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Enantioselectivity of Steroid-Induced γ-Aminobutyric Acid$_A$ Receptor Modulation and Anesthesia

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SUMMARY

Neuroactive steroids have been postulated to cause anesthesia by binding to unique steroid recognition sites on γ-aminobutyric acid (GABA) receptors and modulating GABA receptor function. Steroids interact with these sites diastereoselectively, but it is unknown whether steroid sites show enantioselectivity. To address this issue, we synthesized enantiomers to (+)-3α-hydroxy-5α-androstane-17β-carbonitrile and (+)-3α-hydroxy-5α-pregnan-20-one. In this study, we show that potentiation of GABA-mediated currents and gating of the GABA$_A$ channel by steroids, as well as steroid-induced anesthesia in tadpoles and mice, is enantioselective, with the (+)-enantiomers exhibiting significantly greater potency in all assays. The correlation between the effects of steroid enantiomers on channel behavior and their effects as anesthetics provides strong evidence that GABA$_A$ receptors play a predominant role in steroid-induced anesthesia. The enantiomers also provide a tool to probe the relative contributions of direct chloride channel activation versus potentiation of GABA-elicited currents to the induction of anesthesia. Studies examining the effects of combinations of (+)- and (−)-3α-hydroxy-5α-androstane-17β-carbonitrile were consistent with the hypothesis that potentiation of GABA-activated currents contributes to steroid-induced anesthesia but indicated that direct steroid activation of GABA$_A$ receptors is not mechanistically important in producing anesthesia.

The discovery that steroids enhance GABA$_A$ receptor-mediated chloride currents has generated widespread interest in the study of the pharmacological and physiological consequences of steroid modulation of ion channel function (1, 2). Binding studies have shown that these steroid effects do not result from steroid binding at the barbiturate, benzodiazepine, picrotoxin, or GABA sites located on GABA$_A$ receptors (1–6). Thus, a unique binding site for steroids on GABA$_A$ receptors has been proposed as the locus of action (7–9). Although the current lack of a suitable radioligand precludes the measurement of direct binding to steroid recognition sites, numerous structure-activity studies that correlate the behavioral or physiological effects of steroids with steroid structure support the hypothesis that unique steroid binding sites exist on these receptors (2–6, 10–12).

Particularly relevant to the studies described herein are previous results that examined the stereoselectivity of steroid action on GABA$_A$ receptor function. For example, the actions of (+)-DHP and (+)-3β-DHP on GABA$_A$ receptor function are markedly different. Whereas (+)-DHP is a potent positive allosteric modulator, (+)-3β-DHP is devoid of this activity (10, 11). Hence, it is clear that the actions of steroids on GABA$_A$ receptors are stereoselective. However, the type of stereoselectivity that has been demonstrated is diastereoselectivity, not enantioselectivity.

Because the steroid (+)-DHP has eight chiral centers (C-3, C-17, and the six carbons at the ring fusions), as many as 256 different stereoisomers (128 racemic or enantiomeric pairs) are theoretically possible for this compound. All but one of these stereoisomers are diastereomers of (+)-DHP that arise from changing the absolute configuration of up to any seven of the eight chiral centers in (+)-DHP. These diastereomers, of which (+)-3β-DHP is one, have physical properties that differ from (+)-DHP and interact differently with the chiral environment of a protein receptor binding site and the achiral environment of a lipid bilayer. Hence, diastereomers cannot be used to discriminate between effects caused by interactions with chiral versus achiral sites. Thus, (+)-DHP and (+)-3β-DHP differ not only in their actions at GABA$_A$ receptors but also in their ability to perturb lipid bilayers. The ability of steroids to perturb lipid bilayers has led to the alternative hypothesis that the anesthetic effects of (+)-DHP result from membrane effects (13).

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ABBREVIATIONS: (+)-ACN, (+)-3α-hydroxy-5α-androstane-17β-carbonitrile; (+)-DHP, (+)-3α-hydroxy-5α-pregnan-20-one; (+)-3β-DHP, (+)-3β-hydroxy-5α-pregnan-20-one; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LRR, loss of righting reflex.

