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Loreclezole modulates [^{35}S]*t*-butylbicyclophosphorothionate and [^{3}H]flunitrazepam binding via a distinct site on the $GABA_A$ receptor complex

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Abstract

The allosteric modulation of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) and [^{3}H]flunitrazepam binding was utilized to evaluate the actions of loreclezole at the $GABA_A$ receptor complex in the rat brain. Loreclezole was observed to allosterically inhibit the binding of [^{35}S]TBPS in a dose-dependent manner with micromolar potency (IC_{50} = 1 μM). Loreclezole was found to have an additive effect on neuroactive steroid modulation of [^{35}S]TBPS binding, but merely potentiated the effect of Ro5-4864 (4″-chlorodiazepam) modulation of [^{35}S]TBPS binding. These observation suggest that loreclezole modulates [^{35}S]TBPS binding through a site independent of the neuroactive steroid and Ro5-4864 sites on the $GABA_A$ receptor complex. The enhancement of [^{3}H]flunitrazepam binding to the benzodiazepine receptor by loreclezole as well as the effect of loreclezole on CL218872/[^{3}H]flunitrazepam dose-response curves suggest that loreclezole does not act through the benzodiazepine site on the $GABA_A$ receptor complex, nor does it selectively modulate benzodiazepine receptor subtypes. The potency of loreclezole as an inhibitor of [^{35}S]TBPS binding in rat brain was regionally dependent and GABA-sensitive. Loreclezole modulation of [^{35}S]TBPS binding showed greater potency and GABA sensitivity in the cerebellum and thalamus when compared to other brain regions such as the cortex, hippocampus and striatum. This finding is consistent with previous reports of the selectivity of loreclezole for $GABA_A$ receptor complex's containing β_2 and β_3 subunits. These β subunit isoforms predominate in the cerebellum and thalamus. Collectively the evidence suggests that loreclezole modulates [^{35}S]TBPS and [^{3}H]flunitrazepam binding through a site distinct from benzodiazepine, neuroactive steroid, Ro5-4864 and GABA sites on the $GABA_A$ receptor complex.

Keywords: Loreclezole; $GABA_A$ receptor; Neuroactive steroid; Benzodiazepine receptor

1. Introduction

Loreclezole (R 72063), (*Z*)-[2-chloro-2-(2,4-dichlorophenyl)ethenyl)-1*H*-1,2,4-triazole) is a novel compound with 'broad spectrum' anticonvulsant activity. It has been shown to inhibit seizure spread and increase seizure threshold in all species tested (Ashton et al., 1992; Pohl and Mares, 1990; Wauquier et al., 1990). The precise mechanism of the anticonvulsant action of loreclezole is unclear. Proposals have been made to suggest an action of loreclezole on the benzodiazepine receptor on the $GABA_A$ receptor complex based on observations that the benzodiazepine inverse agonist CGS 8216 can reverse the effect of loreclezole on seizure threshold (Vaught and Wauquier, 1991; Ashton et al., 1992). Surprisingly, the anticonvulsant effect of loreclezole is insensitive to the prototypical benzodiazepine receptor antagonist, flumazenil (Ro15-1788) (Wauquier et al., 1990; Dawson et al., 1994). This observation suggests that the effect of CGS 8216 may be a physiological and not a pharmacological antagonism, the former effect of CGS 8216 being derived from its negative efficacy. Consequently, whether or not loreclezole acts at the benzodiazepine receptor or a subset of benzodiazepine receptors remains to be clarified. Recently, receptor binding studies have demonstrated that loreclezole inhibited [^{3}H]*t*-butylbicycloorthobenzoate ([^{3}H]TBOB) binding to the $GABA_A$ receptor complex in rat brain with an IC_{50} of 3 μM (Wafford et al., 1994). Both [^{3}H]TBOB and [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) may label similar sites at or near the GABA-gated chloride channel. Correspondingly, a similar IC_{50} for loreclezole displacement of [^{35}S]TBPS binding to recombinatly expressed

