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Characterization of Steroid Interactions with γ-Aminobutyric Acid Receptor-Gated Chloride Ion Channels: Evidence for Multiple Steroid Recognition Sites

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SUMMARY

The potentiation of γ-aminobutyric acid (GABA) receptor-mediated $^{36}Cl^-$ uptake by various steroids has been characterized in rat cerebral cortical synaptoneurosomes. Several of these steroids, including 3α-hydroxy-5α-pregnan-20-one (3α-OH-DHP) and 3α,21-dihydroxy-5α-pregnan-20-one (THDOC), increase the potency of muscimol to stimulate $^{36}Cl^-$ uptake in a concentration-dependent and stereospecific manner. Concentration-response curves for 3α-OH-DHP, THDOC, 3α-hydroxy-pregn-4-en-20-one, and pentobarbital enhancement of muscimol-stimulated $^{36}Cl^-$ uptake are biphasic, with Hill coefficients significantly less than 1.0. Computer-modeling (ALLFIT analysis) of these curves suggests that these steroids and pentobarbital interact with multiple binding sites on $GABA_A$ receptor(s). In contrast, the concentration-response curve for THDOC 21-mesylate is monophasic, with a smaller maximal response, and yields a Hill coefficients of 1.0. In addition to modulating GABA receptor-mediated $^{36}Cl^-$ uptake, THDOC enhanced the ability of the benzodiazepine clonazepam to potentiate muscimol-stimulated $^{36}Cl^-$ uptake. The central benzodiazepine antagonist Ro15-1788 failed to inhibit THDOC-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake, although it has been previously reported to inhibit some of the behavioral actions of THDOC. In contrast to the A ring-reduced metabolites and analogues of progesterone and deoxycorticosterone, glucocorticoids had no effect on muscimol-stimulated $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes at concentrations between 20 nM and 5 μM.

Several major metabolites of progesterone and deoxycorticosterone, including 3α-OH-DHP (allopregnanolone) and THDOC (tetrahydrodeoxycorticosterone) have been shown to interact with the $GABA_A$ receptor chloride channel complex in the rat central nervous system (1–6). In the presence of low concentrations of GABA, these steroids compete with [^{35}S]TBPS binding to the $GABA_A$ receptor complex with very high affinity (K_i values in the low nanomolar range) (7) and with somewhat lower affinity for [^{35}S]TBPS binding in the absence of GABA (K_i = 200–500 nM) (1, 7). Allopregnanolone and THDOC also enhance benzodiazepine receptor binding *in vitro* in a manner similar to barbiturates (1), suggesting that these steroids bind to sites on the $GABA_A$ receptor complex that interact allosterically with benzodiazepine as well as GABA recognition sites. In electrophysiological experiments, both allopregnanolone and THDOC have been shown to augment GABA receptor-mediated chloride ion conductance in cultured rat hippocampal neurons (1). Recently, we have demonstrated that both allopregnanolone and THDOC enhance GABA receptor-mediated chloride ion uptake into rat cerebral cortical synaptoneurosomes at concentrations as low as 25–1000 nM (4, 8).

In behavioral studies, THDOC posesses anti-conflict (9) and hypnotic effects when administered parenterally to rats (10), as well as anti-aggressive properties in mice (11). Allopregnanolone has been shown to produce analgesic effects in mice when administered intracerebroventricularly (12). Because allopregnanolone and THDOC are endogenous steroid metabolites (13, 14) and potently modulate GABA receptor-mediated inhibitory neurotransmission in the central nervous system, we have proposed that these steroids may be naturally occurring anxiolytic and/or hypnotic agents (1, 4, 8). The importance of understanding the pharmacological properties and functional interactions of these steroid metabolites with the GABA receptor complex is supported by evidence that levels of progesterone and deoxycorticosterone and their metabolites are altered in various physiological states, including stress (14–18).

Recent studies on steroid and barbiturate binding to the [^{35}S]TBPS recognition site on the $GABA_A$ receptor complex have suggested that steroids and barbiturates interact with distinct binding sites labeled by [^{35}S]TBPS (3, 5, 19). To further delineate the nature of steroid interactions with the GABA receptor complex, we have characterized the ability of various steroid

ABBREVIATIONS: 3α-OH-DHP, 3α-hydroxy-5α-pregnan-20-one; THDOC, 3α,21-dihydroxy-5α-pregnan-20-one; GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; 3α-OH-P, 3α-hydroxypregn-4-en-20-one.

derivatives to potentiate GABA receptor-mediated $^{36}Cl^-$ uptake into a subcellular vesicle preparation (the synaptoneurosome) from rat cerebral cortex. Several of these steroids enhance the potency of muscimol in stimulating $^{36}Cl^-$ uptake in a concentration-dependent and stereospecific manner. Structure activity experiments demonstrate that a 3α-configuration, in either the 5α- or the 5β-reduced metabolites, is essential for pharmacological activity (8, 20). Computer modeling of the concentration-response curves for steroid-enhanced muscimol-stimulated $^{36}Cl^-$ uptake suggests that the endogenous steroid metabolites interact with multiple binding sites or conformational states. Pseudo-Hill coefficients for steroid potentiation of muscimol-stimulated $^{36}Cl^-$ uptake ranged from 0.5 to 0.7, further suggesting heterogeneous receptor interactions with the GABA receptor complex by these steroids. Moreover, THDOC 21-mesylate appears to interact selectively with one of the steroid binding sites, producing a smaller maximal response, monophasic concentration-response curves, and a Hill slope approximating 1.0. These data suggest the presence of multiple distinct steroid recognition sites or conformational states on $GABA_A$ receptor(s).