One stereoisomer of (+)-DHP, in which the absolute configuration of all eight chiral centers is inverted, is the enantiomer or mirror image of (+)-DHP. Because it is an enantiomer, this compound, (−)-DHP, has physical properties identical to those of (+)-DHP and will perturb membranes in a manner identical to (+)-DHP. Any differences in the modulation of $GABA_A$ receptors by (+)-DHP and (−)-DHP can be attributed to differences in interactions with receptor proteins. In this study, we report the actions of two enantiomeric pairs of steroids, (+)-DHP and (−)-DHP and (+)-ACN and (−)-ACN on $GABA_A$ receptor function and a correlation of these receptor-mediated actions with the production of anesthesia.

Materials and Methods

Steroid Synthesis

The (−)-enantiomers of (+)-ACN and (+)-DHP were made via total steroid synthesis (14, 15) (Fig. 1). Full synthetic details will be reported elsewhere. The (−)-enantiomers had NMR and IR spectra identical to those of the (+)-enantiomers. The (+)- and (−)-enantiomers were checked for enantiomeric purity using optical rotation measurements. Optical rotations of the enantiomeric pairs were recorded in $CHCl_3$ and gave the following values: (−)-ACN, $[\alpha]_D^{25} = -56.8°$; (+)-ACN, $[\alpha]_D^{25} = +57.4°$; (−)-DHP, $[\alpha]_D^{24} = -98.4°$; (+)-DHP, $[\alpha]_D^{24} = +101.5°$.

Electrophysiological Recording

Hippocampal neurons were cultured from 1- to 2-day-old Sprague-Dawley albino rats using methods described previously (16). After 4 to 7 days in culture, neurons were voltage clamped at −70 mV using whole-cell patch clamp recording techniques. The extracellular recording solution contained 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3. Recording pipettes were filled with a solution containing 140 mM CsCl, 4 mM NaCl, 5 mM EGTA, 0.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM HEPES, pH 7.3. GABA and steroids were applied for 500 msec using a pressure (20 psi air) ejection drug delivery system with patch pipettes positioned approximately 5 μm from the recorded neuron. This system allowed reliable drug application while minimizing exposure of neurons to steroids. The concentrations reported are those in the pipette and are an upper limit for the concentrations reaching the cell. Steroids were prepared in a DMSO stock at 10–100 mM and diluted so that the final concentration of DMSO was less than 0.5%.

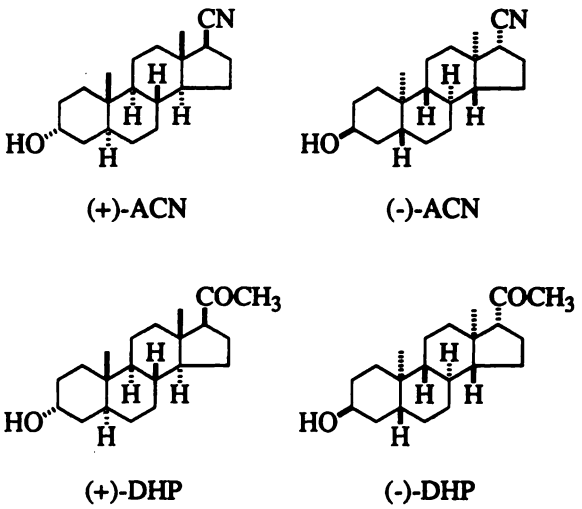

Fig. 1. The structures depict the enantiomers of ACN and DHP used in this study.

DMSO, at this concentration, had no effect on GABA responses. Dose-response data were fit to an equation of the form

$$\text{response} = \text{response}_{max} \times \{[\text{conc}]^{n_H}/([\text{conc}]^{n_H} + EC_{50}^{n_H})\}$$

where response_{max} is the maximum effect, [conc] is the drug concentration, EC_{50} is the half-maximal effective concentration, and n_H is the Hill coefficient.

Anesthesia Assays

Tadpole assay. Groups of 10 early prelimb-bud stage *Xenopus laevis* tadpoles (Nasco, Fort Atkinson, WI) were placed in 100 ml of oxygenated Ringer's solution containing various concentrations of compound. Compounds were added from a 10-mM DMSO stock (final concentration of DMSO in test solutions < 0.2%). After equilibrating at room temperature for 3 hr, tadpoles were evaluated using the LRR behavioral endpoint. LRR was defined as failure of the tadpole to right itself within 5 sec after being flipped by a smooth glass rod. In all cases, the tadpoles regained their righting reflex when placed in fresh oxygenated Ringer's solution. Control beakers containing up to 0.6% DMSO produced no LRR in tadpoles.

