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Studies on the Mechanism of Interactions between Anesthetic Steroids and γ-Aminobutyric Acid$_A$ Receptors

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SUMMARY

Functional interactions between steroidal anesthetics and γ-aminobutyric acid$_A$ (GABA$_A$) receptors have been examined with $^{36}Cl^-$ uptake measurements in rat cerebrocortical synaptoneurosomes. The primary effect of the steroids was to enhance the affinity of GABA for its receptors without much effect on the maximal uptake rate; the ED_{50} for GABA decreased from 66.4 ± 5.7 to 8.9 ± 1.2 μM in the presence of 20 μM 3α,21-dihydroxy-5α-pregnan-20-one. Stimulation of $^{36}Cl^-$ uptake by high concentrations of the anesthetic steroid in the absence of exogenous GABA was not due to direct stimulation of GABA$_A$ receptors, as currently proposed, but is due to enhanced action of endogenous GABA, inasmuch as the steroid markedly increases GABA affinity for the receptors. Typically, endogenous GABA was maintained at near 1 μM by a Na^+-dependent GABA transport system in the synaptoneurosomes. Elevation of its level with nipecotic acid, a specific inhibitor of the GABA transport system, or reduction with GABase, a GABA-scavenging system, increased or decreased, respectively, the steroid-induced bicuculline-sensitive $^{36}Cl^-$ uptake. At low concentrations of GABA (<2 μM), the stimulatory effect of 3α,21-dihydroxy-5α-pregnan-20-one was markedly potentiated by pentobarbital but antagonized by 3α-hydroxy-5β-pregnan-20-one, a partial agonist of higher affinity. These observations, along with the structure-activity relationships of steroid analogs, strongly suggest the existence of a specific binding site for the steroids in GABA$_A$ receptors and led us to propose a minimal model in which two key common functional groups of anesthetic steroids, 3α-OH- and 17β-polar substituents, interact with GABA$_A$ receptors (probably through hydrogen bondings) while their hydrophobic backbone remains in contact with the fatty acyl chains of membrane phospholipids.

Progesterone and its reduced analogs have been shown to induce sedation and anesthesia in experimental animals (1) and this led to the development of clinically useful steroidal anesthetics (2–4). Earlier electrophysiological and neuropharmacological studies showed that effects of the anesthetic steroids on the central nervous system were similar to those of barbiturates (4, 5). Recently, in confirmation of the earlier view, progesterone metabolites have been shown to stimulate $^{36}Cl^-$ uptake through GABA receptors in isolated brain vesicles (6, 7), to increase muscimol binding (8, 9), to inhibit the binding of TBPS to the receptors (10), and to potentiate the inhibitory action of GABA in cultured rat hippocampal and spinal cord neurons (6, 8, 11, 12). Careful examinations of the binding characteristics and sensitivities to various inhibitors, however, indicate no common binding site for the steroids and barbiturates (9, 10, 13, 14). Although the target of the anesthetic steroids has been clearly identified to be GABA$_A$ receptors (15–17), their exact modes of interactions are not clear at present. Fesik and Makriyannis (18) proposed from their NMR studies with active and inactive steroids that perturbation of membrane lipid structure by the steroids may be transmitted to GABA$_A$ receptors. Yet, the observed biological activity of the anesthetic steroids at nanomolar concentrations (7) and their stringent SARs including stereospecificity favor the modulation of GABA$_A$ receptors via a specific binding site (8, 10, 12, 19, 20). Also, diverse modes of interactions have been reported between anesthetic steroids and other ligands for GABA$_A$ receptors. The steroids have been reported to potentiate GABA and pentobarbital in inducing Cl^- conductance through GABA$_A$ channels in electrophysiological studies (11, 12) and also to cause direct activation of GABA$_A$ channels (6, 12, 17). In equilibrium binding studies, barbiturates have been reported to be only additive to the steroid effect on muscimol binding (9) but to potentiate their inhibitory effect on TBPS binding (14). Clearly, further efforts are needed to clarify the mechanism of action for anesthetic steroids.

In this study, we have attempted to examine interactions between GABA, pentobarbital, and anesthetic steroids with $^{36}Cl^-$ uptake measurements in rat cerebrocortical synaptoneurosomes. This report will describe that 5α-THDOC, a prototype anesthetic, markedly enhanced the affinity of GABA for its receptors and that the steroid action was markedly potentiated by pentobarbital, at low GABA, but antagonized by 3α-hydroxy-5β-pregnan-20-one, a partial agonist, suggesting the ex-

ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; 5α-THDOC, 3α,21-dihydroxy-5α-pregnan-20-one; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DIDS, 4,4′-diisothiocyantostilbene-2,2′-disulfonic acid; SAR, structure-activity relationship.

istence of a unique binding site for the steroid in $GABA_A$ receptors.

