0026-895X/95/020354-08$03.00/0

MOLECULAR PHARMACOLOGY, 47:354–362, 1995.

Selective Actions of Certain Neuroactive Pregnanediols at the γ-Aminobutyric Acid Type A Receptor Complex in Rat Brain

LINDA D. McCAULEY,[1] VINCENT LIU, JIE-SHEN CHEN, JON E. HAWKINSON, NANCY C. LAN, and KELVIN W. GEE

Department of Pharmacology, College of Medicine, University of California, Irvine, California 92717 (L.D.M., V.L., K.W.G.), and CoCensys, Inc., Irvine, California 92718 (J.-S.C., J.E.H., N.C.L.)

Received May 13, 1994; Accepted October 25, 1994

SUMMARY

Certain endogenous pregnanediols (5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol) were observed to have limited efficacy as allosteric modulators of *t*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) and [^{3}H]flunitrazepam binding to sites on the γ-aminobutyric acid $(GABA)_A$ receptor complex in rat brain. In contrast, 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) and 3α-hydroxy-5β-pregnan-20-one (3α,5β-P) have full efficacy. Moreover, 3α,5β-P but not 3α,5α-P recognizes high (nanomolar) and low (micromolar) affinity neuroactive steroid sites in these allosteric modulatory assays. The concentration-response curve for 3α,5α-P modulation of [^{35}S]TBPS binding was shifted rightward in the presence of these pregnanediols and GABA. The maximum shift produced by these pregnanediols never exceeded the concentration-response curve obtained with 3α,5α-P alone in the absence of GABA. Additionally, neither 5α-pregnan-3α,20α-diol nor 5β-pregnan-3α,20β-diol had any effect on the site recognized by 3α,5α-P in the absence of GABA. The difference in the affinities of the two apparent sites (29 nM versus 152 nM in the presence and absence of GABA, respectively) recognized by 3α,5α-P is only ~5-fold. In contrast, the difference between the high (30 nM) and low (7 μM) affinity sites discriminated by 3α,5β-P is >200-fold. Thus, the selective interaction between the high affinity site recognized by 3α,5β-P and these pregnanediols can be clearly observed. A saturating concentration of 5β-pregnan-3α,20β-diol selectively eliminated the high affinity component recognized by 3α,5β-P, whereas 5α-pregnan-3α,20α-diol did not completely abolish the high affinity site. 5α-Pregnan-3α,20α-diol recognized only a portion of the high affinity sites discriminated by 3α,5β-P, relative to 5β-pregnan-3α,20β-diol, whereas the two pregnanediols recognized a similar population of sites mediating 3α,5α-P inhibition of [^{35}S]TBPS binding. Collectively, these studies provide evidence that the limited efficacy of certain pregnanediols as allosteric modulators of [^{35}S]TBPS binding may be explained in part by selectivity for the high affinity site recognized by 3α,5β-P. Data collected from 5α-pregnan-3α,20α-diol modulation of [^{35}S]TBPS binding to recombinantly expressed receptors suggest that the subunit composition of the $GABA_A$ receptor complex may contribute to pregnanediol selectivity. The relative contributions of neuroactive steroid receptor subtypes and/or different affinity states of the same receptor to the apparent receptor heterogeneity observed remain to be determined.

The effect of GABA on chloride ion conductance can be allosterically modulated by several distinct but interacting sites that have been identified on the hetero-oligomeric GRC. Among the well known modulatory sites on the GRC are the central BZ site, the barbiturate site, the convulsant or TBPS site, and the most recently identified neuroactive steroid site (1–3). In contrast to their traditional hormonal effects, certain pregnane steroids acting at this neuroactive steroid site exert rapid and reversible effects on GABA-gated chloride channel conductance. The unique structure-activity requirements and rapid/potent pharmacological effects mediated by this neuronal, membrane-bound, steroid site distinguish these recognition sites from the cytosolic hormonal steroid receptor and its ligands.

Numerous GRC-active derivatives of progesterone have been evaluated for their ability to allosterically modulate known recognition sites on the GRC. One of the most interesting ones, 5α-pregnan-3α,20α-diol, has an apparent partial agonist profile in both radioligand (3) and functional (4) assays. The maximum potentiation of GABA-stimulated $^{36}Cl^-$ uptake by 5α-pregnan-3α,20α-diol in rat cortical synaptoneurosomes was significantly less than that produced by 3α,5α-P. Furthermore, 5α-pregnan-3α,20α-diol antagonized, in a concentration-dependent fashion, the enhancement of $^{36}Cl^-$ uptake elicited by 3α,5α-P. Those studies provided the initial evidence that 3α,5α-P and 5α-pregnan-3α,20α-diol may act with different degrees of efficacy, through a common site of action, to modulate $^{36}Cl^-$ conductance. Indeed, the majority of binding studies to date support a

This work was presented in part at the 1993 Society for Neuroscience Annual Meeting in Washington D. C. and was supported by grants from the United States Public Health Service (NS25986) and CoCensys, Inc.

[1] This work was in partial fulfillment of dissertation requirements for the Ph.D. degree in Pharmaceutical Sciences at the University of Southern California.

ABBREVIATIONS: GABA, γ-aminobutyric acid; GRC, γ-aminobutyric acid type A receptor complex; BZ, benzodiazepine; TBPS, *t*-butylbicyclophosphorothionate; 3α,5α-P, 3α-hydroxy-5α-pregnan-20-one; 3α,5β-P, 3α-hydroxy-5β-pregnan-20-one; FLU, flunitrazepam; ANOVA, analysis of variance.

single population of neuroactive steroid sites with stringent structure-activity requirements and neuroactive steroids that act at these sites with different degrees of apparent efficacy. In addition to partial agonism as a basis for apparent limited efficacy, some of these differences in apparent efficacy may be related in part to differences in the subunit composition of the GRC, as has been demonstrated recently for the BZs (5). An additional complication may be that the observed differences in efficacy may be a reflection of neuroactive steroid site heterogeneity. Such a notion is entirely conceivable, given that GRCs can potentially be derived from >15 variants belonging to six subunit families, as identified by molecular cloning (for reviews, see Refs. 6 and 7). Transfection assays have shown that the subunit composition of transiently expressed recombinant GRCs influences the recognition properties of neuroactive steroid sites, raising the possibility that neuroactive steroid sites are heterogeneous (8–12).

We have recently reported on the ability of 3α,5β-P to discriminate high affinity and low affinity neuroactive steroid sites, using allosteric modulation of [^{35}S]TBPS and [^{3}H]FLU binding in bovine brain membranes (13). This apparent heterogeneity of neuroactive steroid receptors is observed with 3α,5β-P but not with its stereoisomer 3α,5α-P. The relative abundance of apparent receptor subtypes defined by 3α,5β-P was regionally dependent and GABA sensitive, as detected by allosteric modulation of [^{35}S]TBPS and [^{3}H]FLU binding. In the present report, four endogenous pregnanediols were evaluated as modulators of [^{35}S]TBPS and [^{3}H]FLU binding in rat brain homogenates. Two of the pregnanediols with limited efficacy as allosteric modulators of TBPS and BZ binding selectively interact with the high affinity site recognized by 3α,5β-P and a GABA-dependent site recognized by 3α,5α-P. Additionally, experiments evaluating the efficacy and potency of 5α-pregnan-3α,20α-diol, in the presence of GABA, as an inhibitor of [^{35}S]TBPS binding to recombinantly expressed GRCs suggest that both efficacy and potency are influenced by GRC subunit composition.