Mouse assay. Balb/Cj mice (Sprague Dawley, Harlan Bioproducts for Science, Indianapolis, IN) weighing 20–30 g each were placed under a heat lamp for 1–2 min. Compounds were injected intravenously through a tail vein in a 5% ethanol, 8% Cremophor EL solution (Sigma Chemical, St. Louis, MO) at a rate of 50–250 μl per 5–10 sec. Sleep time was measured from the moment mice displayed LRR until they were able to right themselves. All mice recovered fully without observable neurologic deficits. Control solutions containing 5–8% ethanol and 8–16% Cremophor EL in volumes of 100–300 μl were administered with no observable neurobehavioral effect.

Results

Steroid effects on $GABA_A$ currents. At a fixed concentration of 10 μM, the (+)-enantiomers of ACN and DHP exhibit significantly greater potentiation of 2 μM GABA-mediated chloride currents than their (−)-enantiomeric counterparts (Fig. 2). The (+)-enantiomers show a clear concentration-dependent potentiation of 2 μM GABA-induced currents with an EC_{50} value of 1.4 ± 0.2 μM and 2.2 ± 0.03 μM for (+)-ACN and (+)-DHP, respectively (Fig. 2B). (+)-ACN and (+)-DHP achieve their maximal effects, about 15-fold potentiation of 2 μM GABA-mediated currents, near 100 μM. In contrast, in the concentration range of 100 nM to 100 μM, the (−)-enantiomers exhibit a maximal response with a peak amplitude that is 2-fold larger than that of 2 μM GABA alone.

The (+)-enantiomers also show a concentration-dependent relationship for direct chloride channel gating (i.e., activation of chloride currents in the absence of GABA) with an EC_{50} value of 5.0 ± 0.3 μM and 23 ± 2 μM and Hill coefficients of 1.0 ± 0.4 and 1.0 ± 0.1 for (+)-ACN and (+)-DHP, respectively (Fig. 3). These are only approximate estimates of the EC_{50} values for channel activation because solubility limitations preclude obtaining complete concentration-response curves. Unlike the (+)-enantiomers, which clearly activate chloride currents in the absence of GABA at concentrations ≥ 1 μM, the (−)-enantiomers do not directly activate chloride currents at concentrations up to 100 μM. Despite the failure of the (−)-enantiomers to gate macroscopic Cl^- currents, these agents diminish the currents elicited by the (+)-enantiomers (Fig. 4A). When a range of (+)-ACN concentrations were studied in the presence of 20 μM (−)-ACN, the inhibition

