ELSEVIER

Pharmacology Biochemistry & Behavior, Vol. 55, No. 3, pp. 323–326, 1996

0091-3057/96 $15.00 + .00

PII S0091-3057(96)00100-1

Evaluation of In Vivo Interactions in Mice Between Flurazepam and Two Neuroactive Steroids

S. I. DEUTSCH,*†[1] R. B. ROSSE,*† K. STEINBERG,* C. MORN,* L. KOETZNER,* R. RIGGS* AND J. MASTROPAOLO*

*Psychiatry Service (116A), Department of Veterans Affairs Medical Center, 50 Irving Street, N.W., Washington, DC 20422
†Department of Psychiatry, Georgetown University School of Medicine, 3800 Reservoir Road, N.W., Washington, DC 20007

Received 16 October 1995; Accepted 9 February 1996

DEUTSCH, S. I., R. B. ROSSE, K. STEINBERG, C. MORN, L. KOETZNER, R. RIGGS AND J. MASTROPAOLO. *Evaluation of in vivo interactions in mice between flurazepam and two neuroactive steroids.* PHARMACOL BIOCHEM BEHAV **55**(3) 323–326, 1996.—The development of neuroactive steroids as anticonvulsant medications may be useful both as a primary treatment and as an adjuvant to other anticonvulsants. They may be limited, however, by sedative and ataxic side effects. In the current study, 3_{alpha}-hydroxy-5_{beta}-pregnan-20-one and alfaxalone, two prototypic neuroactive steroids, were shown to potentiate the ability of flurazepam to antagonize electrically precipitated tonic hindlimb extension in mice at doses that by themselves had little antiseizure efficacy. While alfaxalone alone lacked motor incoordinating effects at a dose (18.0 mg/kg) that potentiated the antiseizure efficacy of flurazepam, the same dose of 3_{alpha}-hydroxy-5_{beta}-pregnan-20-one possessed both the ability to potentiate flurazepam's anticonvulsant effect and disrupt mouse rotorod performance. The data suggest that allosteric interactions that have been described in vitro between neuroactive steroids and other modulators of the $GABA_A$ receptor complex may have relevance for the intact animal. Finally, the data also suggest that neuroactive steroids could be developed as short-lived adjuvant antiseizure medications in certain critical situations (e.g., medication-refractory status epilepticus). However, the motor incoordinating effects resulting from the combination of neuroactive steroids and flurazepam suggest that their usefulness as adjuvant medications in the chronic therapy of seizure disorders may be limited.

Neuroactive steroids GABA Seizures Rotorod

NATURALLY occurring 3_{alpha}-hydroxy ring-A reduced metabolites of progesterone and deoxycorticosterone have been shown to act as allosteric modulators of the $GABA_A$ receptor complex (2,8). These neuroactive steroids bind to a distinct site within the hydrophobic channel domain of this pentameric protein complex. Steroids that are positive allosteric effectors potentiate GABA-stimulated chloride ion conductance, enhance ^{3}H-muscimol and ^{3}H-flunitrazepam binding, and inhibit the binding of ^{35}S-*t*-butylbicyclophosphorothionate (^{35}S-TBPS), a channel ligand that is a specific marker of the GABA-associated chloride ionophore (3). There is considerable interest in the development of these GABA-positive neuroactive steroids as anticonvulsants, as well as anxiolytic, sedative, and anesthetic medications (1).

There are preliminary data suggesting that naturally occurring and synthetic neuroactive steroids antagonize seizures at doses that are associated with neurotoxicity (i.e., sedation and motor incoordination) (6). Moreover, the anticonvulsant efficacy of neuroactive steroids in mice appears to be more easily detected by the assessment of their abilities to protect against pentylenetetrazol (PTZ)-induced seizures, rather than their abilities to protect against tonic hindlimb extension in the maximal electroshock seizure (MES) test (6). Therefore, we wondered if the incremental electroconvulsive shock (IECS) seizure paradigm might afford a better opportunity to detect efficacy against electrically precipitated tonic hindlimb extension than the MES test. Moreover, we studied doses of the two prototypic neuroactive steroids that would be theoreti-

[1] To whom requests for reprints should be addressed.

cally less likely to be associated with sedation and motor incoordination (i.e., doses less than 20 mg/kg) for their abilities to not only protect from seizures alone, but also to potentiate flurazepam's antiseizure efficacy (6). Other investigators reported that the TD_{50} (i.e., the dose that resulted in at least 50% of the animals showing motor impairment in the horizontal screen test) for a series of nine neurosteroids was above 20 mg/kg (6).

