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Interaction of Pregnanolone and Pregnenolone Sulfate With Ethanol and Pentobarbital

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MELCHIOR, C. L. AND P. M. ALLEN. *Interaction of pregnanolone and pregnenolone sulfate with ethanol and pentobarbital.* PHARMACOL BIOCHEM BEHAV 42(4)605-611, 1992. — 3-α-Hydroxy-5-β-pregnan-20-one [pregnanolone (Pa)] and 3-β-hydroxy-5-pregnen-20-one 3-sulfate [pregnenolone sulfate (PS)] are steroids that have been shown in biochemical studies to be active at the GABA-benzodiazepine-chloride receptor complex, Pa as a "barbiturate-like" agonist and PS as a "picrotoxin-like" antagonist. Since other compounds that are active at this site interact with the effects of pentobarbital and ethanol, the behavioral effects of these steroids alone and in combination with pentobarbital and ethanol were tested. Pa blocks the convulsions caused by pentylenetetrazole (PTZ) and increases motor activity when given alone in low doses. In combination with either pentobarbital or ethanol, it enhances the depression in motor activity, hypothermia, and hypnosis. In contrast, PS has no effect on PTZ convulsions and depresses motor activity by itself. With pentobarbital, PS enhances the depression in motor activity but has no effect on hypothermia or hypnosis. With ethanol, PS enhances the hypothermia but does not affect motor activity or hypnosis. Therefore, Pa and PS show different but not opposite effects in interacting with compounds active at the GABA-benzodiazepine-chloride receptor complex.

Pregnanolone Pregnenolone sulfate Alcohol Pentobarbital Neurosteroid Benzodiazepine
Pentylenetetrazole Motor activity Hypothermia Sleep time

MANY of the behavioral effects of ethanol are thought to be related to the actions of ethanol on the GABA-benzodiazepine-chloride receptor complex. Acutely, the incoordinating and anesthetic effects of ethanol are enhanced by GABA-mimetic agents and reduced by GABA antagonists. Reciprocally, the incoordinating effects of GABA mimetics are enhanced by ethanol while the actions of GABA antagonists are blocked by ethanol (26). The depressant actions of benzodiazepines and barbiturates are enhanced by ethanol, and cross-tolerance between these substances and ethanol has been demonstrated (36).

A great deal of attention has been focused on the benzodiazepine receptor inverse agonist Ro 15-4513. This compound has been shown to reverse some, but not all, of the effects of ethanol. It has been noted to be particularly effective in modifying the behavioral effects of ethanol in tests of locomotor activity, anxiety, exploration, and hypnosis, but not hypothermia (3,18,19). In many tests, other inverse agonists are also capable of influencing the response to ethanol (18,33).

Recently, several steroids have been shown in biochemical and electrophysiological studies to interact with the GABA-benzodiazepine-chloride receptor complex (9,20-22,24,25,27, 28,37). Majewska et al. (24) characterized these steroids as "barbiturate-like," with the notable exception of 3-β-Hydroxy-5-pregnen-20-one 3-sulfate [pregnenolone sulfate (PS)], which acts as a "picrotoxin-like" antagonist at the chloride channel and counteracts the electrophysiological responses to pentobarbital (9,27). In vivo, Majewska et al. (23) found that PS antagonized barbiturate-induced hypnosis in rats. In contrast, many of the barbiturate-like steroids are known to be effective hypnotic agents (2,6,12,31).

In considering a possible interaction of these steroids with ethanol, Majewska (9) demonstrated that the increase in [^{3}H]muscimol binding in rat cortical synaptosomal membranes produced by the barbiturate-like steroid 3-α-hydroxy-5-α-pregnan-20-one (3A-OH-DHP) is markedly reduced in the presence of 50 mM ethanol. Ethanol also alters the effects of PS, which has biphasic effects on muscimol binding. In the presence of 50 mM ethanol, the phase of augmentation of binding is abolished and the decrement of binding is somewhat enhanced. At 5 mM ethanol, similar effects may occur (21).