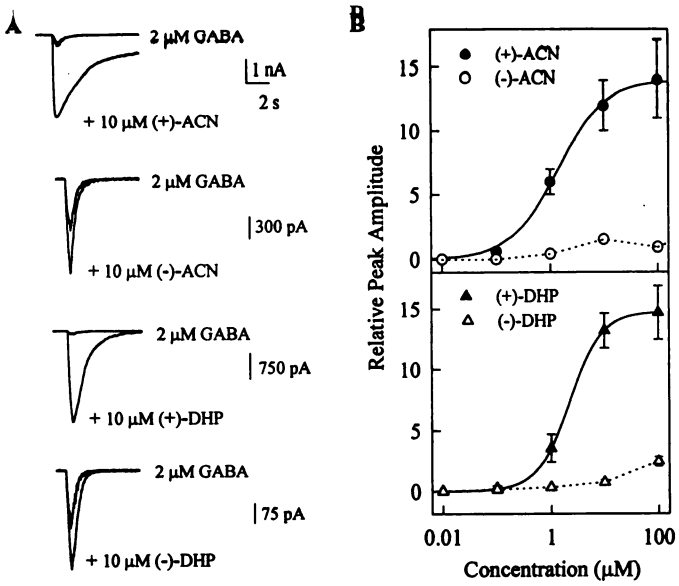

Fig. 2. ACN and DHP potentiate GABA-mediated chloride currents enantioselectively in rat hippocampal neurons. A, Potentiation of 2 μM GABA-mediated chloride currents by 10 μM (+)-ACN and (+)-DHP relative to the potentiation produced by the same concentration of (−)-ACN and (−)-DHP. B, Concentration-response curves for the relative increase in peak currents gated by 2 μM GABA in the presence of various concentrations of (+)-ACN, (−)-ACN, (+)-DHP, and (−)-DHP. *0*, no change in the response to 2 μM GABA; *circles*, ACN enantiomers (*top*); *triangles*, DHP enantiomers (*bottom*); *points*, mean ± standard error of five or more cells (connected by *dotted line*); *solid lines*, fit of the data to the dose-response equation described under Methods.

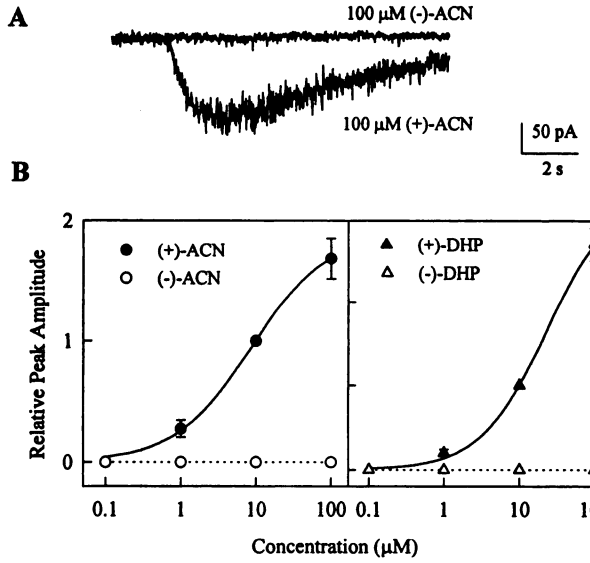

Fig. 3. ACN and DHP directly activate GABA$_A$ receptors enantioselectively in rat hippocampal neurons. A, Response of a single neuron voltage clamped at −70 mV to 500 msec applications of 100 μM (+)-ACN and 100 μM (−)-ACN in the absence of GABA. B, Gating concentration-response curves for ACN and DHP (● and ▲, (+)-enantiomer; ○ and △, (−)-enantiomer); *points*, mean ± standard error of five or more cells (connected by *dotted lines*); *solid lines*, fits of the dose-response equation.

of (+)-ACN chloride channel activation by (−)-ACN was noncompetitive (Fig. 4B).

Anesthesia bioassays. The ACN and DHP enantiomeric pairs were also studied in two widely used anesthesia assays (17–20) that measure LRR in tadpoles and mice. Experiments were performed in tadpoles because concentration-response relationships could be examined, allowing comparisons between the physiology and anesthesia assays. This is possible because the concentration of a nonpolar drug will equilibrate between the tadpole extracellular fluid and the water in which the tadpole swims. The concentration-response curves for LRR in tadpoles were fit to the logistic Hill equation, yielding EC_{50} values of 0.07 ± 0.01 μM and 0.7 ± 0.01 μM and Hill slopes of 1.0 ± 0.1 and 1.4 ± 0.2 for (+)-ACN and (−)-ACN, respectively (Fig. 5A). The (+)- and (−)-enantiomers of ACN achieve the same maximal effect (100% LRR) in this assay. Similarly, the concentration-response curves for (+)-DHP and (−)-DHP in the tadpole assay reveal enantioselectivity in anesthetic action (Fig. 5A). A best fit of these data results in EC_{50} values of 0.39 ± 0.04 μM and 1.1 ± 0.1 μM and Hill coefficients of 2.2 ± 0.5 and 1.4 ± 0.1 for (+)-DHP and (−)-DHP, respectively.

