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Allosteric Effects of a GABA Receptor-Active Steroid Are Altered by Stress

STEPHEN I. DEUTSCH,*†[1] CHAN H. PARK* AND ANA HITRI*†

*VA-NIDA Research Unit and Psychiatry Service, 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

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DEUTSCH, S. I., C. H. PARK AND A. HITRI. *Allosteric effects of a GABA receptor-active steroid are altered by stress.* PHARMACOL BIOCHEM BEHAV 47(4) 913–917, 1994. – Recently, ring A reduced metabolites of naturally occurring steroids have been shown to act as allosteric modulators of GABA-gated chloride ion conductance. Specifically, 5α-pregnane-3α,21-diol-20-one (allotetrahydrodeoxycorticosterone; 5α-THDOC) was shown to be a positive allosteric effector. For example, 5α-THDOC enhances the specific binding of [^{3}H]flunitrazepam, a benzodiazepine receptor agonist, among other pharmacological actions. Swim stress has been shown to reduce the ability of flurazepam, a prototypic benzodiazepine agonist, to antagonize the electrical precipitation of tonic hindlimb extension in mice. This stress-induced reduction in flurazepam's antiseizure efficacy persists for up to 72 h and is associated with alterations in the specific binding of ligands to the $GABA_A$ receptor complex. In the current study, the potentiation of [^{3}H]flunitrazepam binding by 5α-THDOC was greater in cerebral cortical membranes prepared from stressed mice compared with unstressed controls. Moreover, nanomolar concentrations of 5α-THDOC that were ineffective in potentiating the specific binding of [^{35}S]TBPS in cerebral cortical membranes prepared from unstressed control mice were capable of potentiating this binding in membranes prepared from stressed animals. Specifically, 50 nM 5α-THDOC caused a 23% increase in the specific binding of [^{35}S]TBPS in membranes from stressed mice, whereas it was without any significant effect in unstressed controls. This apparent ability of 5α-THDOC to distinguish between the binding of [^{35}S]TBPS to crude membranes prepared from stressed and unstressed control mice was eliminated in the presence of a 5 μM concentration of GABA. The data are consistent with an effect of stress on the ability of a GABA-positive steroid to allosterically modulate the binding of both a benzodiazepine agonist and $GABA_A$-associated channel ligand. These data provide additional support for a stress-induced alteration of the $GABA_A$ receptor complex.

Stress $GABA_A$ Receptor complex Steroids

THE ability of flurazepam to antagonize the electrical precipitation of tonic hindlimb extension is reduced 24 h after mice are forced to swim for up to 10 min in cold (6°C) water (4). This stress-induced reduction in flurazepam's antiseizure efficacy persists for up to 72 h and is not due to a nonspecific reduction in the threshold voltage for seizure production per se (9). Flurazepam is a prototypic benzodiazepine receptor agonist that binds to a distinct site on the $GABA_A$ receptor complex, an example of a receptor-gated channel. Benzodiazepine agonists increase the likelihood that gamma-aminobutyric acid (GABA) will be effective in promoting channel opening and chloride ion conductance (2,13). The $GABA_A$ receptor complex is a pentameric protein complex constructed from at least five polypeptide subunits, each of which has four transmembranous hydrophobic domains. Multiple molecular forms of specific subunit classes have been identified (e.g., several different alpha subunits); although these multiple molecular forms are homologous, they are encoded by alleles that have distinct loci and different chromosomal localizations (1,3). The $GABA_A$ receptor complex has multiple domains or binding sites that interact with each other in an allosteric fashion. The biochemical properties of these binding sites are determined by the unique combination of specific polypeptide subunits.

The stress-induced reduction of flurazepam's antiseizure efficacy may reflect an alteration in a population of $GABA_A$ receptor complexes due to the selective transcription of specific polypeptide subunits and their insertion into functional

[1] Requests for reprints should be addressed to Stephen I. Deutsch, M.D., Ph.D., Chief, Psychiatry Service (116A), Department of Veterans Affairs Medical Center, 50 Irving Street, N.W., Washington, DC 20422.