The purpose of the present studies was to examine the behavioral effects of both a barbiturate-like steroid, 3-α-hydroxy-5-β-pregnan-20-one [pregnanolone (Pa)], and the picrotoxin-like steroid, PS, alone and in combination with ethanol. For comparison, the interaction of these steroids with pentobarbital was also evaluated.

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METHOD

Subjects

Male C57Bl/6 mice obtained from NCI and weighing 21–30 g were used for all experiments. Mice were housed in groups of five to six per cage and maintained in the Animal Research Facilities under a 12 L:12 D cycle. Except in the activity studies, all injections were administered between 0930 and 1130 h. Within the experiments on activity and temperature, mice were tested more than once (see below).

Drugs

Drugs were prepared in a vehicle of saline containing 0.4% w/v Tween-80. Except where noted, drugs were administered as a single injection of 0.01 ml/g body weight. Drugs used were PS (Sigma Chemical Co., St. Louis, MO), PA (Sigma), 95% ethanol (Gold Shield, Hayward, CA), pentobarbital (Nembutal; Abbott, North Chicago, IL), and pentylenetetrazole (PTZ; Sigma). PS and Pa were suspended in saline/Tween, then sonicated 10–15 min and mixed with a vortex, resulting in a fine dense suspension. Ethanol was prepared with distilled water as a 20% w/v solution. When administered with Pa and PS, the steroids were dissolved in this concentration of ethanol.

Convulsions

Because the ability of some compounds to interact with the effects of ethanol has been attributed to their convulsant properties, and because the biochemical profile of PS suggests that it could be a convulsant, both Pa and PS were tested for pro- and anticonvulsant activity with PTZ.

Presence or absence, as well as latency to onset, of myoclonic seizures was noted for animals injected intraperitoneally with 50 mg/kg PTZ given alone or in combination with a dose of Pa or PS. To assess the possibility that PS might have a proconvulsant effect, it was also tested with a dose of 25 mg/kg PTZ.

Activity

Activity measurements were made in an open-field setting with a 10 × 18-in. plastic cage marked into six squares, set on white paper in a brightly lit room. Immediately after drug injection, the animal was placed in one corner and the number of squares traveled in 5-min periods for 30 min were counted. Data are presented as the sum of counts for the period 5–30 min after injection. Experiments were conducted between 0800 and 1400 h. Over the days of testing, the hour of testing for each drug and dose was randomized to reduce any contribution by time of day. Various doses of Pa and PS were tested alone and in combination with pentobarbital and ethanol.

For this experiment, individual mice were tested six times with at least 1 week between trials. The first test trial for each mouse was performed with a saline injection (this data was not included in the analyses) to reduce a novelty effect. Drugs were administered in random order, with no animal receiving the same drug combination more than once.

Temperature

Mice were anesthetized with 65 mg/kg pentobarbital to implant Mini-Mitters (Mini-Mitter Co., Sun River, OR) in the peritoneal cavity. Experiments were begun approximately 1 week after surgery.

Mice were placed individually in cages set on receiver platforms so that temperature could be recorded by the Dataquest computer program every 2 min for 3 h. The first hour was used to establish baseline. A dose-response curve for Pa and PS was determined in one set of mice. In another set of mice, animals were injected intraperitoneally with 50 mg/kg Pa or PS, 2.0 g/kg ethanol, 20 mg/kg pentobarbital, or a combination of these doses of steroid and ethanol or steroid and pentobarbital.

Each mouse in this experiment was utilized five times with at least 3 days between tests. Prior to any drug testing, each mouse was given a trial with saline to reduce the influence of novelty of the environment. Drugs were administered in random order, with no animal receiving the same drug combination more than once.

Sleep Time

Sleep time was defined as the time between loss of righting reflex and the time at which the mouse was again able to right itself three times in 30 s. Duration of loss of righting reflex was monitored following an injection of 50 mg/kg pentobarbital or 3.5 g/kg ethanol. To replicate Majewska et al.'s (23) procedures, for one experiment PS was injected 15 min after pentobarbital. In the other experiments, 50 mg/kg PS or Pa was injected in combination with ethanol or pentobarbital.

