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Differential Cross Talk of ROD Compounds with the Benzodiazepine Binding Site

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ABSTRACT

We have recently identified a novel class of allosteric modulators of $GABA_A$ receptors, the ROD compounds that are structurally related to bicuculline. Here, the relationship of their site of action relative to other known modulatory sites of this receptor was investigated. Two types of ROD compounds, R1 (ROD164A, ROD185) and R2 (ROD222 and ROD259) could be differentiated. R1 compounds competitively inhibited binding of benzodiazepines in $\alpha1\beta2\gamma2$ receptors, and their functional effects were partially inhibited by the benzodiazepine antagonist Ro15-1788 in a noncompetitive manner. The enhancement by an R1 compound was not additive with that by diazepam. R2 compounds in contrast failed to inhibit binding of benzodiazepines; the R2 compounds' functional effects were not inhibited by the benzodiazepine antagonist. The enhancement by an R2 compound was additive with that by diazepam. In contrast to benzodiazepines, both R1 and R2 type compounds were still able to enhance $\alpha1\beta2$ receptors. ROD164A in $\alpha1\beta2\gamma2$ receptors was found to be partially antagonized by Ro15-1788 in a noncompetitive way. ROD178B did not affect γ-aminobutyric acid induced currents, but was able to inhibit both enhancement by R1 and R2 type compounds as well as enhancement by diazepam. R1 and R2 type compounds as well as diazepam enhanced pentobarbital-induced currents in a Ro15-1788-sensitive way. We conclude that R1 type compounds act at the benzodiazepine binding site and additionally at a different R1 site, and that the R1, but not the R2 site is allosterically coupled to the benzodiazepine binding site. ROD178B is a competitive antagonist at the R1 site in that it shows allosteric interaction with the benzodiazepine binding site and displacement of benzodiazepines, and a negative allosteric modulator at the R2 site.

Several types of drug are known to exert their effect via the inhibitory neurotransmitter $GABA_A$ receptor (Sieghart, 1995). Among them are the benzodiazepines, whose site of action has recently been reviewed (Sigel and Buhr, 1997). Enhancement of the action of the $GABA_A$ receptor results in sedation/hypnosis, muscle relaxation, anxiolysis, and prevention of convulsions, whereas inhibition of the $GABA_A$ receptor results in opposite effects. $GABA_A$ receptors and their molecular biology and relatedness to other ligand-activated ion channels has been extensively reviewed (Burt and Kamatchi, 1991; Dunn et al., 1994; Macdonald and Olsen, 1994; Rabow et al., 1995; Barnard et al., 1998).

Recently, a novel stimulatory ligand of the $GABA_A$ receptor, (+)-ROD188, with a novel site of action has been described (Thomet et al., 2000). (+)-ROD188 has been synthesized as described by Razet et al. (2000a,b) along with about 60 structurally similar substances in the search for novel ligands of the GABA binding site. None of the compounds acted at the GABA binding site, but many of them were positive allosteric modulators of the $GABA_A$ receptor. Because we could not investigate all 60 compounds, we selected two families of similar structure (Fig. 1). The first family of compounds, here termed type R1 (ROD164A, ROD185), seemed to interact with the benzodiazepine binding site, as indicated by [^{3}H]flunitrazepam displacement in binding studies and inhibition by a benzodiazepine antagonist. The other family of compounds, here termed type R2 (ROD222 and ROD259), did not seem to interact with the benzodiazepine binding site. In this study, we investigated the relationship of their binding sites with the benzodiazepine binding site.

Materials and Methods

Construction of Receptor Subunits. The cDNAs encoding the $\alpha1$, $\beta2$, and $\gamma2S$ subunits of the rat $GABA_A$ receptor channel have been described elsewhere (Lolait et al., 1989; Malherbe et al., 1990a,b). For cell transfection, the cDNAs were subcloned into the polylinker of pBC/CMV. This expression vector allows high-level expression of a foreign gene under control of the cytomegalovirus

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEK, human embryonic kidney; TBPS, *t*-butylbicyclophosphorothionate.

promoter and an SP6 promotor for in vitro transcription. The α subunit was cloned into the *Eco*RI and the β and γ subunits were subcloned into the *Sma*I site of the polylinker by standard techniques.

Expression and Functional Characterization. *Xenopus laevis* oocytes were prepared, injected, defolliculated, and currents recorded as described previously (Sigel, 1987; Sigel et al., 1990). Briefly, oocytes were injected with 50 nl of cRNA dissolved in 5 mM K-HEPES, pH 6.8. This solution contained the transcripts coding for the different subunits at a concentration of 10 nM for α1, 10 nM for β2, and 50 nM for γ2 for the triple subunit combination, and of 75 nM for each α1 and β2 for the dual subunit combination. RNA transcripts were synthesized from linearized plasmids encoding the desired protein using the message machine kit (Ambion, Austin, TX) according to the recommendation of the manufacturers. A poly(A) tail of about 300 residues was added to the transcripts by using yeast poly(A) polymerase [U.S. Biochemical Corp. (Cleveland, OH) or Amersham Pharmacia Biotech (Piscataway, NJ)]. The cRNA combinations were coprecipitated in ethanol and stored at −20°C. Transcripts were quantified on agarose gels after staining with Radiant Red RNA Stain (Bio-Rad, Richmond, CA) by comparing staining intensities with various amounts of molecular mass markers (RNA-Ladder; Life Technologies, Grand Island, NY). Electrophysiological experiments were performed by the two-electrode voltage-clamp method at a holding potential of −80 mV. The medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Na-HEPES, pH 7.4. GABA was applied for 20 s without or in combination with other drugs and a washout period of 4 min was allowed to ensure full recovery from desensitization. The perfusion solution (6 ml/min) was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte. The rate of solution change under our conditions has been estimated 70% within less than 0.5 s (Sigel et al., 1990). A GABA concentration that elicited 1 to 5% (50–500 nA) of the maximal current amplitude was used. Currents were also elicited with 200 μM pentobarbital in the absence of GABA. They had an amplitude in the range of 30 to 180 nA. The perfusion system was cleaned between drug applications by washing with dimethyl sulfoxide to avoid contamination. Data are given as mean ± S.E.M. (number of experiments).

