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Steroid inhibition of [^{3}H]SR 95531 binding to the GABA$_A$ recognition site

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Abstract

The interaction of three types of steroids with the GABA$_A$ recognition site labeled by the antagonist ligand [^{3}H]SR 95531 was evaluated in rat brain cortical membranes. The first type is the GABA site antagonist RU 5135, which potently (IC_{50} 7 nM) but also incompletely (I_{max} 82%) displaced [^{3}H]SR 95531. RU 5135 probably binds only to high affinity [^{3}H]SR 95531 sites recognized by GABA and unlabelled SR 95531. The second type are the neuroactive steroids which act as positive allosteric modulators, including 3α-hydroxy-5β-pregnan-20-one (3α,5β-P) and 5β-tetrahydrodeoxycorticosterone (5β-THDOC), which inhibited [^{3}H]SR 95531 binding with limited efficacy (IC_{50} 460 nM and 1.4 μM, I_{max} 41 and 31%, respectively). In contrast, 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) was inactive. The third type are the neurosteroids acting as negative allosteric modulators, such as pregnenolone sulfate, which inhibited [^{3}H]SR 95531 binding with limited efficacy (IC_{50} 10 μM, I_{max} 23%). In the presence of a saturating concentration of pregnenolone sulfate, 3α,5β-P further inhibited [^{3}H]SR 95531 binding suggesting that these two steroids act through different sites or, possibly, at different populations of GABA$_A$ receptors. The allosteric modulation was selective for steroids since benzodiazepines and barbiturates were inactive up to 100 μM. Taken together, these data suggest that 3α,5β-P and 5β-THDOC modulate [^{3}H]SR 95531 binding by interacting with a unique site on the GABA$_A$ receptor complex distinct from the sites for 3α,5α-P, pregnenolone sulfate, GABA, benzodiazepines, and barbiturates.

Keywords: SR 95531; Neuroactive steroid; Neurosteroid; GABA$_A$ receptor; RU 5135; Pregnenolone sulfate; 3α-Hydroxy-5β-pregnan-20-one

1. Introduction

Three types of steroids interact with the GABA$_A$ receptor (Fig. 1). Two of these types are neurosteroids which are synthesized in brain, and include both positive and negative allosteric modulators. Positive modulators, exemplified by 3α-hydroxy-5α-pregnan-20-one (3α,5α-P), potentiate GABA-evoked currents (Peters et al., 1988) and agonist-stimulated $^{36}Cl^-$ uptake (Morrow et al., 1990). They also allosterically modulate the binding of the GABA recognition site agonist [^{3}H]muscimol, the benzodiazepine agonist [^{3}H]flunitrazepam, and the chloride channel ligand [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) (Gee et al., 1988; Goodnough and Hawkinson, 1995; Hawkinson et al., 1994a,b). These steroids are thought to mediate their effects by binding to a unique site on the GABA$_A$ receptor complex (Gee et al., 1988; Peters et al., 1988). A second type is comprised of the negative modulators dehydroepiandrosterone sulfate (Demirgoren et al., 1991) and pregnenolone sulfate (Mienville and Vicini, 1989) which antagonize GABA-evoked currents, although their exact site of action has not been determined. Binding studies indicate that pregnenolone sulfate and the positive steroidal modulators do not share a common site of action (Gee et al., 1989). The third type of steroid interacting with the GABA$_A$ receptor is the synthetic amidine steroid RU 5135, which acts primarily as a potent GABA site antagonist (Cadoni and Gee, 1992).

The pyridazinyl GABA derivative SR 95531 (gabazine) is a competitive GABA$_A$ receptor antagonist (Hamann et al., 1988) and [^{3}H]SR 95531 is thought to label the GABA recognition site with the receptor in an antagonist conformation (Heaulme et al., 1987). The interaction of steroids

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with the GABA agonist radioligand [^{3}H]muscimol has previously been evaluated (Goodnough and Hawkinson, 1995). In the present study, the interaction of steroids with [^{3}H]SR 95531 binding to fresh rat brain cortical membranes is evaluated and compared to other GABAergic modulators.