In a replication of the ethanol experiments, 20-μl trunk blood samples were obtained at the time righting reflex was regained in mice given 3.5 g/kg ethanol alone or in combination with 50 mg/kg PS or Pa for the determination of blood ethanol levels by a gas chromatographic procedure (35).

Statistical Analysis

An analysis of variance (ANOVA) followed by a Dunnett test was employed in the dose-response activity measures. Student's t-test was used to analyze motor activity, temperature, sleep time, and convulsion latency. For the nonparametric data of seizure occurrence, a Fisher Exact Probability Test was used. A level of $p < 0.05$ was considered significant. Values are expressed as means ± SEM.

TABLE 1

SEIZURES WITH 50 mg/kg PENTYLENETETRAZOLE

Dose Steroid	No. Seizing/ No. Mice	Latency (min)
0 mg/kg	12/12	2.48 ± 0.21
12.5 mg/kg Pa	6/6	3.88 ± 0.57*
25.0 mg/kg Pa	2/6†	
50.0 mg/kg Pa	0/5†	
0 mg/kg	5/5	2.42 ± 0.07
50.0 mg/kg PS	6/6	2.65 ± 0.21
100.0 mg/kg PS	6/6	2.89 ± 0.77

Pa, pregnanolone; PS, pregnenolone sulfate.
*$p < 0.05$, Student's t-test.
†$p < 0.05$, Fisher Exact Probability Test.

RESULTS

Convulsions

PTZ causes myoclonic seizures in all animals given 50 mg/kg but none in animals given half that dose. Table 1 shows that 25 or 50 mg/kg Pa protected animals from seizures induced by 50 mg/kg PTZ and at 12.5 mg/kg Pa delayed the onset of seizures. PS at 50 or 100 mg/kg had no effect on seizures, neither protecting against seizures produced by 50 mg/kg PTZ (Table 1) nor initiating seizures when given with 25 mg/kg PTZ (data not shown).

Activity

A bell-shaped dose-response curve was obtained with Pa (Fig. 1). Concentrations of Pa from 12.5-50 mg/kg cause an increase in activity while 100 mg/kg results in a decrease in activity. At 50 and 100 mg/kg, some mice exhibit a high-frequency, low-amplitude tremor. For each dose, activity levels were maximal within the first 15 min, then returned toward the levels exhibited by control animals.

Increasing doses of PS from 25 to 100 mg/kg shows a progressive decrease in activity (Fig. 1). The nadir in activity at each dose occurred within 15 min, after which activity returned to the level of control mice.

In the presence of 20 mg/kg pentobarbital plus 50 mg/kg either of the steroids, the level of activity becomes significantly less than that seen in mice given saline, pentobarbital, or the respective steroid alone. The increased activity caused by Pa alone is clearly abolished in the presence of pentobarbital (Fig. 2).

While 1.0 g/kg ethanol has no effect on motor activity in this paradigm, 2.0 g/kg causes a significant depression in activity (Fig. 3). Both doses of ethanol reduce the hyperactivity caused by Pa. At 2.0 but not at 1.0 g/kg ethanol, the level of activity with Pa is significantly less than that produced by ethanol alone. In contrast, the reduced level of activity resulting from 50 mg/kg PS is not further diminished by ethanol.

Temperature

Both steroids as well as ethanol and pentobarbital cause a fall in body temperature. Unlike the other compounds, with Pa the temperature did not begin to drop until approximately 15 min after injection (Figs. 4-6). As shown in Fig. 4, a dose-dependent hypothermic response occurred with injections of Pa. Following PS, a small hypothermic response of similar magnitude occurred with doses of 50 and 100 mg/kg. Since 50 mg/kg of either steroid produced an effect on temperature, this dose was selected for testing in combination with pentobarbital and ethanol.

Pa in combination with 20 mg/kg pentobarbital (Fig. 5) produced a greater fall in temperature than either compound alone. In contrast, PS given with pentobarbital did not show any differences from either substance given alone. When the dose of pentobarbital was increased to 50 mg/kg to generate a greater fall in temperature, PS again failed to interact with the hypothermic response (data not shown).