Materials and Methods

Preparation of synaptoneurosomes. The ability of various steroids to potentiate the effects of the GABA agonist muscimol were studied in a subcellular vesicle preparation, the synaptoneurosome. This preparation contains pre- and postsynaptic vesicles, including intact synapses between vesicles, and is essentially devoid of whole cells (21, 22). Synaptoneurosomes were prepared as previously described (23). Briefly, cerebral cortices from adult male Sprague-Dawley rats were dissected free from white matter, placed in ice-cold HEPES-Tris buffer (HEPES, 20 mM; NaCl, 118 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; D-glucose, 10 mM, pH 7.4 at 25°), and homogenized by hand (7–10 strokes) using a glass-glass homogenizer. The homogenate was diluted to 30 ml/g of tissue and filtered through three layers of nylon mesh (160μm) and subsequently through Millipore filters (10-μm) using Millipore Swinex filter holders. The filtrate was then washed twice by centrifugation (100 × g for 15 min) and the final pellet was suspended in buffer to a final protein concentration of approximately 10 mg/ml.

Measurement of $^{36}Cl^-$ uptake. $^{36}Cl^-$ was measured as previously described by Morrow and Paul (24) using a modification of the method of Schwartz *et al.* (23). Synaptoneurosomes (1.0 mg of protein/tube) were preincubated for 20 min in HEPES-Tris buffer at 30°. Drugs or vehicle and 0.5 μCi of $^{36}Cl^-$ were added simultaneously and uptake was terminated after 5 sec by the addition of 5 ml of ice-cold buffer containing picrotoxin (100 μM). Synaptoneurosomes were recovered by filtration under vacuum onto glass fiber filters (Schleicher & Schuell No. 30). The filters were washed twice with 5 ml of ice-cold buffer and counted by liquid scintillation counting in 5 ml of RediSolv (Beckman Instruments, Fullerton, CA) at an efficiency of >95%. Protein determinations were made using the method of Lowry *et al.* (25).

Materials. All of the steroids tested were synthesized by R. H. Purdy (20) and the purity of the steroids was established by high pressure liquid chromatography. Allopregnanediol was purchased from Steraloids (Wilton, NH). Steroids were dissolved in 100% dimethyl sulfoxide and diluted in assay buffer to a final dimethyl sulfoxide concentration of <0.5%. Glucocorticoids and mineralcorticoids were purchased from Sigma Chemical Co. (St. Louis, MO). $^{36}Cl^-$ (specific activity, 12–15 mCi/g) was purchased from New England Nuclear (Boston, MA).

Data analysis. Data are expressed as net $^{36}Cl^-$ uptake or potentiation in nmol/mg of protein. Net uptake represents total uptake in the presence of drugs minus uptake in the absence of drug (basal). Potentiation represents the increase in $^{36}Cl^-$ uptake over and above, but not including, that produced by the GABA agonist muscimol. In some cases, steroids produced a direct effect on $^{36}Cl^-$ uptake at high concentrations. If direct steroid effects were detected then the amount of directly stimulated $^{36}Cl^-$ uptake was subtracted from the value determined for steroid potentiation. This calculation assured that potential direct effects of steroids on $^{36}Cl^-$ uptake did not contribute to the analysis of their allosteric effects. Concentration-response curves were analyzed using ALLFIT, a weighted, nonlinear curve-fitting program based on a four-parameter logistic equation to describe sigmoidal curves (26). The curve that produced the smallest residual variance between the actual data and the logistic equation was used to estimate the parameters of E_{max} and EC_{50}. Pseudo-Hill coefficients (n_H) were calculated according to the equation:

$$\log \frac{E}{E_{max} - E} = n_H \log A - \log K$$

where E = potentiation of muscimol-stimulated $^{36}Cl^-$ uptake, E_{max} = the maximal potentiation, and A = the concentration of steroid.

Results

We have previously reported that allopregnanolone significantly enhances muscimol-stimulated $^{36}Cl^-$ uptake at concentrations as low as 20 nM (4). The concentration-response curve for allopregnanolone was shallow and biphasic, suggesting a complex interaction of this steroid with the GABA receptor complex. The potentiation of muscimol-stimulated $^{36}Cl^-$ uptake by THDOC exhibits a similar biphasic concentration-response relationship (Fig. 1). Using ALLFIT, the data for potentiation of $^{36}Cl^-$ uptake by both steroids do not fit a model of a single receptor binding site interaction. Pseudo-Hill coefficients for THDOC, as well as allopregnanolone and 3α-OH-P are significantly less than 1.0 (Table 1). In contrast, the concentration-