GABA-gated channels in discrete regions of mouse brain (11). The change in flurazepam's ability to antagonize seizure production was associated with alterations in the binding of [^{3}H]Ro15-1788, a benzodiazepine receptor antagonist in hippocampus and cerebellum (11). Thus, stress may stimulate the selective transcription of polypeptides that result in $GABA_A$ receptor complexes whose benzodiazepine binding site is in the antagonist-preferring state.

Reduced ring A metabolites of naturally occurring steroids have been shown to modulate GABA-gated chloride ion conductance [for review see (5)]. As reviewed, these GABA-active steroids bind to a site within the hydrophobic channel domain of the $GABA_A$ receptor complex. GABA-positive steroids (e.g., 5α-pregnane-3α,21-diol-20-one; allotetrahydrodeoxycorticosterone; 5α-THDOC) 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. In the current study, the ability of 5α-THDOC to enhance [^{3}H]flunitrazepam binding, and to inhibit the binding of [^{35}S]TBPS in the absence and presence of GABA was studied in crude brain membranes prepared from stressed and control mice. Thus, we wondered whether stress was associated with an alteration of the allosteric effects of a GABA-positive steroid, reflecting a possible change in the hydrophobic domain of this GABA-gated chloride ion channel. 5α-THDOC was also selected for study because its levels were shown to increase in brain and plasma of rats subjected to an ambient temperature swim stress (12).

METHOD

Chemicals

5α-Pregnane-3α,21-diol-20-one (allotetrahydrodeoxycorticosterone) was purchased from the Sigma Chemical Company and prepared as a stock solution (1 mM) in dimethyl sulfoxide (DMSO). The stock solution was diluted with DMSO. Unlabelled TBPS was purchased from Research Biochemicals Incorporated (Natick, MA) and was prepared as a 5% acacia gum suspension in deionized water. Other chemicals were also purchased from the Sigma Chemical Company and were of the highest grade available.

Subjects

An outbred strain of experimentally naive, male NIH Swiss mice weighing approximately 30 g and housed in hanging wire cages (five mice/cage) were used throughout the experiments. Animals were maintained in a temperature-controlled vivarium on a 12 L : 12 D cycle and had free access to food and water.

Stress Procedure

Mice were forced to swim in cold water (6°C) for up to 10 min. Twenty-four hours after the session of cold water swim stress, the animals were killed by decapitation in parallel with a group of unstressed control mice. This procedure was approved by the Animal Studies Subcommittee and Research Committee of this Department of Veterans Affairs Medical Center. Brains were immediately removed and dissected on ice. Cerebral cortices, hippocampi, and cerebella were stored frozen at −70°C prior to preparation of tissue homogenates.

Tissue Preparation

Crude membranes were prepared from cerebral cortex according to the procedure described by Havoundjian et al. (6) with only slight modification. Briefly, tissue was homogenized in 50 vol. of 50 mM Tris-HCl buffer (pH 7.4) with a Brinkmann Polytron (5 s at maximal speed). The homogenate was then centrifuged at 20,000 × *g* for 20 min (4°C). The resulting pellets were washed four times in 50 vol. of 50 mM Tris-citrate buffer (pH 7.4) containing 100 mM NaCl. The final pellet was resuspended in 50 vol. of 50 mM Tris-citrate buffer (pH 7.4). The concentration of protein in the final tissue suspension was determined by the method of Lowry et al. (7).

[^{3}H]Flunitrazepam Binding

The specific binding of [^{3}H]flunitrazepam (specific activity 93 Ci/mmol; New England Nuclear, Boston, MA) was studied in an incubation volume of 1 ml using 100 μl of a [^{3}H]flunitrazepam solution, 100 μl of tissue homogenate (approximately 0.1 mg protein), and Tris-citrate buffer to the final volume (11). Nonspecific binding was determined with the addition of 100 μl midazolam (final concentration 10 μM) and represented less than 10% of the total binding. The assay was initiated with the addition of tissue homogenate and proceeded for 60 min at 4°C. In the experiments examining the ability of allotetrahydrodeoxycorticosterone to potentiate [^{3}H]flunitrazepam binding, tissue homogenate was incubated in the presence of 1.5 nM [^{3}H]flunitrazepam and a range of allotetrahydrodeoxycorticosterone concentrations (10^{-8} to 10^{-5} M). In all instances, 20 μl of either steroid solution in DMSO or DMSO was added to the final incubation.