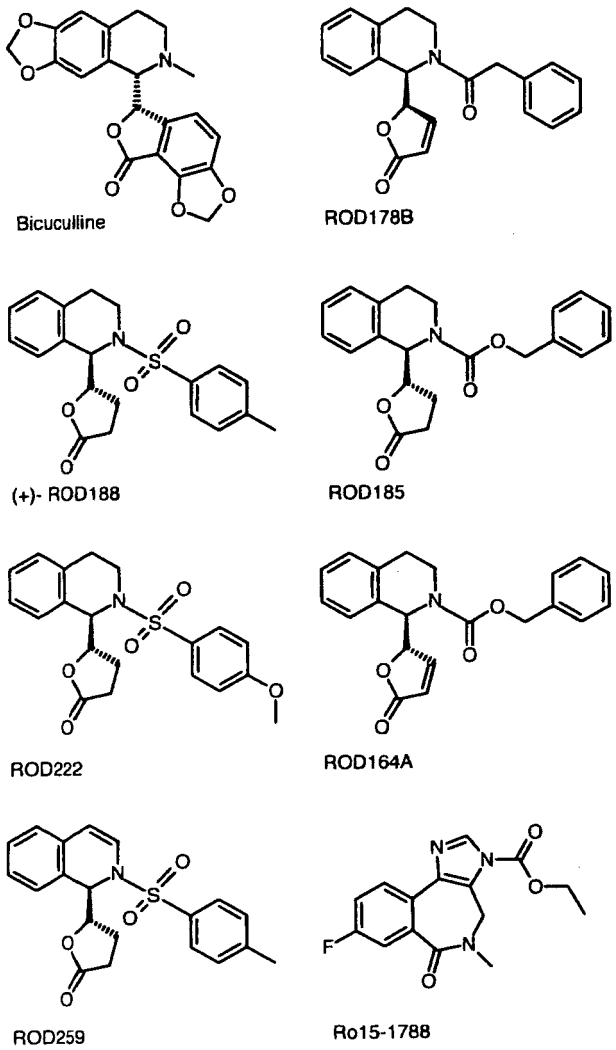

Fig. 1. Chemical structures of the compounds used.

Binding Assays. For binding studies HEK-293 cells (CRL 1573; American Type of Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum (JHR Biosciences, Lenexa, KS), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 units/ml penicillin G, and 100 μg/ml streptomycin in 75-cm^2 Petri dishes by using standard cell culture techniques.

HEK-293 cells (3×10^6) were transfected with a total of 21 μg of cDNA encoding for the rat α1, β2, and γ2 subunits (ratio 1:1:1) subcloned individually into pCDM8 expression vectors, using the calcium phosphate precipitation method (Chen and Okayama, 1988). The medium was changed 20 h after transfection and the HEK-293 cells were harvested 48 h after transfection by scraping into phosphate-buffered saline. Cells were centrifuged at 12,000*g* for 10 min and the cell pellet was homogenized in 50 mM Tris-citrate buffer, pH 7.4, by using an Ultraturrax, followed by three centrifugation (200,000*g* for 20 min) resuspension cycles, and were then used for ligand binding studies or were stored at −20°C.

For binding assays, membranes from rat forebrain or membranes from transiently transfected HEK-293 cells were centrifuged and resuspended in 50 mM Tris-citrate buffer, pH 7.4, at a protein concentration of about 1 mg/ml as measured by the bicinchoninic acid-protein assay kit of Pierce (Rockford, IL) with bovine serum albumin as standard. Membranes (0.5 ml) were then incubated in a total of 1 ml of a solution containing 50 mM Tris-citrate buffer, pH 7.4, 150 mM NaCl, and various concentrations of [^{3}H]flunitrazepam, 5 nM [^{3}H]muscimol, or 2 nM [^{35}S]TBPS in the absence or presence of 10 μM diazepam, 10 μM GABA, or 10 μM TBPS or various concentrations of the ROD compounds for 90 min at 4°C (Zezula et al., 1996). Membranes were then filtered through Whatman GF/B filters. The filters were rinsed twice with 5 ml of ice-cold 50 mM Tris-citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after addition of 3.5 ml of Hydrofluor (National Diagnostics, Manville, NJ) scintillation fluid. Nonspecific binding determined in the presence of 10 μM unlabeled compounds was subtracted from total [^{3}H]flunitrazepam, [^{3}H]muscimol, or [^{35}S]TBPS binding, respectively, to result in specific binding.

Results

Two Families of ROD Compounds, R1 and R2, Allosterically Stimulate $GABA_A$ Receptors. In the search for novel ligands of the GABA binding site of $GABA_A$ receptors, we investigated numerous compounds with a structure similar to the competitive antagonist of GABA, bicuculline. The structure of some selected compounds is shown in Fig. 1. None of these compounds was able to interact with the GABA binding site as indicated by the lack of displacement of [^{3}H]muscimol from rat brain membranes (data not shown). However, some, but not all compounds were able to displace [^{3}H]flunitrazepam. Although ROD164A, ROD185, and ROD178B were active in the low micromolar range, (+)-

TABLE 1

Binding properties of ROD compounds

Data (means ± S.E.M. from at least three experiments performed in triplicates) are from rat forebrain membranes. The concentration of [^{3}H]flunitrazepam or [^{35}S]TBPS is 2 nM.