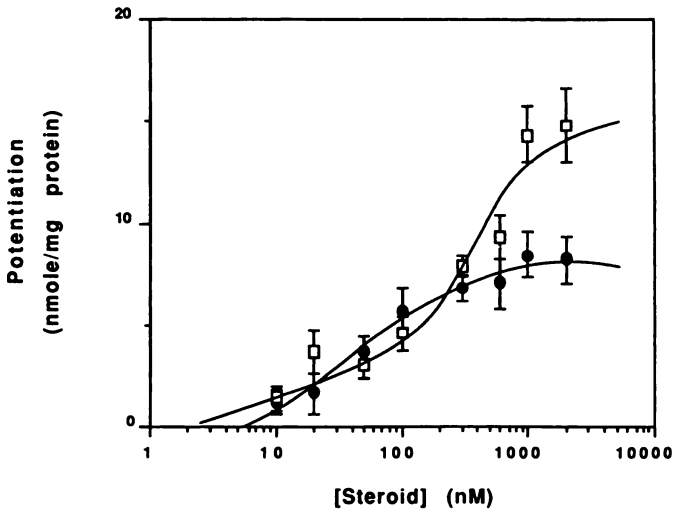

Fig. 1. Concentration-response curves for THDOC (□)- and THDOC 21-mesylate (●)-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in rat cerebral cortical synaptoneurosomes. Increasing concentrations of steroid (10 nM to 2 μM) were added simultaneously with muscimol (3 μM) and uptake was measured for 5 sec. Potentiation represents the increase in $^{36}Cl^-$ uptake over that produced by muscimol alone. In four experiments, THDOC and THDOC 21-mesylate concentration curves were conducted simultaneously in the same tissue preparation. The E_{max} for THDOC potentiation was significantly greater than the E_{max} for THDOC 21-mesylate ($p < 0.001$; Student's t test). Data shown are the mean ± standard error of five to eight separate experiments, each conducted in quadruplicate.

response curve for the 21-mesylate derivative of THDOC is steep and monophasic and exhibits a pseudo-Hill coefficient close to 1.0 (Fig. 1 and Table 1). The data for THDOC 21-mesylate-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake fit well to a single-site model using ALLFIT. The E_{max} for potentiation by THDOC 21-mesylate is approximately 60–70% of that produced by allopregnanolone or THDOC itself (Table 1). Pentobarbital enhances muscimol-stimulated $^{36}Cl^-$ uptake with a similar biphasic concentration-response curve (4) Pseudo-Hill coefficients for pentobarbital-induced enhancement are less than 1.0 and pentobarbital is considerably less potent than the endogenous steroids (Table 1).

Allopregnanolone enhances the potency of the GABA agonist muscimol to stimulate $^{36}Cl^-$ uptake. Fig. 2 shows that allopregnanolone (500 nM) shifts the concentration-response curve for muscimol stimulation of $^{36}Cl^-$ uptake to the left, decreasing the EC_{50} from 3.0 to 1.0 μM, with no effect on the maximal stimulation. Thus, the potency of muscimol in the presence of allopregnanolone (500 nM) is increased approximately 3-fold.

The ability of steroid hormone metabolites to potentiate GABA receptor-mediated chloride ion flux is stereospecific for the 3α-hydroxyl group. Concentration-response curves for 3α-OH-P [a progesterone metabolite produced by Sertoli cells (26)] and its 3β-OH isomer are shown in Fig. 3. The 3β-OH isomer of this steroid is inactive at concentrations up to 5 μM, whereas the 3α-OH compound significantly enhances muscimol-stimulated $^{36}Cl^-$ uptake at concentrations as low as 30 nM. Similar stereospecificity for the 3α- versus the 3β-configurations of THDOC and 3α-OH-DHP has also been observed for muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes.

Table 2 compares the activity of several steroid derivatives in potentiating GABA receptor-mediated $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes. All of the steroids were tested at 1 μM, a concentration that produces the maximal response by THDOC and allopregnanolone Figs. 1. Several of the steroids tested were significantly less efficacious as allosteric modulators than the prototypic steroids THDOC and allopreg-

TABLE 1

Concentration-response curves generated for steroid hormone- or pentobarbital-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake into rat cerebral cortical synaptoneurosomes

Steroids (10 nM to 1 μM) and muscimol (3 μM) were added simultaneously and $^{36}Cl^-$ uptake was terminated after 5 sec. 3α-OH-5α-androstane-17β-carbonitrile was also assayed at 2 and 5 μM. Pentobarbital was tested at nine concentrations from 1 to 100 μM. There was no significant direct effect of any of these steroids (1 μM) in the absence of muscimol. Data were analyzed as described in Materials and Methods. The parameters for THDOC 21-mesylate and 3α-OH-5α-androstane-17β-carbonitrile were estimated by ALLFIT analysis. The data for the remaining steroids did not produce a statistically valid fit using the ALLFIT analysis. Therefore, the E_{max} was estimated from the concentration-response curve and the EC_{50} was estimated by log-probit analysis. Each value represents the mean of three to eight independent determinations.

Steroid	E_{max}	EC_{50}	Hill slope (n_H)
	nmol/mg of protein	*nM*	
THDOC 21-mesylate	8.2	52	1.0
Allopregnanolone	10.9	110	0.56
THDOC	14.2	344	0.67
3α-OH-P	11.3	430	0.76
3α-OH-5α-androstane-17β-carbonitrile	9.1	833	2.2
Pentobarbital	12.9	20,000	0.70