[^{35}S]TBPS Binding

The binding of 2 nM [^{35}S]TBPS (approximately 140–160 Ci/mmol; New England Nuclear) was performed according to the method described by Havoundjian et al. (6) with only slight modification. Binding was studied in an incubation volume of 1.0 ml consisting of 100 μl of [^{35}S]TBPS solution, 500 μl tissue homogenate (approximately 0.4–0.5 mg protein), 100 μl 2 M NaCl, and buffer to final volume. Nonspecific binding was determined using 10 μM picrotoxinin and represented less than 15% of the total binding. The reaction was initiated with the addition of [^{35}S]TBPS and proceeded at 25°C for 100 min. The reaction was terminated by filtration through Whatman GF/B glass fiber filters. The filters were washed twice with 5 ml of ice-cold Tris-citrate buffer (pH 7.4) and counted using liquid scintillation spectrometry. Saturation studies of [^{35}S]TBPS binding to cerebral cortical membranes prepared from stressed and control mice were performed using unlabelled TBPS (0 to 500 nM). In some experiments, the ability of allotetrahydrodeoxycorticosterone (10^{-8} to 10^{-5} M) to inhibit the binding of [^{35}S]TBPS in the absence and presence of GABA (5 μM) was studied. In experiments examining the effect of 5α-THDOC on [^{35}S]TBPS binding, 20 μl of either steroid solution in DMSO or DMSO was added to the final incubation.

Data Analysis

The equilibrium binding constants were determined by Scatchard analysis. The raw data expressed as disintegrations per minute (dpm) were subjected to Equilibrium Binding Data Analysis (EBDA), using the Collection of Radioligand Binding Analysis Programs (10). The program provided a mean and the standard error (SEM) for replicate determinations at each point. For each point the program calculated the amount

of bound (total, specific) and unbound (free) ligand concentration. The initial estimates of the dissociation constants (K_d), and maximum number of binding sites (B_{max}) were obtained from Scatchard plots using EBDA, and for the final estimate of the constants the LIGAND program was used.

RESULTS

In a dose-dependent manner, 5α-THDOC increased the specific binding of [^{3}H]flunitrazepam to cerebral cortical membranes prepared from stressed and unstressed control mice (Fig. 1). In contrast to our previous results showing that there is no effect of stress on the specific binding of [^{3}H]flunitrazepam in the absence of 5α-THDOC (11), the current data suggest that stress may affect [^{3}H]flunitrazepam binding in the presence of 5α-THDOC. The two-way ANOVA indicated significant main effects for both stress, $F(1, 79) = 3.99$, $p = 0.046$, and the concentration of 5α-THDOC, $F(7, 79) = 12.36$, $p = 0.0001$. There was no significant main effect for the interaction between stress and the concentration of 5α-THDOC. Using Tukey's protected t-test, post hoc comparisons showed that in control animals there was no significant effect of low 5α-THDOC concentrations (10 to 500 nM) on the specific binding of [^{3}H]flunitrazepam, whereas this binding was potentiated at higher concentrations of the steroid [1 μM = 46% potentiation ($t = 3.44$, $p = 0.01$); 5 μM = 53% potentiation ($t = 3.96$, $p = 0.01$); 10 μM = 54% potentiation ($t = 4.03$, $p = 0.01$)]. In contrast to unstressed controls, submicromolar concentrations of 5α-THDOC significantly potentiated [^{3}H]flunitrazepam binding [100 nM = 35% potentiation ($t = 2.60$, $p = 0.05$); 500 nM = 39.7% potentiation ($t = 2.94$, $p = 0.01$); 1 μM = 51% potentiation ($t = 3.81$, $p = 0.01$); 5 μM = 59% potentiation ($t = 4.40$, $p = 0.01$); 10 μM = 67% ($t = 4.95$, $p = 0.01$)].