The current study evaluated the abilities of 3_{alpha}-hydroxy-5_{beta}-pregnan-20-one (3a5B-P) and alfaxalone, prototypic naturally occurring and synthetic neuroactive steroids, respectively, to antagonize electrically precipitated seizures in mice using an IECS procedure (10). Furthermore, the abilities of these two compounds to potentiate the antiseizure efficacy of flurazepam, a benzodiazepine agonist representing another class of positive allosteric effector of the $GABA_A$ receptor complex, in this paradigm were also examined. Also, the motor incoordinating effects of these neuroactive steroids alone and in combination with flurazepam were evaluated at a dose of each that was devoid of a significant anticonvulsant effect (i.e., 18.0 mg/kg). The latter experiments were performed to evaluate potential sedative-ataxic effects of the neuroactive steroids alone and in combination with flurazepam; sedative-ataxic effects would limit the clinical usefulness of the neuroactive steroids as adjuvant maintenance antiseizure medications.

METHOD

Animals

An outbred strain of experimentally naive, male NIH Swiss mice purchased from the National Cancer Institute was used throughout all experiments. Animals weighed approximately 30 g and were housed in hanging wire cages (five mice/cage). Animals were maintained in a temperature and humidity controlled vivarium with a 12 L:12 D cycle with free access to food and water. Animals were transported to the laboratory on the day of the experiment.

Drugs

3_{alpha}-Hydroxy-5_{beta}-pregnan-20-one (3a5B-P; a generous gift of Dr. Nancy C. Lan, CoCensys, Inc., Irvine, CA) and alfaxalone (a generous gift of Glaxo Group, Middlesex, UK) were dissolved in 4.5% w/v 2-hydroxypropyl-beta-cyclodextrin in distilled water. Flurazepam HCl (Hoffmann–LaRoche, Nutley, NJ) was dissolved in 0.9% saline. All drugs were injected intraperitoneally in a volume of 0.01 ml/g of body weight. 3a5B-P was always injected 10 min prior to the IECS or rotorod procedures. When studied alone, alfaxalone was injected 20 min prior to the IECS or rotorod procedures; however, when its interaction with flurazepam was studied, it was injected 5 min before flurazepam. Flurazepam was always injected 20 min prior to the IECS or rotorod procedures.

Incremental Electroconvulsive Shock (IECS) Procedure

In the IECS procedure, a Hittman electroconvulsive shock generator (Medcraft model B24-II) was utilized to administer 0.3 s of voltage via earclip electrodes (4,9). To determine the threshold voltage for the precipitation of tonic hindlimb extension, the procedure began with 70 V and was increased in 10-V increments every 2 s until maximal tonic hindlimb extension occurred or 170 V was reached. A voltage of 180 was recorded for animals that did not show tonic hindlimb extension.

Rotorod Procedure

To evaluate possible sedative/ataxic properties of 3a5B-P and alfaxalone alone and in combination with flurazepam, we used a rotorod apparatus (Biological Research Apparatus, Model 7600, Comerio Varese, Italy) rotating at a constant speed of 12 rpm. Rotorod assessment was divided into two phases: a baseline period of 5 min, during which the animals were replaced on the rotating rod in the event of failure to maintain balance. Subsequent to this, a 5-min testing period began following the administration of either 3a5B-P, alfaxalone, or their vehicle, in combination with a dose of flurazepam or flurazepam vehicle.

Analysis

In all experiments, groups of at least 12 mice were tested in each of the experimental conditions. Data from the experiments were analyzed with either a one-way or two-way analysis of variance (ANOVA) and subsequent post hoc tests when appropriate. All reports of statistical significance were based upon a value of $p < 0.05$.