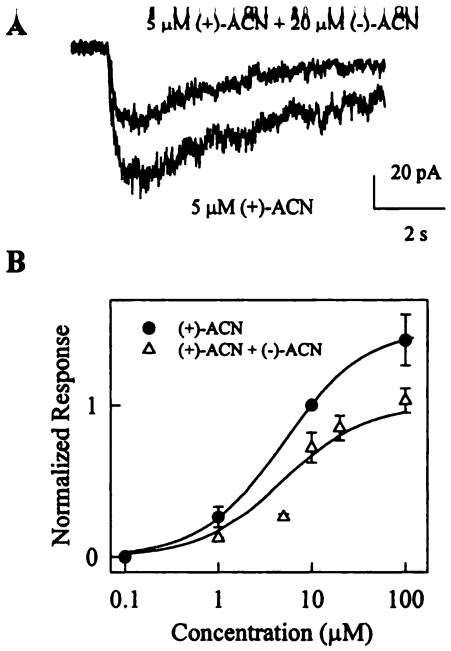

Fig. 4. A, Inhibition of gating by 5 μM (+)-ACN by a higher but nongating concentration (20 μM) of its enantiomer when they are coapplied. B, Effects of coapplication of 20 μM (−)-ACN on currents gated by various concentrations of (+)-ACN (△) compared with the effects of (+)-ACN alone (●). *Curve through* △, fit of a noncompetitive inhibition model to the data. A competitive inhibition model failed to provide an adequate fit.

Dose-response relationships for steroid-induced LRR were also examined in mice because anesthetic endpoints in rodents are more easily compared with humans and because behavioral data obtained in mice facilitate comparison to the rat GABA$_A$ receptor data. When injected into mice, (+)-ACN produced anesthesia in a dose-dependent manner, whereas (−)-ACN was ineffective in producing LRR, even at doses 10 times greater than those used for (+)-ACN (Fig. 5B). (+)-DHP was an effective anesthetic in mice, as previously shown (7, 8), but (−)-DHP was not tested because of the large amount of drug required for these studies.

Interactions between (+)- and (−)-enantiomers. Because (−)-ACN antagonizes the direct gating actions of (+)-ACN (Fig. 4), this provides an opportunity to determine whether direct channel activation is necessary for steroid anesthesia. If it is, then (−)-ACN should shift the (+)-ACN

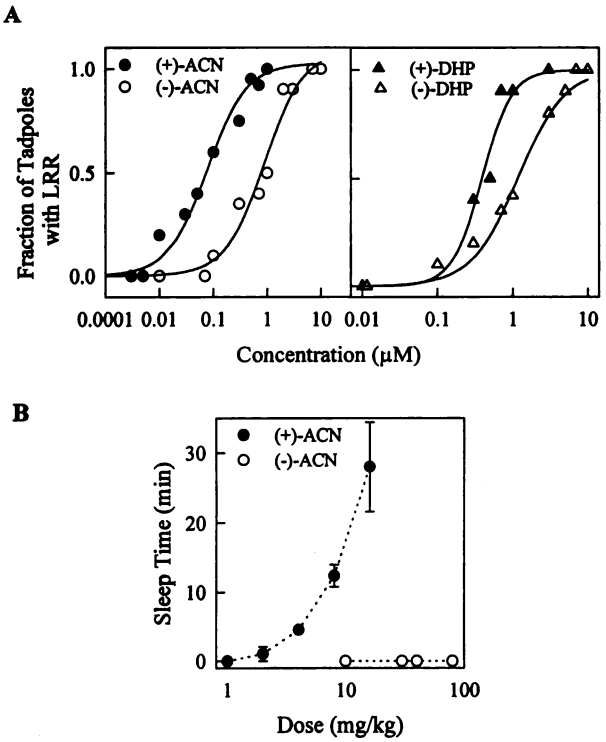

Fig. 5. ACN and DHP act enantioselectively in tadpole and mouse anesthesia assays. A, Concentration-response curves for LRR in tadpoles show the differential effects of (+)-ACN and (+)-DHP (●) versus their (−)-enantiomers (○). *Points* on the tadpole concentration-response *curves* represent mean response of 10–50 animals, scored quantally. Dose-response *curves* were fit to the logistic equation. B, Intravenous injection of (+)-ACN (●) into mice produced dose-dependent LRR (sleep times) ranging from 0–30 min, whereas injection of (−)-ACN (△) did not produce LRR at doses up to 80 mg/kg. *Points* (connected by *dotted line*) on the mice dose-response *curve* for (+)-ACN and (−)-ACN represent the average sleep time from 4–6 animals and 2–3 animals, respectively.