Materials and Methods

Tissue preparation. The cortex, thalamus, or cerebellum from male Sprague-Dawley rats (160–200 g; Simonsen Laboratories, Gilroy, CA) was removed immediately after sacrifice and dissected over ice. A P_2 homogenate was prepared for radioligand binding assays as described previously (2). Briefly, the tissue was gently homogenized (with a Teflon pestle) as a 10% (w/v) suspension in 0.32 M sucrose, followed by centrifugation at 1000 × *g* for 10 min at 0–4°. The supernatant was collected and centrifuged at 9000 × *g* for 20 min at 0–4°. The resultant pellet was washed three times in 100 volumes of ice-cold phosphate-buffered saline (50 mM sodium/potassium phosphate, 200 mM NaCl, pH 7.4) by centrifugation at 9000 × *g* for 10 min and was resuspended as a 10% (w/v) homogenate for immediate use in binding assays.

Expression of GRCs. Human embryonic kidney 293 cells were seeded on 10-cm dishes the day before transfection. The cells were transfected with a total of 20 μg of cDNA/dish, using a modified high-efficiency calcium phosphate precipitation method described previously (14). DNA used for the experiments was a mixture of equal amounts of cloned cDNA encoding the human GRC α_1 (α_2 or α_3), β_1, and γ_{2L} subunits. The cells were washed with growth medium 48 hr after the transfection and were harvested the following day. The harvested cells were washed twice with phosphate-buffered saline and frozen at −70° or immediately used in binding assays.

[^{35}S]TBPS and [^{3}H]FLU binding assays. Because certain neuroactive steroids have been reported to influence chloride channel conductance in the absence of GABA (1, 15–17) and because neuroactive steroid efficacy in the absence of exogenous GABA, as measured in the [^{35}S]TBPS binding assay, may be correlated with these direct effects, the actions of neuroactive steroids in both the presence and the absence of added GABA were evaluated. Furthermore, neuroactive steroid enhancement of [^{3}H]FLU binding was measured only in the presence of GABA, because of the absolute dependence of BZs on GABA for efficacy as modulators of the chloride channel. Aliquots (100 μl) of P_2 or cell membrane homogenate were incubated with 2 nM [^{35}S]TBPS (75–130 Ci/mmol; New England Nuclear, Boston, MA) in the presence or absence of various concentrations of neuroactive steroids dissolved in dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO). The concentration of dimethylsulfoxide never exceeded 1%. Similar experiments were conducted in the presence or absence of 5 μM GABA (the IC_{50} for GABA inhibition of [^{35}S]TBPS binding under the conditions used) dissolved in assay buffer. Nonspecific binding was defined as binding in the presence of 2 μM TBPS (Research Biochemicals, Natick, MA) and ranged from 20% to 30% of the total binding. The incubation mixture was brought to a final volume of 1 ml with assay buffer. Assays were terminated after a 90-min incubation at 25°, by rapid filtration through no. 32 glass fiber filters (Schleicher and Schuell, Keene, NH). The filters were washed three times with 3 ml of ice-cold phosphate buffer and the filter-bound radioactivity was quantified by liquid scintillation counting.

[^{3}H]FLU binding assays were performed under conditions identical to those used in the [^{35}S]TBPS binding assay, with the following modifications. Various concentrations of neuroactive steroids were incubated with 0.5 nM [^{3}H]FLU (87.3 Ci/mmol; New England Nuclear) in the presence of 1 μM GABA, a concentration of GABA sufficient to potentiate allosteric effects without maximally enhancing [^{3}H]FLU binding alone. Nonspecific binding was defined as binding in the presence of 1 μM clonazepam and did not exceed 10% of the total binding. Assays were terminated after 60 min at 25°, by the same method.

Data analysis. The data were evaluated by computerized nonlinear regression (InPlot, GraphPAD, San Diego, CA), using a one-component (three-parameter) or two-component (five-parameter) model to obtain IC_{50} (concentration at which half-maximal inhibition of radioligand binding occurs) or EC_{50} (concentration at which half-maximal enhancement of radioligand binding occurs) values and, when indicated, the percentage of sites comprising high and low affinity components. In each case, the *F* test was used to compare the more complex model (the two-site model) with the less complex model (the one-site model), to determine whether the inclusion of additional parameters significantly improved the goodness of fit of the data set to the regression curve (18). The data collected from expressed receptor assays were analyzed by ANOVA and *post hoc t* tests (Newman-Keuls, $p \leq 0.05$) when warranted.

Results

Apparent partial efficacy of four endogenous pregnanediols as modulators of [^{35}S]TBPS and [^{3}H]FLU binding. The structures of the four endogenous pregnanediols evaluated differ in two respects. The first difference is the spatial orientation of the steroid A-ring (5α- or 5β-reduced), and the second is the configuration (α or β) of the hydroxyl group at C20 (Fig. 1). Interestingly, as the potency decreases, there is generally a concomitant rise in the efficacy of the pregnanediol, as measured by the modulation of [^{35}S]TBPS and [^{3}H]FLU binding in both cortical (Table 1) and thalamic (Table 2) homogenates. It is interesting to note the influence of GABA on the efficacy and potency of these pregnanediols as inhibitors of [^{35}S]TBPS binding. In the absence of GABA, only 5α-pregnan-3α,20α-diol became virtually inactive, whereas the other compounds showed reduced potency and efficacy. In the

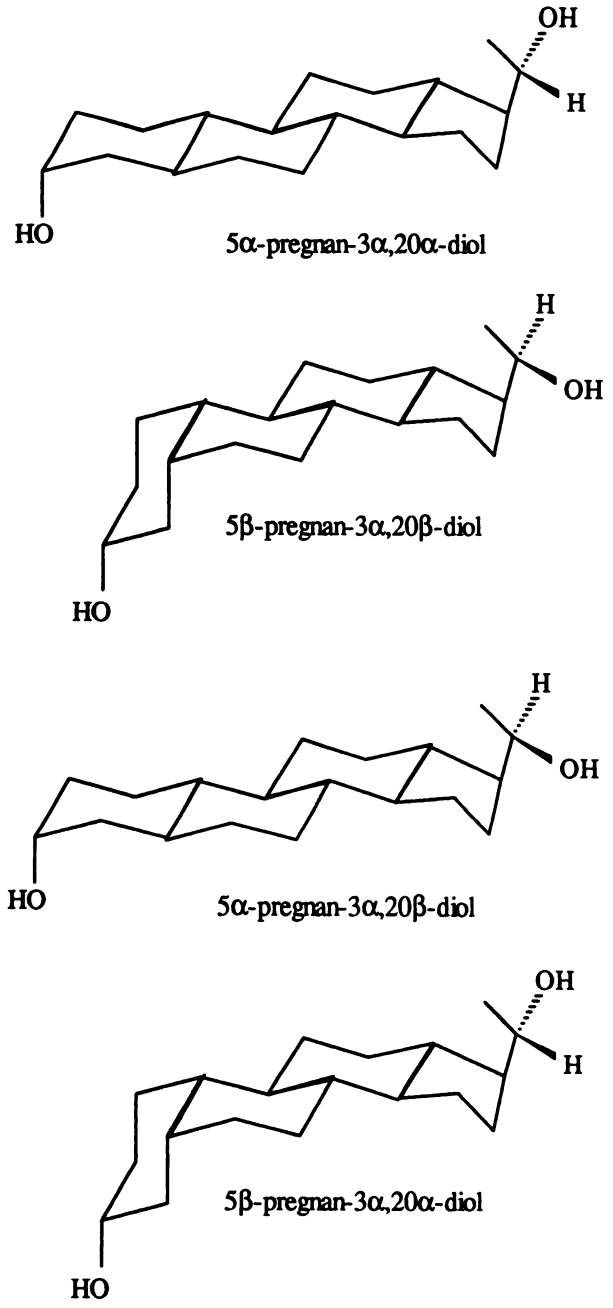

Fig. 1. Structures of four endogenous pregnanediols, i.e., 5α-pregnan-3α,20α-diol, 5β-pregnan-3α,20β-diol, 5α-pregnan-3α,20β-diol, and 5β-pregnan-3α,20α-diol.

presence of 5 μM GABA, 5α-pregnan-3α,20α-diol was more potent than the other three pregnanediols in the [^{35}S]TBPS binding modulation assay. The efficacy and potency of these pregnanediols as measured by the enhancement of [^{3}H]FLU binding in the presence of 1 μM GABA (5 μM GABA enhanced [^{3}H]FLU binding by >50%, limiting further stimulation of binding by neuroactive steroids) are consistent with those observed in the [^{35}S]TBPS binding assay (Tables 1 and 2). The most potent but least efficacious pregnanediol was 5α-pregnan-3α,20α-diol.