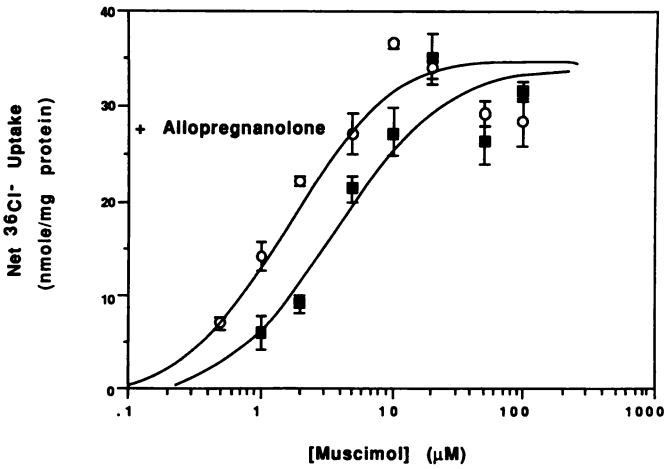

Fig. 2. Allopregnanolone shifts the muscimol concentration-response curve to the left, decreasing the EC_{50} but not the E_{max} of muscimol-stimulated $^{36}Cl^-$ uptake. Muscimol (1–100 μM) and allopregnanolone (500 nM) or vehicle (0.1% dimethyl sulfoxide) were added simultaneously. Uptake was terminated after 5 sec. In the presence of allopregnanolone, the EC_{50} for muscimol-stimulated $^{36}Cl^-$ uptake was reduced from 2.6 to 0.9 μM (65%, $p < 0.05$; Student's t test). Data are from a representative experiment conducted in quadruplicate and repeated three times.

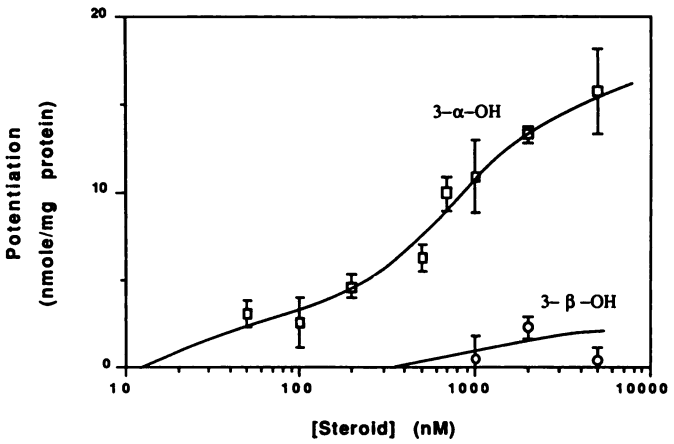

Fig. 3. Stereospecificity of 3α-OH-P-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake. Concentration-response curves for 3α-OH-P (20–1000 nM) potentiation of muscimol-stimulated $^{36}Cl^-$ uptake were conducted. Steroid and muscimol (3 μM) were added simultaneously and incubated for 5 sec. 3β-OH-P is inactive at all concentrations tested. Data shown are from a representative experiment carried out in quadruplicate and repeated twice.

TABLE 2

Potentiation of muscimol-stimulated $^{36}Cl^-$ uptake by pregnane steroids in rat cerebral cortical synaptoneurosomes

Steroid (1 μM) and muscimol (3 μM) were added simultaneously and uptake was conducted for 5 sec. Potentiation represents the increase in $^{36}Cl^-$ uptake over that produced by muscimol alone. Allopregnandiol did not significantly enhance muscimol-stimulated $^{36}Cl^-$ uptake. Data represent the mean of 5–10 independent determinations.

Steroid (1 μM)	Potentiation	Increase
	nmol/mg of protein	%
THDOC	13.1 ± 1.1	119
3α-Hydroxy-5α-androstane-17β-carbonitrile	11.0 ± 2.6	118
THDOC 21-acetate	12.2 ± 1.1	111
Allopregnanolone	10.9 ± 2.2	99
3α-OH-P	10.4 ± 1.0	94
5β-THDOC 21-acetate	10.3 ± 0.6	93
THDOC 21-mesylate	7.9 ± 0.7*	72
5β-THDOC	7.7 ± 0.2*	70
3α-Hydroxy-5α-androstan-17-one	6.9 ± 2.3*	63
3α,20α-Dihydroxy-5α-pregnane	1.1 ± 1.2*	10

* Significantly less effective than THDOC (analysis of variance, $p < 0.05$; Dunnett's t test, $p < 0.05$.

nanolone (Table 2). In order to determine whether any of these steroids behave as partial agonists, we tested whether the ability of THDOC to enhance muscimol-stimulated $^{36}Cl^-$ uptake was inhibited by a high concentration of each of these steroids. Table 3 illustrates that maximal concentrations of THDOC 21-mesylate and 3α-hydroxy-5α-androstane-17β-carbonitrile had no inhibitory effect on the maximal potentiation produced by THDOC, indicating that these steroids are not partial agonists. In addition, 3α,21-dihydroxy-5β-pregnan-20-one (5 μM) and 3α,20α-dihydroxy-5α-pregnane (allopregnanediol) (5 μM) showed no significant inhibition of the THDOC (2 μM)-induced potentiation, suggesting that these steroids also lack partial agonist activity, despite their reduced efficacy in potentiating muscimol stimulation at a concentration of 1 μM. Further studies are required to determine whether these steroids interact selectively with a single population of steroid recognition sites.

