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Differential Modulation of the γ-Aminobutyric Acid Type C Receptor by Neuroactive Steroids

KENDALL D. W. MORRIS, CHARLES N. MOOREFIELD, and JAHANSHAH AMIN

Department of Pharmacology and Therapeutics, Department of Physiology and Biophysics, The Institute for Biomolecular Science (K.D.W.M., J.A.), and The Center for Molecular Design and Recognition (C.H.N.), University of South Florida, Tampa, Florida

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ABSTRACT

γ-Aminobutyric acid type C receptor channels ($GABA_CRs$) composed of ρ subunits are pharmacologically distinct from $GABA_A$ receptor channels ($GABA_ARs$). This difference is illustrated by the insensitivity of homo-oligomeric ρ_1 receptor channels to many known modulators of $GABA_ARs$, such as barbiturates and benzodiazepines. A number of endogenous metabolites of corticosterone and progesterone, known as neuroactive steroids, compose yet another class of compounds that can modulate $GABA_ARs$. Here, several neuroactive steroids are shown to also modulate the ρ_1 receptor channel. 5α-Pregnane-3α,21-diol-20-one (allotetrahydrodeoxycorticosterone), 5α-pregnane-3α-ol-11,20-dione (alphaxalone), and 5α-pregnane-3α-ol-20-one (allopregnanolone) potentiated the GABA-evoked currents from ρ_1 receptor channels and concomitantly altered the deactivation kinetics by prolonging the decay time. In contrast, 5β-pregnane-3α-ol-20-one (pregnanolone), 5β-pregnane-3,20-dione (5β-dihydroprogesterone), and 5β-pregnane-3α,21-diol-20-one (tetrahydrodeoxycorticosterone), all potentiators of $GABA_ARs$, inhibited the GABA-elicited currents of the ρ_1 receptor channel. In comparison to $GABA_ARs$, the modulation of ρ_1 receptor channels by these neuroactive compounds occurred with relatively high concentrations of the neuroactive steroids and was more prominent in the presence of low concentrations of GABA, equivalent to fractions of the EC_{50} value of the ρ_1 receptor channel. Structural comparison of these six neuroactive steroids reveals that the key parameter in determining the mode of modulation for the ρ_1 receptor channel is the position of the hydrogen atom bound to the fifth carbon, imposing a *trans*- or *cis*-configuration in the backbone structure. This is the first demonstration of isomeric compounds that can differentially modulate the activity of the ρ_1 receptor channel.

The interplay of neurotransmitters and their corresponding ligand-gated ion channels plays a pivotal role in inhibition or excitation of synaptic transmission. In the central nervous system (CNS), inhibitory transmission is mediated predominantly through interactions of the neurotransmitter γ-aminobutyric acid (GABA) with two classes of receptor-chloride channel complexes: γ-aminobutyric $acid_A$ receptor channels ($GABA_ARs$) and γ-aminobutyric $acid_C$ receptor channels ($GABA_CRs$; Macdonald and Olsen, 1994). These receptor channels are differentially distributed within the CNS, with $GABA_ARs$ ubiquitous throughout the CNS, whereas $GABA_CRs$ are found primarily within the retina (Enz et al., 1995; Lukasiewicz, 1996). The main criteria for distinguishing between these two classes of receptor channels are their differential responses to drugs. For instance, the barbiturates and the benzodiazepines can modulate $GABA_ARs$ by increasing the magnitude of the GABA-induced current (Macdonald and Olsen, 1994), whereas $GABA_CRs$ are insensitive to these two classes of drugs (for reviews, see Johnston, 1996; Lukasiewicz, 1996; Feigenspan and Bormann, 1998).

Metabolites of the stress hormone corticosterone and the female sex hormone progesterone compose another class of $GABA_AR$ modulators: the neuroactive steroids (Harrison and Simmonds, 1984; Callachan et al., 1986, 1987; Majewska et al., 1986; Barker et al., 1987; Harrison et al., 1987; Morrow et al., 1987; Gee et al., 1988; Peters et al., 1988; Turner et al., 1989; Paul and Purdy, 1992; Twyman and Macdonald, 1991; Lambert et al., 1995; Le Foll et al., 1997). The concentrations of these metabolites can increase markedly within the CNS after stress and can vary during menstrual cycles (Purdy et al., 1990, 1991; Paul and Purdy, 1992; Negri-Cesi et al., 1996). Two metabolites of corticosterone, allotetrahydrodeoxycorticosterone (5α-THDOC) and tetrahydrodeoxycorticosterone (5β-THDOC), are positive modulators of $GABA_ARs$, with the 5α compound being the more efficacious of the two (Harrison et al., 1987; Peters et al., 1988; Im et al., 1990;

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ABBREVIATIONS: CNS, central nervous system; GABA, γ-aminobutyric acid; $GABA_AR$, γ-aminobutyric $acid_A$ receptor channel; $GABA_CR$, γ-aminobutyric $acid_C$ receptor channel; 5α-THDOC, allotetrahydrodeoxycorticosterone; 5β-THDOC, tetrahydrodeoxycorticosterone; 5β-DHP, 5β-dihydroprogesterone.

Kokate et al., 1994; Xue et al., 1997). The progesterone metabolites pregnanolone, 5β-dihydroprogesterone (5β-DHP), and allopregnanolone also enhance GABA-induced current from $GABA_AR$ (Harrison et al., 1987; Poisbeau et al., 1997; Reith and Sillar, 1997; Maitra and Reynolds, 1999). In nanomolar concentrations, these neuroactive steroids can potentiate the GABA-elicited currents, and at higher concentrations, they can act as partial agonists on the $GABA_AR$ (Harrison and Simmonds, 1984; Barker et al., 1987; Morrow et al., 1990; Puia et al., 1990; Wittmer et al., 1996; Le Foll et al., 1997).

Thus far, no potentiators have been reported for $GABA_CRs$ (ρ_1). Here, the two-electrode voltage-clamp technique of an oocyte expression system is used to study the effects of neuroactive steroids on ρ_1 receptor channels. Several metabolites of corticosterone and progesterone, as well as a synthetic steroid alphaxalone (Harrison and Simmonds, 1984; Cottrell et al., 1987), are demonstrated to modulate the activity of homo-oligomeric ρ_1 receptor channels in a positive or negative fashion. In view of these findings, correlation between the structure of these neuroactive steroids and their differential effect on the ρ_1 receptor channel is presented.