Preliminary experiments were performed to evaluate the influence of receptor subunit composition on the efficacy and potency of 5α-pregnan-3α,20α-diol. Differential effects of this neuroactive steroid on [^{35}S]TBPS binding (K_d ~ 30 nM with recombinant receptors) were observed, depending on the α subunit present. The maximal degrees of inhibition of [^{35}S]TBPS binding exhibited by 5α-pregnan-3α,20α-diol with α_1, α_2, or α_3 plus $\beta_1\gamma_{2L}$ recombinant receptors were all significantly different [$F(2,8) = 66.9$, $p \leq 0.0001$], with the greatest efficacy being observed in α_1-containing GRCs (Fig. 2). The influences of the α subunit on potency and efficacy were similar. The IC_{50} values for the inhibition by 5α-pregnan-3α,20α-diol of [^{35}S]TBPS binding to both α_2- and α_3-containing recombinant GRCs were significantly lower than for α_1-containing receptors [$F(2,8) = 10.5$, $p < 0.006$].

Effect of 3α,5α-P and 3α,5β-P on [^{35}S]TBPS and [^{3}H]FLU binding in different brain regions. The primary structural difference between the endogenous pregnanediols tested and 3α,5α-P and 3α,5β-P is the nature of the C20-position. 3α,5α-P and 3α,5β-P have a 20-ketone, whereas the pregnanediols have an hydroxyl group at the C20-position (Fig. 3). Numerous studies have reported the complete allosteric inhibition by 3α,5α-P of [^{35}S]TBPS binding to rat cortical membranes, through a single population of neuroactive steroid sites (3, 16, 17). Similarly to results observed in the cortex, 3α,5α-P inhibited [^{35}S]TBPS binding in the thalamus and cerebellum with nanomolar potency (Table 3). The addition of 5 μM GABA shifted the 3α,5α-P concentration-response curves to the left (Fig. 4). In the presence of 1 μM GABA, 3α,5α-P produced concentration-dependent enhancement of [^{3}H]FLU that fit a one-site model with nanomolar potency in each brain region. In contrast, identical experiments with 3α,5β-P, which differs structurally from 3α,5α-P only in the spatial orientation of the steroid A-ring, produced concentration-response curves consistent with effects mediated through two sites (Table 4). In cortical tissues, analysis of the binding data showed a significantly better fit to a two-site inhibition model in both the presence [$F(2,13) = 221$, $p < 0.0001$] and the absence [$F(2,13) = 18.4$, $p = 0.0002$] of exogenous GABA (Fig. 5). The addition of GABA did result in a small increase of potency at both sites and a large increase in the proportion of high affinity sites observed. With membranes derived from the thalamus as the receptor source, the data were consistent with a two-site model of inhibition in both the presence [$F(2,13) = 18$, $p = 0.0002$] and the absence [$F(2,12) = 8$, $p < 0.01$] of 5 μM GABA (Table 4). Here again, we observed both a nanomolar site and a micromolar site. The notable effect of GABA was to increase the proportion of sites defining the high affinity component while reducing the low affinity component. In the cerebellum, the data obtained in the presence and absence of 5 μM GABA were similar to those obtained in the thalamus. In the absence of added GABA, the data were fit to the same two-site model [$F(2,11) = 8.3$, $p = 0.0064$] as for the other brain regions (Table 4). The addition of 5 μM GABA to the assay resulted in a shift to a greater number of neuroactive steroid sites of the high affinity type [$F(2,10) = 17.5$, $p = 0.0005$].

Consistent with the two-site inhibition by 3α,5β-P of [^{35}S]TBPS binding in the cortex, this neuroactive steroid enhanced [^{3}H]FLU binding in a concentration-dependent manner (Table 4) in cortical homogenates, in the presence of GABA, through two sites [$F(2,8) = 9.1$, $p < 0.01$]. The percentages of high and low affinity components and their respective EC_{50} values (48%, 31 nM; 52%, 840 nM) were similar to the IC_{50} values and proportions obtained for the modulation by 3α,5β-P of [^{35}S]

TABLE 1

Effects of four pregnanediols on 2 nM [^{35}S]TBPS and 0.5 nM [^{3}H]FLU binding in washed rat cortical P_2 homogenates

I_{max} or E_{max} indicates the maximum inhibition or enhancement, respectively, observed under the conditions used. All values represent the mean ± standard error of washed (three times) P_2 homogenates in three or four independent experiments.

Steroid	[^{35}S]TBPS				[^{3}H]FLU + 1 μM GABA	
	Control		+5 μM GABA			
	IC_{50}	I_{max}	IC_{50}	I_{max}	EC_{50}	E_{max}
	μM	%	nM	%	nM	%
5α-Pregnan-3α,20α-diol	NA[a]	6 ± 1	69 ± 12	42 ± 2	85 ± 13	25 ± 2
5β-Pregnan-3α,20β-diol	5 ± 4	24 ± 4	289 ± 22	58 ± 4	430 ± 40	49 ± 3
5β-Pregnan-3α,20α-diol	3 ± 1	54 ± 2	411 ± 115	80 ± 6	494 ± 53	70 ± 4
5α-Pregnan-3α,20β-diol	4 ± 1	66 ± 1	716 ± 74	90 ± 3	688 ± 36	60 ± 3

[a] NA, such low efficacy that no IC_{50} could be calculated.

TABLE 2

Effects of four pregnanediols on 2 nM [^{35}S]TBPS and 0.5 nM [^{3}H]FLU binding in washed rat thalamic P_2 homogenates

I_{max} or E_{max} indicates the maximum inhibition or enhancement, respectively, observed under the conditions used. All values represent the mean ± standard error of washed (three times) P_2 homogenates in three or four independent experiments.

Steroid	[^{35}S]TBPS + 5 μM GABA		[^{3}H]FLU + 1 μM GABA	
	IC_{50}	I_{max}	EC_{50}	E_{max}
	nM	%	nM	%
5α-Pregnan-3α,20α-diol	77 ± 9	31 ± 2	64 ± 10	18 ± 2
5β-Pregnan-3α,20β-diol	299 ± 14	42 ± 4	475 ± 57	50 ± 4
5β-Pregnan-3α,20α-diol	602 ± 65	74 ± 5	497 ± 83	58 ± 6
5α-Pregnan-3α,20β-diol	759 ± 168	76 ± 3	986 ± 217	54 ± 4