Materials and Methods

Rat cerebrocortical synaptoneurosomes were prepared following the procedure of Hollingsworth *et al.* (21). Briefly, cerebral cortices from four male Sprague-Dawley rats were suspended in 30 ml of ice-cold solution containing 118 mM NaCl, 5 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 20 mM HEPES/Tris, pH 7.0 (buffer A), and 10 mM D-glucose and were homogenized in a Dounce homogenizer by eight strokes with a loose-fitting pestle and then by five strokes with a tight-fitting pestle. The suspension was diluted to 160 ml with buffer A (NaCl buffer) and filtered through three layers of nylon cloth and a 10-μm Millipore filter (LC type). The filtrates were centrifuged at 3000 × *g* for 15 min. The membranous pellets were washed once with buffer A and resuspended to a final protein concentration of 8 mg/ml.

$^{36}Cl^-$ uptake in the synaptoneurosomes was measured by a rapid filtration technique using Whatman GF/B glass microfiber filters (22), with some modifications. A typical incubation medium contained 0.2 μCi of $Na^{36}Cl$/ml (NEN), 118 mM NaCl, 5 mM KCl, 1.8 mM $MgSO_4$, and 20 mM HEPES/Tris, pH 7.0, with or without test drugs. Steroids were added in concentrated methanolic solutions. The final concentration of methanol did not exceed 0.8% and was maintained constant in all tubes. Under our experimental conditions, methanol at 0.8% stimulated $^{36}Cl^-$ uptake up to 0.5 ± 0.8 nmol/5 sec·mg of protein, which did not exceed the range of experimental variations in our measurements. Replacing methanol with 0.4% dimethyl sulfoxide showed no noticeable effect on the degree of $^{36}Cl^-$ uptake in the presence of 5α-THDOC (10 μM), suggesting no apparent interaction between methanol and the anesthetic steroid. The membrane suspensions were preincubated for 5 min at 30°. The reaction was initiated by mixing equal volumes (125 μl) of the membrane suspension (1 mg of protein) and the reaction mixture containing $^{36}Cl^-$ at 30°. After 5 sec, unless indicated otherwise, the reaction was terminated by adding ice-cold buffer A containing 1 mM DIDS. DIDS, when added with GABA and $^{36}Cl^-$, simultaneously inhibited GABA-induced $^{36}Cl^-$ uptake with an IC_{50} of 200 μM. DIDS also has been reported to block both GABA-induced and basal efflux of $^{36}Cl^-$ from rat brain synaptoneurosomes (23). The mixture was filtered over a GF/B filter under vacuum, and the filters were washed four times with 5 ml of ice-cold buffer A. The radioactivity was counted after the filters were mixed with 15 ml of Instagel.

In attempts to reduce the level of endogenous GABA in the medium, the GABase system from *Pseudomonas fluorescence* (Sigma) was employed. The membrane suspensions (125 μl) were incubated for 20 min at 30° in the presence of GABase cocktail (10 μl), which contained GABA-glutamic transaminase (0.02 unit), succinic semialdehyde dehydrogenase (0.02 unit), NADP (5 mM), mercaptan (10 mM), and α-ketoglutarate (10 mM) in 25 mM sodium phosphate buffer, pH 7.2. For control, the membrane suspensions were treated equally except that the GABase cocktail was added to the reaction mixture containing $^{36}Cl^-$.

Bicuculline-sensitive $^{36}Cl^-$ uptake refers to the portion inhibited in the presence of 500 μM bicuculline methiodide. Protein was determined by the method of Lowry *et al.* (24), using bovine serum albumin as standard.

$Na^{36}Cl$ was obtained from New England Nuclear. Bicuculline methiodide, DIDS, nipecotic acid, and GABase were purchased from Sigma. Steroid analogs 5α- and 5β-THDOC were provided by Dr. Robert H. Purdy (Southwest Foundation for Biomedical Research San Antonio, TX) and others were obtained from the Biological Screening Office at The Upjohn Company.