The combination of Pa with 2.0 mg/kg ethanol (Fig. 6) resulted in a greater fall in temperature than observed with either compound alone. In this experiment, six of the eight mice given Pa plus ethanol lost righting reflex. PS at 50 mg/kg plus ethanol (Fig. 6) also generated a larger fall in temperature than with either substance alone.

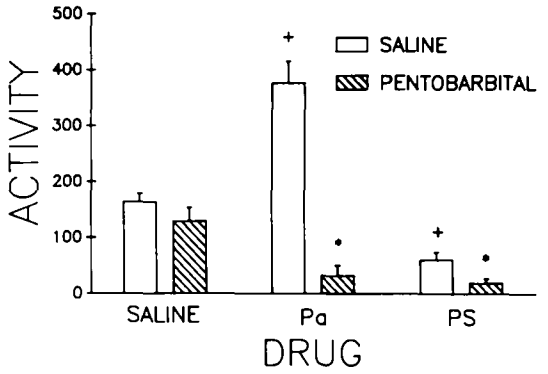

FIG. 2. Motor activity of mice given 20 mg/kg pentobarbital in combination with saline, 50 mg/kg pregnanolone (Pa), or 50 mg/kg pregnenolone sulfate (PS). n = 8-12. $^{*}p < 0.05$, t-test compared to steroid or pentobarbital alone; $^{+}p < 0.05$, t-test compared to saline alone.

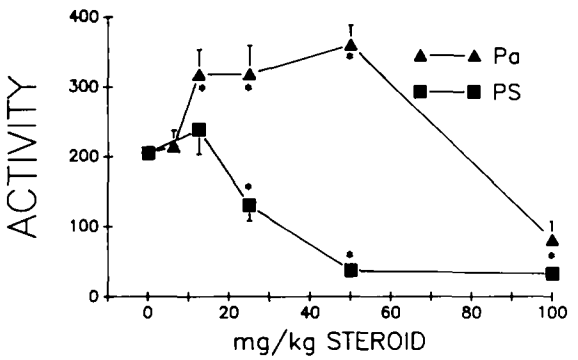

FIG. 1. Motor activity of mice following various doses of pregnanolone (Pa) or pregnenolone sulfate (PS). n = 6-25 except for the control group. Since saline mice run on different days were not different, the groups were pooled for an n = 67. $^{*}p < 0.05$, Dunnett test compared to no steroid.

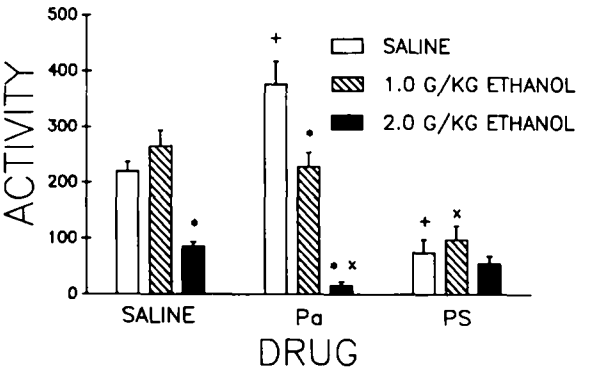

FIG. 3. Motor activity of mice given ethanol in combination with saline, 50 mg/kg pregnanolone (Pa), or 50 mg/kg pregnenolone sulfate (PS). n = 7-21. $^{*}p < 0.05$, t-test compared to the same drug without ethanol; $^{+}p < 0.05$, t-test compared to saline alone; $^{\times}p < 0.05$, t-test compared to the same dose of ethanol alone.