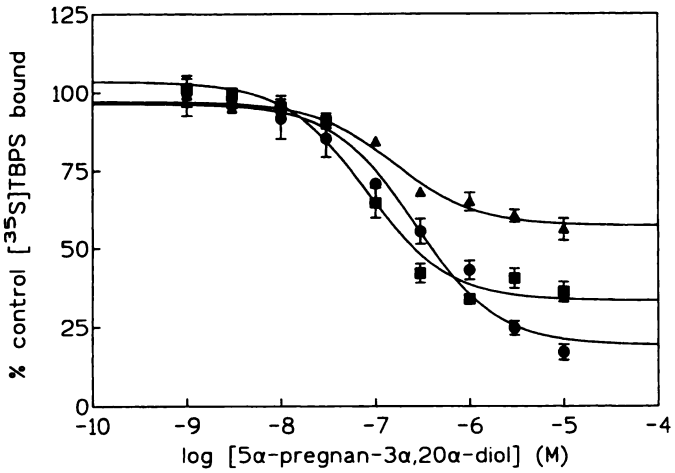

Fig. 2. Influence of the α subunit on the inhibition by 5α-pregnan-3α,20α-diol, in the presence of 5 μM GABA, of 2 nM [^{35}S]TBPS binding to recombinantly expressed receptors. ●, $\alpha_1\beta_1\gamma_{2L}$ (IC_{50} = 288 ± 36.5 nM, I_{max} = 83 ± 2.4%); ■, $\alpha_2\beta_1\gamma_{2L}$ (IC_{50} = 84 ± 17.7 nM, I_{max} = 68 ± 1.0%); ◆, $\alpha_3\beta_1\gamma_{2L}$ (IC_{50} = 168 ± 21.8 nM, I_{max} = 45 ± 2.5%). Each *point* represents the mean ± standard error of at least three independent experiments. All I_{max}values are significantly different from each other (ANOVA, $p \leq 0.0001$). $\alpha_2\beta_1\gamma_{2L}$ and $\alpha_3\beta_1\gamma_{2L}$ IC_{50} values are significantly lower than the $\alpha_1\beta_1\gamma_{2L}$ IC_{50} (ANOVA, $p \leq 0.006$).

TBPS binding in the presence of GABA (63%, 29 nM; 37%, 6 μM). In contrast, the thalamus and cerebellum yielded reasonably potent EC_{50} values (68 nM and 36 nM, respectively) that were derived from single-site enhancement curves.

Selective interaction of 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol with the high affinity sites recognized by 3α,5α-P and 3α,5β-P modulation of [^{35}S] TBPS binding. Previous observations of the interaction between 5α-pregnan-3α,20α-diol and 3α,5α-P suggested either that 5α-pregnan-3α,20α-diol was a partial agonist at the neuroactive steroid site recognized by 3α,5α-P (4) or that 5α-pregnan-3α,20α-diol recognized only one of two apparent sites mediating the inhibition by 3α,5α-P of [^{35}S]TBPS binding. In cortex, 3α,5α-P inhibited [^{35}S]TBPS binding with IC_{50} values of 29 nM and 152 nM in the presence and absence of 5 μM GABA, respectively (Table 3). In the presence of 5 μM GABA, 1 μM 5α-pregnan-3α,20α-diol shifted the 3α,5α-P inhibition curve to the right (Fig. 6A). The IC_{50} obtained (127 nM) was similar to that derived from 3α,5α-P inhibition of [^{35}S]TBPS binding in the absence of exogenous GABA. Concentrations of 5α-pregnan-3α,20α-diol above 1 μM produced no further shifts in the 3α,5α-P versus [^{35}S]TBPS binding concentration-response curve (data not shown). In addition, 1 μM 5α-pregnan-3α,20α-diol was incapable (IC_{50} = 166 nM) of shifting the concentration-response curve for 3α,5α-P inhibition of [^{35}S] TBPS binding in the absence of 5 μM GABA (Fig. 6B). In the presence of 5 μM GABA 3 μM 5β-pregnan-3α,20β-diol shifted the 3α,5α-P/[^{35}S]TBPS binding inhibition curve to the right (IC_{50} = 148 nM) (Fig. 7A), whereas in the absence of added GABA 5β-pregnan-3α,20β-diol did not produce any notable effect (IC_{50} = 139 nM) (Fig. 7B). These data suggest that 3α,5α-P recognizes two sites, i.e., a high affinity site (29 nM) in the presence of 5 μM GABA and a low affinity site (127–152 nM) in the absence of GABA or the presence of 5α-pregnan-3α,20α-diol or 5β-pregnan-3α,20β-diol.

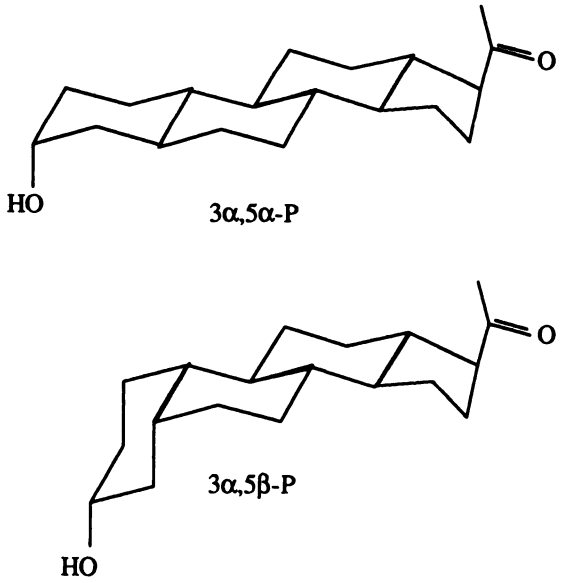

Fig. 3. Structures of 3α,5α-P and 3α,5β-P.

TABLE 3

Effects of 3α,5α-P on 2 nM [^{35}S]TBPS and 0.5 nM [^{3}H]FLU binding in washed rat brain P_2 homogenates

I_{max} or E_{max} indicates the maximum inhibition or enhancement, respectively, observed under the conditions used. All values represent the mean ± standard error of washed (three times) P_2 homogenates in three or four independent experiments.

Brain region	[^{35}S]TBPS				[^{3}H]FLU + 1 μM GABA	
	Control		+5 μM GABA			
	IC_{50}	I_{max}	IC_{50}	I_{max}	EC_{50}	E_{max}
	nM	%	nM	%	nM	%
Cortex	152 ± 12	87 ± 2	29 ± 2	93 ± 2	75 ± 9	83 ± 3
Thalamus	193 ± 61	93 ± 4	55 ± 15	99 ± 1	81 ± 24	53 ± 2
Cerebellum	67 ± 23	98 ± 2	29 ± 11	96 ± 4	29 ± 7	50 ± 2

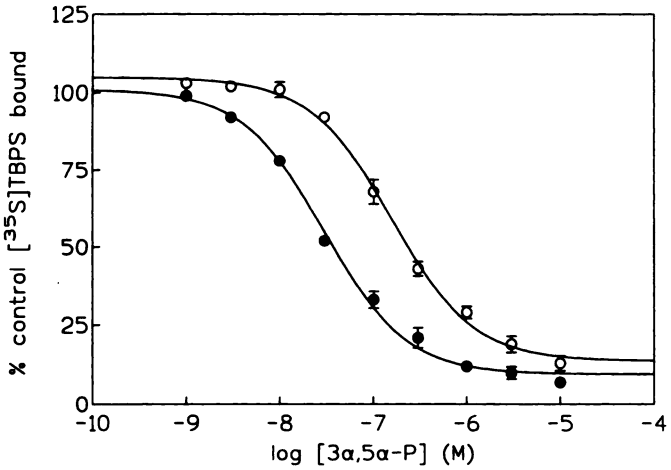

Fig. 4. Concentration-dependent inhibition by 3α,5α-P of 2 nM [^{35}S]TBPS binding, in the presence (●) and absence (○) of 5 μM GABA, in washed cortical P_2 homogenates. Each *point* represents the mean ± standard error of at least five independent experiments.

In contrast to the ~5-fold difference between the IC_{50} values of the two apparent sites discriminated by 3α,5α-P, the >200-fold difference between the two sites differentiated by 3α,5β-P in the presence of 5 μM GABA provides a better opportunity to test the hypothesis that the two pregnanediols with the lowest efficacy in modulating radioligand binding to the GRC interact selectively with one of the two apparent sites discriminated by 3α,5β-P. Thus, the ability of a fixed concentration of pregnanediol to selectively block one of the sites mediating 3α,5β-P inhibition of [^{35}S]TBPS binding was evaluated. As shown in Fig. 8A, the presence of 3 μM 5β-pregnan-3α,20β-diol essentially eliminated the high affinity component associated with 3α,5β-P inhibition of [^{35}S]TBPS binding. Analysis of the data resulted in a "best fit" to a one-site model with an IC_{50} of 10 μM. This effect of 5β-pregnan-3α,20β-diol was independent of the addition of GABA to the incubation mixture, inasmuch as a single site with an IC_{50} of 16 μM was derived from data obtained in the absence of GABA (Fig. 8B).