	[^{3}H]Flunitrazepam (IC_{50})	[^{35}S]TBPS (IC_{50})
	μM	
ROD178B	3.6 ± 0.5	>100
ROD164A	0.42 ± 0.08	36.6 ± 1.8
ROD185	0.16 ± 0.01	35.4 ± 0.5
(+)-ROD188	33.1 ± 3.5[a]	N.D.
ROD259	No effect	>100
ROD222	No effect	85.6 ± 4.9

N.D., not determined.
[a] Determined with 2 nM [^{3}H]Ro15-1788.

ROD188 had a reduced activity and ROD222 and ROD259 none at all (Table 1). Scatchard analysis of [^{3}H]flunitrazepam binding to recombinant $\alpha1\beta2\gamma2$ receptors in the absence or presence of ROD185 or (+)-ROD188 indicated a competitive interaction of these compounds with the [^{3}H]flunitrazepam binding site (Fig. 2). Based on the ability to displace [^{3}H]flunitrazepam we named ROD164A and ROD185 R1 compounds and ROD222 and ROD259 R2 compounds.

ROD164A and ROD185 were able to displace [^{35}S]TBPS binding at micromolar concentrations, indicating an allosteric effect on $GABA_A$ receptors at concentrations higher than that interacting with the benzodiazepine binding site. ROD178B, ROD222, and ROD259 exhibited only a weak displacing activity at the [^{35}S]TBPS binding site (Table 1).

To investigate functional effects of these compounds, recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors were expressed in *X. laevis* oocytes. None of the investigated compounds at a concentration of 100 μM opened the channel by itself, but all compounds except ROD178B allosterically enhanced $GABA_A$ receptors in a concentration-dependent way (Fig. 3). With ROD164A and ROD185 significant enhancement was seen at 0.1 μM, and for (+)-ROD188, ROD222, and ROD259 at 1 μM.

We next investigated the effect of the benzodiazepine antagonist Ro15-1788 on this current enhancement. Although the enhancement by ROD164A (Fig. 4), ROD185, and (+)-ROD188 was partially sensitive to 1 μM Ro15-1788, the enhancement by ROD222 and ROD259 was largely resistant to the benzodiazepine antagonist (Fig. 5; Table 2). Quantitative comparison of the degree of inhibition cannot be made because different concentrations of ROD compounds were used. The standard concentration was 20 μM. Despite the higher concentration of ROD164A, inhibition by Ro15-1788 was larger than for (+)-ROD188, ROD222, and ROD259. ROD185 with its high affinity to the benzodiazepine binding site was used at a lower concentration to allow competition with Ro15-1788 to occur.

Diazepam enhances currents elicited by GABA via the benzodiazepine binding site. We were interested whether this enhancement was additive to the enhancement by ROD compounds. Although the current enhancement by ROD185 was not additive with the one by diazepam, enhancement by ROD222 was additive (Table 3). Interestingly, (+)-ROD188 potentiated the effect of diazepam. Whether this effect was caused by its interaction with the ROD222 site or with an additional site cannot be decided by the data available.

R1 Type Compounds Act at an Additional Site Beside the Benzodiazepine Binding Site. We next tested the properties of the different ROD compounds on $\alpha1\beta2$ $GABA_A$ receptors. Ligands of the benzodiazepine binding site are known to depend on the presence of a γ subunit (Pritchett et al., 1989; Sigel et al., 1990; Günther et al., 1995) for their interaction with $GABA_A$ receptors. Unexpectedly, not only R2 type compounds but also R1 type compounds strongly enhanced $\alpha1\beta2$ $GABA_A$ receptor-mediated currents (Fig. 6). We therefore analyzed the inhibition by the benzodiazepine antagonist Ro15-1788 of the stimulatory effect of ROD164A on GABA-induced chloride current in $\alpha1\beta2\gamma2$ receptors in more detail. To determine whether we are dealing here with competitive or noncompetitive inhibition, it would be desirable to construct a Schild plot. However, due to solubility