Modification of the C ring by incorporating a carbonitrile group of the C_{17} position significantly increases the activity of 3α-hydroxy-5α-androstane-17-one. This is a novel modification, which has not previously been shown to have GABA receptor activity by radioligand binding or electrophysiological techniques. 3α-Hydroxy-5α-androstane-17β-carbonitrile enhances GABA-mediated Cl^- flux, producing a very steep concentration-response curve (pseudo-Hill slope = 2.2) (Table 1) and a maximal response of 8.78 nmol/mg of protein. Fig. 4 illustrates concentration-response curves for 3α-hydroxy-5α-androstane-17β-carbonitrile and its 17α-derivative. The 17α-configuration is inactive at concentrations up to 2 μM, whereas the 17β-carbonitrile is active (EC_{50} = 833 nM).

Glucocorticoids have been reported to have antagonist-like interactions with [^{35}S]TBPS binding sites in cerebral cortex (28). In contrast, cortisol has been shown to potentiate GABA-mediated contraction in the ileum with extremely high potency (pM) and to inhibit at higher concentrations (0.1–1.0 μM) (29). In cerebral cortical synaptoneurosomes, both corticosterone and cortisol had no effect on muscimol-stimulated $^{36}Cl^-$ uptake at concentrations between 20 nM and 5 μM. Table 4 illustrates the effect of muscimol (3 μM) on $^{36}Cl^-$ uptake in the presence of various glucocorticoids, mineralcorticoids, and progesterone. Using $^{36}Cl^-$ flux measurements, there was no evidence for potentiation or inhibition of GABA receptor-mediated chloride channel function by any of these steroids.

Several of the behavioral effects of THDOC have been reported to be inhibited, in part, by the central benzodiazepine receptor antagonist Ro15-1788 (11, 12). Because these steroid hormone metabolites are not believed to interact directly with benzodiazepine binding sites on the GABA receptor complex, we investigated whether Ro15-1788 would inhibit steroid-induced potentiation of GABA receptor-mediated $^{36}Cl^-$ uptake *in vitro*. Ro15-1788 (2 μM) had no effect on THDOC-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake (Fig. 5). This concentration of Ro15-1788 has been shown to block the potentiation of muscimol-stimulated $^{36}Cl^-$ uptake by benzodiazepines (24). In addition, the partial benzodiazepine receptor inverse agonist and ethanol antagonist Ro15-4513 (0.5 μM) had no significant effect on THDOC-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake (Fig. 5). This concentration of Ro15-4513 is approximately 5 times greater than the concentration required to completely inhibit ethanol-induced potentiation of GABA-mediated $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes (30).

Steroid hormone metabolites have been shown to allosterically modulate benzodiazepine receptor binding *in vitro* (1, 2). Benzodiazepines have no direct effect on GABA receptor-mediated chlorine ion flux; however, they enhance the effect of muscimol by increasing the potency of the agonist to stimulate $^{36}Cl^-$ uptake (24). We, therefore, investigated whether the steroid metabolite THDOC altered benzodiazepine-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake. Fig. 6 illustrates that a low concentration of THDOC (10 nM), which has no significant effect on muscimol-stimulated $^{36}Cl^-$ uptake, enhances the ability of clonazepam (0.5 μM) to potentiate the effect of muscimol. Higher concentrations of THDOC (100 nM) had an additive effect with clonazepam (0.5 μM) in potentiating muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes (data not shown).

Discussion

These data suggest that allopregnanolone, THDOC, 3α-OH-P, and pentobarbital interact with more than one recognition site (or conformational state) of the $GABA_A$ receptor complex to enhance GABA receptor-mediated chloride ion uptake in cerebral cortical synaptoneurosomes. The shallow biphasic concentration-response curves for steroid- and barbiturate-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake cannot be fit to a sigmoidal curve (e.g., ALLFIT analysis) characteristic of a single population of binding sites. In addition, pseudo-Hill coefficients for these compounds are significantly less than 1, consistent with the presence of multiple binding sites or conformational states involved in the potentiation of GABA receptor-mediated $^{36}Cl^-$ uptake. THDOC 21-mesylate appears to interact with only one of these steroid recognition sites, because it exhibits a steep monophasic concentration-response curve

TABLE 3

Reduced efficacy in potentiation of muscimol-stimulated $^{36}Cl^-$ uptake is not correlated with inhibition of THDOC-induced potentiation

In order to determine whether the steroid agonists having reduced efficacy acted as partial agonists, we investigated their ability to inhibit the maximal effect of THDOC-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake. Muscimol (3 μM) and steroids were added simultaneously to synaptoneurosomes and incubated for 5 sec. Potentiation of muscimol-stimulated $^{36}Cl^-$ uptake by each of the steroids is shown at their maximally effective concentrations. The maximal potentiation for THDOC was significantly greater than the other steroids (analysis of variance, $p < 0.0001$; Dunnett's t test, $p < 0.05$). No significant inhibition of THDOC-induced potentiation was observed (analysis of variance, $F = 0.91$, $p = 0.47$). Data are the mean ± standard error of five experiments conducted in quadruplicate.