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$GABA_A$ receptor complex was also observed (Wafford et al., 1994). Furthermore, loreclezole did not inhibit [^{3}H]muscimol binding thus ruling out direct action on the $GABA_A$ receptor. GABA-induced currents in *Xenopus* oocytes potentiated by a maximally effective concentration of loreclezole (10 μM) is further potentiated by the neuroactive steroid 3α-hydroxy-5α-pregnan-20-one and the barbiturate pentobarbital (Wafford et al., 1994). Collectively these results indicate that loreclezole may act on a site distinct from GABA, neuroactive steroids and barbiturate sites on the $GABA_A$ receptor complex. Studies on recombinantly expressed in *Xenopus* oocytes and transfected cells have demonstrated that loreclezole is highly receptor subunit-selective. Loreclezole has a 300-fold greater potency in the potentiation of GABA-induced current on GABA receptor complexes containing the β_2 or β_3 subunits when compared to those containing the β_1 subunit (Wafford et al., 1994). Furthermore, it was found that a single amino acid, β_2 Asn^{289} or β_3 Asn^{290}, located at the carboxyl-terminal end of the putative channel-lining domain TM2, confers sensitivity to the modulatory effects of loreclezole on $GABA_A$ receptor complex function (Wingrove et al., 1994). Together these reports provide compelling evidence that loreclezole acts at a previously unidentified site with a unique receptor subunit selectivity. The present study confirms and extends, through the use of allosteric modulatory assays, the proposal that loreclezole interacts with the $GABA_A$ receptor complex in a regionally specific manner and acts at a hitherto unknown site. It also determines the presence and type of interaction between loreclezole and the putative neuroactive steroid and Ro5-4864 sites on the $GABA_A$ receptor complex.

2. Materials and methods

2.1. Tissue preparation

The brains from male Sprague-Dawley rats (150–200 g, Simonsen Laboratories, Gilroy, CA, USA) were removed immediately after killing and gently dissected over ice. A P_2 homogenate of the cortex, hippocampus, striatum, thalamus and cerebellum was prepared for radioligand binding assays as previously described (Gee et al., 1986). Briefly, the tissue was homogenized with a teflon pestle in 0.32 M sucrose (10% w/v) followed by centrifugation at 1000 × *g* for 10 min at 0–4°C. The resultant P_2 fraction was washed 3 × in 100 volumes of ice-cold 50 mM Na/K phosphate-buffer (pH 7.4) containing 200 mM NaCl by centrifugation at 9000 × *g* for 10 min. The final pellet was resuspended in the same buffer as a 10% (w/v) homogenate immediately before use.

2.2. [^{35}S]TBPS binding assay

Two nanomolar [^{35}S]TBPS (60–120 Ci/mmol; New England Nuclear, Boston, MA, USA) was incubated with 100-μl aliquots of P_2 homogenate derived from the cortex, cerebellum, hippocampus, striatum or thalamus in the presence and absence of various concentrations of loreclezole, Ro5-4864, 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol. All test drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St Louis, MO, USA) and added to the incubation mixture in 5-μl aliquots. The incubation mixture was brought to a final volume of 1 ml with assay buffer. Nonspecific binding was defined as binding in the presence of 2 μM TBPS (Research Biochemicals, Natick, MA, USA). The binding assays were performed in the presence or absence of 5 μM GABA (the IC_{50} for GABA inhibition of [^{35}S]TBPS binding under the conditions used). The incubation (90 min, 25°C) was terminated by rapid filtration through glass fiber filters (No. 32; Schleicher and Schuell, Keene, NH, USA). The filters were washed 3 times with 3 ml of ice-cold phosphate buffer and the filter-bound radioactivity was quantitated by liquid scintillation spectrophotometer. Protein concentration was determined by the method of Lowry et al. (1951).