Results

We have examined the effect of 5α-THDOC (10 μM) and pentobarbital (200 μM) on GABA-mediated $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes (Fig. 1). As the concentration of exogenous GABA was raised from 0 to 1, 2, 3, and 4 μM, the steroid and the barbiturate brought about marked stimulation of the $^{36}Cl^-$ uptake with linear dependence on GABA concentrations; the uptake rate per μM GABA increased from 0.6 nmol/5 sec·mg of protein with GABA alone to 3.5 and 7.1 nmol/5 sec·mg of protein with 5α-THDOC and pentobarbital, respectively. Also note that the two drugs stimulated $^{36}Cl^-$ uptake to considerable degrees in the absence of exogenous GABA. This stimulation, in previous reports (6, 17), has been attributed to direct activation of the receptor channel by the drugs, but it appears to arise from potentiation of endogenous GABA, on the basis of the following observations. First, the steep linear dependence of their stimulatory effect on exogenous GABA could be extrapolated to a common intercept on the GABA-axis, seemingly indicating the presence of endogenous GABA (1.4 μM) (Fig. 1A). It has been observed that the synaptoneurosomes under our experimental conditions constantly release endogenous GABA into the medium, which in turn is scavenged by the Na^+-dependent GABA transport system in the presence of a proper inwardly directed electrochemical Na^+ gradient.[1] This process maintains a low level of endog-

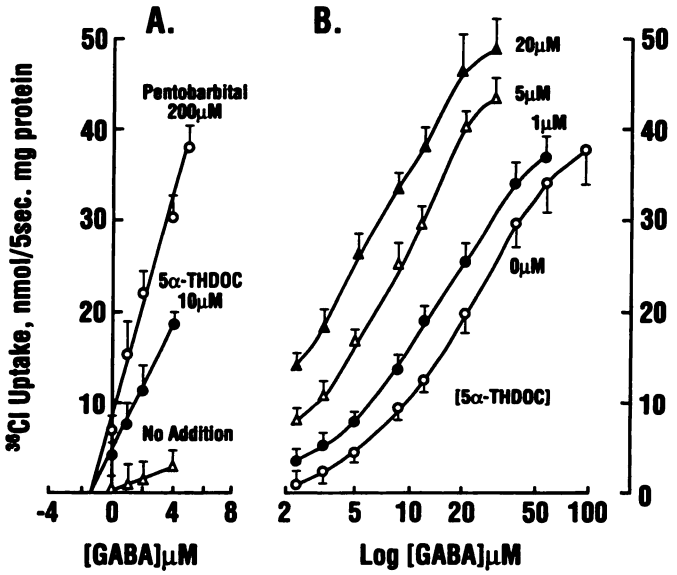

Fig. 1. Dose-response profiles of GABA-induced $^{36}Cl^-$ uptake in the presence of 5α-THDOC or pentobarbital. *A*, An aliquot (125 μl) of rat cerebrocortical synaptoneurosome suspension was preincubated for 5 min at 30° and then mixed with an equal volume of $^{36}Cl^-$NaCl buffer containing the indicated level of exogenous GABA (Δ), with 10 μM 5α-THDOC (●), or with 200 μM pentobarbital (○) at 30°. The reaction was terminated 5 sec later. The level of $^{36}Cl^-$ radioactivity associated with the synaptoneurosomes was determined by rapid filtration techniques. The GABA-induced $^{36}Cl^-$ uptake was computed by subtracting the value obtained under the same conditions but without exogenous GABA and the drugs. Estimation of endogenous GABA level was obtained from extrapolation of the linear dose-response profile in the presence of 5α-THDOC and pentobarbital to the zero $^{36}Cl^-$ uptake level (see text). The data represent the mean and standard deviation of three experiments, each consisting of duplicate measurements. *B*, The GABA-mediated $^{36}Cl^-$ uptake was measured, as described above, at varying concentrations of exogenous GABA from 0 to 100 μM in the presence of 0, 1, 5, and 20 μM 5α-THDOC. For the plotting, GABA concentrations represent the sums of the endogenous (1.4 μM) and added GABA. The data represent the mean and standard deviation of three experiments consisting of duplicate measurements.

[1] Unpublished observations.

enous GABA in the medium. Second, brief incubation of the synaptoneurosomes with nipecotic acid, a specific inhibitor of the Na^+-dependent GABA transport system (25), led to marked stimulation of $^{36}Cl^-$ uptake by pentobarbital and the steroid without exogenous GABA (Table 1). For example, in the synaptoneurosomes preincubated for 0 (nipecotic acid added simultaneously with $^{36}Cl^-$ reaction buffer), 30 and 90 sec with 5 mM nipecotic acid, the ^{36}Cl uptake rate in the presence of 5α-THDOC (10 μM) increased from 5.6 to 6.7, 19.1, and 25.4 nmol/5 sec·mg of protein, respectively. Similarly, the stimulatory effect of pentobarbital rose from 7.9 to 9.3, 24.2, and 30.1 nmol/5 sec·mg protein. The marked stimulation by the steroid and the barbiturate in the synaptoneurosomes treated with nipecotic acid for 90 sec was completely blocked by bicuculline (500 μM) and disappeared upon washing of the nipecotic acid-treated synaptoneurosomes. Finally, incubation of the synaptoneurosomes for 20 min with a GABA-scavenging system, which includes GABAse, α-ketoglutarate, and NADP, reduced the stimulatory effect of the steroid and pentobarbital by about 30%. This moderate reduction is likely due to the slow reaction rate of the GABAse system, which takes 45 min at room temperature to completely convert GABA to succinate at subsaturating doses.