Steroid	Potentiation	Additivity with THDOC
	nmol/mg of protein	
THDOC (2 μM)	12.95 ± 0.86	
THDOC 21-mesylate (2 μM)	7.39 ± 1.9	11.91 ± 0.8
5β-THDOC (5 μM)	9.80 ± 1.3	11.20 ± 1.0
3α-OH-5α-Androstane-17β-carbonitrile (2 μM)	9.00 ± 1.2	12.39 ± 1.4
Allopregnanediol (5 μM)	0.04 ± 1.2	11.76 ± 1.4

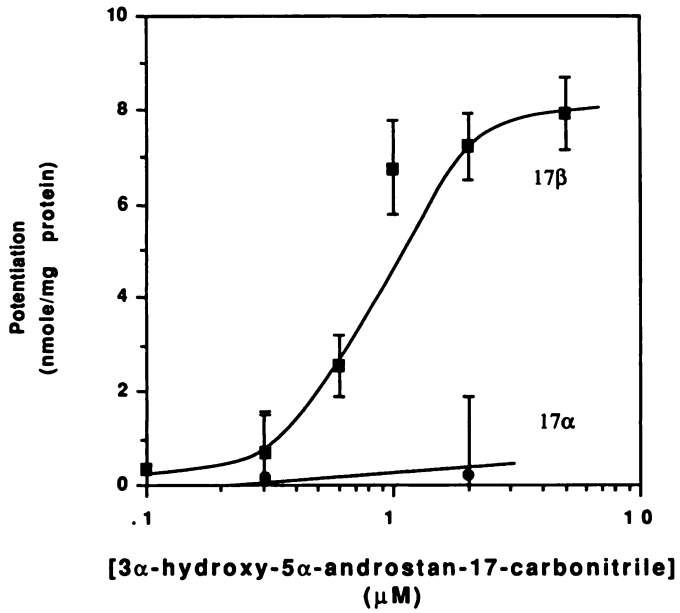

Fig. 4. Concentration-response curve for 3α-hydroxy-5α-androstane-17β-carbonitrile potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes. The activity of the 17α-derivative was tested at three concentrations (0.1, 0.3, and 2 μM). Steroid (0.1–5 μM) and muscimol were incubated for 5 sec. Data were analyzed using ALLFIT as shown and described in Table 1.

TABLE 4

Glucocorticoids, mineralcorticoids, and progesterone do not potentiate or inhibit muscimol-stimulated $^{36}Cl^-$ uptake in rat cerebral cortical synaptoneurosomes

Steroids (1 μM) and muscimol (3 μM) were added to synaptoneurosomes simultaneously and incubated for 5 sec. There was no significant effect of any of these steroids at concentrations up to 5 μM (data is shown for 1 μM). Each data point represents the mean ± standard error of three to five independent determinations.

Drug	$^{36}Cl^-$ uptake
	nmol/mg of protein
Muscimol (3 μM)	11.04 ± 1.2
+Cortisol	12.07 ± 1.6
+Corticosterone	11.84 ± 0.5
+Deoxycorticosterone	12.65 ± 0.4
+Aldosterone	10.86 ± 1.6
+Progesterone	9.49 ± 1.8

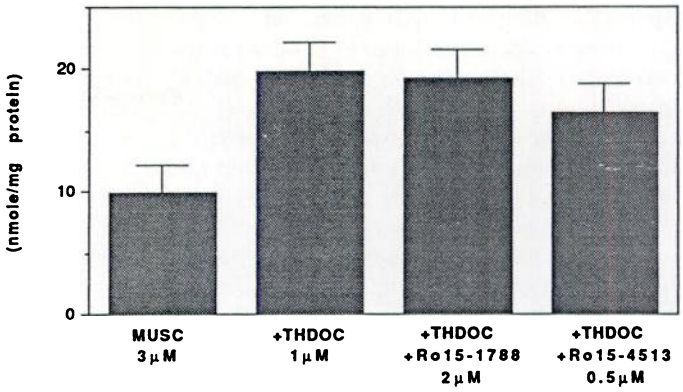

Fig. 5. Effect of Ro15-1788 and Ro15-4513 on THDOC-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake. Muscimol (*MUSC*) (3 μM) and THDOC (1 μM) were incubated in the presence or absence of Ro15-1788 (2 μM) or Ro15-4513 (0.5 μM) for 5 sec. There was no significant inhibition of the THDOC-induced potentiation by either drug. Data shown represent the mean ± standard error of two experiments conducted in quadruplicate.

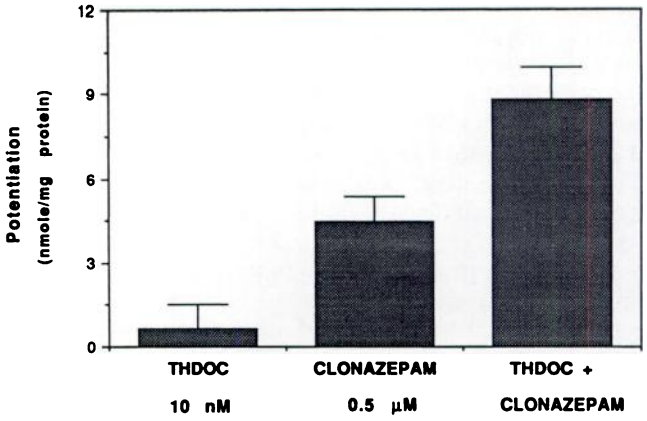

Fig. 6. THDOC augments clonazepam-induced potentiation of muscimol-stimulated $^{36}Cl^-$ uptake. Muscimol (3 μM) and clonazepam (0.5 μM) were incubated in the presence or absence of THDOC (10 nM). All drugs were added simultaneously to synaptoneurosomes. THDOC (10 nM) had no significant effect on muscimol-stimulated $^{36}Cl^-$ uptake but enhanced the effect of clonazepam ($p < 0.05$; Student's t test). Data shown are from a representative experiment conducted in quadruplicate and repeated three times.

with a Hill slope of 1 (Table 1). This mesylate derivative of THDOC is a very potent enhancer of GABA response (EC_{50} = 60 nM) and does not exhibit partial agonist activity when tested in additivity experiments with THDOC (Table 3). Thus, it appears that THDOC 21-mesylate selectively binds to a single population of steroid recognition sites on $GABA_A$ receptor(s), whereas the endogenous steroid metabolites appear to interact with multiple binding sites to potentiate $^{36}Cl^-$ uptake.