2.3. [^{3}H]Flunitrazepam binding assay

To label benzodiazepine sites under conditions similar to those used for the [^{35}S]TBPS binding assays, 0.5 nM [^{3}H]flunitrazepam (83.9 Ci/mmol, New England Nuclear, Boston, MA, USA) was used. Briefly, 5 μl of loreclezole and other test drugs were dissolved in DMSO and added to the incubation mixture. [^{3}H]Flunitrazepam binding was performed in the presence of 1 μM GABA (the EC_{50} concentration for GABA under the conditions used). Nonspecific binding was defined as binding in the presence of 1 μM clonazepam and did not exceed 10% of the total binding. After 60 min of incubation at 25°C, the assay was terminated by rapid filtration. The other experiments using [^{3}H]flunittrazepam and CL218,872 (3-methyl-6-[3-triflunitrazepamoromethyl-phenyl]-1.2.4-triazolo[4,3-*b*]pyridazine), the incubation was at 0–4°C for 90 min. The remaining steps of the assay were similar to those described for the [^{35}S]TBPS binding assay.

2.4. Data analysis

The dose-response data were evaluated by computerized nonlinear regression (InPlot, GraphPAD, San Diego, CA, USA), using a one-component (three-parameter) or two-component (five-parameter) model to generate IC_{50} values (concentration at which half-maximal inhibition of binding occurs) and EC_{50} values (concentration at which half-maximal enhancement of binding occurs) and when indicated, the percentage of sites comprising high and low affinity components. In each case, the *F*-test was applied to compare the two-site model with the one-site model to determine whether the inclusion of additional parameters significantly improved the fit of the data set to the regression curve (Boxenbaum et al., 1972). The data collected from

the receptor binding assays in different brain regions were analyzed by one-way analysis of variance (ANOVA) and Newman-Keuls ($P < 0.05$) when warranted.

3. Results

3.1. Heterotropic cooperativity between loreclezole and neuroactive steroids, Ro5-4864 and CL218,872 on the $GABA_A$ receptor complex

Loreclezole inhibits [^{35}S]TBPS binding to the rat cortex in the presence or absence of 5 μM GABA with IC_{50} values of 1.8 μM and 4.5 μM, respectively (Fig. 1). In the presence of 5 μM GABA, loreclezole inhibits [^{35}S]TBPS binding with apparent full efficacy (i.e., 100% inhibition). A previous study using combinations of $GABA_A$ receptor subunits showed that loreclezole modulated $GABA_A$ receptor complex function at a site distinct from those for the neuroactive steroids (Wafford et al., 1994). In view of the apparent heterogeneity of neuroactive steroid sites (Hawkinson et al., 1994; McCauley et al., 1995), it was of interest to study the interaction between loreclezole and certain $GABA_A$ receptor complex-active neurosteroids that discriminate apparent receptor subtypes. Modulation of [^{35}S]TBPS binding (+5 μM GABA) by 5α-pregnan-3α,20α-diol or 5β-pregnan-3α,20β-diol was measured in the presence and absence of 1 μM loreclezole. As previously reported (McCauley et al., 1995), both 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol produced dose-dependent inhibition of [^{35}S]TBPS binding with limited efficacy (Fig. 2A,B). Loreclezole (1 μM) alone produced 50% inhibition of [^{35}S]TBPS binding. The effect of loreclezole (1 μM) was additive to the actions of 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol on [^{35}S]TBPS binding causing a downward shift of the 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol/[^{35}S]-TBPS dose-response curves and no change in their apparent IC_{50}'s (data not shown) (Fig. 2A,B). These results suggest that loreclezole does not modulate [^{35}S]TBPS binding directly through the neuroactive steroid binding sites discriminated by the two pregnanediols.

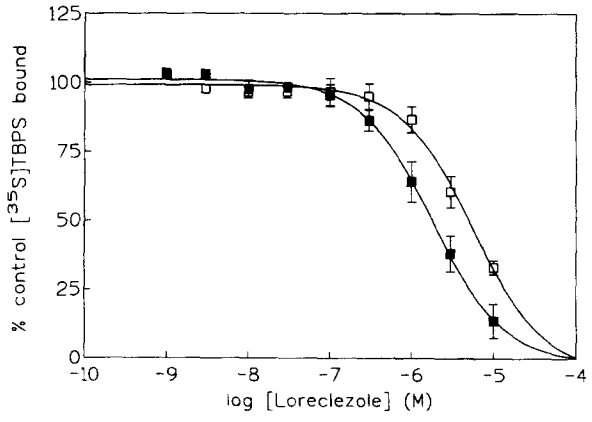

Fig. 1. Concentration-dependent inhibition of 2 nM [^{35}S]TBPS binding by loreclezole in the presence (■) and absence (□) of 5 μM GABA in cortical P_2 homogenates. Each point represents the mean ± S.E.M. of three to five independent experiments.