Similar experiments conducted with 3 μM 5α-pregnan-3α,20α-diol in both the presence and the absence of added GABA revealed an apparently selective interaction of 5α-pregnan-3α,20α-diol with the high affinity component of 3α,5β-P modulation of [^{35}S]TBPS binding. Although the IC_{50} values remained virtually unchanged, the presence of 5α-pregnan-3α,20α-diol resulted in a greater proportion of low affinity sites. In the presence of 5 μM GABA and 3 μM 5α-pregnan-3α,20α-diol (Fig. 9A) it was determined that 3α,5β-P inhibition of [^{35}S]TBPS binding was still mediated through two sites [$F(2,12) = 11.8$, $p = 0.005$], whereas in the absence of added GABA but in the presence of 3 μM 5α-pregnan-3α,20α-diol (Fig. 9B) a single site, with an IC_{50} of 17 μM, was observed.

Discussion

It has been shown that varying the subunit composition of the GRC changes the allosteric modulatory effects of neuroactive steroids on the GRC (8–12). It is also known that very subtle structural changes in the neuroactive steroids produce striking differences in activity as modulators of the GRC (e.g., 3α- versus 3β-hydroxy analogs). In the pregnanediol experiments, we examined the relationship between relative GRC

TABLE 4

Effects of 3α,5β-P on 2 nM [^{35}S]TBPS and 0.5 nM [^{3}H]FLU binding in washed rat brain P_2 homogenates

All values represent the mean ± standard error of washed (three times) P_2 homogenates in three or four independent experiments.

Brain region	High affinity sites		Low affinity sites	
	Proportion	IC_{50}	Proportion	IC_{50}
	%	nM	%	μM
Control [^{35}S]TBPS				
Cortex	15 ± 2	70 ± 21	85 ± 2	14 ± 1
Thalamus	31 ± 4	47 ± 23	69 ± 4	5 ± 1
Cerebellum	27 ± 6	18 ± 10	73 ± 6	8 ± 0
[^{35}S]TBPS + 5 μM GABA				
Cortex	63 ± 2	30 ± 3	37 ± 2	7 ± 1
Thalamus	57 ± 9	12 ± 9	43 ± 9	4 ± 2
Cerebellum	68 ± 15	23 ± 2	32 ± 15	4 ± 2
[^{3}H]FLU + 1 μM GABA				
Cortex	48 ± 6	31 ± 4	52 ± 6	0.84 ± 0.246
Thalamus	100	68 ± 12		
Cerebellum	100	36 ± 6		

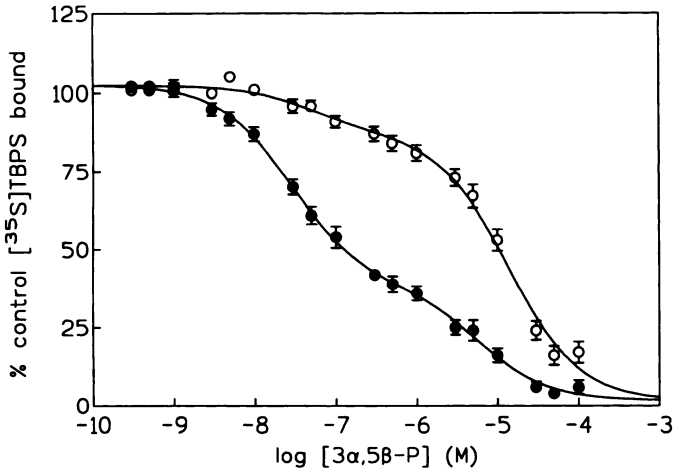

Fig. 5. Concentration-dependent inhibition by 3α,5β-P of 2 nM [^{35}S]TBPS binding, in the presence (●) and absence (○) of 5 μM GABA, in washed cortical P_2 homogenates. Each *point* represents the mean ± standard error of at least five independent experiments.

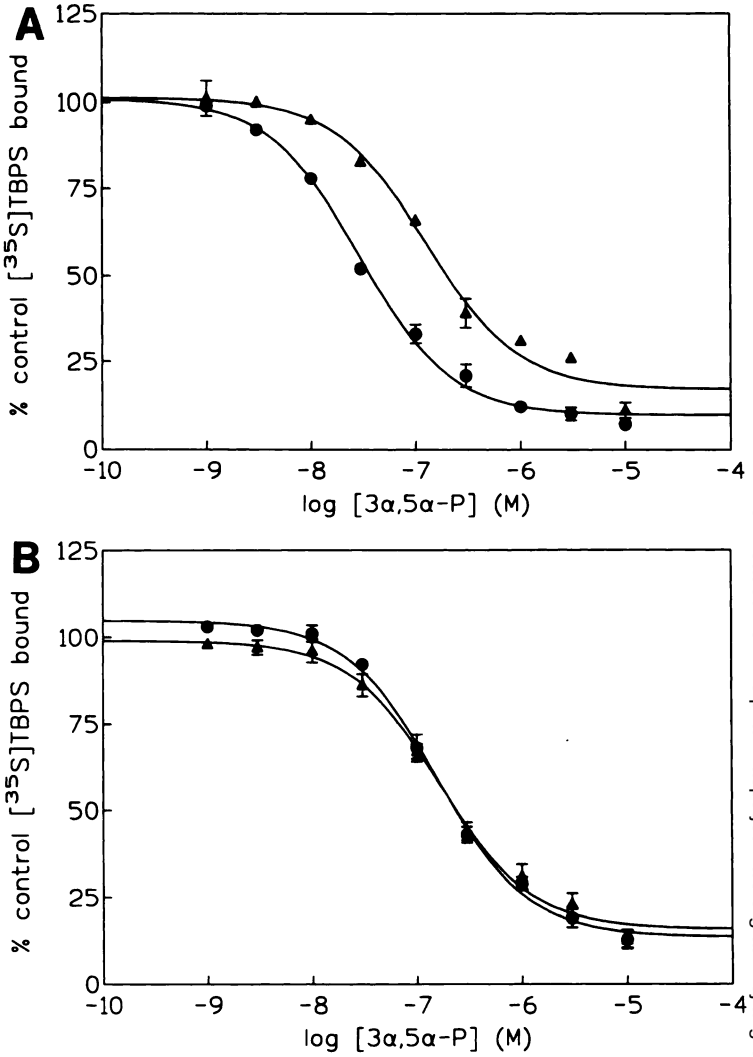

Fig. 6. Concentration-dependent inhibition by 3α,5α-P of 2 nM [^{35}S]TBPS binding, in the presence (▲) and absence (●) of 1 μM 5α-pregnan-3α,20α-diol, in washed cortical P_2 homogenates. A, Incubated in the presence of 5 μM GABA; B, incubated in the absence of GABA. Each *point* represents the mean ± standard error of at least four independent experiments.

subunit composition [i.e., primarily α_1 subunits in thalamus versus many α subunit variants in cortex (for review, see Ref. 19), as well as α subunit composition in recombinantly expressed receptors] and subtle versus dramatic differences in three-dimensional neuroactive steroid structure (i.e., isomers at the C3- and C20-positions versus isomers at the C5-position, respectively), but we observed no remarkable correlations under these conditions. The rank orders of potency and efficacy of the four pregnanediols were almost identical under each of the three conditions studied in cortex and thalamus. Both the differential efficacy and potency of 5α-pregnan-3α,20α-diol as a modulator of [^{35}S]TBPS binding to recombinantly expressed GRCs containing different α subunits suggest that subunit composition may contribute, at least in part, to the apparent receptor heterogeneity. However, the multiple α, β, and γ subunits present in any given brain region make the expression of all of the possible subunit compositions found in a particular region difficult and render a precise correlation between the results of the brain homogenate studies and the expressed receptor binding studies beyond the scope of the present work. Thus, it appears that, although BZ receptor heterogeneity (20) can be readily explained by the α subunit composition of the GRC, the basis of the apparent neuroactive steroid receptor subtypes revealed by the pregnanediols cannot be as clearly defined on the basis of receptor α subunit composition alone.