Note also in Table 1 that extending the preincubation period of the synaptoneurosomes at 30° from 5 to 20 min led to a 70% increase in the stimulatory effects of pentobarbital and the steroid on the $^{36}Cl^-$ uptake, which apparently arose from an increased level of endogenous GABA, because of their sensitivity to the GABAse system. It, thus, appears that the rate of GABA release exceeds that of GABA uptake in the synaptoneurosomes under our experimental conditions. With a limited preincubation period of 5 min for the synaptoneurosomes at 30°, however, the level of endogenous GABA, 1.4 ± 0.17 μM (four experiments), was fairly constant, as estimated by extrapolation of the linear portion of the GABA dose-response profile for $^{36}Cl^-$ uptake (in the presence of the steroid or the barbiturate) to zero chloride uptake. Also, the level of exogenous GABA during the 5-sec exposure to the synaptoneurosomes for $^{36}Cl^-$ uptake appears to be unchanged, because nipecotic acid showed minimal effect on $^{36}Cl^-$ uptake when added with $^{36}Cl^-$ and various levels of exogenous GABA. With this information on total level in the medium (the sum of endogenous and exogenous GABA), we have further examined interactions between 5α-THDOC and GABA on $^{36}Cl^-$ uptake.

Fig. 1B shows the profile for $^{36}Cl^-$ uptake as a function of total GABA concentration (a semilogarithmic plot) in the presence of 5α-THDOC at the concentration of 1, 5, or 20 μM. Apparently, the steroid shifted the GABA dose-response profile to the left. In all the profiles, the Hill coefficient was measured to be 0.97 to 0.99. Also, our analysis of muscimol-induced $^{36}Cl^-$ uptake data in the literature (22) yielded a Hill coefficient of 0.94. This contrasts with the coefficient of 2 observed in GABA-induced whole-cell currents in adrenal chromaffin cells (26). The reasons for the apparent lack of cooperativity in $^{36}Cl^-$ uptake by the synaptoneurosomes could be multiple, probably including our inability to measure true initial uptake rates, which requires a time resolution on the order of micro- or at least milliseconds, and possible variations in the kinetics from the receptor heterogeneities inherent or induced during the broken-cell preparations. In any event, to evaluate the overall effect of the steroid on GABA-induced $^{36}Cl^-$; uptake, the data were fitted to the Michaelis-Menten equation by nonlinear regression. The analysis reveals that the ED_{50} for GABA decreased from 66.4 ± 5.7 μM to 42.3 ± 7.0, 21.3 ± 3.1, and 8.9 ± 1.2 μM in the presence of 1, 5, and 20 μM 5α-THDOC, respectively (Table 2). The maximal uptake, on the other hand, was not significantly affected in the presence of the steroid.

Earlier SARs for steroid anesthetics have been extensively examined by measuring the loss of righting reflex in mice, GABA-mediated chloride currents electrophysiologically, the inhibitory effect on TBPS binding to $GABA_A$ receptors, and $^{36}Cl^-$ uptake in rat synaptoneurosomes (8–10, 12, 19, 20). All these studies pointed out that the 3α-hydroxyl and 17β-acetyl

TABLE 1

Effect of nipecotic acid and GABase system on $^{36}Cl^-$ uptake stimulation by pentobarbital and 5α-THDOC