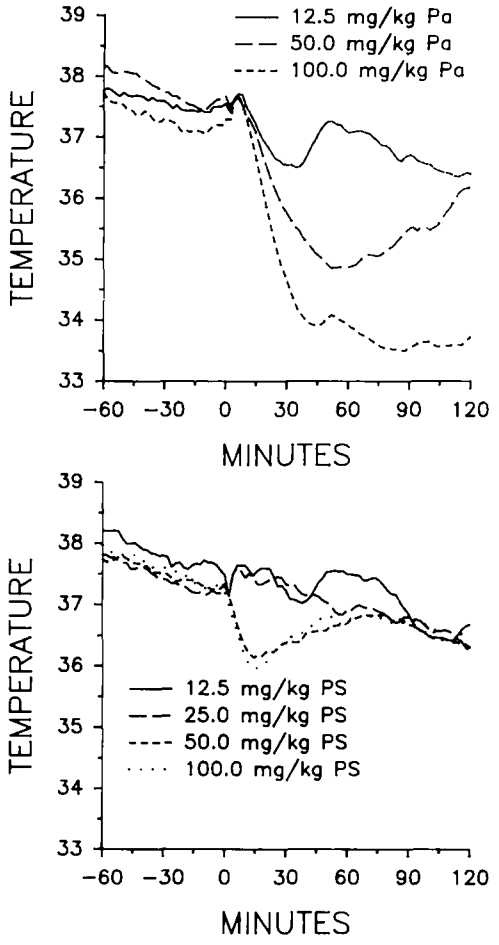

FIG. 4. Mean body temperature (°C) recorded every 2 min for 1 h before and 2 h after an intraperitoneal injection of various doses of pregnanolone (Pa; top) or pregnenolone sulfate (PS; bottom). n = 5-11.

Sleep Time

Pa at 50 mg/kg administered with either 50 mg/kg pentobarbital or 3.5 g/kg ethanol significantly increased sleep time (Fig. 7).

Following the procedure Majewska et al. (23) used with rats, mice were injected with 50 mg/kg pentobarbital, followed 15 min later with 16.8 mg/kg PS or saline. No difference in sleep time was seen. Administering a higher dose of PS, 50 mg/kg, also failed to influence sleep time (Fig. 7). Given with 3.5 g/kg ethanol, 50 mg/kg PS again failed to affect sleep time.

In a separate experiment, blood ethanol levels were determined at regain of righting reflex. The blood ethanol level of animals injected with 50 mg/kg PA, 252.8 ± 9.4 mg/dl (mean ± SEM), was much lower than those of animals given ethanol alone, 383.1 ± 18.0 mg/dl, $t(7) = 5.92$, $p < 0.01$. Mice given 50 mg/kg PS had a blood ethanol level of 369.7 ± 4.8 mg/dl (N = 5) at regain of righting reflex. As with sleep time, this was not different from the group given ethanol alone.

DISCUSSION

The profile of effects of Pa is consistent with previous reports of its anticonvulsant activity (11) and what might be expected of an anesthetic agent. It enhances the depressant effects of both pentobarbital and ethanol on measures of motor activity, body temperature, and hypnosis.

Given by itself at low doses, Pa causes an increase in activity, an effect that has not previously been noted. Both pentobarbital and ethanol, when tested under appropriate conditions, are also known to have a biphasic affect on motor activity (29), with low doses causing an increase in activity and high doses causing sedation. Other studies utilizing Pa tested for hypnotic (2,6,12) or anticonvulsant actions (11) and did not have appropriate conditions for observing increased locomotor activity. It has also been administered in a vehicle such as propylene glycol that could obscure this behavioral effect (12). However, it has been reported that other steroids with similar effects on the GABA-benzodiazepine-chloride receptor complex, namely, 3A-OH-DHP and 3-α,5-α-tetrahydroxycorticosterone (16,17), do not increase locomotor activity.

Blood ethanol levels at regain of righting reflex are a measure of sensitivity to ethanol (1,15). Since the levels of ethanol in mice treated with Pa are lower than controls at regain of