The observation of the apparent partial agonist profile of 5α-pregnan-3α,20α-diol (4) raised the intriguing question of whether 5α-pregnan-3α,20α-diol was a unique neuroactive steroid or whether all endogenous pregnanediols would exhibit limited efficacy or partial agonism. The potency and efficacy of pregnanediol modulation of [^{35}S]TBPS and [^{3}H]FLU binding to the GRC were essentially determined by the spatial orientation of the steroid A-ring and the hydroxyl groups in the C20-position. It is not surprising that such significant differences were observed in both the efficacy and potency of the 5α- versus 5β-reduced derivatives, based on our findings with 3α,5α-P and 3α,5β-P. The A-ring of the 5α-pregnanediols lies approximately in the same plane as the remainder of the molecule, whereas in the 5β-pregnanediols the A-ring is approximately perpendicular to the rest of the molecule. Thus, not only is the absolute distance between the important 3α-hydroxyl group and the 20-hydroxyl group greater in the 5β-pregnanediols, but also the three-dimensional shapes of the neuroactive steroids are different. Indeed, we have revealed some interesting structure-activity requirements of neuroactive steroid receptors recognized by the pregnanediols. A dramatic increase in efficacy and decrease in potency for modulating [^{35}S]TBPS binding were observed with the change from a 20α-hydroxyl group to a 20β-hydroxyl group in the 5α-reduced pregnanediols. For example, 5α-pregnan-3α,20α-diol had limited efficacy and a nanomolar IC_{50}, whereas 5α-pregnan-3α,20β-diol had almost full efficacy and high nanomolar potency. This indicates the importance of the C20-hydroxyl group in determining both efficacy and potency of 5α-reduced pregnanediols. On the other hand, when the effects of 5β-reduced pregnanediols with the C20-hydroxyl group in the α- or β-configuration are compared, the present findings indicate that the configuration of the hydroxyl group in this position influences the efficacy but not the potency, as measured by [^{35}S]TBPS binding to the GRC. These data suggest that both the C20- and C3-hydroxyl groups are important

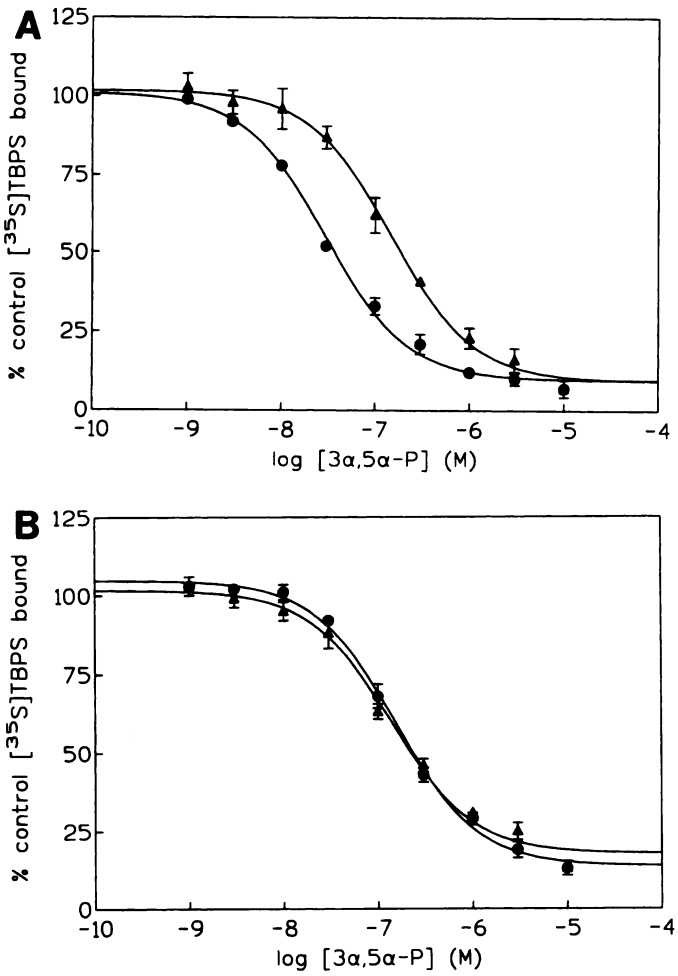

Fig. 7. Concentration-dependent inhibition by 3α,5α-P of 2 nM [^{35}S]TBPS binding, in the presence (▲) and absence (●) of 3 μM 5β-pregnan-3α,20β-diol, in washed cortical P_2 homogenates. A, Incubated in the presence of 5 μM GABA; B, incubated in the absence of GABA. Each *point* represents the mean ± standard error of at least four independent experiments.

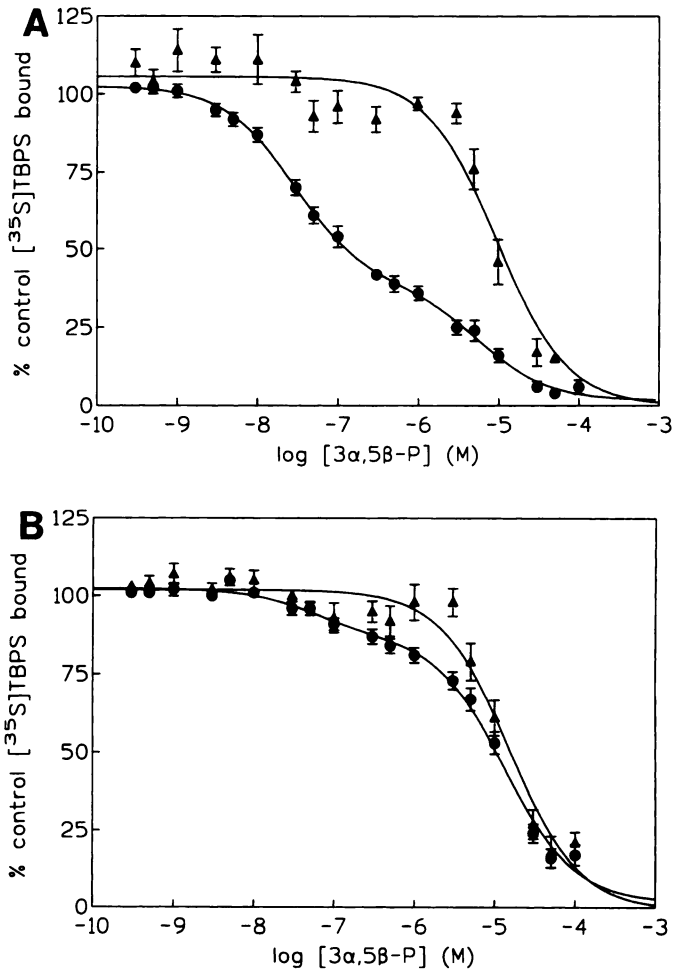

Fig. 8. Concentration-dependent inhibition by 3α,5β-P of 2 nM [^{35}S]TBPS binding, in the presence (▲) and absence (●) of 3 μM 5β-pregnan-3α,20β-diol, in washed cortical P_2 homogenates. A, Incubated in the presence of 5 μM GABA; B, incubated in the absence of GABA. Each *point* represents the mean ± standard error of at least four independent experiments. The apparent proportions and IC_{50} values of the sites are as follows: in the presence of GABA: under control conditions, 63% at 30 nM and 37% at 7 μM; in the presence of 5β-pregnan-3α,20β-diol, 100% at 10 μM; in the absence of GABA: under control conditions, 15% at 70 nM and 85% at 14 μM; in the presence of 5β-pregnan-3α,20β-diol, 100% at 16 μM.

determinants in pregnanediol/neuroactive steroid receptor interactions.