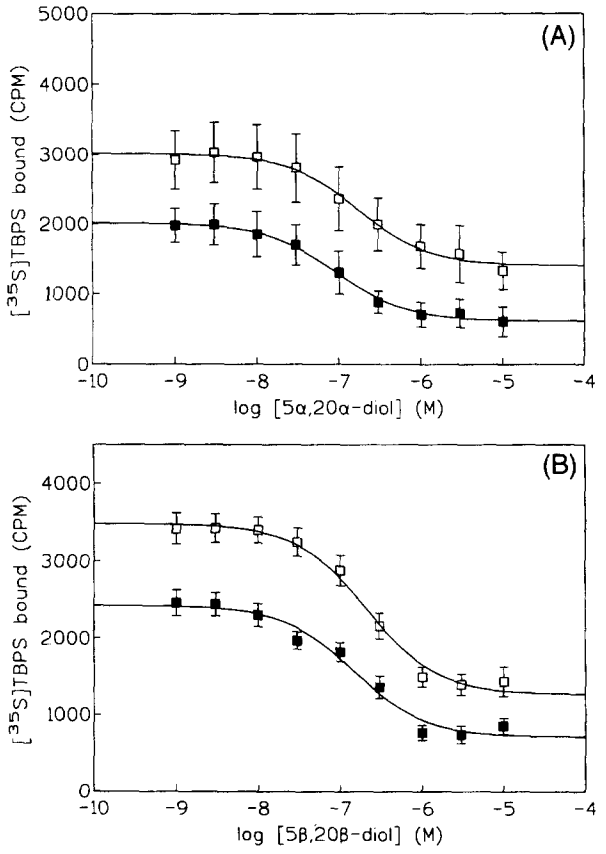

Fig. 2. Effect of a single concentration of loreclezole (1 μM) on 5α-pregnan-3α,20α-diol (A) and 5β-pregnan-3α,20β-diol (B) modulation of 2 nM [^{35}S]TBPS binding (expressed as specific [^{35}S]TBPS bound in counts-per-minute (CPM)) in the presence of 5 μM GABA in rat cortical P_2 homogenates: control (□), +1 μM loreclezole (■). Each point represents the mean ± S.E.M of three or four independent experiments.

The second series of experiments was designed to determine whether loreclezole interacts with the site that mediates Ro5-4864 modulation of [^{35}S]TBPS binding at the $GABA_A$ receptor complex. Ro5-4864 is an atypical benzodiazepine convulsant which modulates [^{35}S]TBPS binding to the $GABA_A$ receptor complex through a novel site and has GABA-negative properties (Gee, 1987). Ro5-4864 potentiates [^{35}S]TBPS binding to the $GABA_A$ receptor complex in the presence of GABA. Similar to previous reports, Ro5-4864 (+5 μM GABA) enhanced [^{35}S]TBPS binding by 40% of control at the maximally effective dose (10 μM) (Gee, 1987; Belelli et al., 1990). Loreclezole (2 μM) significantly potentiated Ro5-4864 enhancement of [^{35}S]TBPS binding from 140% to 200% of control at the maximally effective dose (10 μM) of Ro5-4864 (Fig. 3). Consistent with its GABA-negative properties, Ro5-4864 (1 and 3 μM) increased the IC_{50}'s for loreclezole inhibition of [^{35}S]TBPS binding by shifting the lorecle-