An aliquot (125 μl) of the synaptoneurosome suspensions in 118 mM NaCl, 5 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM D-glucose, 20 mM HEPES/Tris, pH 7.0, was preincubated at 30° for 5 min. Nipecotic acid (final concentration, 5 mM) was added to the suspensions 30 or 90 sec before the end of the preincubation period, or included in the $^{36}Cl^-$-NaCl buffer (0 sec), or not added at all (control). Also the synaptoneurosomes exposed to nipecotic acid for 90 sec were washed with 8 volumes of NaCl buffer (90 sec and washing). Then, the reaction was initiated by adding an equal volume of $^{36}Cl^-$-NaCl buffer containing 20 μM 5α-THDOC or 400 μM pentobarbital. In the case of nipecotic acid for 90 sec and bicuculline, the $^{36}Cl^-$-NaCl buffer containing 1 mM bicuculline was reacted with the synaptoneurosomes that had been exposed to nipecotic acid for 90 sec. For GABase experiments, the membrane suspensions were preincubated for 20 min at 30° in the absence (control) or presence of 10 μl of GABase cocktail, which contained GABA-glutamic transaminase (0.02 unit), succinic semialdehyde dehydrogenase (0.02 unit), NADP (5 mM), mercaptan (10 mM), and α-ketoglutarate (10 mM) in 25 mM Na phosphate buffer, pH 7.2, or the GABase cocktail was included in $^{36}Cl^-$-NaCl buffer (0 sec). The reaction was terminated 5 sec later by the addition of 1 ml of the NaCl buffer containing 1 mM DIDS. The mixture was rapidly filtered over a Whatman GF/B glass fiber filter. The filter was washed four times with 5 ml of ice-cold buffer. The baseline $^{36}Cl^-$ level was obtained from the same sets of experiments without the steroid and pentobarbital and was subtracted from the value obtained with the drugs to yield the stimulated portion of $^{36}Cl^-$ uptake.

Treatments	Stimulation of $^{36}Cl^-$ uptake	
	5α-THDOC	Pentobarbital
	nmol/5 sec·mg of protein	
Control[a]	5.6 ± 1.3	7.9 ± 1.0
Nipecotic acid		
0 sec	6.7 ± 0.9	9.3 ± 1.2
30 sec	19.1 ± 1.7	24.2 ± 2.6
90 sec	25.4 ± 1.4	30.1 ± 2.1
90 sec and bicuculline	0.8 ± 0.2	0.4 ± 1.0
90 sec and washing	7.0 ± 1.9	9.1 ± 1.5
Control[b]	12.3 ± 1.4	16.0 ± 1.5
GABase		
0 sec	11.2 ± 0.9	16.2 ± 1.2
20 min	8.0 ± 0.7	10.6 ± 0.9

[a] The synaptoneurosomes were preincubated for 5 min at 30°.
[b] The synaptoneurosomes were preincubated for 20 min at 30°.

TABLE 2

Kinetic parameters for GABA in GABA-mediated $^{36}Cl^-$ uptake

GABA-mediated $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes was measured with varying concentrations of exogenous GABA from 0 to 100 μM, in the presence of 5α-THDOC at 0, 1, 5, and 20 μM. Nonspecific association of $^{36}Cl^-$ with the membranes was estimated from the similar experiments without GABA and the steroids and was subtracted. The GABA level included not only the exogenous but the endogenous GABA, which was estimated to be 1.4 μM (see text). Analysis of the data (the means of three experiments) was carried out using a computer program of nonlinear regression.

Additions	K_d for GABA	V_{max}
	μM	*nmol of Cl^-/5 sec·mg of protein*
GABA	66.4 ± 5.7	74.6 ± 4.3
GABA + 1 μM 5α-THDOC	42.3 ± 7.0	75.5 ± 9.8
GABA + 5 μM 5α-THDOC	21.5 ± 3.1	82.8 ± 7.9
GABA + 20 μM 5α-THDOC	8.9 ± 1.2	68.0 ± 4.8

side chains are the key functional groups for the steroidal action. This has also been confirmed from our current study, where we examined the effect of various steroid analogs at 20 μM on the $^{36}Cl^-$ uptake in the presence of 5 μM exogenous GABA (Table 3). In addition, we have observed quantitatively different effects of several functional groups of the steroids on $^{36}Cl^-$ uptake. For example, the 5β- analogs appear to be less active than the 5α- ones, if otherwise equal in their structure (compare 5β- and 5α-THDOC). Introduction of 11-keto and 21-OH groups produced more active compounds. Several polar substituents at C-17, other than the acetyl group, were active, i.e., U-57030 and U-6036, containing 17β-hydroxyl and 17-keto groups, respectively. The activity of the latter has also been reported earlier (14). On the other hand, U-2598, containing a bulky hydrophobic substituent at 17β-, was inactive. It appears that the presence of electronegative atoms at the 17-position is necessary for the steroidal action.

