligand efficacy was confirmed in two species using GABA, as well as muscimol (Table 1). These

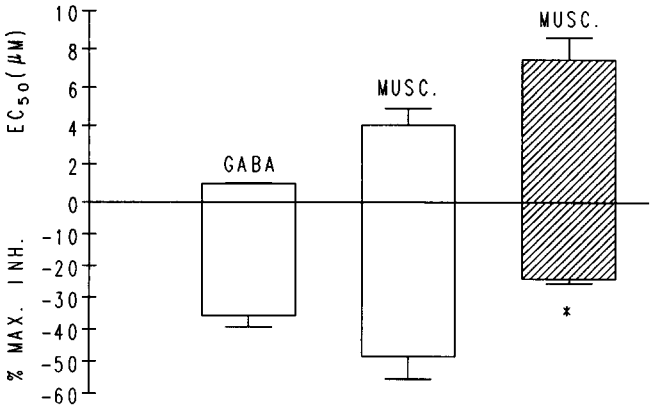

FIG. 2. [^{3}H]βCCM and [^{3}H]Ro 15-4513 binding to mouse cerebellar membranes: Effects of GABA or muscimol. Ligand binding (2 nM) was performed in the presence of GABA (1–100 μM) or muscimol (0.1–10 μM) as described in the Methods Section. Since diazepam (10 μM) and Ro 15-1788 (10 μM) displaced 90–95% of total [^{3}H]βCCM binding, the values represented for [^{3}H]βCCM are DS binding. Δ_{max} values are expressed as the % maximum inhibition of ligand binding. Values illustrated are mean ± SEM from three separate experiments. The maximum inhibition of [^{3}H]Ro 15-4513 by muscimol (hatched bar) was significantly less (*$p < 0.05$) than [^{3}H]βCCM.

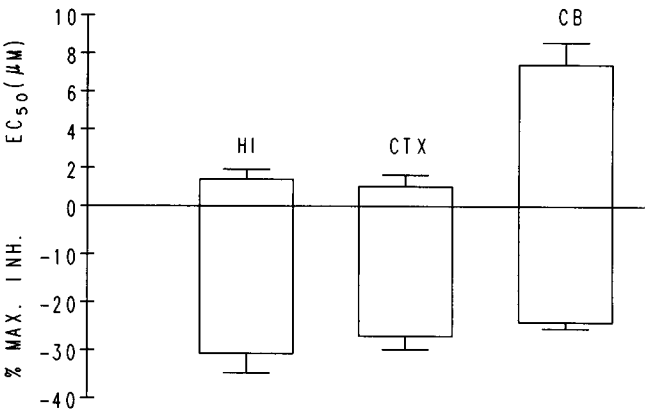

FIG. 3. Inhibition of [^{3}H]Ro 15-4513 binding to DS BzR by GABA and muscimol in different brain areas. Diazepam (10 μM) and Ro 15-1788 (10 μM) inhibited [^{3}H]Ro 15-4513 binding to the same extent in mouse cerebral cortex and hippocampus, indicating a lack of DI BzRs in these tissues. [^{3}H]Ro 15-4513 (2 nM) binding was determined in the presence of GABA (HI, hippocampal; CTX, cortical) or muscimol (CB, cerebellar) as described in the Methods Section. EC_{50} values are expressed as μM GABA or muscimol. Δ_{max} values are expressed as the % maximum binding inhibited by the GABAmimetic. SEM bars from three separate experiments are shown. No statistically significant differences were found in the Δ_{max} among the three brain areas.

differences in ligand efficacy at DS and DI BzR were not due to differences in the affinity of [^{3}H]Ro 15-4513 since the K_d of this ligand was not significantly different (Fig. 1). These findings indicate the apparent dependence of ligand efficacy on receptor conformation is neither species specific nor dependent upon the GABAmimetic employed. Consistent with these findings, Malminiemi and Korpi (9) reported that at 100 μM GABA Ro 15-4513 was GABA neutral at DI and GABA negative at DS in cerebellar membranes from 14-day-old rat pups using incubation conditions similar to those reported here. Since the GABA shift (17) is widely used to predict ligand efficacies in vivo, the behavior of Ro 15-4513 at DS and DI prompted us to compare it to the "full inverse" agonist [^{3}H]β-CCM (2,3,13). [^{3}H]βCCM binding to DI was not detected (Fig. 2), consistent with the finding that this compound has a very low affinity (K_i > 2000 nM) for the $\alpha_6\beta_2\gamma_2$ configuration in transfected cells (8). Nonetheless, the magnitude of the negative GABA shift at DS was significantly greater for βCCM than for Ro 15-4513. The observation that ligand efficacy is dependent upon subunit composition is also consistent with the report of Pritchett et al. (12), who demonstrated an approximate twofold difference in the efficacy of GABA to enhance [^{3}H]diazepam binding in cell lines transfected with the $\alpha_3\beta_1\gamma_2$ subunit combinations when compared with those transfected with $\alpha_1\beta_1\gamma_2$ or $\alpha_2\beta_1\gamma_2$ subunit combinations.

If the GABA shift is predictive of ligand efficacy in vivo, the present findings indicate Ro 15-4513 behaves as an antagonist and inverse agonist at DS and DI, respectively. While Ro 15-4513 has been shown to antagonize the actions of ethanol in brain regions that do not appear to possess DI BzR [(19); reviewed in (8)], the high affinity and specificity of Ro 15-4513 for DI in cerebellum (4,8,17) has led to the suggestion that this compound antagonizes the alcohol-induced motor impairment at DI (8). If this hypothesis is correct [but, see (4)], the demonstration that Ro 15-4513 behaves as a GABA-neutral ligand at DI indicates that an inverse agonist action per se is neither necessary nor sufficient for these effects. Moreover, the demonstration that subunit composition effects ligand efficacy may explain the anxioselective actions of some BzR ligands (16).

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