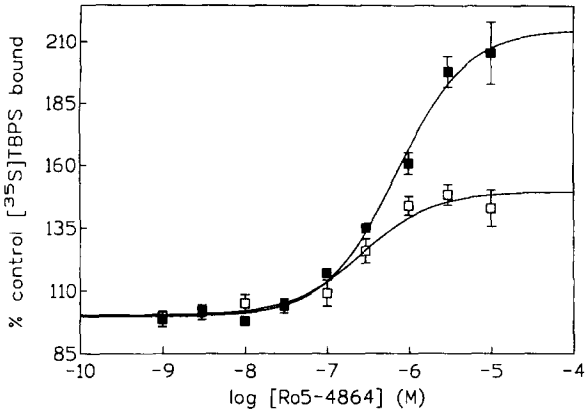

Fig. 3. Concentration-dependent enhancement of 2 nM [^{35}S]TBPS binding by Ro5-4864 in the presence (■) and absence (□) of 2 μM loreclezole in cortical P_2 homogenates. All assays were performed in the presence of 5 μM GABA. Each point represents the mean ± S.E.M. of three to four independent experiments.

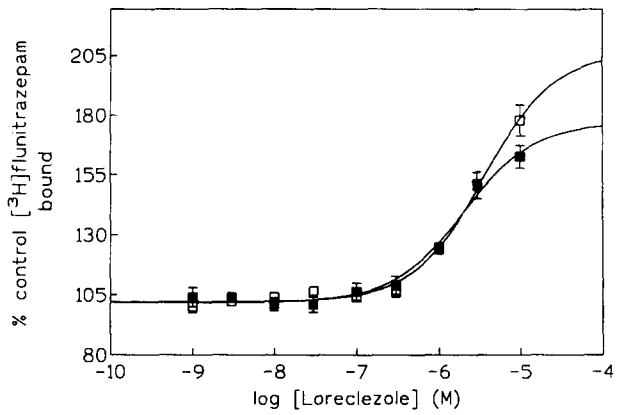

Fig. 5. Concentration-dependent enhancement of 0.5 nM [^{3}H]flunitrazepam binding by loreclezole in the presence (■) and absence (□) of 1 μM GABA in cortical P_2 homogenates. Each point represents the mean ± S.E.M. of at least three experiments.

zole/[^{35}S]TBPS dose-response curve to the right (Fig. 4). Increasing concentrations up to 10 μM of Ro5-4864 produced no additional rightward shifts in the lorcelezole/[^{35}S]TBPS dose-response curve (data not shown).

In addition to the [^{35}S]TBPS binding studies, we examined the effect of loreclezole on the benzodiazepine receptor site labeled by the [^{3}H]flunitrazepam in the rat cortex. Loreclezole caused dose-dependent enhancement of [^{3}H]flunitrazepam binding in a GABA-independent manner. The potency and efficacy of loreclezole enhancement of [^{3}H]flunitrazepam binding was insensitive to GABA (1 μM) (Fig. 5). In order to determine whether loreclezole has any selective actions on the apparent benzodiazepine receptor subtypes discriminated by CL218,872, its effect on the CL218,872/[^{3}H]flunitrazepam dose-response curve was examined. CL218,872 produced dose-dependent inhibition of [^{3}H]flunitrazepam binding (in the presence of 1 μM GABA) consistent with the effects mediated through two sites [$F(2,15) = 16.1$, $P < 0.001$]. In the presence of 2 μM of loreclezole, the CL218,872/[^{3}H]flunitrazepam binding dose-response curve was unchanged. The percentage of high and low affinity components and their respective IC_{50} values were no different from those obtained under control conditions (Table 1).