2. Materials and methods

2.1. Compounds

[^{3}H]SR 95531 (48.5–51 Ci/mmol) was obtained from NEN and unlabeled SR 95531 was from RBI. GABA, dehydroepiandrosterone sulfate, pregnenolone sulfate, pentobarbital, and phenobarbital were from Sigma. 3α,5β-P was purchased from Diosynth. RU 5135 was a gift from Roussel-UCLAF and methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) was a gift from Ciba-Geigy. 3α,5α-P was synthesized at CoCensys.

2.2. [^{3}H]SR 95531 binding assay

[^{3}H]SR 95531 binding was examined in fresh rat brain cortical membranes using the method of Goodnough and Hawkinson (1995). Briefly, cortices from male Sprague-Dawley rats were homogenized in 15 vols. of 0.32 M sucrose and centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged for 20 min at $8000 \times g$. The supernatant and white buffy layer were collected and centrifuged at $250\,000 \times g$ for 20 min. The pellet was washed once with water and once with binding buffer (100 mM KCl/40 mM KH_2PO_4, pH 7.4) by centrifugation at $250\,000 \times g$ for 20 min. The washed pellet was resuspended in 35 ml binding buffer, incubated at 37°C for 30 min, and centrifuged at $31\,000 \times g$ for 20 min. The final pellet was resuspended in 10 vols. binding buffer (~ 2 mg protein/ml). Aliquots of membrane suspension (100 μl) were incubated with 5 nM [^{3}H]SR 95531 and 5 μl dimethylsulfoxide (DMSO) or compound dissolved in DMSO in a final volume of 1.0 ml binding buffer. Non-specific binding was defined using 100 μM unlabeled SR 95531 and ranged from 8 to 12% of total binding. Following incubation for 60 min at 4°C, the incubations were filtered over glass fiber filters, rinsed with 3 × 3 ml ice-cold buffer, and filter-bound radioactivity was determined by liquid scintillation spectrometry. Protein was measured with a modified Lowry assay (Peterson, 1977).

2.3. Data analysis

Non-linear curve fitting of the concentration-effect curves was done using Prism (GraphPad). The data were fit to one and two component models. The two component model was chosen if the sum of squares for this fit was significantly lower than for the one component fit as determined by F test. The data were fit to a partial (bottom plateau > 0) instead of a full (bottom plateau = 0) inhibition model if the sum of squares was significantly lower. The IC_{50} is the concentration of test compound producing