Materials and Methods

The plasmid vector containing the human ρ_1 cDNA was linearized using restriction enzyme *Ssp*I. This restriction site is located a few hundred bases downstream from the stop codon, which during synthesis of the cRNA results in incorporation of additional sequences at the 3′ end of the cRNA. These auxiliary sequences may enhance the stability of the synthesized cRNA within the oocyte. The resulting DNA template was in vitro transcribed into cRNA using the T7 Megascript in vitro transcription kit (Ambion, Austin, TX). The quality of the cRNA was determined using electrophoresis of set dilutions of the products on a 1% agarose gel containing formaldehyde.

Xenopus laevis (Xenopus I, Ann Arbor, MI) were anesthetized via hypothermia, and oocytes were surgically removed and placed into oocyte Ringer's solution (OR2; 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $NaPO_4$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin, pH 7.5). The oocytes were then dissociated in 82.5 mM NaCl, 2.5 mM KCl, 1 mM $NaPO_4$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5, plus 0.3% collagenase A (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for ~2 h. After separation, the oocytes were washed thoroughly with OR2. Finally, stage VI oocytes were isolated and maintained in OR2 containing 2% horse serum at 18°C.

Micropipettes for cRNA injection were made on a Sutter P87 horizontal puller (Sutter Instrument Co., Novato, CA), and the tips were cut off using microscissors. cRNA for injection was drawn up into the micropipette using negative pressure and injected into the oocytes by the application of positive pressure using a PICO-SPRITZER II (General Valve Corporation, Fairfield, NJ).

At 2 to 3 days postinjection, the oocytes were placed on a nylon mesh suspended in a recording chamber (volume, ~50 μl). This recording chamber has an inlet in the top and an outlet in the bottom that allowed continuous perfusion of control or drug solution with ~2 ml/min. Twenty separate reservoirs (100-ml glass containers) were connected to four six-way valves, and the outlet of each of these six-way valves (the sixth position was connected to the reservoir containing control solution) was connected to one four-way valve. The outlet of the four-way valve lead to the chamber. In this way, up to 20 different solutions could be introduced to an individual oocyte. The exchange time (dead time plus equilibration time in the chamber), which is ~7 s, is accounted for in the $T_{1/2}$ of deactivation measurements. Switching between the different solutions was controlled manually. The oocytes were constantly perfused with recording OR2 (OR2 lacking Na_2HPO_4, Na pyruvate, and antibiotics) and switched to test solutions containing GABA or GABA plus steroid.

The neuroactive steroids were purchased from Sigma Chemical Co. (St. Louis, MO), Research Biochemicals Inc. (Natick, MA), and Steraloids (Newport, RI) to compare batch-to-batch variation. No significant variability in the outcome of the results was noted when

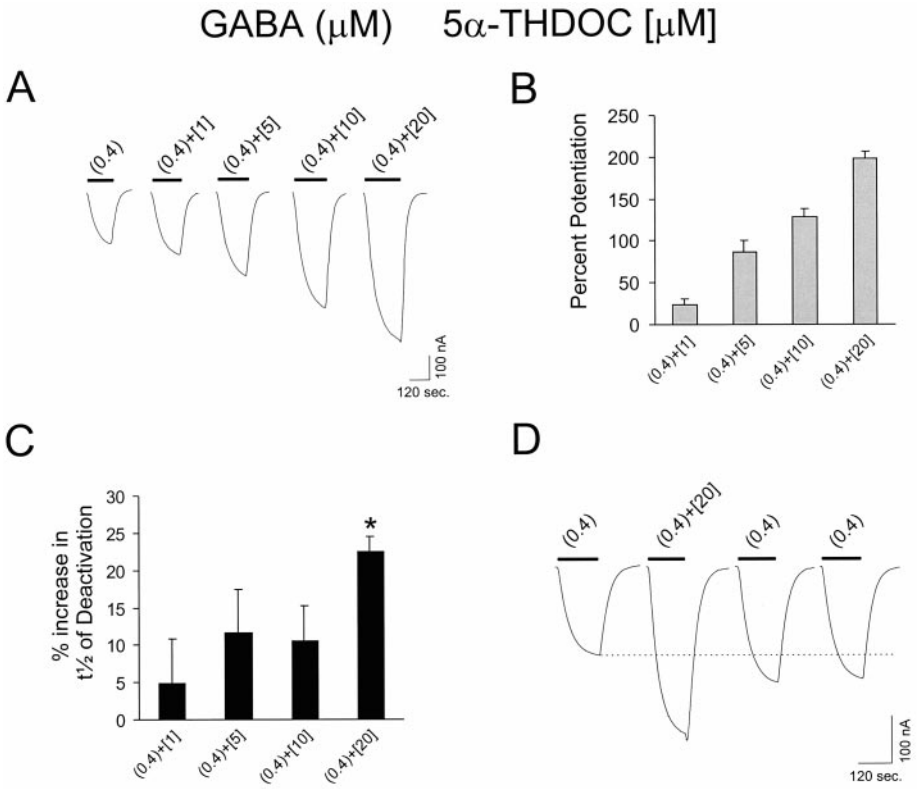

Fig. 1. 5α-THDOC potentiation of GABA-evoked currents from ρ_1 receptor channels. A, representative current traces from application of GABA or coapplication of GABA with different concentrations of 5α-THDOC. Thick lines above current trace represent the duration of application of either GABA or GABA plus steroid. B, the bar graph represents percent potentiation of GABA-evoked currents in the presence of increasing concentrations of 5α-THDOC (n = 4). The error bars are the S.E.M. C, average percent increase in $T_{1/2}$ of deactivation with increasing concentration of 5α-THDOC (n = 4). Note the increase in the decay time of the GABA-induced current with coapplication of 5α-THDOC. The asterisk denotes a $T_{1/2}$ of deactivation value significantly different from that of GABA alone ($P < .05$, one-tailed Student's nonpaired t test). D, residual effect of 5α-THDOC on subsequent GABA applications. The return of the GABA-induced current to the control level occurred over several applications of GABA. The times between the drug applications are ~4 min.

samples of the same steroid obtained from different sources were compared. The stock solutions of 10 mM steroids were made in dimethyl sulfoxide. Test solutions containing drugs were made by adding the steroid stock solutions to rapidly stirring recording OR2. Given that these neuroactive steroids are highly hydrophobic, the maximum feasible concentration of these compounds within the recording OR2 appeared to be ~20 μM. The presence of the vehicle solution dimethyl sulfoxide at the maximum tested concentration (0.2%) did not alter the GABA-induced current from ρ_1 receptor channel.