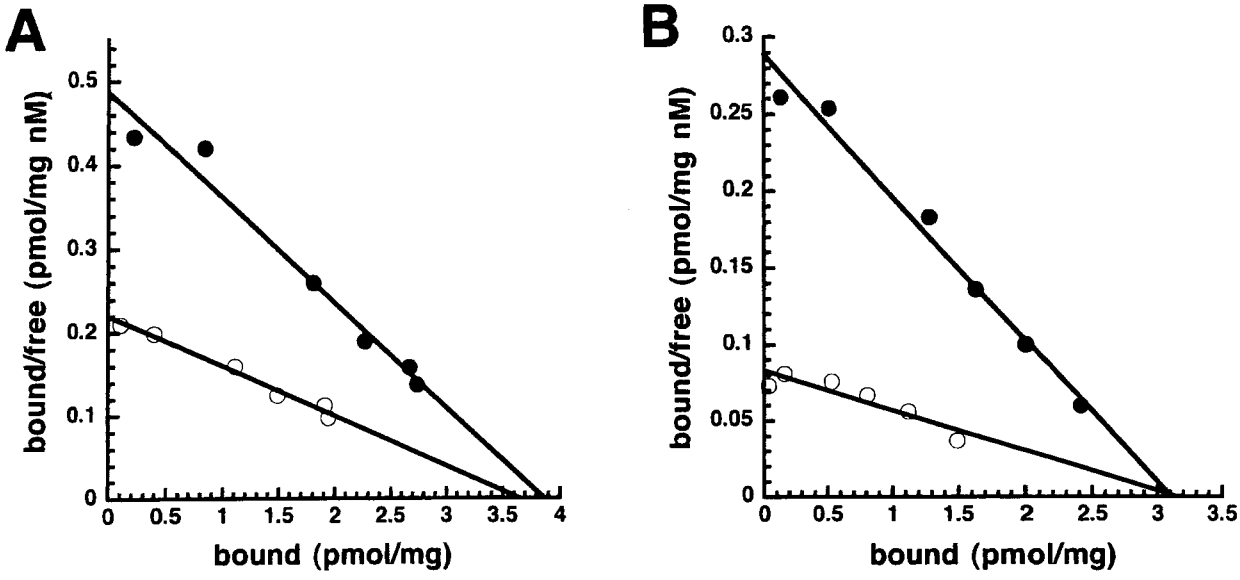

Fig. 2. Scatchard analysis of [^{3}H]flunitrazepam binding in the absence and presence of ROD185 (A) or (+)-ROD188 (B). Membranes from HEK-293 cells transfected with $\alpha1\beta2\gamma2$ $GABA_A$ receptor were incubated with various concentrations of [^{3}H]flunitrazepam in the absence (●) and presence (○) of 100 nM ROD185 (A) or 300 μM (+)-ROD188 (B). Results are from one experiment in triplicates. The experiment was repeated twice with similar results. B_{max} values were not significantly different in the absence or presence of ROD185 or (+)-ROD188.

problems, we were limited in the drug concentration range and were unable to do such an analysis. Therefore, we studied the concentration-dependent inhibition by Ro15-1788 of the GABA-induced current enhanced by several different concentrations of ROD164A. For a competitive inhibition we would expect here Hill slopes of −1 and a parallel shift of the inhibition curves to the right with increasing concentrations of ROD164A. The results shown in Fig. 7 are not compatible with a competitive interaction of ROD164A with Ro15-1788 at a common binding site. The nature of the curves, especially at higher concentrations of ROD164A is difficult to understand. Ro15-1788 completely inhibits the enhancement

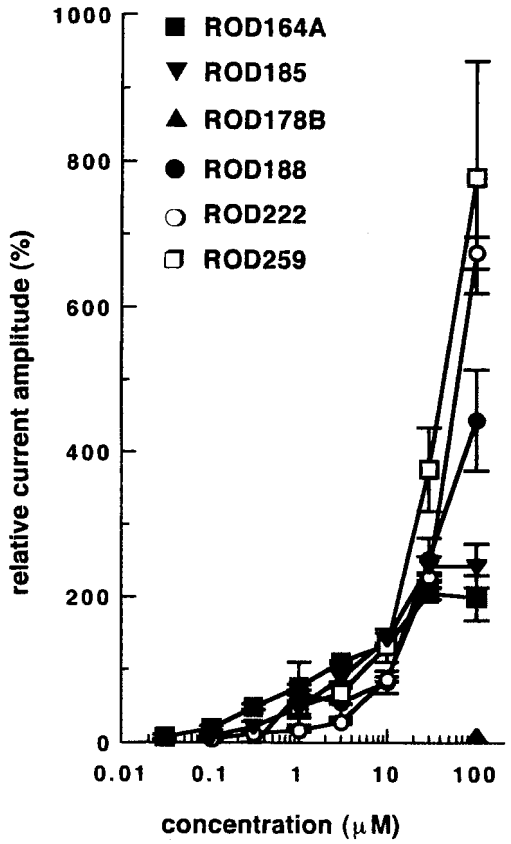

Fig. 3. Allosteric enhancement of currents elicited by GABA in $\alpha1\beta2\gamma2$ $GABA_A$ receptors by ROD164A, ROD185, ROD178B, (+)-ROD188, ROD222, and ROD259. Recombinant rat $GABA_A$ receptors were expressed in *X. laevis* oocytes. Application of 2 μM GABA alone resulted in approximately 5% of the maximal current amplitude. Increasing concentrations of the compounds were coapplied with GABA. The figure shows cumulative concentration response curves. Values are shown as mean ± S.E.M. of three to four oocytes.

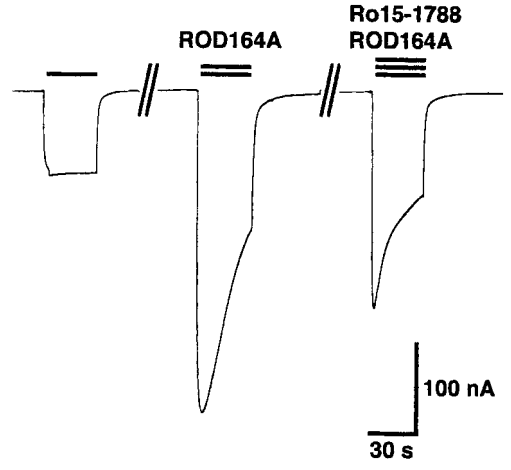

Fig. 4. Enhancement by ROD164A is inhibited by a benzodiazepine antagonist. The concentration of GABA eliciting approximately 5% of the maximal current amplitude at recombinant rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors was determined first. ROD164A (100 μM) markedly enhanced these currents. When 1 μM Ro 15-1788 was coapplied with 100 μM ROD164A, the enhancement was inhibited to a large extent.