RESULTS

As illustrated in Fig. 1a, a one-way ANOVA revealed a significant main effect for alfaxalone in terms of raising the threshold voltage required for the electrical precipitation of tonic hindlimb extension, $F(1, 4) = 4.15$. Post hoc comparisons (Scheffe test) showed that although vehicle and the 5.6 mg/kg dose of alfaxalone did not differ significantly from each other, the 32.0 mg/kg dose of alfaxalone differed significantly from both vehicle and the 5.6 mg/kg dose. As illustrated in Fig. 1b, a one-way ANOVA revealed a statistically significant main effect for 3a5B-P in terms of antagonizing electrically precipitated tonic hindlimb extension, $F(1, 4) = 3.85$. However, post hoc comparisons (Scheffe test) failed to detect statistically significant differences between any of the 3a5B-P doses and vehicle. As illustrated in Fig. 2a, an ineffective or marginally effective dose of alfaxalone (18.0 mg/kg) was able to potentiate the antiseizure efficacy of flurazepam. A two-way ANOVA revealed a significant main effect for flurazepam, $F(2, 5) = 34.28$, a significant main effect for alfaxalone, $F(1, 1) = 36.56$, and the absence of a significant interaction, $F(12, 5) = 2.06$. As illustrated in Fig. 2b, an ineffective dose of 3a5B-P (18.0 mg/kg) potentiated the antiseizure efficacy of flurazepam. A two-way ANOVA revealed a significant main effect for flurazepam, $F(1, 5) = 24.71$, a significant main effect for 3a5B-P, $F(2, 1) = 17.21$, and no significant interaction, $F(12, 5) = 1.57$.

As illustrated in Fig. 3, a one-way ANOVA revealed a significant main effect for flurazepam dose, $F(2, 4) = 128.75$, and a significant main effect for treatment with each of the neuroactive steroids, $F(2, 4) = 18.18$, on rotorod performance. In addition, there were significant interactions between the neuroactive steroids and flurazepam, $F(4, 99) = 6.22$. However, whereas post hoc comparisons (Scheffe test) showed that both 3a5B-P and alfaxalone potentiated the motor incoordinating effects of flurazepam, only 3a5B-P possessed sedative/ataxic effects at a dose of 18.0 mg/kg when administered alone.

DISCUSSION

In addition to the adrenal cortex and gonads, the brain serves as an additional source of neuroactive steroids, endogenous modulators of the $GABA_A$ receptor complex (2,8). These steroids are derived from the translocation of cholesterol

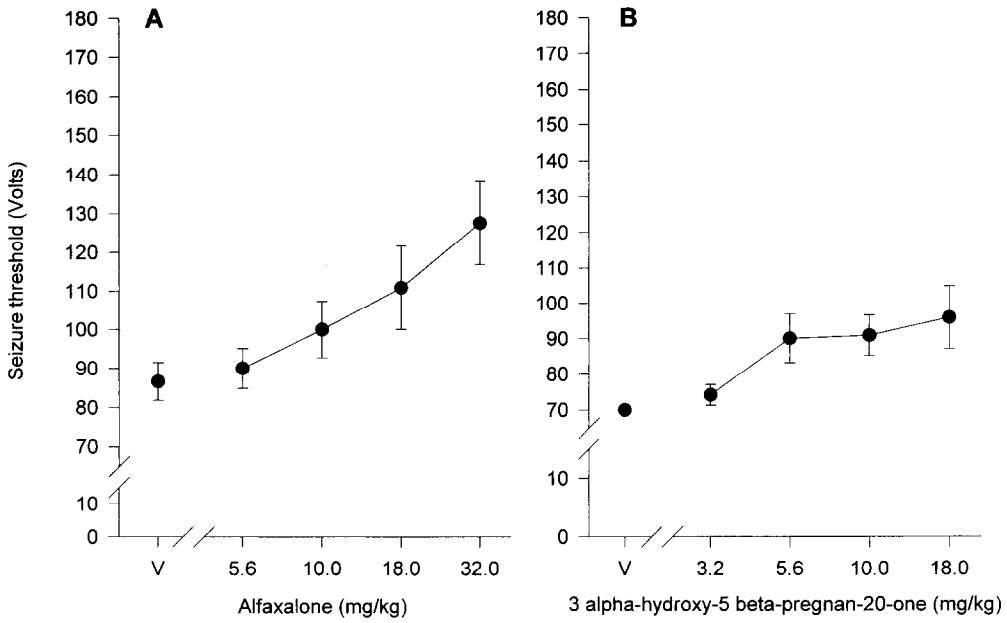

FIG. 1. (A) Depicts the mean seizure voltage (± SEM) of groups of mice injected with cyclodextrin vehicle (point above V) or various doses of alfaxalone. Details of B are identical and portray the data for 3a5B-P.

across the mitochondrial membranes of glial cells to the site of the cytochrome P450 side-chain cleavage enzyme. The rate of this translocation is controlled by the mitochondrial benzodiazepine receptor and is the rate-limiting step for steroid biosynthesis. The mitochondrial benzodiazepine binding site is independent of the GABA-associated chloride ionophore. Neuroactive steroids synthesized locally near sites of the $GABA_A$ receptor complex may provide another intermediate level of regulatory control of GABA-gated chloride ion conductance (2).

