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Modulation of [^{3}H]Flunitrazepam Binding by Natural and Synthetic Progestational Agents

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McAULEY, J. W., P. D. KROBOTH, D. D. STIFF AND I. J. REYNOLDS. *Modulation of [^{3}H]flunitrazepam binding by natural and synthetic progestational agents.* PHARMACOL BIOCHEM BEHAV **45**(1) 77–83, 1993.—Progesterone is metabolized by ring-A reduction with subsequent oxidoreduction to 3α-hydroxy-5α-dihydroprogesterone (3α-OH-5α-DHP), a naturally occurring metabolite that has been shown to enhance [^{3}H]flunitrazepam ([^{3}H]FNZ) binding. Medroxyprogesterone acetate (MPA), a commonly prescribed progestational agent, is a synthetic progesterone derivative that has a metabolic profile similar to that of progesterone. In this study, the effects of MPA and its ring-A reduced metabolites DHMPA and THMPA on [^{3}H]FNZ binding were investigated. While known modulators of specific [^{3}H]FNZ binding demonstrated expected effects in frozen and fresh rat cortical tissue, 3α-OH-5α-DHP enhanced [^{3}H]FNZ binding only in fresh, not frozen, tissue. Neither DHMPA nor THMPA affected binding, while MPA partially inhibited [^{3}H]FNZ binding by 40%. In addition, five test drugs were used to assess the effect of gender and hormonal status on [^{3}H]FNZ binding. Neither gender nor hormonal status influenced binding. Thus, ring-A reduced metabolites of progesterone but not of MPA enhance [^{3}H]FNZ binding. The clinical implications of these in vitro results are currently under investigation.

Benzodiazepine [^{3}H]Flunitrazepam GABA Progesterone Medroxyprogesterone acetate Ring-A reduced metabolites Steroids Progestational agents

GABA is the major inhibitory neurotransmitter that is present in about 30% of mammalian CNS synapses. GABA receptors are divided into two classes based upon sensitivity to the alkaloid bicuculline: $GABA_A$ receptors are sensitive to bicuculline; $GABA_B$ receptors are bicuculline insensitive. Activation of $GABA_A$ receptors by synaptically released GABA stimulates a transient increase in chloride ion conductance that usually results in membrane hyperpolarization and thus reduces the probability of action potential generation. The function of the $GABA_A$ receptor is allosterically modulated by several anxiolytic-hypnotic drugs, including benzodiazepines and barbiturates, that act to enhance and prolong $GABA_A$-mediated synaptic inhibition (6,30,33,39). The various binding sites on the GABA–benzodiazepine receptor complex (GBRC) have been extensively studied using the high-affinity GABA agonist [^{3}H]muscimol, the benzodiazepine agonists [^{3}H]flunitrazepam ([^{3}H]FNZ) and [^{3}H]diazepam, the benzodiazepine antagonist [^{3}H]Ro 15-1788, and the convulsant tert-butyl bicyclophospho[^{35}S]thionate, which binds to the chloride channel.

Several steroid hormones exert pharmacological effects by actions on the CNS (8). Many of the effects of steroid hormones are thought to be due to an interaction with intracellular receptors and gene-controlled changes in protein synthesis. In addition, it has been known for a long time that general anesthesia can be induced by injecting certain steroids (29). The rapid onset of the anesthetic effect suggests a mechanism other than changes in protein synthesis. Recent evidence shows that the synthetic anesthetic steroid, alphaxalone, can potentiate inhibitory responses on the $GABA_A$ receptor (2, 7,12,35). In addition, numerous investigators have reported that naturally occurring steroids modulate the GBRC via a steroid recognition site [for reviews, see (3,9,13,15,25)], which may account for the rapid anesthetic actions of alphaxalone. Certain structure-activity requirements are necessary for this stereospecific steroidal interaction: ring-A reduction, a 3α-hydroxyl group, and a C20 ketone group (11). Specific [^{3}H]FNZ binding is enhanced in the presence of the naturally occurring ring-A reduced metabolite of progesterone, 3α-hydroxy-5α-dihydroprogesterone (3α-OH-5α-DHP) (10,11,16,34).

Medroxyprogesterone acetate (MPA, Provera®), a synthetic derivative of progesterone, is commonly prescribed in combination with estrogen supplementation for hormonal replacement in postmenopausal women. MPA and progesterone are structurally similar and have similar metabolic pathways (1,17) as outlined in Fig. 1. There is limited information in humans that MPA and/or its metabolites interact at the GBRC. Suggestion of interaction at the GBRC is found in a report by Mattson and colleagues; when MPA was added to existing anticonvulsant regimens, 11 of 14 women with uncontrolled seizures had significantly fewer seizures (18). Based

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upon the similarity between MPA and progesterone, our objective was to determine whether MPA and/or its ring-A reduced metabolites interact with the GBRC. To test the 3α-OH, ring-A reduced metabolite of MPA for activity, synthesis from its immediate precursor was necessary. We report the first published in vitro results examining the effect of MPA and/or its ring-A reduced metabolites on specific [^{3}H]FNZ binding. Additional objectives were to determine the effects of tissue storage and of gender and hormonal status on the steroidal-[^{3}H]FNZ interaction.