Initially, both 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol appeared to behave as partial agonists in [^{35}S]TBPS binding modulation assays. This is exemplified by the observations that both were limited efficacy compounds when evaluated alone, as shown in Table 1; however, as seen in Figs. 6 and 7, they acted as antagonists in the presence of a full agonist. These data suggested that the two pregnanediols recognized a similar population of neuroactive steroid sites that was defined by 3α,5α-P inhibition of [^{35}S]TBPS binding in the presence of GABA. The identification of apparent neuroactive steroid receptor subtypes with affinities orders of magnitude apart, as discriminated by 3α,5β-P, has provided a better tool for evaluating whether the limited efficacy of the pregnanediols could be explained in part by apparent receptor subtype selectivity. The present observations indicate that 5β-pregnan-3α,20β-diol is not simply a ligand with partial efficacy at a single neuroactive steroid receptor population but, more likely, is a full agonist ligand at a subpopulation of receptors recognized with high affinity by 3α,5β-P. This is supported by the observation that in the presence of a saturating concentration of 5β-pregnan-3α,20β-diol only the low affinity component of 3α,5β-P inhibition of [^{35}S]TBPS binding remains. Furthermore, the interaction between 5α-pregnan-3α,20α-diol and the two sites revealed by 3α,5β-P suggests a selective interaction with only part of the high affinity component defined by 3α,5β-P. Specifically, a saturating concentration of 5α-pregnan-3α,20α-diol only partially reduces the proportion of sites with high affinity for 3α,5β-P in the presence of GABA. Interestingly, in the absence of exogenous GABA, 5α-pregnan-3α,20α-diol completely eliminates the approximately 15% of high affinity sites discriminated by 3α,5β-P under control conditions. In contrast, the modulation by 5α-pregnan-3α,20α-diol alone of [^{35}S]TBPS binding indicates that 5α-pregnan-3α,20α-diol is inactive in the absence of GABA. It is possible that, although 5α-pregnan-3α,20α-diol is not able to allosterically modulate the chloride channel in the absence of GABA, as is necessary to detect a change in [^{35}S]TBPS binding (i.e., it lacks efficacy), it retains affinity for the receptor and therefore eliminates the high

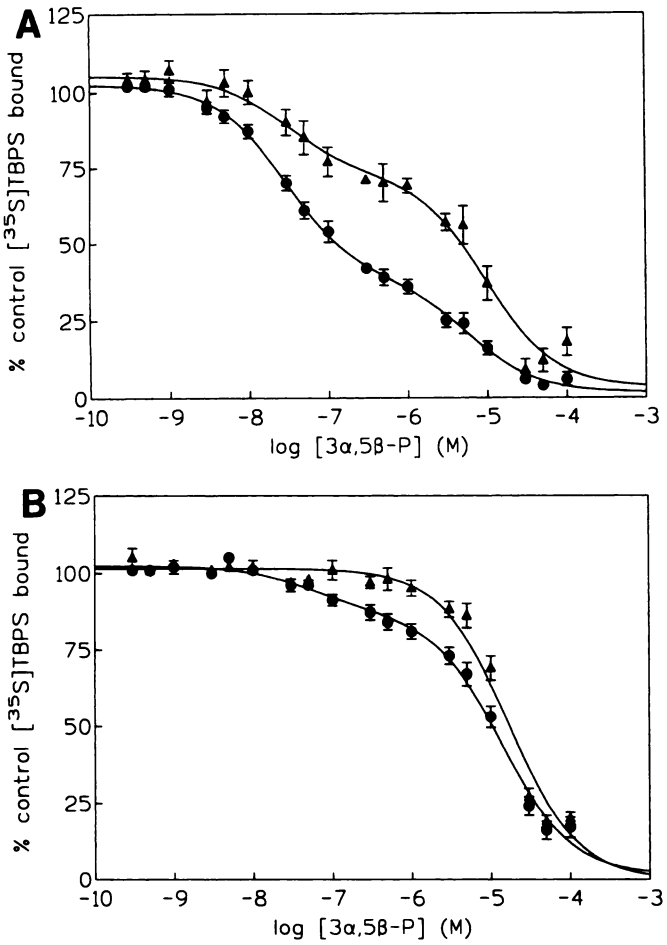

Fig. 9. Concentration-dependent inhibition by 3α,5β-P of 2 nM [^{35}S]TBPS binding, in the presence (▲) and absence (●) of 3 μM 5α-pregnan-3α,20α-diol, in washed cortical P_2 homogenates. A, Incubated in the presence of 5 μM GABA; B, incubated in the absence of GABA. Each *point* represents the mean ± standard error of at least four independent experiments. The apparent proportions and IC_{50} values of the sites are as follows: in the presence of GABA: under control conditions, 63% at 30 nM and 37% at 7 μM; in the presence of 5α-pregnan-3α,20α-diol, 32% at 31 nM and 68% at 10 μM; in the absence of GABA: under control conditions, 15% at 70 nM and 85% at 14 μM; in the presence of 5α-pregnan-3α,20α-diol, 100% at 17 μM.

affinity component of the 3α,5β-P/[^{35}S]TBPS binding inhibition curve by an antagonist-like effect. Together, these data suggest that the low nanomolar site defined by 3α,5α-P inhibition of [^{35}S]TBPS binding in the presence of GABA is part of the population of high affinity sites defined by 3α,5β-P inhibition in the presence of GABA. Furthermore, 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol are selective for this common population of sites, with no effect on the second site observed with either 3α,5α-P (observed in the absence of GABA) or 3α,5β-P (micromolar affinity) inhibition of [^{35}S]TBPS binding.

Additional complexities arise when one considers the dependence on GABA of the two populations of sites differentiated by 3α,5β-P. The discrimination of high and low affinity sites by 3α,5β-P in the allosteric modulation of binding to the TBPS site on the GRC appears to be independent of brain region in rats, because 3α,5β-P but not 3α,5α-P discriminated apparent neuroactive steroid receptor subtypes in all brain regions examined by using modulation of [^{35}S]TBPS binding. However, the proportions of high and low affinity sites recognized by 3α,5β-P, as well as their IC_{50} values, were regionally dependent and GABA dependent. In contrast, 3α,5α-P modulated binding through a single site in each brain region examined. If the neuroactive steroid receptor subtypes are simply a reflection of structurally distinct sites, then a change in potency at one or both of the sites may be expected in the presence of GABA but the relative proportions of the sites defining the components should remain unchanged. This was not observed in the present study. On the contrary, under control conditions 85% of the sites defined by 3α,5β-P in the [^{35}S]TBPS binding assay were low affinity, whereas in the presence of 5 μM GABA 37% of the sites were low affinity. Even if one assumes that the 50% of the sites that are lost with the addition of 5 μM GABA (5 μM GABA inhibits 50% of [^{35}S]TBPS binding) are low affinity, the net result would be ~70% (85% − 50% = 35% × 2 = 70%) low affinity sites and 30% (15% − 0% = 15% × 2 = 30%) high affinity sites. However, in the presence of GABA 63% of the sites were observed to be high affinity. One possible explanation for the apparent increase in high affinity sites is that the addition of 5 μM GABA not only inhibits 50% of [^{35}S]TBPS binding to low affinity sites but also converts ~15% of the remaining low affinity sites to high affinity sites. Specifically, 85% low affinity sites in the absence of GABA minus 50% lost to inhibition by GABA of [^{35}S]TBPS binding equals 35%. Of these 35%, ~15% are converted to high affinity sites. Of the original population of neuroactive steroid sites, 20% low affinity sites remain. In addition to the original 15% high affinity sites in the absence of GABA are added the 15% converted from low affinity sites in the presence of 5 μM GABA. This results in 30% of the original population of neuroactive steroid sites as high affinity sites. When the number of sites is reduced by one half in the presence of GABA, the relative proportions are doubled. Therefore, one may expect that ~60% of the sites are high affinity and ~40% low affinity in the 3α,5β-P inhibition of [^{35}S]TBPS binding in the presence of 5 μM GABA. The interconversion of high and low affinity sites may be only a partial explanation for the sites discriminated by 3α,5β-P in the modulation of [^{35}S]TBPS binding and thus may contribute to the neuroactive steroid site selectivity recognized by 5α-pregnan-3α,20α-diol and 5β-pregnan-3α,20β-diol.