The current data suggest that at doses that are by themselves devoid of antiseizure efficacy in the IECS paradigm, neuroactive steroids may be able to potentiate the antiseizure efficacy of flurazepam. Interestingly, alfaxalone alone was es-

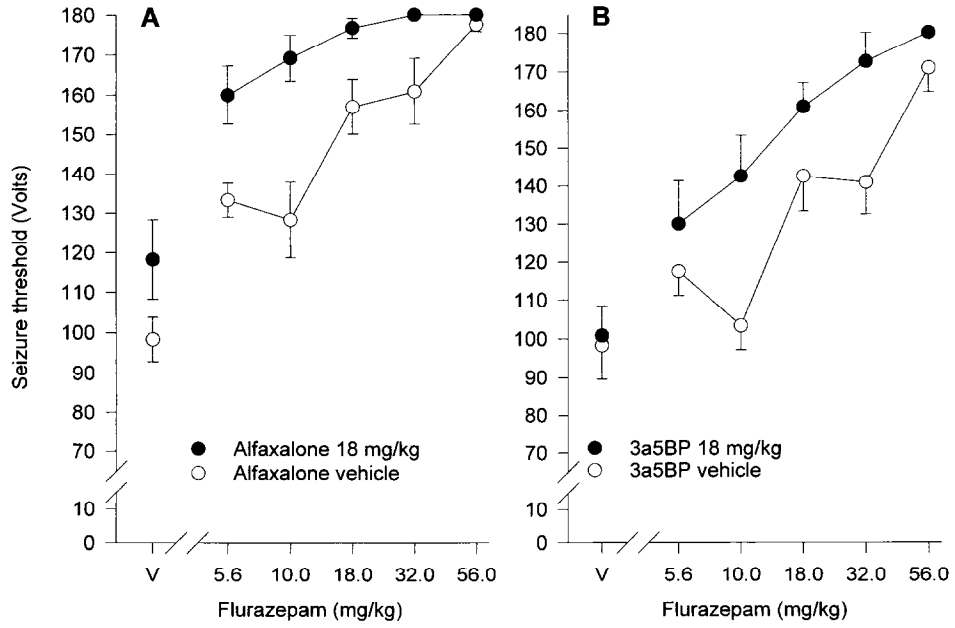

FIG. 2. (A) Depicts the mean seizure voltage (± SEM) of groups of mice injected with cyclodextrin vehicle (closed circles) or 18 mg/kg of alfaxalone (open circles) prior to an injection of saline (points above V) or various doses of flurazepam. Details of B are identical except that closed circles represent groups injected with 3a5B-P and 3a5B-P was injected 10 min after flurazepam or the flurazepam vehicle.

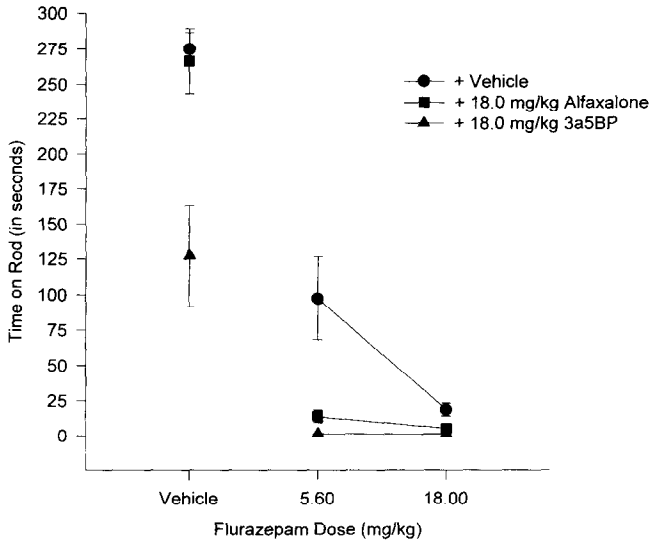

FIG. 3. The figure depicts the mean (± SEM) time on rotorod for groups of animals treated with either alfaxalone (squares), 3a5B-P (triangles) or their vehicle (circles), in addition to an injection of either the flurazepam vehicle (points above vehicle) or one of two doses of flurazepam (5.6 or 18.0 mg/kg).