Recording electrodes were fabricated on a Narishige PP-83 (Narishige Scientific Instrument Lab., Tokyo, Japan). Electrodes were then filled with a solution of 3 M KCl. The oocytes were voltage-clamped at −70 mV using a TURBO TEC-05 npi (Adams and List, Westbury, NY) amplifier, and output was recorded on tape and chart paper by a Gould TA240 chart recorder.

Percent potentiation (PP) and percent inhibition (PI) were calculated as follows:

$$\text{PP or PI} = 100 \times (I_{steroid} - I_{GABA})/I_{GABA}$$

where I_{GABA} is the current elicited by a control application of GABA, and $I_{Steroid}$ is the current with the application of both GABA and steroid. Thus, a 100% potentiation represents a current twice the amplitude of the control GABA-elicited current.

The IC_{50} value and Hill coefficient were determined by fitting the concentration-response relationship to the following logistic equation using Sigma Plot 4.0.

$$I = I_{max}/[1 + (IC_{50}/[S])^n]$$

where I is the peak current at a given concentration of steroid [S], I_{max} is the maximal inhibited current, IC_{50} is the concentration of steroid giving half-maximal inhibition, and n is the Hill coefficient.

The one-tailed Student's t test was used to calculate confidence levels for $T_{1/2}$ of deactivation and concentration dependence of 5β-THDOC inhibition. Nonpaired analysis was used for the $T_{1/2}$ calculation, and paired analysis was used for the inhibition by 5β-THDOC.

Results

5α-THDOC Potentiates GABA-Evoked Currents of ρ_1 Receptor Channels. Figure 1A depicts representative current traces from the application of GABA and GABA plus different concentrations of 5α-THDOC to an oocyte expressing ρ_1 receptor channels. Serial bath application of GABA (0.4 μM; GABA EC_{50} = 1.03 ± 0.26) plus 1, 5, 10, and 20 μM 5α-THDOC resulted in currents that were greater in magnitude than the GABA current alone. With successively higher concentrations, the amplitude of the GABA currents increased without reaching a plateau even in the presence of the highest feasible concentration of 5α-THDOC (20 μM, see *Materials and Methods*). These currents did not exhibit desensitization even in the presence of the highest concentration of 5α-THDOC and GABA. The bar graph representing the mean percent potentiation of GABA currents (±S.E.M) in the presence of different concentrations of 5α-THDOC is shown in Fig. 1B (n = 4). The percentage potentiation of the GABA-evoked currents was 24 ± 7% and 87 ± 14% in the presence of 1 and 5 μM 5α-THDOC, respectively (n = 4). This

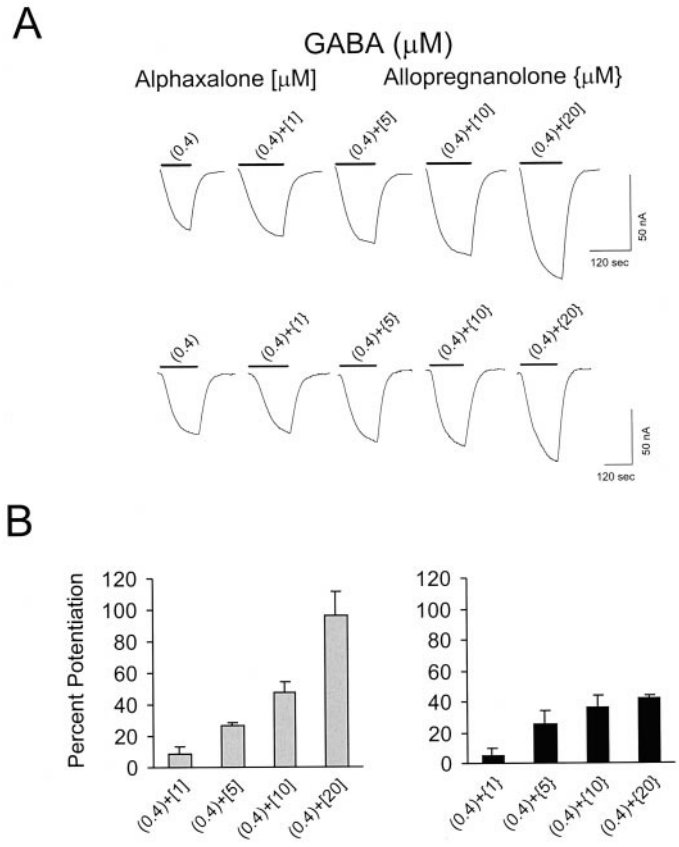

Fig. 2. Alphaxalone and allopregnanolone modulation of ρ_1 receptor channels. A, current traces from application of GABA or coapplication of GABA with either alphaxalone or allopregnanolone. Control currents were scaled to the same peak height to demonstrate the difference in efficacy between these compounds. Thick lines above current trace represent duration of application of either GABA or GABA plus neuroactive steroid. B, average percent potentiation (±S.E.M.) for coapplication of GABA with different concentrations of alphaxalone (n = 3) and allopregnanolone (n = 3).