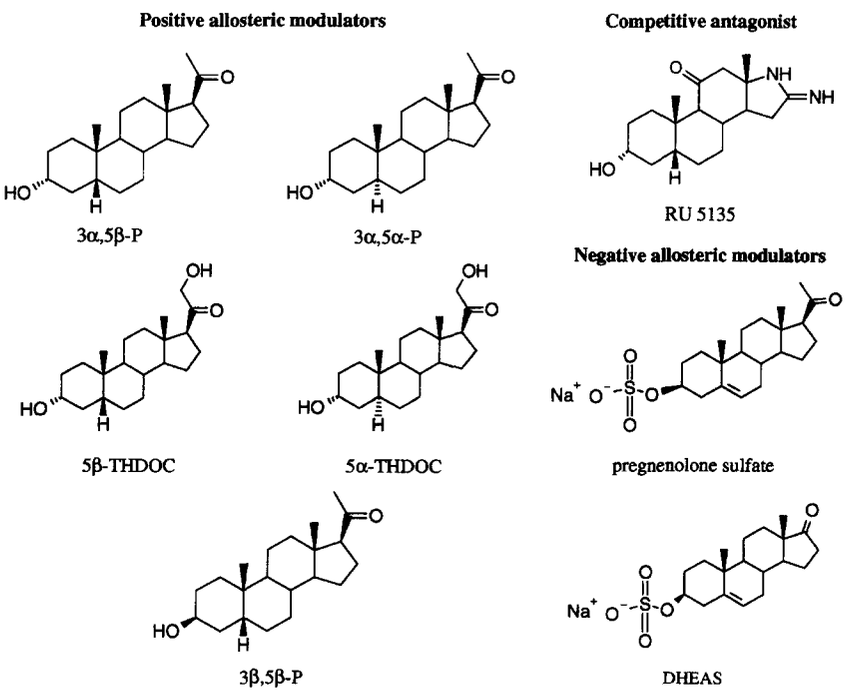

Fig. 1. Structures of steroids acting at the $GABA_A$ receptor. The steroids have three types of action: (1) positive allosteric modulators (3α,5β-P, 3α,5α-P, 5β-THDOC, 5α-THDOC); (2) competitive receptor antagonists (RU 5135); and (3) negative allosteric modulators (pregnenolone sulfate, dehydroepiandrosterone sulfate). 3β,5β-P is inactive at $GABA_A$ receptors.

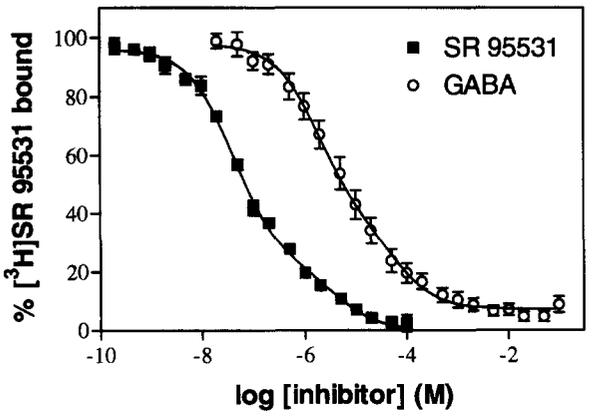

Fig. 2. Two component displacement of [^{3}H]SR 95531 binding by unlabeled SR 95531 and GABA in rat brain cortical membranes. Unlabeled SR 95531 completely displaces specific [^{3}H]SR 95531 binding with high- (43 nM, 76%) and low-affinity (3.9 μM, 24%) components. GABA almost completely displaces specific [^{3}H]SR 95531 binding with high- (1.9 μM, 60%) and low-affinity (58 μM, 31%) components. The symbols represent the mean ± S.E.M. of at least three independent experiments. Affinities and proportions are calculated from the overall data averaged across concentrations.

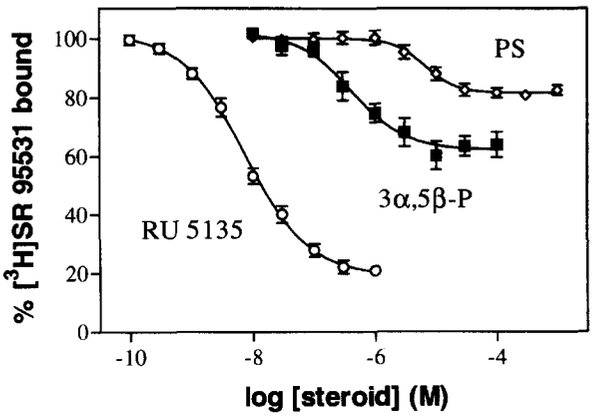

Fig. 3. Limited efficacy inhibition of [^{3}H]SR 95531 binding by RU 5135 (○), 3α,5β-P (■) and pregnenolone sulfate (◇) in rat brain cortical membranes. IC_{50}, I_{max} and Hill values are given in Table 1. The symbols represent the mean ± S.E.M. from three to five independent experiments.

50% inhibition of specific binding. The maximal extent of inhibition (I_{max}) is defined as the difference between the top and bottom plateaus.