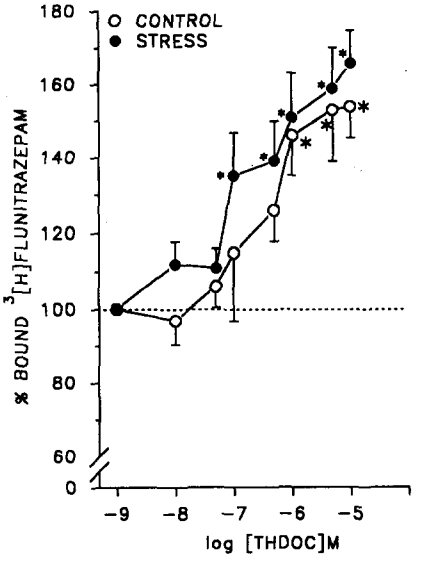

FIG. 1. Effect of 5α-THDOC on [^{3}H]flunitrazepam Binding (1.5 nM) to cortical membranes of stressed and unstressed control mice. Values represent the percent of [^{3}H]flunitrazepam binding observed in the absence of 5α-THDOC. Values represent the mean of three separate experiments performed in duplicate. The two-way ANOVA indicated significant main effects for both stress, $F(1, 79) = 3.99$, $p = 0.046$, and the concentration of 5α-THDOC, $F(7, 79) = 12.36$, $p = 0.0001$. Small asterisks represent significant difference from control values in stressed animals. Large asterisks represent significant difference from control values (100%) in unstressed control mice.

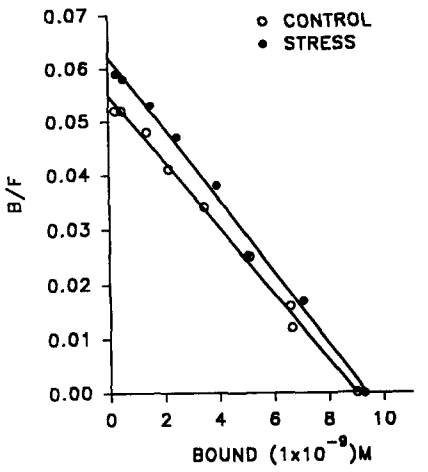

FIG. 2. Scatchard analysis of [^{35}S]TBPS binding (2 nM) to cerebral cortical membranes of stressed and unstressed control mice. The results of a typical experiment are shown. The experiments were conducted with cerebral cortical membranes pooled from two to three mice from each group. The binding of 2 nM [^{35}S]TBPS was performed in the presence of various concentrations of unlabelled TBPS (0–500 nM) using 10 μM picrotoxinin for the determination of nonspecific binding. B_{max} and K_d values did not differ between stressed and unstressed control animals.

As illustrated in Fig. 2, the specific binding of [^{35}S]TBPS did not differ between stressed (K_d = 150.4 nM, B_{max} = 17.1 pmol/mg prot) and unstressed control mice (K_d = 163.3 nM, B_{max} = 16.2 pmol/mg prot). These data show that stress does not alter the specific binding of [^{35}S]TBPS in the absence of 5α-THDOC.

Figure 3 shows that 5α-THDOC has a biphasic effect on the specific binding of [^{35}S]TBPS to cerebral cortical membranes prepared from stressed and unstressed control mice. Submicromolar concentrations of 5α-THDOC potentiated, whereas concentrations in the micromolar range inhibited, the specific binding of [^{35}S]TBPS. The two-way ANOVA showed a significant main effect of 5α-THDOC concentration, $F(7, 79) = 11.2$, $p = 0.0001$, but no significant main effect of either stress, $F(1, 79) = 1.9$, or the interaction of stress and 5α-THDOC. Post hoc comparisons performed with the Tukey's protected t-test showed that 50 and 100 nM 5α-THDOC significantly increased the binding of [^{35}S]TBPS over control values in stressed animals [23% ($t = 2.47$, $p = 0.05$) and 40% ($t = 4.3$, $p = 0.01$), respectively], whereas only the 100 nM 5α-THDOC was effective at potentiating binding in unstressed control mice (20% increase). Thus, stressed membranes may be more sensitive to the ability of nanomolar concentrations of 5α-THDOC to potentiate the binding of [^{35}S]TBPS.