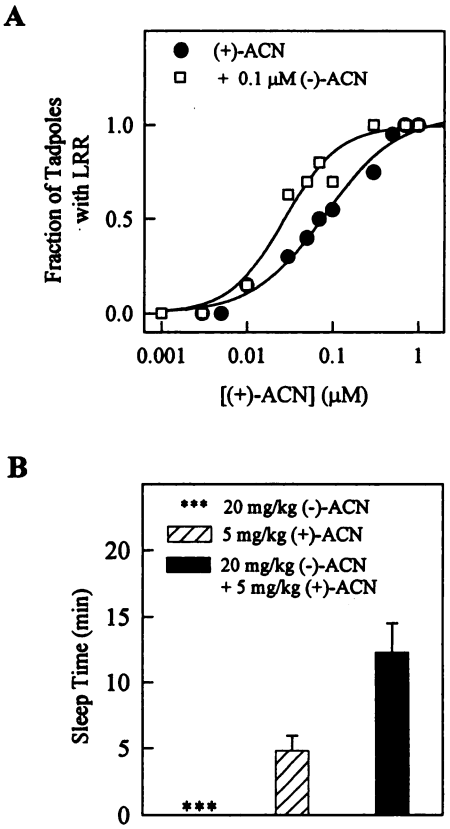

Fig. 6. (−)-ACN enhances (+)-ACN-induced anesthesia in tadpoles and mice. A, An ineffective concentration of (−)-ACN causes a leftward shift in the LRR dose-response *curve* of (+)-ACN when coadministered to tadpoles. B, Correspondingly, an ineffective dose of (−)-ACN (20 mg/kg) augments the effect of a low dose (5 mg/kg) of (+)-ACN when injected intravenously into mice (n = 4).

concentration-response curve for LRR to the right. However, we observed that simultaneous administration of a low concentration of (−)-ACN with (+)-ACN produced a leftward shift in the LRR concentration-response curve in tadpoles (Fig. 6A). The LRR concentration-response curve for (+)-ACN + 0.1 μM (−)-ACN was fit with an EC_{50} value of 0.027 ± 0.003 μM (about one half of that for (+)-ACN alone) and a Hill coefficient of 1.3 ± 0.2. Likewise, in mice, when a low dose of (+)-ACN was administered with an ineffective dose of (−)-ACN, sleep time was increased by at least 2-fold (Fig. 6B). The mean sleep time (n = 4) for mice that were administered only (+)-ACN was found to be statistically less than the mean sleep time for mice that were administered both enantiomers (p = 0.002, two-tailed Student's t test).

These results suggest that if $GABA_A$ receptors play a role in steroid-induced anesthesia, the effect must be mediated by potentiation of GABA responses. If this is the case, one would expect a similar synergistic interaction between the enantiomers in the GABA potentiation assay. In contrast to the high-concentration effects in which (−)-ACN inhibited channel gating by (+)-ACN, simultaneous application of low concentrations of (+)- and (−)-enantiomers resulted in enhanced potentiation of GABA-activated currents. A threshold concentration (1.0 μM) of (−)-ACN had minimal effect on currents mediated by 2 μM GABA (Fig. 7A1). However, when 1.0 μM (−)-ACN was simultaneously applied with a low concentration (0.1 μM) of (+)-ACN, enhanced potentiation of 2 μM GABA-mediated currents was observed. Similar observations were made when comparing the effects of 0.1 μM (+)-ACN alone with that of both 0.1 μM (+)-ACN and 1.0 μM (−)-ACN (Fig. 7A2). Fig. 7B shows a summary of the data from physiology experiments in which both enantiomers were coapplied to hippocampal neurons. Although 1.0 μM (−)-ACN increased 2 μM GABA responses to 121 ± 10% of control (n = 5), the combination of 1.0 μM (−)-ACN and 0.1 μM (+)-ACN produced currents that were 220 ± 20% of control (Fig. 7B, *left*) (p = 0.003, two-tailed t test). Likewise, 0.1 μM (+)-ACN was ineffective in potentiating 2 μM GABA-mediated currents, producing responses that were 107 ± 14% that of 2 μM GABA alone, but 0.1 μM (+)-ACN plus 1.0 μM (−)-ACN yielded currents that were 199 ± 11% of control (Fig. 7B, *right*) (p = 0.003, two-tailed t test). Similar, although smaller, enhancements were observed when higher concentrations of the enantiomers were coapplied. However, at high concentrations, direct gating by (+)-ACN and desensitization make these experiments difficult to interpret.