3. Results

3.1. SR 95531 and GABA are two component inhibitors of [^{3}H]SR 95531 binding

Unlabeled SR 95531 displaced [^{3}H]SR 95531 binding with high affinity (IC_{50} 43 nM, 76%) and low affinity (IC_{50} 3.9 μM, 24%) components (Fig. 2). Although considerably less potent, GABA also displaced [^{3}H]SR 95531 binding with high affinity (IC_{50} 1.9 μM, 60%) and low affinity (IC_{50} 58 μM, 31%) components (Fig. 2). Unlabeled SR 95531 completely and GABA almost completely displaced [^{3}H]SR 95531 binding.

3.2. Three types of steroids inhibit [^{3}H]SR 95531 binding

The GABA site competitive antagonist RU 5135 potently inhibited [^{3}H]SR 95531 binding with an IC_{50} of 7.4 nM (Table 1, Fig. 3). However, RU 5135 incompletely inhibited [^{3}H]SR 95531 binding with an I_{max} of 82%. Four neuroactive steroids which are positive allosteric modulators of the $GABA_A$ receptor were evaluated for activity in the [^{3}H]SR 95531 binding assay. The progesterone metabolite 3α-hydroxy-5β-pregnan-20-one (3α,5β-P) was the most potent and efficacious neuroactive steroid tested (IC_{50} 460 nM, I_{max} 31%) (Table 1, Fig. 3). However, the 5α-reduced isomer 3α,5α-P was inactive (Table 2). The deoxycorticosterone metabolite 5β-tetrahydrodeoxycorticosterone (5β-THDOC) was less potent and efficacious

Table 1
Potencies and efficacies of steroids which inhibit [^{3}H]SR 95531 binding to rat cortical membranes

Compound	IC_{50} (μM)	I_{max} (%)
RU 5135	0.0074 ± 0.0009	82 ± 1
3α,5β-P	0.46 ± 0.11	41 ± 4
5β-THDOC	1.4 ± 0.1	31 ± 3
5α-THDOC	2.9 ± 1.4	11 ± 1
Pregnenolone sulfate	10 ± 4	23 ± 2

Inhibition of 5 nM [^{3}H]SR 95531 binding was determined in fresh rat cortical membranes in the presence of 9 concentrations of test compound ranging up to 10^{-6} M (RU 5135), 10^{-4} M (3α,5β-P, 5β-THDOC, 5α-THDOC) or 10^{-3} M (pregnenolone sulfate). Values are means ± S.E.M. of at least three independent experiments. Hill numbers were not allowed to vary from 1.0 unless a variable Hill number significantly improved the fit of the data ($P < 0.05$). Hill values were significantly different from 1.0 for RU 5135 (0.83 ± 0.02) and pregnenolone sulfate (1.22 ± 0.09).

Table 2
Effect of GABAergic modulators on [^{3}H]SR 95531 binding to rat cortical membranes

Compound	% inhibition at 100 μM
Dehydroepiandrosterone sulfate	0.3 [a]
3β,5β-P	0.5 ± 1.8
3α,5α-P	7 ± 2
Diazepam	11 ± 1
DMCM	26 ± 7
Pentobarbital	−2 ± 2 [b]
Phenobarbital	3 ± 1

The effect of GABAergic modulators on 5 nM [^{3}H]SR 95531 binding was determined in fresh rat cortical membranes in the presence of 9 concentrations of test compound ranging up to 10^{-4} M (3α,5α-P, 3β,5β-P, diazepam, DMCM, phenobarbital), 10^{-3} M (dehydroepiandrosterone sulfate) or 10^{-1} M (pentobarbital). Values are means ± S.E.M. of at least three independent experiments. [a] Mean of two determinations. [b] Due to its water solubility, sufficiently high concentrations of pentobarbital were achieved to calculate IC_{50} and I_{max} values of 15 ± 1 mM and 97 ± 2%, respectively.

than 3α,5β-P, whereas the 5α isomer 5α-THDOC inhibited [^{3}H]SR 95531 binding with only 11% efficacy (Table 1, Fig. 3). 3β-Hydroxy-5β-pregnan-20-one (3β,5β-P), which is inactive as a positive modulator of the $GABA_A$ receptor (Woodward et al., 1992), was also inactive in the [^{3}H]SR 95531 binding assay. The negative allosteric modulator pregnenolone sulfate inhibited [^{3}H]SR 95531 binding with an IC_{50} of 10 μM and an I_{max} of 23% (Table 1, Fig. 3), however the related sulfate ester dehydroepiandrosterone sulfate was inactive.