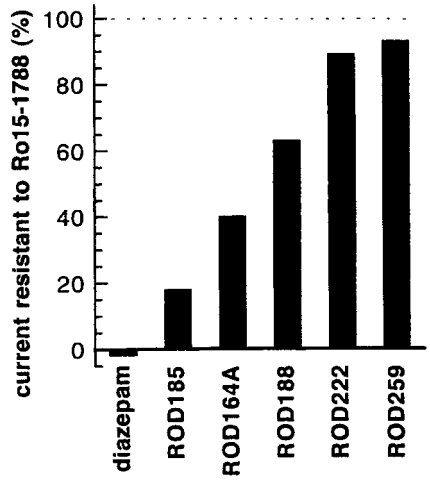

Fig. 5. Inhibition by 1 μM Ro15-1788 of the enhancement by ROD compounds. The current elicited by GABA was enhanced by either ROD164A or ROD185, (+)-ROD188, ROD222, and ROD259 at the concentrations indicated in Table 2. Enhancement was standardized to 100%. Subsequently, the same concentration of the ROD compound was coapplied with 1 μM Ro15-1788 and the residual relative enhancement calculated.

TABLE 2

Inhibition by Ro15-1788

Currents activated by a GABA concentration that elicited 1 to 5% of the maximal current amplitude were standardized to 100%. The relative current amplitude in the presence of drug after subtraction of the current induced by GABA alone (100%) is given. All experiments were carried out with recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in *X. laevis* oocytes. Peak values were taken except for the numbers in italics, where values were taken at the end of the 20-s drug application period, reflecting a time dependence of drug action.

Substance	Enhancement	Enhancement in Presence of 1 μM Ro15-1788
	%	
		6 ± 3 (n = 3)
0.3 μM Diazepam	162 ± 54 (n = 3)	33 ± 10 (n = 3)
	151 ± 56 (n = 3)	*3 ± 3 (n = 3)*
10 μM ROD185	94 ± 12 (n = 9)	23 ± 12 (n = 6)
100 μM ROD164A	214 ± 30 (n = 9)	98 ± 32 (n = 6)
20 μM (+)-ROD188	207 ± 42 (n = 5)	136 ± 20 (n = 5)
20 μM ROD222	128 ± 8 (n = 4)	120 ± 5 (n = 4)
20 μM ROD259	270 ± 14 (n = 4)	256 ± 8 (n = 4)

TABLE 3

Coenhancement by diazepam and ROD compounds

Currents activated by a GABA concentration that elicited 1 to 5% of the maximal current amplitude were standardized to 100%. The relative current amplitude in the presence of drug after subtraction of the current induced by GABA alone (100%) is given. All experiments were carried out with recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in *X. laevis* oocytes.

Substance	Enhancement	Enhancement in Presence of 0.3 μM Diazepam
		206 ± 9% (n = 9)
10 μM ROD185	94 ± 12% (n = 9)	176 ± 10% (n = 3)
20 μM (+)-ROD188	380 ± 36% (n = 4)	903 ± 96% (n = 30
10 μM ROD222	128 ± 8% (n = 4)	343 ± 14% (n = 3)

by classical benzodiazepines at concentrations less than 0.1 μM (Sigel and Baur, 1988). Judging by the extent of inhibition at these concentrations of Ro15-1788, stimulatory effects of ROD164A via the benzodiazepine binding site amount to less than 20% at all concentrations of ROD164A tested. The inhibition of the current enhancement by ROD164A at higher concentrations of Ro15-1788 thus could have been caused by an allosteric effect of Ro15-1788 on an additional site for ROD164A.

Interaction between Different ROD Compounds. ROD178B only marginally enhanced GABA-induced currents, displaced [^{3}H]flunitrazepam at micromolar concentrations, and exhibited only a weak effect on [^{35}S]TBPS binding.

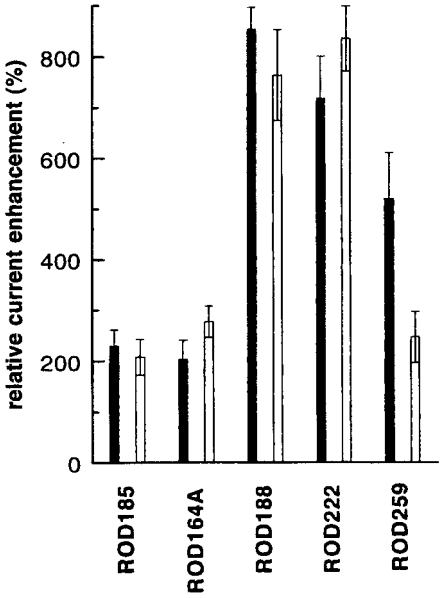

Fig. 6. Enhancement by ROD164A, ROD185, (+)-ROD188, ROD222, and ROD259 in $\alpha1\beta2\gamma2$ (□) and in $\alpha1\beta2$ (■) recombinant $GABA_A$ receptors. The concentration of GABA eliciting approximately 5% of the maximal current amplitude at the corresponding $GABA_A$ receptor was determined first. This concentration was then coapplied with 100 μM the substance to be tested. For all the tested substances enhancement was comparable in $\alpha1\beta2\gamma2$ and $\alpha1\beta2$ receptors.