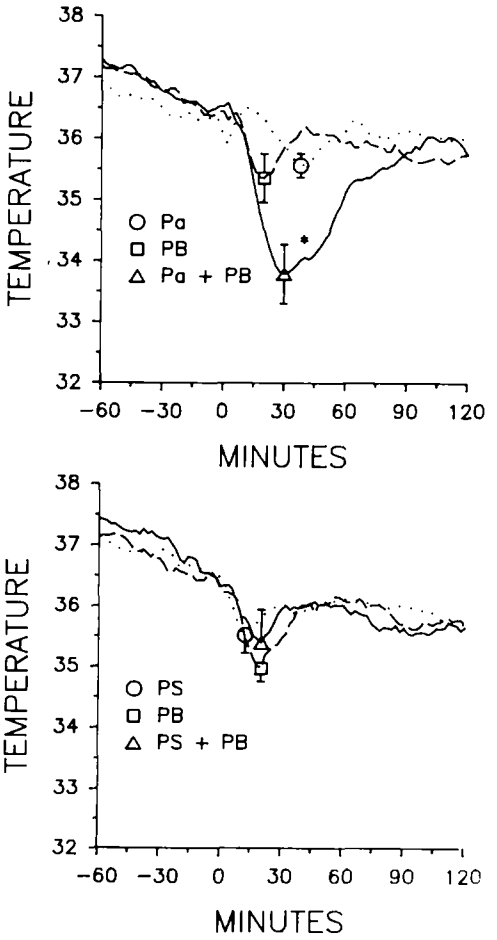

FIG. 5. Mean body temperature (°C) recorded every 2 min for 1 h before and 2 h after an intraperitoneal injection of 50 mg/kg Pregnanolone (Pa; top), 50 mg/kg pregnenolone sulfate (PS; bottom), 20 mg/kg pentobarbital (PB), or 50 mg/kg steroid plus 20 mg/kg PB. Symbols indicate the mean ± SEM of the nadir in temperature. n = 8-9. *$p < 0.01$, t-tests compared to other groups.

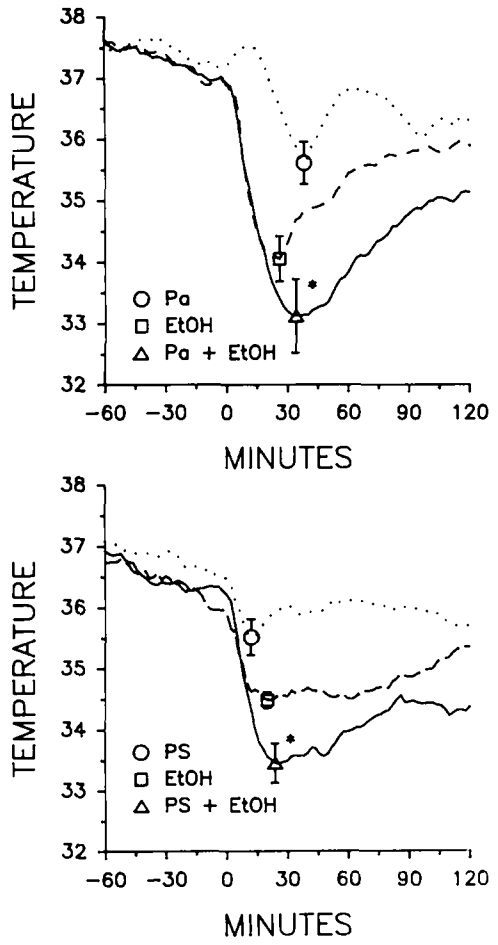

FIG. 6. Mean body temperature (°C) recorded every 2 min for 1 h before and 2 h after an intraperitoneal injection of 50 mg/kg pregnanolone (Pa; top), 50 mg/kg pregnenolone sulfate (PS; bottom), 2.0 g/kg ethanol (EtOH), or 50 mg/kg steroid plus 2.0 g/kg EtOH. Symbols indicate the mean ± SEM of the nadir in temperature. n = 7–10. *$p < 0.01$, t-tests compared to other groups.

righting reflex, Pa increases sensitivity to ethanol. With righting reflex reappearing at a much later time in the Pa group, allowing more time for the elimination of ethanol, it does not appear that Pa alters ethanol metabolism.

A compound such as PS with properties of a picrotoxin-like antagonist at the GABA-benzodiazepine-chloride receptor complex might be expected to have convulsant or proconvulsant effects. However, Gee et al. (5) demonstrated that PS has anticonvulsant activity at 100 mg/kg in mice against convulsions induced by t-butylbicyclophosphorothionate (TBPS). No anticonvulsant or proconvulsant activity was observed in the doses tested in the experiments reported here against convulsions produced by PTZ.