3.3. 3α,5β-P and pregnenolone sulfate inhibit [^{3}H]SR 95531 binding via distinct binding sites

In the presence of a saturating concentration of pregnenolone sulfate (300 μM), 3α,5β-P inhibited [^{3}H]SR 95531 binding to a greater extent than in the control condition suggesting that the inhibition is mediated via distinct sites on the receptor complex or at different populations of receptors labeled by [^{3}H]SR 95531 (Fig. 4A). In contrast, 3α,5β-P does not further inhibit [^{3}H]SR 95531 binding in the presence of a saturating concentration of 5β-THDOC suggesting that the inhibition is mediated via the same site (Fig. 4B).

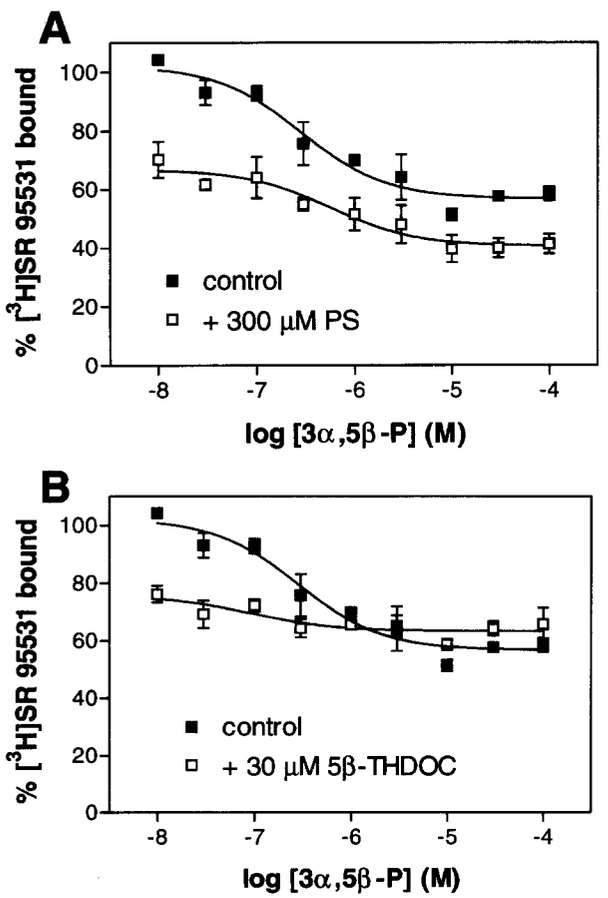

Fig. 4. Interaction of 3α,5β-P with pregnenolone sulfate and 5β-THDOC as inhibitors of [^{3}H]SR 95531 binding in rat brain cortical membranes. (A) Inhibition of [^{3}H]SR 95531 binding by 3α,5β-P in the absence (■) and presence of 300 μM pregnenolone sulfate (□). (B) Inhibition of [^{3}H]SR 95531 binding by 3α,5β-P in the absence (■) and presence of 30 μM 5β-THDOC (□). The symbols represent the mean ± S.E.M. of three independent experiments.

3.4. Benzodiazepines and barbiturates have limited effect on [^{3}H]SR 95531 binding

The benzodiazepine agonist diazepam and inverse agonist DMCM and the barbiturates pentobarbital and phenobarbital had very low potency for inhibiting [^{3}H]SR 95531 binding with IC_{50}s > 100 μM (Table 2). Pentobarbital was evaluated at higher concentrations due to its relatively high solubility in buffer, allowing the estimation of an IC_{50} value of 15 ± 1 mM and an I_{max} of 97 ± 2%.