The synergistic effects of low concentrations of the ACN enantiomers in both physiology and anesthesia assays are inconsistent with the simple action of (−)-ACN as a partial agonist at a single steroid site and suggest that the (+)- and (−)-enantiomers bind to two sites on the same $GABA_A$ receptor. This finding is consistent with previous reports that

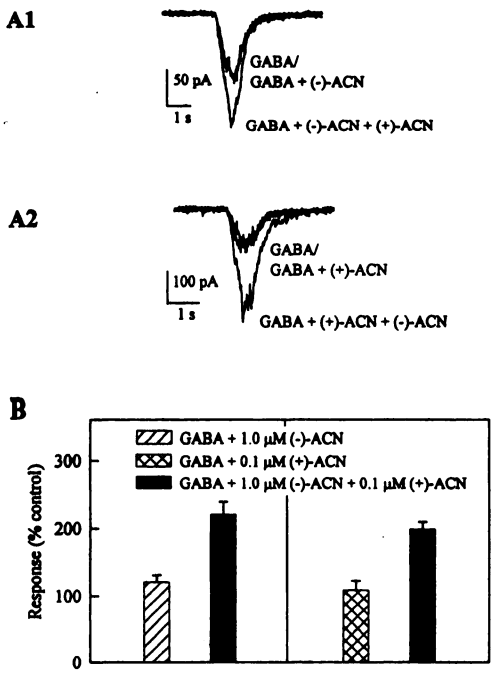

Fig. 7. (−)-ACN enhances the potentiation of GABA-mediated currents by (+)-ACN. A, Physiology experiments in which low concentrations of (+)- and (−)-enantiomers of ACN were coapplied to rat hippocampal neurons show the enhancement of their effects. A1, 1.0 μM (−)-ACN is ineffective in potentiating 2 μM GABA-induced currents, but it synergistically potentiates the effects of 0.1 μM (+)-ACN on GABA-mediated currents. A2, Likewise, 0.1 μM (+)-ACN, which is also ineffective in potentiating 2 μM GABA-induced currents, synergistically potentiates the effects of 1.0 μM (−)-ACN. B, A histogram summarizes the physiology results (*bars*, average response from five neurons, expressed as percentage of control).

steroids act at multiple sites on $GABA_A$ receptors (7, 8, 21). Prior reports indicate that natural enantiomers of pregnane steroids act at a site on the $GABA_A$ receptor that is distinct from benzodiazepine or picrotoxin sites (1–6). Similarly, we found that 1 μM flumazenil, a benzodiazepine receptor antagonist, and 3 mM α-isopropyl-α-methyl-γ-butyrolactone, a picrotoxin site antagonist (22), failed to inhibit the effects of 10 μM (−)-ACN on GABA-mediated currents (data not shown). Thus, it is also unlikely that the (−)-enantiomers bind at these sites. Furthermore, both (+)-ACN and (−)-ACN potentiate currents activated by 500 μM pentobarbital, suggesting that these steroids do not act at the same site as barbiturates. Lower concentrations of (+)-ACN are required to potentiate pentobarbital-gated currents, with 1.0 μM (+)-ACN and 50 μM (−)-ACN yielding responses that are 520 ± 70% (nine experiments) and 230 ± 30% (14 experiments) of pentobarbital alone, respectively.

Discussion

Although both the (+)- and (−)-enantiomers of ACN and DHP potentiate GABA-activated chloride currents in cultured postnatal rat hippocampal neurons, (+)-ACN and (+)-DHP, which have the absolute configuration of naturally occurring steroids, are markedly more potent than their (−)-enantiomers. There also may be differences in the intrinsic efficacies of the (+)- and (−)-enantiomers, but solubility limitations prohibit using sufficiently high concentrations of the (−)-enantiomers to determine maximal effects. Furthermore, unlike the (+)-enantiomers, the (−)-enantiomers are ineffective in gating chloride currents through $GABA_A$ receptors, which indicates that the absolute configuration of the native steroid is required for direct activation of the channel, at least in the concentration range studied. The loss of gating activity for the (−)-enantiomers may be attributed to the lower affinity of these steroids for the binding site(s) involved in gating of $GABA_A$ receptors. These data provide strong support that steroids produce both enhancement of GABA responses and direct channel activation via unique sites on $GABA_A$ receptor proteins.