Although Majewska et al. (23) were able to attenuate pentobarbital-induced sleep time with PS in rats, no affect of PS on either pentobarbital- or ethanol-induced sleep time was noted in this study. The different subjects used, rats vs. mice, may be one explanation for this discrepancy. In Majewska et al.'s experiments, a dose-dependent effect of PS on pentobarbital hypnosis was reported for the doses utilized (8.4, 12.6, and 16.8 mg/kg). Of the doses tested in the present study, 16.8 mg/kg was selected to match the range Majewska et al. tested. When this proved ineffective, a higher dose (50 mg/kg) was tested, but it also had no effect. If an ability to interact with the TBPS receptor site is important for altering anesthetic actions (13), and Gee et al. (5) showed that 100 mg/kg *attenuated* TBPS-induced convulsions in mice, a higher dose would not be likely to inhibit hypnosis in mice.

PS exhibited different interactions with pentobarbital and ethanol on the other measures examined. PS enhanced the depression in motor activity with pentobarbital but not with ethanol. In examining hypothermia, PS did not alter the effect of pentobarbital while the same dose enhanced the fall in temperature caused by ethanol. Biochemical differences in the actions of pentobarbital and ethanol at the chloride channel or on GABA or benzodiazepine binding (1,7,8) may contribute to the different interactions of PS with the behavioral effects of the drugs. Differences in the systems by which pentobarbital and ethanol influence the behavioral outcomes monitored may also contribute to these observations.

Studies of the central mechanisms of the thermic effects of ethanol (14) and the lack of effect of benzodiazepine antagonists and inverse agonists, as well as the GABA antagonist picrotoxin, on ethanol-induced hypothermia in mice (10,26, 34) suggest that the GABA-benzodiazepine-chloride receptor complex is not critical to this action of ethanol. Therefore, the enhanced hypothermia observed when PS was administered with ethanol may reflect the fact that the drugs act at different sites to influence temperature. In contrast, the inhibition of the hypothermic effect of pentobarbital by the benzodiazepine receptor inverse agonist Ro 15-4513 does indicate that this complex may be involved in the hypothermic effect of pentobarbital (10).

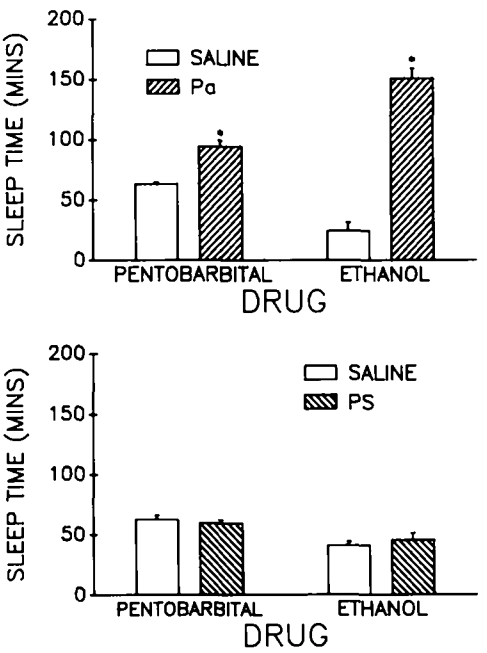

FIG. 7. Sleep time in mice given 50 mg/kg pentobarbital or 3.5 g/kg ethanol in combination with 50 mg/kg pregnanolone (Pa; top) or 50 mg/kg pregnenolone sulfate (PS; bottom). PS was given 15 min after pentobarbital; in all other experiments, steroid and drug were administered at the same time. n = 9–14. *$p < 0.05$, t-test compared to saline.

Although certain steroids have been characterized as barbiturate- or picrotoxin-like, they have been shown not to act at the same site as barbiturates (4,5). In fact, more than one steroid binding site has been indicated (30). In addition, PS has, for example, been shown to bind to the σ-receptor (32) and to inhibit glycine-induced current in a patch clamp preparation (38). The behavioral effects observed with steroids may therefore be influenced by actions in a variety of systems. Taken together, the behavioral data in mice fail to substantiate the characterization of PS as a picrotoxin-like antagonist at the GABA-benzodiazepine-chloride receptor complex.

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