sentially devoid of motor incoordinating effects, whereas 3a5B-P, at the same dose, disrupted mouse rotorod performance. Thus, a detailed evaluation of structure–activity relations could lead to the design and development of neuroactive steroids with selective therapeutic effects, while devoid of unwanted side effects. For example, the allosteric modulatory properties of 3a5B-P with its *cis*-fused configuration of the steroid A-ring differed in several regions of bovine brain from the molecule with the steroid A-ring in the *trans*-fused configuration (5). The behavioral consequences of these structural changes are under investigation. Furthermore, the interactions between the neuroactive steroids and flurazepam with respect to the potentiation of antiseizure efficacy and motor incoordinating effects suggest that allosteric interactions described using in vitro paradigms may be relevant to the intact animal. Finally, the data support consideration of the development of neuroactive steroids as adjuvant antiseizure medications in certain acute situations (e.g., medication-refractory status epilepticus). Unfortunately, the possibility of allosteric interactions between neuroactive steroids and other modulators of GABA-gated chloride ion conductance may limit the consideration of these combinations in the maintenance pharmacotherapy of seizure disorders.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Veterans Affairs to S.I.D.

REFERENCES

1. Deutsch, S. I.; Mastropaolo, J. Discriminative stimulus properties of midazolam are shared by a GABA-receptor positive steroid. Pharmacol. Biochem. Behav. 46(4):963–965; 1993.
2. Deutsch, S. I.; Mastropaolo, J.; Hitri, A. GABA-active steroids: Endogenous modulators of GABA-gated chloride ion conductance. Clin. Neuropharmacol. 15(5):352–364; 1992.
3. Deutsch, S. I.; Park, C. H.; Hitri, A. Allosteric effects of a GABA receptor-active steroid are altered by stress. Pharmacol. Biochem. Behav. 47(4):913–917; 1994.
4. Deutsch, S. I.; Rosse, R. B.; Huntzinger, J. A.; Novitzki, M. R.; Mastropaolo, J. Profound stress-induced alterations in flurazepam's antiseizure efficacy can be attenuated. Brain Res. 520:272–276; 1990.
5. Hawkinson, J. E.; Kimbrough, C. L.; McCauley, L. D.; Bolger, M. B.; Lan, N. C.; Gee, K. W. The neuroactive steroid 3_{alpha}-hydroxy-5_{beta}-pregnan-20-one is a two-component modulator of ligand binding to the $GABA_A$ receptor. Eur. J. Pharmacol. 269:157–163; 1994.
6. Kokate, T. G.; Svensson, B. E.; Rogawski, M. A. Anticonvulsant activity of neurosteroids: Correlation with gamma-aminobutyric acid-evoked chloride current potentiation. J. Pharmacol. Exp. Ther. 270(3):1223–1229; 1994.
7. Lambert, J. J.; Belelli, D.; Hill-Venning, C.; Peters, J. A. Neurosteroids and $GABA_A$ receptor function. Trends Pharmacol. Sci. 16:295–303; 1995.
8. Mellon, S. H. Neurosteroids: Biochemistry, modes of action, and clinical relevance. J. Clin. Endocrinol. Metab. 78(5):1003–1008; 1994.
9. Norris, D. O.; Mastropaolo, J.; O'Connor, D. A.; Cohen, J. M.; Deutsch, S. I. A glycinergic intervention potentiates the antiseizure efficacies of MK-801, flurazepam, and carbamazepine. Neurochem. Res. 19(2):161–165; 1994.
10. Wieland, S.; Belluzzi, J. D.; Stein, L.; Lan, N. C. Comparative behavioral characterization of the neuroactive steroids 3_{alpha}-OH,5_{alpha}-pregnan-20-one and 3_{alpha}-OH,5_{beta}-pregnan-20-one in rodents. Psychopharmacology (Berlin) 118(1):65–71; 1995.