From the SAR study, we discovered several weakly active steroids. Among these, U-1903, U-2340, and U-2411 were of particular interest because their structures represent minor changes in the two functional groups central to stimulation of GABA-mediated $^{36}Cl^-$ uptake, i.e., the substituents at the 3- and 5-positions. We have examined the relative affinity of these compounds for putative steroid binding site(s) by measuring their ability to depress the stimulatory action of 5α-THDOC on $^{36}Cl^-$ uptake in the presence of 1 μM exogenous GABA. At this GABA level, U-1903, U-2340, and U2411 at 20 μM stimulated $^{36}Cl^-$ uptake by 3.5 ± 1.6, 3.3 ± 0.7, and 2.2 ± 1.4 nmol/5 sec·mg of protein, respectively. Fig. 2A shows that U-2340 dose-dependently blocked the stimulatory effect of 20 μM 5α-THDOC on $^{36}Cl^-$ uptake, with an IC_{50} value of 4 μM, whereas U-1903 and U-2411 showed neither additive nor inhibitory effects even as their concentration was raised to 20 μM. In Fig. 2B, a dose-response curve for U-2340 shows its stimulatory effect on $^{36}Cl^-$ uptake reaching near saturation as its concentration was raised to 10 μM or higher concentrations. U-2340, thus, appears to be a partial agonist of higher affinity for the steroid binding site, as compared with 5α-THDOC.

On the other hand, pentobarbital markedly potentiated the stimulatory effect of 5α-THDOC on $^{36}Cl^-$ uptake at the micromolar range of GABA (in the absence of exogenous GABA). Fig. 3 shows the dose-response profile of 5α-THDOC for stimulation of $^{36}Cl^-$ uptake in the presence of 200 μM pentobarbital, as compared with a hypothetical response profile if their effects are additive. 5α-THDOC alone dose-dependently stimulated $^{36}Cl^-$ uptake with an apparent V_{max} of 14.2 ± 1.9 nmol/5 sec·mg of protein and a half-maximal steroid concentration of 13.9 ± 2.8 μM. Pentobarbital alone at 200 μM increased the $^{36}Cl^-$ uptake by 7.9 nmol/5 sec·mg of protein. When combined, their maximal stimulatory rate reached 43.4 ± 1.1 nmol/5 sec·mg of protein, with a half-maximal steroid concentration of 2.2 ± 0.2 μM, which is only 1/7 of that observed without pentobarbital. This observation is in agreement with the earlier proposal that the steroid and pentobarbital do not share a common binding site (10, 13, 14) and further points out an allosteric interaction(s) between the two drugs.

TABLE 3

Stimulation of GABA-induced $^{36}Cl^-$ uptake by various progesterone metabolites and their analogs

An aliquot (125 μl) of the synaptoneurosome suspension preincubated for 5 min at 30° was mixed with an equal volume of $^{36}Cl^-$NaCl buffer containing 5 μM exogenous GABA and 20 μM various steroids, at 30°. The reaction was terminated 5 sec later by the addition of 1 ml of ice-cold NaCl buffer and the mixture was filtered over a Whatman GF/B glass fiber filter. The filter was washed four times with 5 ml of ice-cold NaCl buffer and counted for radioactivity. The degree of stimulation by the steroids was computed by subtracting the value obtained in the presence of 5 μM exogenous GABA without the steroids. The data represent the mean and standard deviation of three experiments, each consisting of triplicate measurements.

		Stimulation of GABA-induced $^{36}Cl^-$ uptake
		nmol/5 sec·mg of protein
U-0949	3β-Hydroxy-5α-pregnan-20-one	0.9 ± 0.8
U-1843	3α,17α-Dihydroxy-5β-pregnane-11,20-dione	0.2 ± 0.4
U-1894	3α-Hydroxy-5β-pregnane-11,20-dione	12.3 ± 1.8
U-1903	3β-Hydroxy-5α-pregnane-11,20-dione	5.9 ± 1.5
U-2340	3α-Hydroxy-5β-pregnan-20-one	5.5 ± 0.6
U-2411	5β-Pregnane-3,20-dione	1.2 ± 0.6
U-2598	24,24-Diphenyl-3α-hydroxy-5β-chol-23-en-11-one	0.4 ± 0.3
U-6036	3α-Hydroxy-5α-androstan-17-one	16.9 ± 1.8
U-57030	3-Methyl-5α-androstane-3α,17β-diol	8.6 ± 1.1
U-57031	3-Methyl-5α-androstane-3β,17β-diol	1.3 ± 0.8
5α-THDOC	3α,21-Dihydroxy-5α-pregnan-20-one	22.5 ± 3.7
5β-THDOC	3α,21-Dihydroxy-5β-pregnan-20-one	9.3 ± 1.3

Discussion

In this study, we have proposed that the stimulatory effect of anesthetic steroids on $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes without exogenous GABA arises from potentiation of endogenous GABA, rather than their direct activation of $GABA_A$ receptors, as currently reported in the literature (6, 17). Our proposal is based on (a) the enhanced GABA effect on the $^{36}Cl^-$ uptake in the presence of the steroid, which allowed us to examine the contribution by endogenous GABA in the medium to the Cl^- uptake (Fig. 1), and (b) predictable changes in the stimulatory effect of the steroids on $^{36}Cl^-$ uptake upon manipulation of the endogenous GABA level in the medium with GABase, a GABA-scavenging system, or with nipecotic acid, a specific inhibitor of the Na^+-dependent GABA uptake system (see Table 1). Recently, Huidobro-Toro *et al.* (27) and Turner *et al.* (14) have reported that steroid anesthetics produced little potentiation of basal $^{36}Cl^-$ flux in the absence of GABA agonists. This suggests that the endogenous external GABA level could be variable from one preparation to another.