4. Discussion

Prior to the evaluation of GABAergic modulators, the displacement of [^{3}H]SR 95531 by GABA and unlabeled SR 95531 was examined in rat brain membranes. Unlabeled SR 95531 displaces [^{3}H]SR 95531 binding in a two component manner, consistent with the two component Scatchard plots (Heaulme et al., 1987; Ito et al., 1992) and biphasic dissociation (Maksay, 1988) reported for [^{3}H]SR 95531. GABA also displaces [^{3}H]SR 95531 with two components and similar proportions of high and low affinity components as unlabeled SR 95531. These data suggest that the two components represent two populations of receptors which have similar relative affinities and densities for agonists (GABA) and antagonists (SR 95531). Alternatively, the two component displacement may represent two affinity states of a single population of binding sites which do not discriminate agonists and antagonists.

The competitive GABA receptor antagonist RU 5135 (Cadoni and Gee, 1992) potently inhibits [^{3}H]SR 95531 binding with a maximal displacement of 82%. The incomplete inhibition of [^{3}H]SR 95531 binding by RU 5135 suggests that a proportion (~ 20%) of the [^{3}H]SR 95531 binding detected in rat cortical membranes is to components other than the $GABA_A$ receptor. In this regard, Luque et al. (1994) have provided autoradiographic evidence that SR 95531 is a substrate inhibitor of monoamine oxidase type A in the locus coeruleus. On autoradiograms, the selective monoamine oxidase-A inhibitor clorgyline (10 nM) was found to inhibit [^{3}H]SR 95531 binding by 26% in the locus coeruleus. However, in fresh rat cortical membranes, clorgyline and pargyline inhibited [^{3}H]SR 95531 binding less than 5% at concentrations up to 10 μM (data not shown). In addition, unlabeled SR 95531 retained two component inhibition and RU 5135 did not completely inhibit [^{3}H]SR 95531 binding in the presence of 10 μM clorgyline (data not shown). These findings indicate that the high and low affinity [^{3}H]SR 95531 binding sites are unrelated to monoamine oxidase-A in this membrane

preparation. Moreover, the nearly complete inhibition by GABA indicates that > 90% of [^{3}H]SR 95531 binding in this membrane preparation is to GABA receptors. Thus, the limited efficacy inhibition by RU 5135 can best be explained if this steroid binds only to high affinity [^{3}H]SR 95531 sites and is essentially inactive at low affinity [^{3}H]SR 95531 sites.

The neuroactive steroid 3α,5β-P inhibits [^{3}H]SR 95531 binding with limited efficacy. The 3β-hydroxy epimer 3β,5β-P is inactive, indicating that the effect of 3α,5β-P is stereospecific. 3β,5β-P is also inactive as a positive allosteric modulator (Woodward et al., 1992). 3α,5α-P, the 5α isomer of 3α,5β-P, is also inactive indicating that only certain steroids which are positive allosteric modulators of the $GABA_A$ receptor are inhibitors of [^{3}H]SR 95531 binding. Other neuroactive steroids which inhibited [^{3}H]SR 95531 binding include 5β-THDOC, the 21-hydroxy analog of 3α,5β-P, and 5α-THDOC, although this steroid had very low efficacy (11%). The negative allosteric modulator pregnenolone sulfate inhibited [^{3}H]SR 95531 binding with limited efficacy, whereas the related sulfate ester neurosteroid dehydroepiandrosterone sulfate was inactive.