The heterogeneous interactions of steroids and barbiturates with GABA receptor-mediated chloride ion flux may be due to the existence of multiple $GABA_A$ isoreceptors. Different populations of $GABA_A$ receptors, composed of homologous but unique subunits, may have different affinities for steroids and barbiturates. Recent studies characterizing the transient and stable expression of $GABA_A$ receptor α, β, and γ subunits in *Xenopus* oocytes (31) or cultures human embryonic cells (32) suggest that different combinations of subunits result in $GABA_A$ receptors with distinct gating properties and affinities for GABA (31, 32). Because the cerebral cortical synaptoneurosome preparation used in our experiments may contain multiple $GABA_A$ isoreceptors, the possibility that steroids interact with different affinities with the various isoreceptors must be considered. Studies on the interaction of steroids with these recombinantly expressed $GABA_A$ isoreceptors could determine whether steroids interact with multiple binding sites on a single $GABA_A$ receptor or distinct sites on different $GABA_A$ isoreceptors.

An alternative explanation for the biphasic concentration-response curves elicited by THDOC, allopregnanolone, 3α-OH-P, and pentobarbital invokes the existence of multiple conformations of a single steroid recognition site or a desensitized state of the GABA receptor. Agonist-induced conformational states of a single receptor binding site have been demonstrated for numerous other receptors, including adrenergic (33), muscarinic cholinergic (34), and dopaminergic (35). This explanation seems unlikely to account for the complex characteristics of allopregnanolone and THDOC interactions with the GABA receptor, because two steroids, THDOC 21-mesylate and 3α-

hydroxy-5α-androstane-17β-carbonitrile, do not discriminate multiple conformational states or steroid binding sites in their activity on GABA receptor-mediated chloride ion flux. The identification of a steroid antagonist selective for a single steroid binding site would provide more convincing evidence that the complex agonist interactions that potentiate GABA-mediated $^{36}Cl^-$ uptake are mediated by distinct recognition sites rather than conformational states.

In previous studies, allopregnanolone and pentobarbital inhibited [^{35}S]TBPS binding, with pseudo-Hill coefficients of approximately 1.0 (3, 36). These data provide indirect evidence that [^{35}S]TBPS labels only one steroid/barbiturate recognition site, whereas our data on the effects of allopregnanolone, THDOC, and pentobarbital on muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes indicate that at least one other recognition site appears to mediate the actions of steroids and barbiturates. Furthermore, there is increasing evidence that steroids and barbiturates interact with the $GABA_A$ receptor at different sites. Turner *et al.* (5) have suggested that the steroid anesthetic alphaxalone and the barbiturate pentobarbital inhibit [^{35}S]TBPS binding via separate sites, because the effects of the steroid and the barbiturate are additive. Likewise, Gee *et al.* (2) demonstrated that saturating concentrations of allopregnanolone and pentobarbital additively accelerate the dissociation of [^{35}S]TBPS, which would not be expected if both compounds acted at a single site. Further, the enhancement of [^{3}H]muscimol binding to GABA receptors by pregnanolone and secobarbital is differentially inhibited by antagonists (19). Perhaps the simplest model to account for these data would suggest the presence of two steroid binding sites on the GABA receptor complex and one distinct barbiturate binding site, which recognizes barbiturates exclusively. Further studies will be necessary to resolve the relationship between the steroid- and barbiturate-mediated effects on [^{35}S]TBPS binding and $GABA_A$ receptor-mediated Cl^- channel function.

The maximal potentiation of muscimol-stimulated $^{36}Cl^-$ uptake by allopregnanolone, THDOC, 3α-OH-P, and THDOC 21-mesylate was achieved at a concentration of approximately 1 μM. In our screen of other steroid derivatives, their relative activity (shown in Table 2) probably reflects their maximal efficacy to a greater extent than their potency, because they were tested at this steroid concentration (1 μM). Several of these derivatives were significantly less active than allopregnanolone and THDOC. Allopregnanediol and 3α,21-dihydroxy-5β-pregnane-20-one have nearly the same affinity as allopregnanolone for the [^{35}S]TBPS binding site (2, 3) but significantly lower activity in potentiating muscimol-stimulated chloride ion flux (Table 2). In contrast to a recent report by Belelli and Gee (37), we found that allopregnanediol does not enhance muscimol-stimulated $^{36}Cl^-$ uptake at concentrations up to 5 μM and does not inhibit the maximal response produced by THDOC (Table 3). Given the similarities in the membrane preparations and methods used by both laboratories, the explanation for these discrepancies is unclear at present. 3α,21-Dihydroxy-5β-pregnane-20-one exhibits reduced agonist activity compared with THDOC and does not exhibit the inhibitory properties expected of partial agonists (Table 3). Thus, allopregnanediol and 3α,21-dihydroxy-5β-pregnane-20-one may interact selectively with one steroid binding site, the [^{35}S]TBPS-labeled site. Alternatively, the reduced activity of these steroids compared with allopregnanolone or THDOC may simply indicate that their ability to potentiate muscimol-stimulated $^{36}Cl^-$ flux is not related to their affinities for [^{35}S]TBPS binding.