Our finding that 5α-THDOC decreases the ED_{50} for GABA without a significant effect on V_{max} for the $^{36}Cl^-$ uptake contrasts with the report by Peters *et al.* (9). They have reported that "Scatchard analysis of the ligand binding data suggested an apparent increase in the number, rather than the affinity, of detectable [^{3}H]muscimol binding sites as the principal action of the active steroid isomers." Also, their other observation that "in all combinations of concentration tested, the effect of secobarbitone and 5β-pregnan-3αl-ol-20 one on [^{3}H]muscimol binding was additive" again differs from our data showing a marked functional potentiation between the steroid and barbiturates. These differences, however, are not readily resolved because of marked differences in experimental conditions between the ligand binding and the $^{36}Cl^-$ uptake studies; the

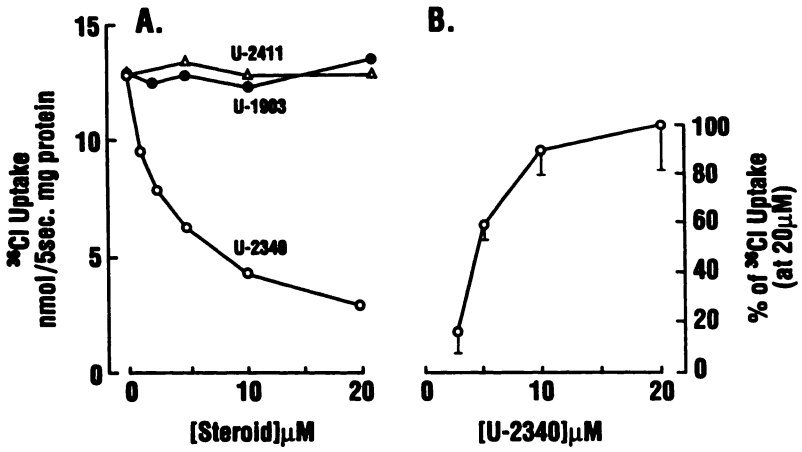

Fig. 2. Plots showing differences in the ability of three steroid analogs to depress the stimulatory effect of 5α-THDOC on $^{36}Cl^-$ uptake and the concentration-response curve for U-2340. *A*, An aliquot (125 μl) of rat cerebrocortical synaptoneurosomes preincubated for 5 min at 30° was mixed with an equal volume of the $^{36}Cl^-$-NaCl buffer containing 1 μM exogenous GABA, 20 μM 5α-THDOC, and the indicated levels of U-1903, U-2340, or U-2411. The reaction was terminated 5 sec later. The level of $^{36}Cl^-$ radioactivity associated with the synaptoneurosomes was determined by rapid filtration techniques. The $^{36}Cl^-$ uptake levels represent the steroid-induced portion, which was obtained from the differences between the $^{36}Cl^-$ uptake with and without steroids. *B*, The $^{36}Cl^-$ uptake in the synaptoneurosomes was measured in the presence of 2, 5, 10, and 20 μM U-2340, as described above. The data were plotted as percentage of the $^{36}Cl^-$ uptake stimulation observed at 20 μM U-2340, which ranged between 2.6 and 4.0 nmol/5 sec·mg of protein.