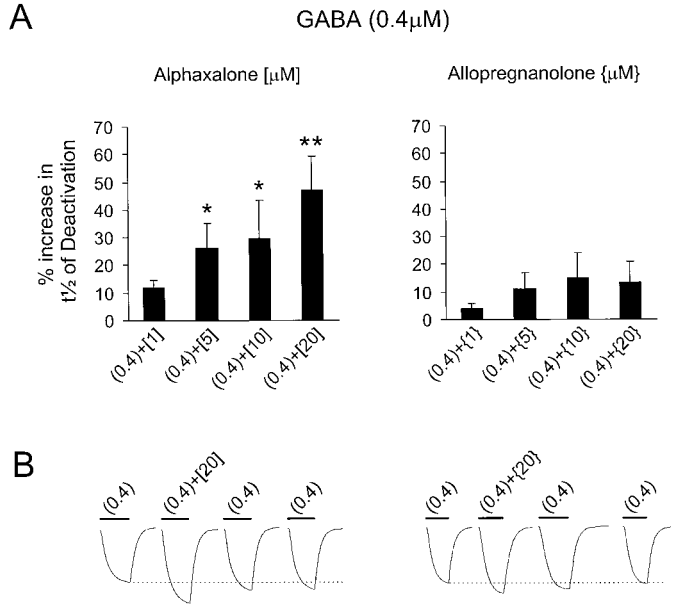

Fig. 3. Coapplication of either alphaxalone or allopregnanolone with GABA lengthens the $T_{1/2}$ of deactivation and confers long-lasting effects. A, average percent increase in $T_{1/2}$ of deactivation over control obtained from coapplication of either alphaxalone (n = 3) or allopregnanolone (n = 3) with GABA. Alphaxalone caused the greatest increase in the $T_{1/2}$ of deactivation. Significant difference between $T_{1/2}$ of deactivation in the presence and absence of steroid (*P < .05, **P < .01). B, current traces showing the prolonged effect of alphaxalone or allopregnanolone on ρ_1 receptor channels. The time between the drug applications is ~4 min.

value was further increased to 129 ± 10% and 198 ± 9% (n = 4) with the coapplication of 10 and 20 μM 5α-THDOC, respectively. Fitting of the Hill equation to the concentration-response data did not yield an EC_{50} value or a Hill coefficient for 5α-THDOC because an extrapolated maximum could not be calculated. The agonistic action of 5α-THDOC alone was also examined. This steroid at a concentration as high as 20 μM did not activate ρ_1 receptor channels (data not shown).

The deactivation time of the GABA-induced currents for ρ_1 receptor channels was prolonged in the presence of 5α-THDOC. Figure 1C shows the percentage increase (n = 4) in time for the current to decay to half of the maximum current ($T_{1/2}$ of deactivation) for the coapplication of GABA and 5α-THDOC, over the $T_{1/2}$ of deactivation for the GABA application alone. This increase in deactivation time was dependent on the concentration of 5α-THDOC because the $T_{1/2}$ values were extended with increasing concentrations of 5α-THDOC. For GABA alone, the $T_{1/2}$ of deactivation was 18 ± 1 s, whereas this value increased by 22 ± 2% for bath application of GABA and 20 μM 5α-THDOC. The times to peak for these currents were prolonged with the coapplication of 5α-THDOC and GABA (data not shown). Thus, it appears that in the presence of 5α-THDOC, the activation and deactivation kinetics of the ρ_1 receptor channel are prolonged.

The effects of 5α-THDOC coapplication on the ρ_1 receptor channel were also long lasting. After steroid application, the magnitude of the currents elicited by subsequent applications of GABA alone remained above the initial control current. Figure 1D shows the traces from the current evoked from two subsequent applications of GABA at ~4-min intervals. As shown, the GABA-elicited currents after the treatment with 20 μM 5α-THDOC are larger in magnitude than the initial control current. After multiple applications of GABA, the current magnitude returned to the control level.

Finally, after removal of GABA and 5α-THDOC at the highest concentration, the current rose before returning to the baseline level (Fig. 1, A and D). This effect of 5α-THDOC at the highest concentration is consistent with a partial channel block.

Alphaxalone and Allopregnanolone Potentiation of ρ_1 Receptor Channels. Additional neuroactive steroids (alphaxalone and allopregnanolone) were also tested with the above protocol. Figure 2 shows the current traces as well as the mean percent potentiation of GABA (0.4 μM) responses for ρ_1 receptor channels in the presence of 1, 5, 10, and 20 μM alphaxalone and allopregnanolone. Both compounds were found to be positive modulators of ρ_1 receptor channels. The GABA-evoked currents in the presence of alphaxalone or allopregnanolone display characteristics similar to 5α-THDOC in that they did not exhibit desensitization even in the presence of the highest concentration of the neuroactive steroids. The times to peak for these currents were also prolonged with increasing concentrations of these compounds.

The maximum potentiation reached in the presence of 20 μM alphaxalone was 96 ± 15% (n = 3). This value is approximately half that of 5α-THDOC at the same concentration. In comparison, 20 μM allopregnanolone induced 42 ± 2% (n = 3) potentiation of the GABA-elicited currents. As with 5α-THDOC, the current magnitude did not plateau at the highest concentrations of allopregnanolone or alphaxalone. As a result, the EC_{50} and the Hill coefficient values could not be obtained because the fitting of the Hill equation to the concentration-response data did not predict a maximum. Neither allopregnanolone nor alphaxalone directly activated ρ_1 receptor channels even at the highest concentration tested (20 μM).