In an attempt to clarify the sites of action of steroid modulators, concentration-effect curves for 3α,5β-P were performed in the presence of saturating concentrations of pregnenolone sulfate or 5β-THDOC. In the presence of pregnenolone sulfate, 3α,5β-P inhibited [^{3}H]SR 95531 binding to a greater extent than 3α,5β-P alone suggesting that these steroids act via different sites on the receptor complex. Alternatively, pregnenolone sulfate and 3α,5β-P may bind to different populations of $GABA_A$ receptors labeled by [^{3}H]SR 95531. In contrast, 3α,5β-P in the presence of 5β-THDOC did not inhibit [^{3}H]SR 95531 binding to a greater extent than 3α,5β-P alone indicating that these steroids bind to identical sites. Further attempts to clarify the interaction between 3α,5β-P and [^{3}H]SR 95531 were unsuccessful since: (1) 3α,5β-P (10 μM) did not eliminate the two component inhibition of [^{3}H]SR 95531 by unlabeled SR 95531; (2) RU 5135 did not completely displace [^{3}H]SR 95531 binding in the presence of 10 μM 3α,5β-P; and (3) clorgyline (10 μM) had no effect on 3α,5β-P inhibition of [^{3}H]SR 95531 binding (data not shown).

The relatively low potency and low efficacy inhibition of [^{3}H]SR 95531 binding by 3α,5β-P is reminiscent of the low affinity component of 3α,5β-P modulation of [^{35}S]TBPS, [^{3}H]flunitrazepam, and [^{3}H]muscimol binding to the chloride channel, benzodiazepine and GABA recognition sites, respectively (Hawkinson et al., 1994a; Goodnough and Hawkinson, 1995). Furthermore, 3α,5α-P inhibits [^{35}S]TBPS and [^{3}H]flunitrazepam binding with a single component (Hawkinson et al., 1994a) and does not modulate [^{3}H]SR 95531 binding. These findings suggest that [^{3}H]SR 95531 may detect only low affinity modulation of the $GABA_A$ receptor by 3α,5β-P. However, 5β-THDOC, which inhibits [^{3}H]SR 95531 binding, is a limited efficacy inhibitor of [^{35}S]TBPS binding (Hawkinson et al., 1994b; Gee and Lan, 1991), i.e. it has no detectable low affinity component in these assays. Thus, the relationship between the low affinity modulation of [^{35}S]TBPS, [^{3}H]flunitrazepam, and [^{3}H]muscimol binding by 3α,5β-P and its inhibition of [^{3}H]SR 95531 binding remains unclear.

The allosteric modulation of [^{3}H]SR 95531 binding by 3α,5β-P and pregnenolone sulfate suggests that SR 95531 has negative efficacy rather than simply blocking the binding of GABA to its recognition site. The allosteric enhancement of [^{3}H]muscimol binding by 3α,5β-P (Goodnough and Hawkinson, 1995) and pregnenolone sulfate (Majewska et al., 1985) is consistent with their allosteric inhibition of [^{3}H]SR 95531 binding since muscimol (agonist) and SR 95331 (antagonist) have opposite pharmacological profiles. Thus, the inhibition of [^{3}H]SR 95531 binding by 3α,5β-P and pregnenolone sulfate implies that SR 95531 has efficacy at the GABA recognition site and that it shifts the receptor into an antagonist conformation.

In conclusion, certain steroids that are positive allosteric modulators of $GABA_A$ receptors, including 3α,5β-P and 5β-THDOC, inhibit [^{3}H]SR 95531 binding with limited efficacy, although other steroids of this type, e.g. 3α,5α-P, are inactive. The negative allosteric modulator pregnenolone sulfate also inhibits [^{3}H]SR 95531 binding with limited efficacy. 3α,5β-P interacts with the same site as 5β-THDOC but with a site distinct from that of pregnenolone sulfate to modulate [^{3}H]SR 95531 binding. The allosteric inhibition of binding by these steroids suggest that [^{3}H]SR 95531 has efficacy at the GABA recognition site. Benzodiazepine receptor ligands (diazepam, DMCM) and barbiturates (pentobarbital, phenobarbital) are inactive at 100 μM, consistent with previous reports (Heaulme et al., 1987; McCabe et al., 1988). Taken together, these data suggest that 3α,5β-P and 5β-THDOC modulate [^{3}H]SR 95531 binding by interacting with a unique site on the $GABA_A$ receptor distinct from the 3α,5α-P, pregnenolone sulfate, GABA, benzodiazepine, and barbiturate sites.

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