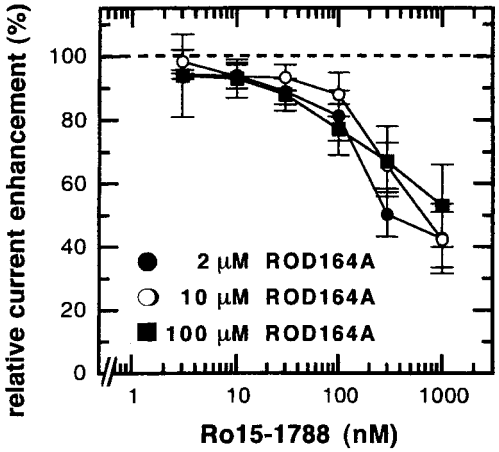

Fig. 7. Concentration-dependent inhibition in $\alpha1\beta2\gamma2$ receptors by Ro15-1788 of the enhancement by different concentrations of ROD164A. Currents elicited by GABA were enhanced by either 2, 10, or 100 μM ROD164A in separate experiments and inhibited by increasing concentrations of Ro15-1788. Each of the three cumulative inhibition experiments was carried out with three oocytes, thus each point represents mean ± S.E.M. (n = 3).

We hypothesized that ROD178B might be an antagonist of stimulatory ROD compounds. Figure 8 shows that the enhancement by ROD164A is indeed strongly inhibited by ROD178B. Table 4 summarizes results on the inhibition by ROD178B of the enhancement by different ROD compounds. R2 type compounds are affected to a smaller extent compared with ROD164A, whereas ROD185 is almost unaffected during the early phase of the exposure to GABA. For ROD185 relative inhibition increased during time of drug application and was much larger 20 s after beginning of the application (Table 4). The reason for this property that was not observed for type R2 compounds is not clear. Presumably, the open conformation of the channel accelerates the action of ROD178B. ROD178B surprisingly also inhibited the enhancement by diazepam very strongly. From their respective concentrations and from their respective affinity to the benzodiazepine binding site, an inhibition of less than 50% would have been expected. This inhibition must therefore be partially due to an allosteric effect of ROD178B on the benzodiazepine binding site or on channel gating.

We further investigated the mode of inhibition by ROD178B of the current enhancement by ROD164A and ROD259. This investigation is complicated by the fact that neither the affinity of ROD164A and ROD259 nor the affinity of ROD178B for the receptor is known. Figure 9A shows the concentration-dependent enhancement by ROD164A in the absence of ROD178B and in its presence at different concentrations. The data are compatible with a competitive action of ROD164A and ROD178B. Figure 9B shows the concentration-dependent enhancement by ROD259 in the absence of ROD178B and in its presence at different concentrations. These data are not compatible with a competitive interaction of ROD178B and ROD259. When a fixed concentration of ROD259 was applied repetitively to the same oocyte in the presence of increasing concentrations of ROD178B, the observed inhibition was very small compared with the values given in Table 4 (data not shown). The same phenomenon was observed with ROD164A although to a smaller degree. The reason for this discrepancy is not clear, but obviously repetitive application to an oocyte is not possible in this case.

We also investigated the relationship between type R1

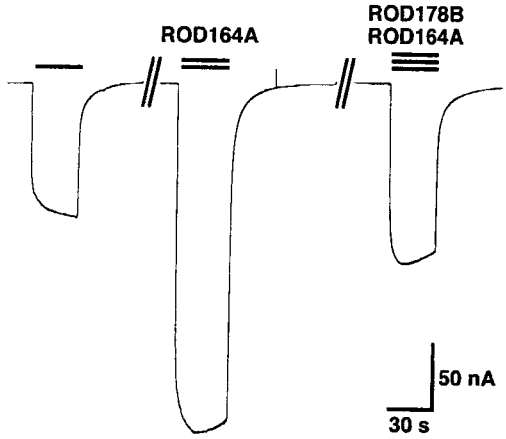

Fig. 8. Enhancement by ROD164A is inhibited by ROD178B. The concentration of GABA eliciting approximately 5% of the maximal current amplitude at recombinant rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors was determined first. ROD164A (10 μM) markedly enhanced these currents. When 100 μM ROD178B was coapplied with 10 μM ROD164A, the enhancement was inhibited to a large extent.

compounds and type R2 compounds. Currents induced by GABA were enhanced with ROD185, (+)-ROD188, or ROD222 alone and subsequently in combination with ROD185 in the latter two cases. Concentrations of the compounds were chosen for a submaximal enhancement. Current enhancement by ROD185 was clearly not additive with that by (+)-ROD188 or ROD222, indicating a common site of action or a common way of influencing channel gating (Table 5). The data on the inhibition by ROD178B of current enhancement by ROD164A and ROD259 argue for the second possibility.

Relation to the Pentobarbital Binding Site. Previously, it has been shown that (+)-ROD188 is able to enhance pentobarbital-induced currents (Thomet et al., 2000). Here, we demonstrate that currents elicited by pentobarbital in the absence of GABA were also enhanced by both ROD185 and ROD259 (Table 6). The enhancement by ROD185, but not that by ROD259, could be significantly inhibited by 1 μM Ro15-1788, a benzodiazepine antagonist. Similarly, diazepam enhanced the currents elicited by pentobarbital in a benzodiazepine antagonist-sensitive way (Table 6). The concentration used for the benzodiazepine antagonist is more than 1000-fold in excess of the concentration used for the half-maximal occupancy of the benzodiazepine binding site. Interestingly, 1 μM Ro15-1788 by itself inhibited pentobarbital-induced currents. This indicates that Ro15-1788 is not simply a benzodiazepine antagonist, but at higher concentrations can allosterically modulate currents elicited by pentobarbital. This is an important observation that is reminiscent to the work by Ueno et al. (1997), who observed an inhibition of these currents by the competitive GABA antagonist bicuculline. It may be concluded from our observation that barbiturate action is not only allosterically coupled to the GABA site but also to the benzodiazepine binding site.