In conclusion, receptor subtype selectivity and partial agonist activity are not mutually exclusive, and the combination of these characteristics may ultimately explain why certain neuroactive steroids have limited efficacy. The use of 3α,5β-P as a two-site modulator provides another tool to explore the basis of the apparent limited efficacy of certain pregnanediols and other neuroactive steroids as modulators of the GRC-coupled chloride channel. Relative contributions of the high and low affinity sites to the overall receptor population vary according to the brain region studied and the presence of GABA. The increased proportion of high affinity sites observed in the presence of GABA can be accounted for in part by the conversion of low affinity sites to high affinity sites, suggesting that the selectivity of the pregnanediols *in vivo* would be dependent upon the local synaptic GABA concentrations. However, the physiological/functional significance of these two sites remains to be determined and may ultimately be explained in part on the basis of GRC subunit composition. The *in vivo* administration of neuroactive steroids leads to a wide range of behavioral effects consistent with GABA-facilitory actions at the GRC

(21, 22). The identification of ligands, such as 5β-pregnan-3α,20β-diol, with apparent selectivity for a particular population of neuroactive steroid sites suggests that it may eventually be possible to assign certain behavioral effects to a preferential activation of neuroactive steroid receptor subtypes. This information would be invaluable for understanding the physiological role of this remarkable class of neuroactive steroids and their receptors, as well as their pharmacological effects.

Acknowledgments

The authors would like to thank Drs. Hasan Tahir and John Drewe for helpful discussions. All neuroactive steroids used in these experiments were generous gifts from CoCensys, and clonazepam was a gift from Hoffmann-La Roche. The authors would like to thank Dr. Peter Seeburg for providing the cDNAs used in these experiments.

References

1. Harrison, N. L., M. D. Majewska, J. W. Harrington, and J. L. Barker. Structure-activity relationships for steroid interaction with the γ-aminobutyric acid-A receptor complex. *J. Pharmacol. Exp. Ther.* **241**:346–353 (1987).
2. Gee, K. W., R. E. Brinton, W. C. Chang, and B. S. McEwen. *Gamma*-aminobutyric acid-dependent modulation of the chloride ionophore by steroids in rat brain. *Eur. J. Pharmacol.* **136**:419–423 (1987).
3. Gee, K. W., M. B. Bolger, R. E. Brinton, H. Coirini, and B. S. McEwen. Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**:803–821 (1988).
4. Belelli, D., and K. W. Gee. 5α-Pregnan-3α,20α-diol behaves like a partial agonist in the modulation of GABA-stimulated chloride ion uptake by synaptoneurosomes. *Eur. J. Pharmacol.* **167**:173–176 (1989).
5. Knoflach, F., U. Drescher, L. Scheurer, P. Malherbe, and H. Mohler. Full and partial agonism displayed by benzodiazepine receptor ligands at recombinant γ-aminobutyric acid$_A$ receptor subtypes. *J. Pharmacol. Exp. Ther.* **266**:385–391 (1993).
6. Olsen, R. W., and A. J. Tobin. Molecular biology of GABA$_A$ receptors. *FASEB J.* **4**:1469–1480 (1990).
7. Burt, D. R., and G. L. Kamatchi. GABA$_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–2923 (1991).
8. Lan, N. C., J.-S. Chen, D. Belelli, D. B. Pritchett, P. H. Seeburg, and K. W. Gee. A steroid recognition site is functionally coupled to an expressed GABA$_A$-benzodiazepine receptor. *Eur. J. Pharmacol.* **188**:403–406 (1990).
9. Shingai, R., M. L. Sutherland, and E. A. Barnard. Effects of subunit types of the cloned GABA$_A$ receptor on the response to a neurosteroid. *Eur. J. Pharmacol.* **206**:77–80 (1991).
10. Lan, N. C., K. W. Gee, M. B. Bolger, and J. S. Chen. Differential responses of expressed recombinant human GABA$_A$ receptors to neurosteroids. *J. Neurochem.* **57**:1818–1821 (1991).
11. Puia, G., S. Vicini, P. H. Seeburg, and E. Costa. Influence of recombinant γ-aminobutyric acid$_A$ receptor subunit compositions on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. *Mol. Pharmacol.* **39**:691–696 (1991).
12. Hadingham, K. L., P. B. Wingrove, K. A. Wafford, D. Bain, J. A. Kemp, K. J. Palmer, A. W. Wilson, A. S. Wilcox, J. M. Sikela, C. I. Ragan, and P. J. Whiting. Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.* **44**:1211–1218 (1993).
13. Hawkinson, J. E., C. K. Kimbrough, L. D. McCauley, M. B. Bolger, N. C. Lan, and K. W. Gee. The neuroactive steroid 3α-hydroxy-5β-pregnan-20-one is a two component modulator of ligand binding to the GABA$_A$ receptor. *Eur. J. Pharmacol.* **269**:157–163 (1994).
14. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel GABA$_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
15. Majewska, M. D., N. L. Harrison, R. D. Schwartz, J. L. Barker, and S. M. Paul. Steroid metabolites are barbiturate-like modulators of the GABA receptor. *Science (Washington D. C.)* **232**:1004–1007 (1986).
16. Turner, D. M., R. W. Ranson, J. S.-J. Yang, and R. W. Olsen. Steroid anesthetics and naturally occurring analogs modulate the *gamma*-aminobutyric acid receptor complex at a site distinct from barbiturates. *J. Pharmacol. Exp. Ther.* **248**:960–966 (1989).
17. Lambert, J. J., J. A. Peters, N. C. Sturgges, and T. G. Hales. Steroid modulation of the GABA$_A$ receptor complex: electrophysiological studies. *Ciba Found. Symp.* **153**:56–70 (1990).
18. Boxenbaum, H. G., S. Riegelmon, and R. M. Elashoff. Statistical estimation in pharmacokinetics. *J. Pharmacokinet. Biopharmacol.* **2**:123–147 (1972).
19. Vicini, S. Pharmacologic significance of the structural heterogeneity of the GABA$_A$ receptor-chloride ion channel complex. *Neuropsychopharmacology* **4**:9–15 (1991).
20. Pritchett, D. B., H. Luddens, and P. H. Seeburg. Type 1 and type 2 GABA$_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
21. Belelli, D., and K. W. Gee. Anticonvulsant profile of the progesterone metabolite 5α-pregnan-3α-ol-20-one. *Eur. J. Pharmacol.* **166**:325–329 (1989).
22. Wieland, S., N. C. Lan, S. Mirsadeghi, and K. W. Gee. Anxiolytic activity of the progesterone metabolite 5α-pregnan-3α-ol-20-one. *Brain Res.* **565**:263–268 (1991).

Send reprint requests to: Kelvin W. Gee, Department of Pharmacology, College of Medicine, University of California, Irvine, CA 92717.