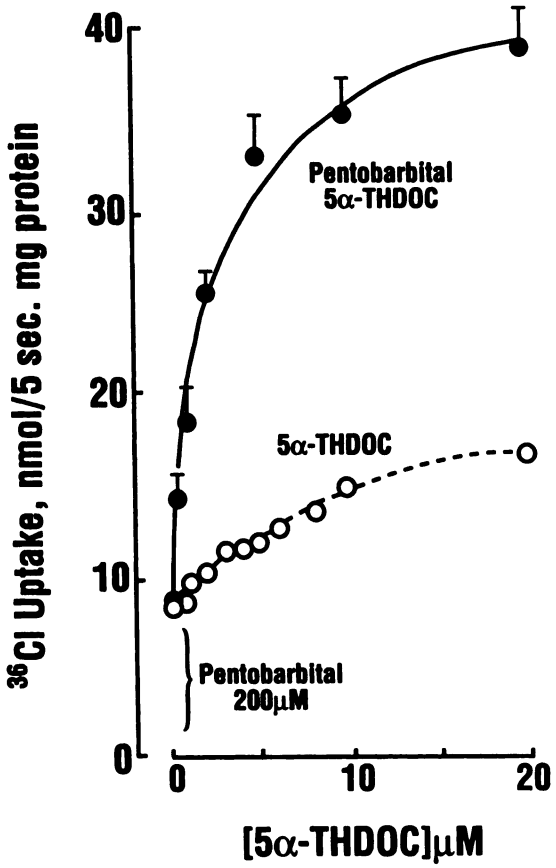

Fig. 3. Plots showing potentiation between pentobarbital and 5α-THDOC, an anesthetic steroid, in $^{36}Cl^-$ uptake. An aliquot (125 μl) of rat cerebrocortical synaptoneurosomes preincubated for 5 min at 30° was mixed with an equal volume of the $^{36}Cl^-$-NaCl buffer containing no exogenous GABA, 200 μM pentobarbital, and the indicated concentrations of 5α-THDOC. The reaction time was 5 sec, and the level of $^{36}Cl^-$ radioactivity associated with the synaptoneurosomes was determined by rapid filtration techniques as already described. The drug-induced $^{36}Cl^-$ uptake represents the differences between those measured with or without the indicated drugs. A hypothetical line (– – –) is shown to indicate the supposed additive effect between the barbiturate and the steroid. ——, The experimental data obtained with the indicated mixture of the two drugs. The data represent the mean of triplicate measurements with standard deviation.

differences in the amount of protein (~50 μg for the binding and 1 mg for the $^{36}Cl^-$ uptake), in the quality of protein (dialyzed synaptosomal membranes for the binding and neuronal sacs for the uptake), and in the reaction time (60 min for equilibrium binding and 5 sec for the uptake). At present, it is difficult to assess how these differences in the experimental conditions contribute to the qualitative and quantitative discrepancies. Nevertheless, the marked difference in the ED_{50} value for muscimol, 18 nM for binding and 4 μM for the $^{36}Cl^-$ uptake, suggests that occupancy of the low affinity GABA site brings about opening of GABA$_A$ chloride channels. The receptor has already been reported to possess a low affinity GABA binding site for GABA that is sensitive to a number of benzodiazepines (28).

Several studies have established that anesthetic steroids share no common binding site with the barbiturates in GABA$_A$ receptors (9, 13), but the nature of the steroidal interactions with the receptors is not clear. Recently, Fesik and Makriyannis (18) proposed from their NMR studies with alphaxalone and inactive Δ^{16}-alphaxalone that perturbation of the membrane lipid structure by the steroids could be transmitted to GABA$_A$ receptors and led to a modification of their function. This proposal, however, is not compatible with several observations in this study. (a) The marked reduction in the half-maximal stimulatory dose of 5α-THDOC (from 14 to 2 μM) in the presence of pentobarbital may arise from the drug-induced inter- or intrapolypeptide interactions. (b) A highly selective and potent antagonistic action of U-2340 against a prototype anesthesic, 5α-THDOC, suggests the presence of a specific binding site for anesthetic steroids in GABA receptors. Finally, Fesik and Makriyannis (18) proposed that anesthetic steroids are accommodated lengthwise between two fatty acyl chains of membrane phospholipids, like cholesterol. The presence of a polar 17-substituent (in addition to a 3α-OH) in anesthetic steroids, however, makes the cholesterol-like interaction with phospholipids very unfavorable. Rather, the two polar groups of hypnotic steroids may interact with polar head groups of phospholipids or polypeptides, while their hydrophobic backbone interacts with the methylene residues of the fatty acyl chains near the membrane surface. On the strength of these considerations, it is reasonable to propose that anesthetic steroids may have two points of contact (probably formation of hydrogen bonding) with GABA$_A$ receptors through the two polar functional groups and their hydrophobic moieties remain

in contact with the fatty acyl chains of phospholipids surrounding $GABA_A$ receptors. Such interactions could demand stringent structural requirements for the ligands, because their two polar groups have to be positioned in a particular spacial orientation. One may, then, observe the disappearance or reduction of anesthetic actions with some chemical modifications of the steroids that lead to distortion of the relative spacial configurations of the two polar groups. Such modifications may include not only the residues near the functional groups but also introduction of hydrophilic groups in the hydrophobic backbone of the steroid, which cause destabilization of their hydrophobic interactions and eventually lead to profound changes in overall configuration of the compounds. This model will be further tested by computational modelings of the active steroids and by preparation of anesthetics with novel structures.

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