The $T_{1/2}$ of deactivation for the GABA-induced current in the presence of alphaxalone and allopregnanolone was also extended (Fig. 3A). Among the three neuroactive steroids tested, the coapplication of 20 μM alphaxalone was the most effective in delaying the return of the current to the baseline, raising the $T_{1/2}$ of deactivation above the control by 47 ± 12%. In comparison, the deactivation $T_{1/2}$ for allopreg-

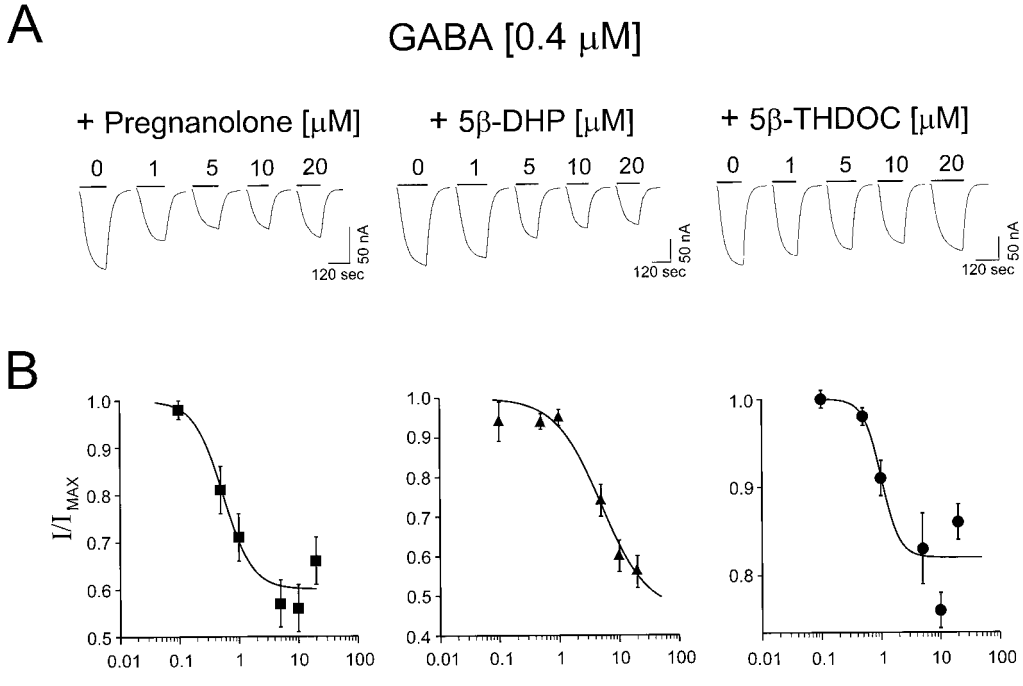

Fig. 4. Pregnanolone, 5β-THDOC, and 5β-DHP inhibition of ρ_1 receptor channels. A, representative current traces from application of GABA or coapplication of GABA and steroid. Control currents were scaled to the same peak height to demonstrate the relative efficacies of pregnanolone, 5β-THDOC, and 5β-DHP. Thick lines above current trace represent duration of application of either GABA or GABA plus steroid. B, concentration-response curves for the three inhibitory neuroactive steroids. I_{max} is the current amplitude in the absence of the neuroactive steroids, and I is the current in the presence of steroid. As shown, pregnanolone is the most potent of these compounds.

nanolone (20 μM) was less than that of 5α-THDOC (20 μM), increasing by only 13 ± 6% over the $T_{1/2}$ of deactivation for the GABA-evoked current.

The effects of alphaxalone and allopregnanolone treatments on the oocytes expressing ρ_1 receptor channels were also long lasting because the currents resulting from subsequent applications of GABA alone remained elevated over the initial control current (see Fig. 3B). The time course of the residual effect, however, was different between these two neurosteroids. For alphaxalone, the time taken for the current to return to control level was similar to that for 5α-THDOC. Several applications of GABA were required for the GABA-elicited current to return to the control level (data not shown). The prolonged effect of allopregnanolone pretreatment on the subsequent GABA-elicited currents was the least among the tested neuroactive steroids. For allopregnanolone experiments, the amplitude of the eventual GABA-evoked current returned to the initial control level after only two or three applications of GABA alone (4–5 min apart). In addition, the $T_{1/2}$ of deactivation remained above the control level in the subsequent GABA applications and gradually decreased with successive GABA applications.

Inhibition of ρ_1 Receptor Channels by Pregnanolone, 5β-THDOC, and 5β-DHP. In contrast to the above neuroactive steroids, pregnanolone, 5β-THDOC, and 5β-DHP were found to inhibit ρ_1 receptor channels. Figure 4A shows the current trace from bath application of either GABA (0.4 μM) alone or GABA (0.4 μM) and 1 to 20 μM concentrations of pregnanolone to an oocyte expressing ρ_1 receptor channels. The GABA (0.4 μM)-evoked currents were inhibited by pregnanolone in a concentration-dependent manner. Pregnanolone at concentrations of 0.5 and 1 μM decreased GABA-evoked currents by 18 ± 5 and 29 ± 5% (n = 4), respectively (Fig. 4B). The currents were further reduced by 45 ± 5 and 46 ± 5% with coapplication of 5 and 10 μM concentrations of this steroid. The inhibition appeared to reach its maximum around 5 μM pregnanolone. At a concentration of 20 μM, pregnanolone caused a partial reversal of the inhibition (32 ± 5% reduction in 0.4 μM GABA-evoked current). The concentration-response relationship for pregnanolone is shown in Fig. 4C. Fitting these data points to the Hill equation yielded an IC_{50} value of 0.55 μM and a Hill coefficient value of 1.8 for pregnanolone.

For ρ_1 receptor channels, 5β-DHP was also found to be inhibitory, with efficacy similar to that of pregnanolone (Fig. 4, A–C). The highest inhibition for 5β-DHP, however, occurred at 20 μM, decreasing the current by 44 ± 4% of the control. Fitting of the Hill equation to the 5β-DHP data points yielded an IC_{50} value of 5.02 μM with a Hill coefficient of 1.15 (Fig. 4C).

Figure 4 also shows the representative current traces, percent inhibition, and concentration-response relationship for GABA and GABA plus 1 to 20 μM 5β-THDOC. The GABA-evoked currents were reduced by 17 ± 4% and 24 ± 2% by 5 and 10 μM 5β-THDOC (n = 3), respectively, with the 10 μM concentration producing the maximum inhibition (Fig. 4B). Similar to pregnanolone, the 20 μM concentration of 5β-THDOC partially reversed the inhibition process. Application of the Hill equation to these data points yielded an IC_{50} value of 1.02 μM and a Hill coefficient of 2.91 for 5β-THDOC.