Modulation of [^{3}H]flunitrazepam binding to rat cerebral cortical homogenates (1, 3) by steroid hormone metabolites suggests that occupancy of the steroid binding site(s) allosterically enhances the affinity of benzodiazepines for the benzodiazepine receptor. This observation is supported by our finding that low concentrations of THDOC potentiate the effect of clonazepam on GABA-mediated chloride uptake (Fig. 6). Higher concentrations of THDOC produced a response that was additive with the effect of clonazepam, consistent with evidence that steroid and benzodiazepine enhancement of GABA is mediated by distinct binding sites on the GABA receptor complex (1). The lack of effect of the central benzodiazepine antagonist Ro15-1788 or the weak inverse agonist Ro15-4513 also confirms the specificity of steroid interactions *in vitro*. The mechanism for inhibition of the behavioral sequelae of steroid administration by Ro15-1788 (11, 12) is unclear.

At submicromolar concentrations, allopregnanolone increases the potency of muscimol-stimulated $^{36}Cl^-$ uptake but has no effect on the maximal response. Benzodiazepines have a similar effect on muscimol-stimulated $^{36}Cl^-$ uptake, increasing the potency of muscimol approximately 2-fold with no effect on the E_{max} (24). Barbiturates, on the other hand, increase both the potency and the maximal response for muscimol-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes (38). The ability of barbiturates to increase the maximal response produced by muscimol may be related to their ability to stimulate $^{36}Cl^-$ uptake directly, with an E_{max} of 15–20 nmol/mg of protein (23). Allopregnanolone (4) and THDOC have a very small direct effect (less than 6 nmol/mg of protein) on $^{36}Cl^-$ uptake in synaptoneurosomes (4), even at concentrations up to 10 μM[1]. The difference in the maximal efficacy of direct steroid- versus barbiturate-stimulated $^{36}Cl^-$ uptake further suggests that different molecular mechanisms underlie steroid and barbiturate actions on $GABA_A$ receptor function.

Introduction of a carbonitrile group at the 17β-position confers high potency and increased efficacy relative to 3α-hydroxy-5α-androstane-17-one. The steep monophasic concentration-response curve and Hill slope of approximately 2.0 suggest that the 17β-carbonitrile may bind to two steroid recognition sites with identical affinity. This interaction is stereoselective, the 17α-derivative being completely inactive. Previous reports have suggested that a C_{20} ketone is necessary for high potency on GABA receptor-mediated chloride conductance (3) and complete inhibition of [^{35}S]TBPS binding activity (2). Interestingly, this 17β-carbonitrile has both high potency and efficacy, suggesting that a hydrogen bond acceptor is also essential for pharmacological activity. Using molecular modeling techniques, we have recently suggested that the distance between the heteroatoms, O_3-N for the 17β-carbonitrile or O_3-O_{20} for allopregnanolone, is important for maximal activity in potentiating GABA-mediated chloride uptake in synaptoneurosomes (20).

Our observations that glucocorticoids are inactive either as potentiators or inhibitors of GABA receptor-mediated $^{36}Cl^-$ uptake in synaptoneurosomes are surprising, considering the recent report that glucocorticoids both stimulate and inhibit

[1] Unpublished data.

[^{35}S]TBPS binding in the rat cerebral cortex (28). The reasons for these discrepancies are unclear, but it is conceivable that higher concentrations of these steroids may alter [^{35}S]TBPS binding via nonspecific chaotropic actions. Interestingly, pregnenolone sulfate-induced potentiation of [^{35}S]TBPS binding (39) also fails to correlate with its pharmacological activity on TBPS-induced seizures in mice (2), suggesting that stimulatory effects on [^{35}S]TBPS binding are not a good predictor of pharmacological activity.

The ability of picomolar concentrations of cortisol to modulate GABA receptor-mediated contraction in the ileum (29) suggests an important difference between the central and peripheral $GABA_A$ receptors. Thus, the effects of stress-induced release of glucocorticoids on GABA receptor function in the periphery may be immediate, whereas the central effects of stress on $GABA_A$ receptor function may be dependent on the catalytic reduction of progesterone or deoxycorticosterone to allopregnanolone or THDOC. Recently, we have measured allopregnanolone in the blood and various brain regions of male and female rats using a specific and sensitive radioimmunoassay[2]. Basal serum levels of allopregnanolone are undetectable in male rats but increase markedly following acute swim stress. Because stress induces the release of progesterone (15, 16) and deoxycorticosterone (14), the conversion of these hormones to their ring A-reduced metabolites may serve an important adaptive function in returning the central nervous system to a less excitable state following stress.

The physiological significance of the interactions of selective steroids with a single steroid binding site on the GABA receptor complex may be related to the dissociation of their recently reported anxiolytic (9) and sedative/hypnotic properties (10). Consequently, it may be possible to identify selective steroid metabolites having anxiolytic activity but lacking strong hypnotic properties. Anxiety and seizure disorders associated with the menstrual cycle have been associated with altered levels of allopregnandiol (40), suggesting an important role for steroid metabolites in the modulation of central nervous system excitability. Thus, pregnane steroids or their analogues may be potential therapeutic agents for anxiety-related symptoms associated with premenstrual syndrome and catamenial epilepsy.

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