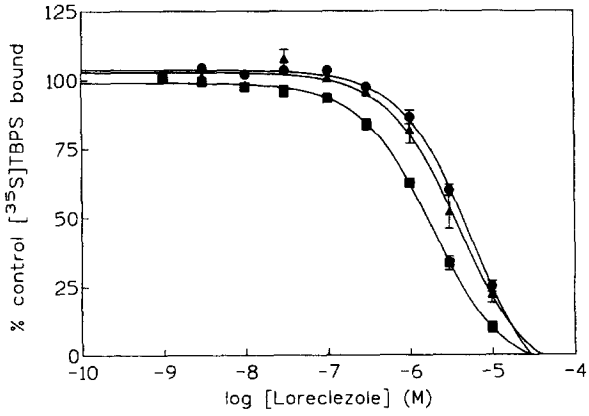

Fig. 4. Effect of 1 μM and 3 μM Ro5-4864 on loreclezole inhibition of [^{35}S]TBPS binding in the presence of 5 μM GABA in rat cortical P_2 homogenates. Loreclezole (■), loreclezole plus 1 μM Ro5-4864 (▲) and loreclezole plus 3 μM Ro5-4864 (●). Each point represents the mean ± S.E.M. of three to four independent experiment. The IC_{50} values are 1.9 μM with loreclezole alone and 4.0 μM and 3.5 μM in the presence of 1 μM and 3 μM Ro5-4864, respectively.

3.2. Modulation of [^{35}S]TBPS binding by loreclezole is regionally dependent

In light of earlier reports that loreclezole shows $GABA_A$ receptor complex receptor subunit selectivity (Wafford et al., 1994) and the differential distribution of $GABA_A$ receptor complex subunits in the brain (Levitan et al., 1988; Wisden et al., 1992), it was of interest to determine the regional dependence of loreclezole modulation of [^{35}S]TBPS binding. Loreclezole inhibited [^{35}S]TBPS binding in all brain regions examined (Table 2). Interestingly, the potency of lorecelezole in the cerebellum and thalamus was significantly greater than the cortex in the presence of GABA. Also, loreclezole modulation of [^{35}S]TBPS binding showed regional differences in GABA-dependent po-

Table 1
Effect of 2 μM loreclezole on benzodiazepine receptor subtypes discriminated by CL218,872 in rat cortical P_2 homogenates

Treatment	High affinity sites		Low affinity sites	
	Proportion (%)	IC_{50} (nM)	Proportion (%)	IC_{50} (μM)
Control	70 ± 2	79 ± 19	30 ± 2	2.3 ± 0.5
2 μM loreclezole	62 ± 11	68 ± 30	38 ± 12	2.6 ± 1.3

All values represent the means ± S.E.M., in three to four independent experiments.

Table 2
Modulation of [^{35}S]TBPS binding by loreclezole in different brain regions

Region	IC_{50} (μM)		
	(−) GABA	(+) GABA	IC_{50} ratio ((−)/(+) 5 μM GABA)
Cortex	4.5 ± 0.4	1.8 ± 0.3	2.5
Striatum	2.9 ± 0.5	1.7 ± 0.3	1.7
Hippocampus	4.3 ± 0.6	1.3 ± 0.2	3.3
Thalamus	4.2 ± 1.0	0.8 ± 0.2 [a]	5.3
Cerebellum	7.1 ± 2.5	0.6 ± 0.3 [a]	11.8

The assays were performed in the presence or absence of 5 μM GABA in P_2 homogenates from different rat brain regions. All values represent the means ± S.E.M. in three to five independent experiments. [a] $P < 0.05$ compared to cortex (+GABA).

tency. The IC_{50} ratios for the inhibition of [^{35}S]TBPS binding in the presence or absence of GABA indicated that the cerebellum and the thalamus were more sensitive to GABA than the cortex and striatum (Table 2).

4. Discussion

The present study measured allosteric modulation of [^{35}S]TBPS and [^{3}H]flunitrazepam binding by loreclezole to further characterize the site through which this drug modulates the $GABA_A$ receptor complex. [^{35}S]TBPS binds to a site on or near the chloride ionophore, and its relative affinity is believed to be related to the conformational state (i.e., open vs. closed) of the chloride channel (Squires et al., 1983; Gee et al., 1988). The present results agree with previous studies, showing that loreclezole allosterically inhibits [^{35}S]TBPS binding in a dose-dependent manner with high nanomolar to micromolar potency in rat brain. In order to further explore the site through which loreclezole modulates [^{35}S]TBPS binding, the interactions between loreclezole and the sites for the prototypical neuroactive steroids and the atypical benzodiazepine Ro5-4864 were examined. Previous investigators have demonstrated that loreclezole does not share the same site recognized by the $GABA_A$ receptor complex-active neurosteriod, 3α-hydroxy-5α-pregnan-20-one (Wafford et al., 1994). However, it was of interest to determine whether neuroactive steroids with apparent receptor subtype selectivity show differential interactions with loreclezole in the modulation of [^{35}S]TBPS binding (Hawkinson et al., 1994; McCauley et al., 1995). As was the case with 3α-hydroxy-5α-pregnan-20-one, the effect of loreclezole (1 μM) was additive to those of 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol in the modulation of [^{35}S]TBPS binding. These results also suggest that loreclezole has no selective actions on the apparent neuroactive receptor subtypes discriminated by the two pregnanediols.