Figure 4 shows that in the presence of a 5 μM concentration of GABA, the ability of 5α-THDOC to inhibit the specific binding of [^{35}S]TBPS does not differ between stressed and unstressed control mice. Interestingly, in the presence of 5 μM GABA, the ability of nanomolar concentrations of 5α-THDOC to potentiate the binding of [^{35}S]TBPS is eliminated. As indicated by the steep inhibition curves with Hill coefficients close to unity, the addition of 5 μM GABA rendered the 5α-THDOC inhibition of [^{35}S]TBPS binding monophasic

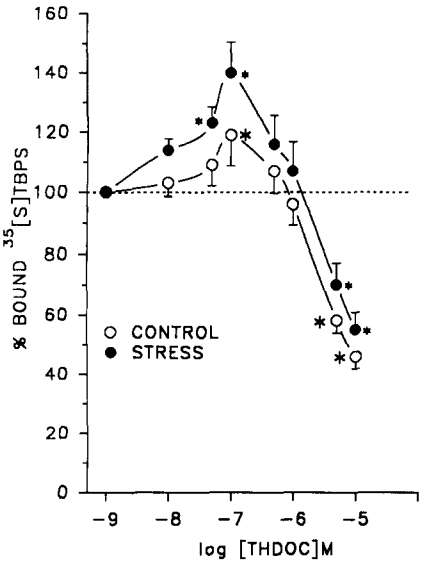

FIG. 3. Effect of 5α-THDOC on [^{35}S]TBPS binding (2 nM) to cerebral cortical membranes of stressed and unstressed control mice. Values represent the percent of [^{35}S]TBPS binding observed in the absence of 5α-THDOC. Values represent the mean of three separate experiments performed in duplicate. Small asterisks represent significant difference from control values in stressed animals. Large asterisks represent significant difference from control values (100%) in unstressed control mice.

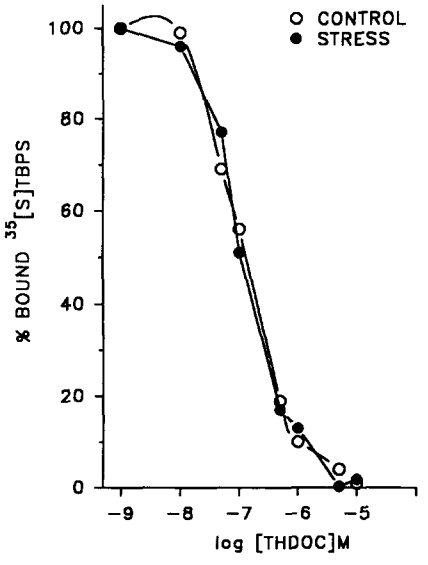

FIG. 4. Effect of 5α-THDOC on [^{35}S]TBPS binding (2 nM) to cerebral cortical membranes of stressed and unstressed control mice in the presence of GABA (5 μM). Values represent the percent of [^{35}S]TBPS binding observed in the absence of 5α-THDOC. Values represent the mean of three separate experiments performed in duplicate. In both groups, the addition of 5 μM GABA potentiated the inhibitory effect of 5α-THDOC on [^{35}S]TBPS binding; moreover, in the presence of GABA, 5α-THDOC inhibited binding in a monophasic manner. Stressed and unstressed control mice did not differ with respect to the ability of 5α-THDOC to inhibit the binding of [^{35}S]TBPS in the presence of 5 μM GABA.

in both groups (Fig. 4). Also, the IC_{50} of 5α-THDOC's inhibition of [^{35}S]TBPS binding did not differ between stressed (0.11 μM ± 0.004, n_H = 1.00 ± 0.08) and unstressed control mice (0.18 μM ± 0.07, n_H = 0.82 ± 0.12).