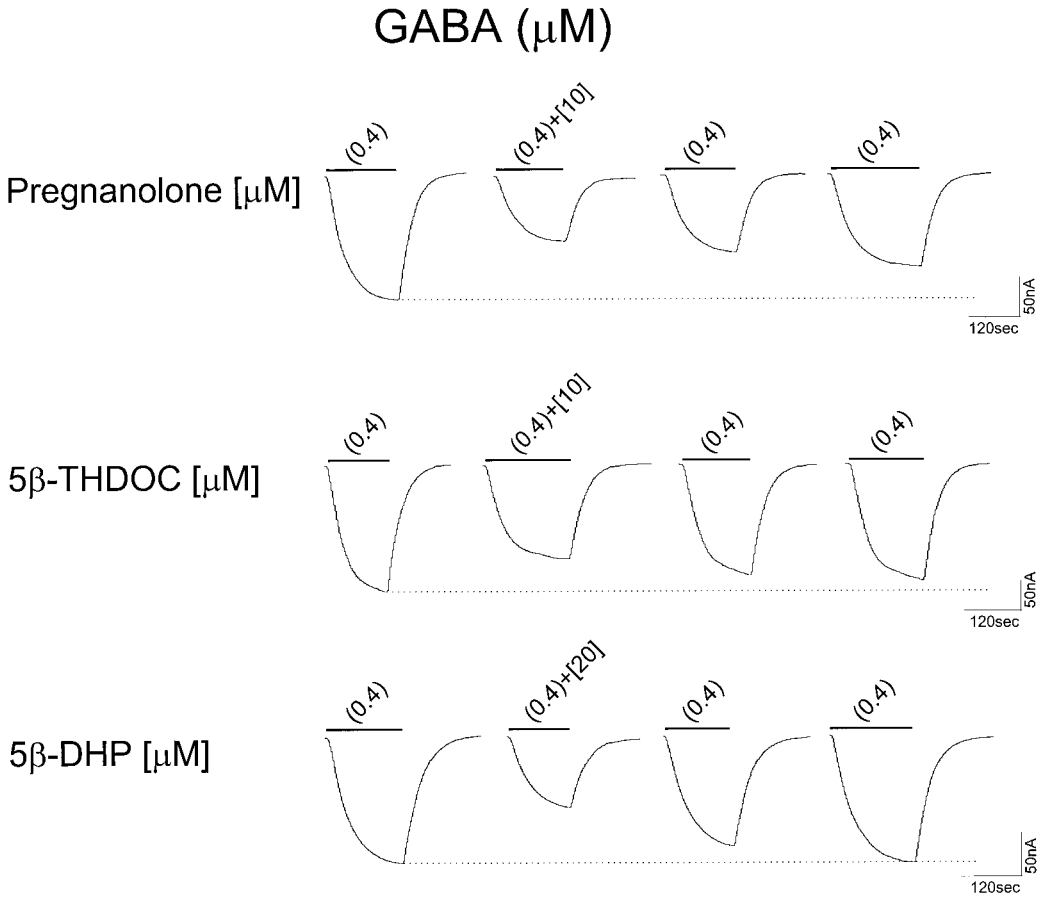

Fig. 5. Long-lasting effects of pregnanolone, 5β-DHP, and 5β-THDOC. Thick lines above current trace represent application time of either GABA or GABA plus neuroactive steroid. Concentrations of pregnanolone (10 μM) and 5β-THDOC (10 μM) that elicited the maximum inhibition were used for this experiment. Pregnanolone, the most potent inhibitor, also required the greatest period of time for recovery. The time between each drug applications is ~4 min.

These data indicate that 5β-THDOC is median in potency in comparison to pregnanolone and 5β-DHP and is the least efficacious among the three inhibitors tested.

Unlike the potentiators previously discussed, pregnanolone and 5β-DHP did not significantly alter the $T_{1/2}$ of deactivation for the GABA-induced current. In comparison, 5β-THDOC at maximal concentration (20 μM) increased the $T_{1/2}$ of deactivation for the GABA-induced currents, although at lower concentrations, it had no significant effect (data not shown). As with the potentiators, the effects of these steroids were also long lasting because after the neuroactive steroid treatment, the GABA-induced currents did not return to the control level (Fig. 5). The GABA-elicited currents remained depressed for all three inhibitors, returning to control level only after several applications of GABA, 4 to 5 min apart.

Modulation by 5α-THDOC and 5β-THDOC Is GABA Concentration Dependent. Figure 6 shows the degree of potentiation by 5α-THDOC (10 μM) in the presence of 0.2, 0.4, 0.8, 1, 2, 4, and 10 μM GABA. These GABA concentrations range from fractions of to nearly 10 times the GABA EC_{50} value for ρ_1 receptor channels (1.03 ± 0.26 μM). There was a significant potentiation in the presence of 0.2 μM GABA. In the presence of low concentrations of GABA (0.2 and 0.4 μM), 5α-THDOC (10 μM) caused a significant potentiation (216 ± 10% and 95 ± 7%, respectively) in the magnitude of the GABA-evoked current (Fig. 6B, $n = 4$). The effects of 5α-THDOC on GABA-induced currents, however, decreased with increasing concentrations of GABA. Furthermore, there was a slight inhibition when concentrations of GABA greater than the EC_{50} value were used. For example, coapplication of 10 μM 5α-THDOC and 4 or 10 μM GABA reduced the GABA-induced currents by 8 ± 4% and 10 ± 4% of the control value, respectively.

Figure 6 also shows the degree of inhibition by 5β-THDOC (10 μM) in the presence of increasing concentrations of GABA ranging from 0.2 to 10 μM. The effects of 5β-THDOC on ρ_1 receptor channels decreased in the presence of increasing concentrations of GABA, albeit to a lesser extent than that shown for 5α-THDOC. The coapplication of 5β-THDOC reduces the GABA-induced current (0.4 μM) by 30 ± 4% of GABA alone ($n = 5$). This inhibition, however, was not overcome even in the presence of a significantly greater concentration of GABA (18 ± 3% inhibition at 10 μM; $n = 6$), indicating that 5β-THDOC is a noncompetitive antagonist for ρ_1 receptor channels.