Ro5-4864, an atypical benzodiazepine, has been reported to act on a unique and relatively low-affinity binding site on the $GABA_A$ receptor complex to inhibit GABA-induced responses (Gee, 1987; Gee et al., 1988; Belelli et al., 1990; Puia et al., 1989). Interestingly, loreclezole at 2 μM (the IC_{50} for loreclezole inhibition of [^{35}S]TBPS binding) significantly increased the efficacy of Ro5-4864 enhancement of [^{35}S]TBPS binding. Ro5-4864 also shifted the loreclezole/[^{35}S]TBPS binding dose-response curve to the right. Both experiments showed the GABA-positive and GABA-negative properties of loreclezole and Ro5-4864, respectively. These results argue against a common site of action for Ro5-4864 and loreclezole. Furthermore, loreclezole does not show selective interactions with [^{3}H]flunitrazepam binding to benzodiazepine receptor subtypes discriminated by CL218,872 (Gee and Yamamura, 1983; Klepner et al., 1979).

Another major finding of the present study is the regional differences in the potency of lorecezole as an inhibitor of [^{35}S]TBPS binding. Lorecezole showed greater potency in the cerebellum and thalamus than in the cortex. This effect is consistent with previous reports on recombinant $GABA_A$ receptors containing β_2 and β_3 subunits which show greater sensitivity to loreclezole modulation than receptors containing β_1 subunits. Based on the distribution of $GABA_A$ receptor subunit mRNAs in rat brain, the cerebellum and the thalamus are enriched with β_2 or β_3 subunits with extremely low levels of β_1 subunits. In contrast, the cortex, the striatum and the hippocampus contain β_1, β_2 and β_3 subunits (Vicini, 1991; Wisden et al., 1992). The predicted contribution of the $GABA_A$ receptor complex containing β_1 subunit would be to lower the apparent potency of loreclezole in modulation of [^{35}S]TBPS binding. In the absence of GABA, the rank order of potency for loreclezole modulation of [^{35}S]TBPS binding in the brain region was striatum > thalamus = hippocampus = cortex > cerebellum which was different from that observed in the presence of GABA. This phenomenon may be explained in part by variation in the GABA dependence of each brain region for loreclezole modulation of [^{35}S]TBPS binding. For example, in the cerebellum 5 μM GABA significantly increased the potency of loreclezole by reducing the IC_{50} over 10-fold from 7 μM to 0.6 μM. In contrast to the cerebellum, loreclezole inhibition of [^{35}S]TBPS binding in the striatum was much less dependent on GABA, reducing the IC_{50} by less than 2-fold.

In conclusion, the present results confirm that loreclezole allosterically modulates [^{3}H]flunitrazepam and [^{35}S]TBPS binding to the $GABA_A$ receptor complex. The interaction between loreclezole and the $GABA_A$ receptor complex does not occur through the Ro5-4864 site which provides further support for a novel site of action on this receptor complex. This study has by no means eliminated all of the possible sites where loreclezole may act. For example, the sites recognized by other allosteric modulators such as avermectin, γ-butyrolactores, etazolate and etomidate have not been evaluated for interactions with

loreclezole at the $GABA_A$ receptor complex. The regional differences in sensitivity to loreclezole inhibition of [^{35}S]TBPS binding is not a result of differential interaction with benzodiazepine or neuroactive steroid receptor subtypes. Instead, this apparent regional selectivity may be explained in part by loreclezole selectivity for $GABA_A$ receptor complexes with certain subunit compositions.

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