DISCUSSION

Reduced ring A metabolites of naturally occurring steroids may serve as endogenous modulators of GABAergic transmission regulating levels of CNS excitability (5). Allotetrahydrodeoxycorticosterone binds to a distinct site within the hydrophobic domain of the GABA-associated chloride ion channel; its binding to this site influences GABA's ability to gate chloride ion conductance (5). The sensitivity of the $GABA_A$ receptor complex to the action of benzodiazepine agonists, another class of allosteric modulator of GABA-gated chloride ion conductance, is reduced 24 h after a 10-min session of cold water swim stress (4). For example, for up to at least 72 h after a single session of cold water swim stress, the ability of flurazepam to antagonize the electrical precipitation of tonic hindlimb extension in mice is reduced (9). This stress-induced reduction in flurazepam's antiseizure efficacy is associated with alterations in biochemical parameters of the $GABA_A$ receptor complex (11,14). Presumably, the biochemical changes reflect the selective transcription of specific molecular subunit forms of individual polypeptides, which confer unique pharmacological properties on the newly inserted GABA-gated channels.

The current data show that the ability of 5α-THDOC to modulate allosterically the binding of both [^{3}H]flunitrazepam and [^{35}S]TBPS may be altered 24 h after a single session of cold water swim stress. Consistent with our previous results (11), in the absence of 5α-THDOC, there is no difference in the specific binding of [^{3}H]flunitrazepam to cerebral cortical membranes prepared from stressed and unstressed control mice (see Fig. 1). Whereas nanomolar concentrations of 5α-THDOC alone potentiated the binding of [^{35}S]TBPS (Fig. 3), in the presence of 5 μM GABA, nanomolar concentrations of 5α-THDOC inhibited the binding of this channel ligand (Fig. 4). Interestingly, the ability of 5α-THDOC to distinguish between the binding of [^{35}S]TBPS to crude membranes prepared from stressed and unstressed control mice was eliminated in the presence of GABA. In previous work, we have shown that GABA's ability to inhibit the specific binding of [^{35}S]TBPS to cerebral cortical membranes was not different in membranes prepared from stressed and unstressed control mice. Therefore, the inability of 5α-THDOC to distinguish between the binding of [^{35}S]TBPS to membranes prepared from stressed and unstressed control mice in the presence of 5 μM GABA is not likely to be related to a differential effect of GABA on this binding in stressed animals. GABA has a robust effect on the inhibition of [^{35}S]TBPS binding and, thus, its inhibitory ability may mask the subtle, but significant, effect of the steroid on the potentiation of [^{35}S]TPBS observed in its absence. Of course, there is the unlikely possibility that at least some of our observations might have resulted from unequal concentrations of endogenous GABA between membranes prepared from stressed and control animals.

The data are consistent with complex allosteric interactions between the hydrophobic channel domains responsible for the binding of [^{35}S]TBPS and GABA-positive steroids and the benzodiazepine binding site. Of especial interest, submicromolar concentrations of 5α-THDOC increased, whereas micromolar concentrations inhibited the binding of [^{35}S]TBPS. The biphasic effect of steroids on [^{35}S]TBPS binding has been observed by other investigators (8). On a functional level, the

biphasic effect of alloTHDOC alone on the binding of [^{35}S]-TBPS could be related to the ability of some steroids to possess both excitatory and inhibitory effects on neuronal firing (8). Moreover, the interactions between these various sites are altered in subtle ways by the effects of stress. For example, stressed membranes may be more sensitive to the ability of submicromolar concentrations of 5α-THDOC to increase the binding of [^{35}S]TBPS.

GABA-positive steroids derive naturally from hormones released in response to stress (5,12). These endogenous 3α-reduced ring A steroid metabolites may be involved in modulation of GABAergic transmission in stressed animals. Following the stress procedure used in our study, flurazepam's ability to antagonize the electrical precipitation of tonic hindlimb extension is reduced in mice. Conceivably, GABA-active steroids could be developed as medications designed to enhance the reduced pharmacological activity of benzodiazepine agonists in a defined period after stress.

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