Potentiation and/or gating of chloride currents through $GABA_A$ receptors has been proposed as a mechanism for general anesthesia (23, 24). These data on enantiomeric steroids provide new evidence that steroid recognition sites on $GABA_A$ receptors are responsible for the pharmacological effects of these agents. Correlations between the physiology and anesthesia assays indicate that steroid-induced anesthesia probably results from low-level potentiation of GABA-mediated currents. This conclusion is supported by the following observations: (i) the (−)-enantiomeric steroids are anesthetics in tadpoles, even though their maximal effect is a doubling of the GABA current; (ii) the (+)-enantiomers produce anesthesia in tadpoles at concentrations much lower than those that produce maximal effects in physiological experiments; and (iii) the enantiomers exhibit positive interactions in both the physiology and anesthesia experiments in which threshold doses of the enantiomers can be combined to give an augmented effect. Moreover, it seems unlikely that direct steroid gating of Cl^- currents at the $GABA_A$ receptor is required for anesthesia because LRR in tadpoles can be achieved at concentrations of the (+)- and (−)-enantiomers of ACN and DHP, which are clearly subthreshold for gating macroscopic Cl^- currents. The positive interactions between the ACN enantiomers also support the assertion that direct activation of $GABA_A$-mediated currents is not required for anesthesia, because (−)-ACN would be expected to antagonize the anesthetic effects of (+)-ACN if direct activation of Cl^- currents was important for producing anesthesia.

Several examples of enantioselective general anesthetics have been described, supporting the hypothesis that anesthetics act directly on proteins rather than on membrane lipids. These examples are as follows: an approximately 2-fold difference in potency between barbiturate enantiomers (25, 26), a 2- to 4-fold difference in potency for ketamine enantiomers (27, 28), and a 2-fold difference in potency for isoflurane enantiomers (29–32). For all of these general anesthetics, only a single chiral center is present in each molecule. Therefore, studies of the stereoselectivity of these agents are also studies of their enantioselectivity. By contrast, steroid anesthetics contain eight chiral centers, and both diastereoselectivity and enantioselectivity can be studied.

The finding that (+)-DHP and (+)-3β-DHP are stereoselective in their actions (10, 11) indicates that there is diastereoselectivity at C-3 in the actions of these steroids. Different pairs of steroid diastereomers may or may not be diastereoselective in their actions. For example, inverting the configuration of the carbonitrile at C-17 of (+)-ACN to produce the 17α-diastereomer or epimer [this term indicates that diastereomers of compounds with more than one chiral center differ in configuration at only one chiral center (33)] gives a com-

pound without positive modulatory effects on $GABA_A$ receptors (10, 34), whereas inverting the configuration at C5 of (+)-ACN to produce the 5β-epimer gives a compound with potent modulatory actions (35). As a further example, it should be noted that the 5β-epimer [(+)-3α-hydroxy-5β-pregnan-20-one] of (+)-DHP is also a potent anesthetic and positive allosteric modulator of $GABA_A$ receptor function (3, 4, 6, 36). Accordingly, there seems to be little or no diastereoselectivity at C5 for these steroid actions. Whether there is enantioselectivity for the actions of anesthetic steroids having the 5β configuration remains to be investigated.

In summary, the enantioselectivity described herein is notably more striking than that observed previously with other general anesthetics. There is a logarithmic order difference in potency between the enantiomers of ACN for producing anesthesia in tadpoles and an even greater enantioselectivity for anesthesia in mice. Additional studies of the enantiomers of other anesthetic steroids such as alphaxalone and 3α-hydroxy-5β-pregnan-20-one and of steroids such as dehydroepiandrosterone sulfate and pregnenolone sulfate, which have inhibitory actions at $GABA_A$ receptors (37–39), are needed to determine whether these steroids are also enantioselective in their actions. Such studies may aid in discrimination among the multiple binding sites that have been proposed for steroids on $GABA_A$ receptors.

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