Discussion

In this study, the effects are shown of several neuroactive steroids on the ρ_1 receptor channel. Allopregnanolone, alphaxalone, and 5α-THDOC all potentiated the GABA-induced currents and prolonged the decay time. In contrast, the coapplication of GABA with 5β-THDOC, pregnanolone, or 5β-DHP inhibited the ρ_1 GABA-evoked current. Collectively, the degree of potentiation and, to a lesser extent, inhibition of ρ_1 GABA-elicited currents by these neuroactive steroids were dependent on the GABA concentration. These effects were most prominent in the presence of low concentrations of GABA, equivalent to a fraction of the EC_{50} value. Finally, the effects of the neuroactive steroids on ρ_1 receptor channels were shown to be long lasting because the applications of GABA alone did not return to the control level for several minutes subsequent to neuroactive steroid treatment.

The most striking finding of this study was the differential modulation of ρ_1 receptor channels by neuroactive steroids. The 5α derivatives were potentiators, whereas the 5β com-

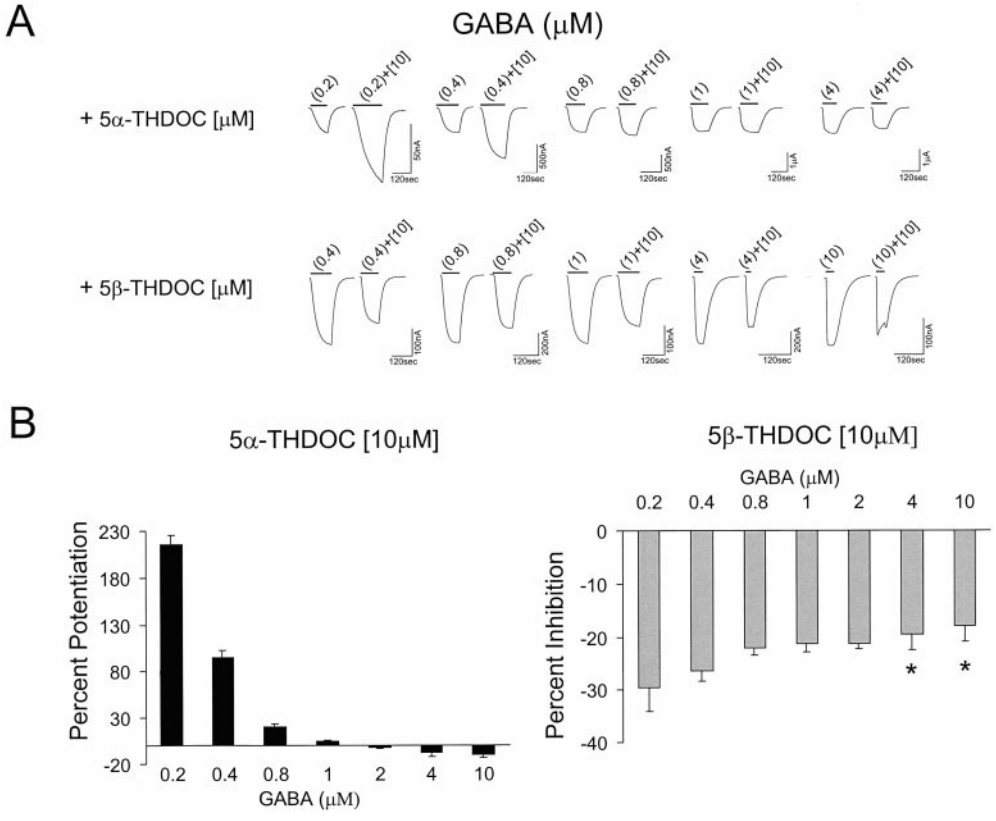

Fig. 6. GABA concentration-dependent modulation of ρ_1 receptor channels by 5α-THDOC and 5β-THDOC. A, representative current traces from application of increasing concentrations of GABA in the presence of 5α-THDOC (10 μM) or 5β-THDOC (10 μM). Control currents for all concentrations of GABA were scaled to same peak height. Note that 5α-THDOC potentiation occurs at concentrations of GABA equivalent to a fraction of the EC_{50} value, whereas at higher concentrations of GABA, 5α-THDOC causes a slight inhibition. Thick lines above current trace represent application time of either GABA or GABA plus steroid. B, average percent potentiation (or inhibition; ±S.E.M.) for 10 μM concentration of either 5α-THDOC or 5β-THDOC in the presence of different concentrations of GABA. The decrease in inhibition at higher concentrations of GABA is statistically significant (*$P < .05$).

pounds were inhibitors of the GABA-evoked currents. This is intriguing because all of the 5α and 5β steroid derivatives examined in this study are known to be potentiators of $GABA_A$Rs (Harrison et al., 1987; Kokate et al., 1994; Le Foll et al., 1997). The ability of the neuroactive steroid to potentiate or inhibit ρ_1 receptor channels depended on the position of the hydrogen atom attached to the fifth carbon (5α versus 5β neuroactive steroids; Fig. 7A). For instance, 5α-THDOC significantly potentiated the GABA-induced current of the ρ_1 receptor channels, whereas 5β-THDOC was inhibitory. A comparison of allopregnanolone and pregnanolone further corroborates this hypothesis given that the only difference in the structure of these two compounds is the position of the hydrogen atom on the fifth carbon (Fig. 7B). A comparison of the architectural differences of these compounds reveals that switching of the fifth carbon hydrogen from the α to the β position induces a *trans*- or *cis*-configuration at the site of the A and B ring fusion. This structural switch can result in multiple physical changes, including ~10% alteration in length (Fig. 7A), as well as a shift in the dipole moment of the molecule. It is tempting to speculate that these physical differences can influence the relative position of these compounds within their effector site, which can then influence the gating components of the ρ_1 ion channel complex in an opposing fashion. In comparison, other structural differences among the neuroactive steroids tested appear to affect only the relative potencies and efficacies for these compounds on the ρ_1 receptor channel. For example, replacement of the hydroxyl bound to the third carbon with a ketone decreased the potency of 5β-DHP in comparison to pregnanolone.