TABLE 4

Inhibition by ROD178B

Currents activated by a GABA concentration that elicited 1 to 5% of the maximal current amplitude were standardized to 100%. The relative current amplitude in the presence of drug after subtraction of the current induced by GABA alone (100%) is given. All experiments were carried out with recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in *X. laevis* oocytes. Peak values were taken except for the numbers in italic, where values were taken at the end of the 20-s drug application period, reflecting a time dependence of drug action.

Substance	Enhancement	Enhancement in Presence of 100 μM ROD178B
	%	
		25 ± 4 (n = 4)
0.3 μM Diazepam	162 ± 14 (n = 4)	33 ± 8 (n = 3)
10 μM ROD185	59 ± 9 (n = 3)	53 ± 6 (n = 3)
	50 ± 8 (n = 3)	*−2 ± 6 (n = 3)*
10 μM ROD164A	174 ± 5 (n = 3)	42 ± 7 (n = 3)
20 μM (+)-ROD188	380 ± 36 (n = 4)	187 ± 18 (n = 4)
20 μM ROD222	123 ± 5 (n = 3)	96 ± 6 (n = 3)
10 μM ROD259	119 ± 8 (n = 5)	47 ± 6 (n = 5)

Discussion

All ROD compounds described here except ROD178B allosterically enhance currents activated by GABA in recombinant $GABA_A$ receptors. It was our aim to analyze the sites of action of these compounds relative to the benzodiazepine binding site. The action of (+)-ROD188 has previously been investigated in more detail (Thomet et al., 2000). It seems to interact with the $GABA_A$ receptor at a site independent of the site for benzodiazepines or loreclezole and of the agonistic site for pentobarbital or neurosteroids.

Novel Enhancers May Be Grouped in R1 and R2 Compounds. Based on their ability to competitively displace [^{3}H]flunitrazepam in binding studies, ROD164A and ROD185 were grouped in the R1 class compounds and compounds ROD222 and ROD259 that are not able to displace [^{3}H]flunitrazepam in binding studies were combined in the R2 class. (+)-ROD188 represents an intermediate. Enhancement of currents elicited by GABA by R1, but not R2 class compounds, was antagonized by the benzodiazepine antagonist Ro15-1788. The enhancement by R2, but not that by R1 compounds was additive to that by the benzodiazepine diaz-

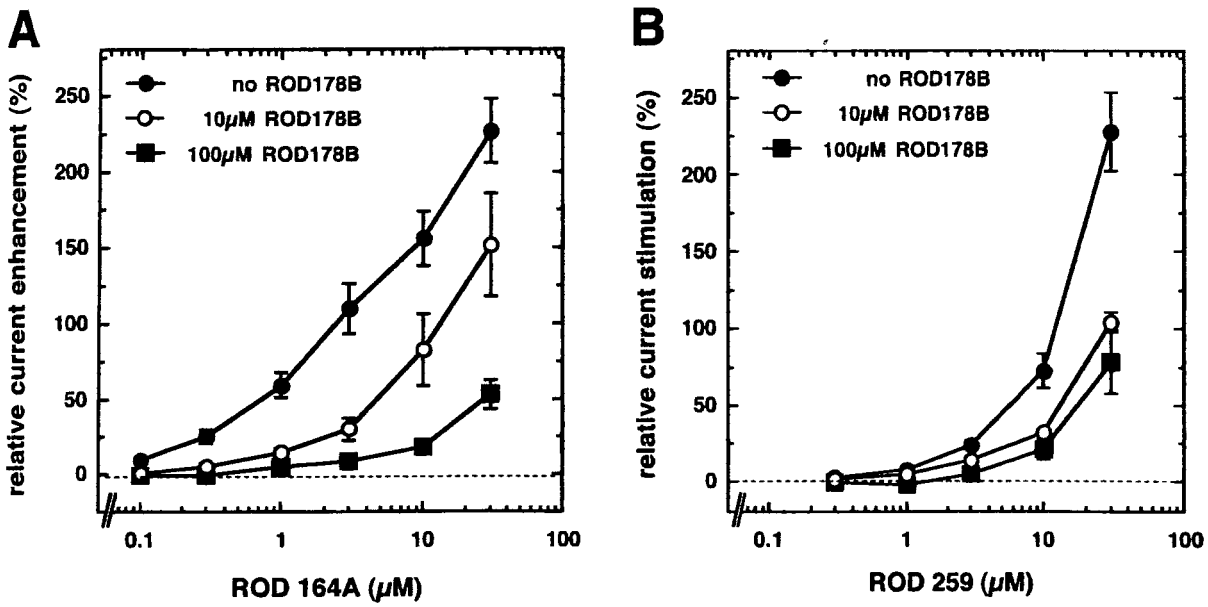

Fig. 9. Mode of inhibition by ROD178B of the current enhancement by ROD164A and ROD259. A, concentration-dependent stimulation by ROD164A in the absence of ROD178B (●) and in its presence at 10 μM (○) and 100 μM (■). B, concentration-dependent enhancement by ROD259 in the absence of ROD178B (●) and in its presence at 10 μM (○) and 100 μM (■).

epam. Thus, based on the observations, it was tempting to postulate that R1, but not R2 compounds are novel ligands of the benzodiazepine binding site.