The concentration of GABA played an important role in the degree of modulation by these steroids. For example, the modulation of ρ_1 receptor channels by 5α-THDOC was dependent on the GABA concentration, yielding potentiation only at exceedingly low concentrations of GABA (below the EC_{50} value) yet causing inhibition at higher concentrations. In comparison, potentiation of the $GABA_A$R by neuroactive steroids occurs over a greater range of GABA concentrations, including concentrations of GABA above the EC_{50} value (Le Foll et al., 1997). Differences in the activation and deactivation kinetics of hetero-oligomeric $GABA_A$Rs and homo-oligomeric ρ_1 receptor channels (Amin and Weiss, 1994, 1996) may explain why potentiation of the latter is more dependent on GABA concentration.

Previous studies with expression of retinal mRNA or cloned ρ_1 subunits within *X. laevis* oocytes have suggested that the bicuculline-insensitive receptor channels ($GABA_C$, ρ_1 receptor channel) do not respond to neuroactive steroids (Woodward et al., 1992). It is important to note that there are no contradictions between results of the previous studies and the data presented here. First, the concentration of GABA used in those studies can dampen the effect of the tested neuroactive steroids. Second, lower concentrations of these steroids were used in those studies, which could in turn result in a less intense response. Finally, the most effective compound used here (5α-THDOC) was not tested in the aforementioned study.

In addition to a contrast in modulation by neuroactive steroids, there are other key differences between the responses of the $GABA_A$R and the ρ_1 receptor channel to neuroactive steroids. Overall, $GABA_A$Rs display higher sensitivity to neuroactive steroids compared with ρ_1 receptor channels. For instance, modulation of the $GABA_A$R is detectable with concentrations of neurosteroids in the nanomolar range (Harrison et al., 1987; Kokate et al., 1994), whereas for ρ1 receptor channel, micromolar concentrations of neuro-

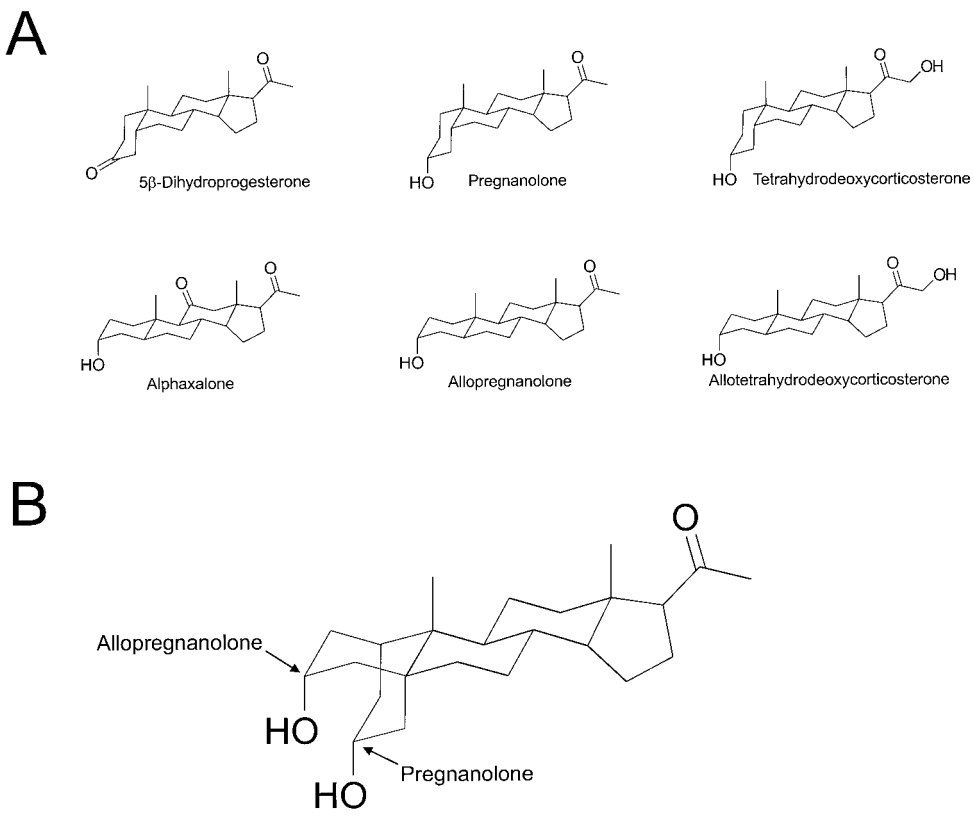

Fig. 7. A, structures of the neuroactive steroids 5α-THDOC, alphaxalone, allopregnanolone, pregnanolone, 5β-THDOC, and 5β-DHP. B, superimposed view of pregnanolone and allopregnanolone. Note the architectural alteration caused by alternative placement of the hydrogen bound to the fifth carbon.

steroids are required to exert an effect. Moreover, these neuroactive compounds can directly activate the $GABA_A$Rs at concentration used in this study but show no agonistic properties on ρ_1 receptor channels. What could account for the difference in the sensitivity between the ρ_1 receptor channel and the $GABA_A$R? It has been demonstrated recently that mutation of a single tryptophan (Trp328) within the third transmembrane domain of the ρ_1 subunit to any hydrophobic residues confers generic pentobarbital sensitivity to ρ_1 receptor channels (Amin, 1999). The converse mutation of the corresponding residue within the α and β subunits of the $GABA_A$R to a Trp residue blocks the action of the general anesthetic enflurane (Mihic et al., 1997). It is possible that the action of neurosteroids on the ρ_1 receptor channel could also be masked, at least in part, by the presence of a large amino acid such as a Trp residue. Other residues within the second transmembrane domain of $GABA_A$R have also been implicated in the action of anesthetics and ethanol (Belelli et al., 1997; Mihic et al., 1997), which could influence the potency of the neuroactive steroids on ρ_1 receptor channels.

Further studies using site-directed mutagenesis and kinetic analysis of ρ_1 and $GABA_A$Rs are needed to explore the mechanisms involved in differential neuroactive steroid action on these two closely related receptor channels.

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Send reprint requests to: Dr. Jahanshah Amin, University of South Florida, College of Medicine, 12901 Bruce B. Downs Blvd., MDC Box 9, Tampa, FL 33612-4799. E-mail: Jamin@pharm.med.usf.edu