R1 Compounds Act Both at the Benzodiazepine and at the R1 Site. The following observations prompted us to postulate an additional site of action for R1 compounds. First, R1 compounds are able to modulate [^{35}S]TBPS binding and to strongly activate GABA-induced currents at much higher concentrations than those necessary for the interaction with the benzodiazepine binding site as predicted from binding studies. Second, R1 (as well as R2) compounds, in contrast to benzodiazepines (Pritchett et al., 1989; Sigel et al., 1990; Günther et al., 1995), enhance currents elicited by GABA in recombinant $\alpha1\beta2$ receptors. Third, results from an analysis of the inhibition of various concentrations of the R1 type compound ROD164A by the benzodiazepine antagonist were not compatible with a competitive interaction. We concluded that R1 type compounds not only act at the benzodiazepine binding site but also at another site that we call R1. ROD178B must be classified as an R1 compound because it is structurally closely related to ROD185 and also has [^{3}H]flunitrazepam displacing activity.

R2 Compounds Use a Separate Site from R1 Compounds. Indicated by the lack of [^{3}H]flunitrazepam displacing activity in binding studies, the lack of antagonism by the benzodiazepine antagonist Ro15-1788 in functional enhancement experiments, by the additivity of enhancement with diazepam, as well as by the finding that R2 compounds are able to modulate $\alpha1\beta2$ receptors, we conclude that R2 type compounds act at a site different from benzodiazepines and have a different effector mechanism.

What is the relationship between the binding sites for R1 and R2 type compounds? ROD178B inhibited the current enhancement by both compounds. Current enhancement by the two types of compound was not additive. Analysis of the inhibition by ROD178B of current enhancement by ROD164A and ROD259 argues for independent sites, because inhibition at the R1 site seemed to be of a competitive nature. In contrast, inhibition at the R2 site seemed to be due to a negative allosteric modulation.

Allosteric Effects of a Benzodiazepine Antagonist on Pentobarbital-Induced Currents. An important observation was made during the study of interaction of the R1 and R2 type compounds with pentobarbital. Namely, it was discovered that currents elicited by pentobarbital in the absence of GABA were inhibited by the benzodiazepine antagonist Ro15-1788. Furthermore, diazepam was able to enhance these currents. This indicates that the actions of barbiturates and those by benzodiazepines are in allosteric interaction. The possibility that Ro15-1788 may induce a conformation different from the resting state of the receptor has been discussed by Williams and Akabas (2000) on the basis of an altered accessibility of a cysteine reagent to the transmembrane region M3 in the presence of this agent. Previously, it has also been shown that competitive GABA antagonists are able to inhibit pentobarbital-induced currents (Ueno et al., 1997). Taken together, the actions of R1 compounds, benzodiazepines, and barbiturates are in allosteric interaction with each other, whereas R2 compounds are not.

Summary. The scheme shown in Fig. 10 summarizes our findings. The benzodiazepine, R1, and R2 binding sites are distinct. Similarly, the R1 and R2 sites must be distinct from the pentobarbital binding site that directly opens chloride ion channels. R1 and R2 type use a common pathway to affect channel gating. From the detailed investigation of the inhibition by Ro15-1788 of the current enhancement by ROD164A, we conclude that R1 and the benzodiazepine binding site interact allosterically. In summary, we provide strong evidence for the presence of additional allosteric sites on the GABA$_A$ receptor. The location of the novel sites remains to be shown.

TABLE 5

Coenhancement by ROD compounds

Currents activated by a GABA concentration that elicited 1 to 5% of the maximal current amplitude were standardized to 100%. The relative current amplitude in the presence of drug after subtraction of the current induced by GABA alone (100%) is given. All experiments were carried out with recombinant $\alpha1\beta2\gamma2$ GABA$_A$ receptors expressed in *X. laevis* oocytes.

Substance	Enhancement	Enhancement in Presence of 100 μM ROD185
	%	
		295 ± 29 (n = 8)
20 μM (+)-ROD188	413 ± 7 (n = 4)	490 ± 39 (n = 4)
100 μM ROD222	899 ± 39 (n = 3)	561 ± 18 (n = 3)

TABLE 6

Modulation of pentobarbital-induced currents

Currents activated by 200 μM pentobarbital was standardized to 100%. The relative current amplitude in the presence of drug after subtraction of the current induced by pentobarbitol alone (100%) is given. All experiments were carried out with recombinant $\alpha1\beta2\gamma2$ GABA$_A$ receptors expressed in *X. laevis* oocytes.

Substance	Enhancement	Enhancement in Presence of 1 μM Ro15-1788
	%	
		−23 ± 3 (n = 4)
20 μM ROD185	125 ± 19 (n = 5)	40 ± 6 (n = 5)
10 μM ROD259	70 ± 13 (n = 4)	48 ± 14 (n = 4)
0.3 μM Diazepam	104 ± 16 (n = 7)	−11 ± 5 (n = 5)

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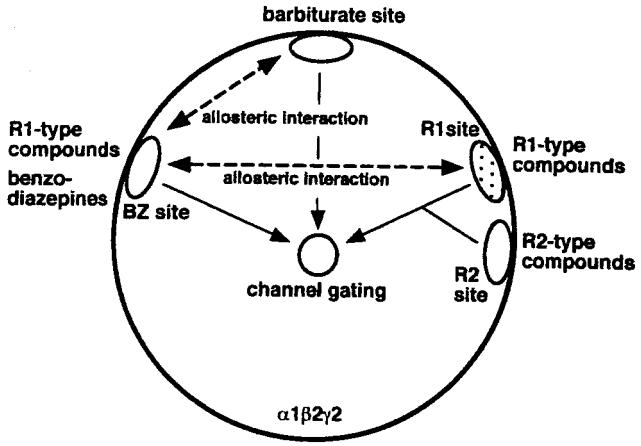

Fig. 10. Scheme summarizing the relationship of the benzodiazepine binding site with the barbiturate site and the R1 and R2 binding sites.

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