METHOD

[^{3}H]FNZ (87.0 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Progesterone, 3,20 pregnanedione, 3α-hydroxy-5α-dihydroprogesterone (3α-OH-5α-DHP), 3α-hydroxy-5β-dihydroprogesterone (3α-OH-5β-DHP), 3β-hydroxy-5α-dihydroprogesterone (3β-OH-5α-DHP), 3β-hydroxy-5β-dihydroprogesterone (3β-OH-5β-DHP), GABA, flunitrazepam, and pentobarbital were purchased from Sigma Chemical Co. (St Louis, MO). Bicuculline was purchased from Research Biochemicals, Inc. (Natick, MN). *N*-Desmethyladinazolam, MPA, and dihydroMPA (DHMPA) were gifts of The Upjohn Company (Kalamazoo, MI). All test drugs were dissolved in ethanol except pentobarbital, which was dissolved in deionized distilled water. The amount of solvent added to the assay had a negligible effect on specific [^{3}H]FNZ binding.

THMPA Synthesis

TetrahydroMPA (THMPA) was synthesized from its precursor DHMPA (17α-acetoxy-6α-methyl-5β-pregnane-20-one) because it was not commercially available. The ketone at the C3 position was reduced to 3α-OH with KS-Selectride (Aldrich Chemical Co., Milwaukee, WI) in a procedure previously described for reduction of similar steroids (21,37). Briefly, DHMPA (25 mg) was placed in a test tube containing 5 ml tetrahydrofuran (THF). The tube was immersed in a dry-ice/acetone bath (−78°C) for 5 min, after which KS-Selectride (100 μl) was added. The mixture was stirred under an argon atmosphere for 1 h and then warmed to 0°C in an ice bath. Five milliliters of TRIS-HCl buffer (0.5 M) was added and the solution stirred for 45 min. The reaction mixture was extracted twice with ethyl acetate (10 ml) and the extracts evaporated to dryness. The residue was reconstituted with MeOH and chromatographed on high-performance silica gel TLC plates with a 9:1 chloroform/ether mobile phase. The plates were sprayed with the steroid-indicating reagent *p*-toluenesulphonic acid (20 g *p*-toluenesulphonic acid in ethanol qs 100 ml) and heated at 95–100°C for 20 min. Two spots appeared on the TLC plates with R_f values of ~3.7 and ~2.6. The spot at R_f 3.7 was the same as that observed for pure DHMPA. Zones corresponding to these two regions, which were not sprayed, were scraped from the plates and the compounds eluted from the silica gel with THF and acetone. The solvents were evaporated and the residues derivatized and analyzed by mass spectrometry. The structure of THMPA was confirmed based upon the report by Martin et al. (17).

[^{3}H]Flunitrazepam Binding

The study protocol was reviewed and approved by the University of Pittsburgh Animal Care and Use Committee. For fresh membrane preparations, male Wistar rats were decapitated and brains rapidly removed and homogenized using a polytron at setting 3 in assay buffer (200 mM KCl, 20 mM KOH, 20 mM KH_2PO_4, pH 7.4) and centrifuged. This step was repeated twice with the supernatant being discarded after centrifugation and replaced with fresh buffer. Frozen rat brains, obtained from Pel-Freez (Rogers, AK), were processed in the same manner. The total 1-ml volume contained ~200 μg protein, test drug as appropriate, assay buffer (containing 300 nM GABA), and 0.1 nM [^{3}H]FNZ. Assays were conducted in duplicate at 22°C for 60 min. Specific binding was obtained as the difference between radioligand bound in the absence and presence of *N*-desmethyladinazolam (30 μM); this benzodiazepine agonist has a high affinity for the benzodiazepine site on the GBRC (26). The incubations were terminated by filtration under vacuum through glass filters using a Brandel cell harvester (Gaithersburg, MD) and two subsequent washes with 4 ml cold buffer. Membrane-bound [^{3}H]FNZ was extracted from filters with 4 ml scintillation cocktail (Ecolite®) and counted on a Beckman LS 1801 scintillation counter; radioactivity was reported as counts per minute. Specific binding was calculated by subtracting nonspecific from total binding; nonspecific binding was less than 10% of the total. The results were expressed as percent inhibition or enhancement of control binding in the absence of test drugs. Protein concentrations were determined by the method of Smith et al. (32).

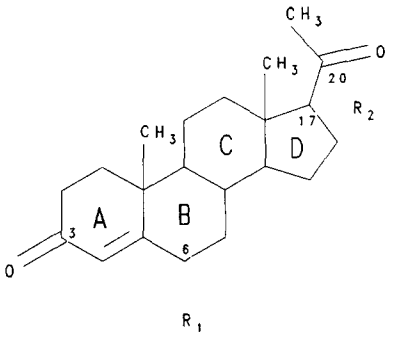
Effect of Gender and Hormonal Status

Cortical tissue was obtained from male, oophorhysterectomized female, and pregnant rats; there were four animals per group. The effects of gender and of hormonal status on specific [^{3}H]FNZ binding were tested utilizing 3α-OH-5α-DHP, flunitrazepam, MPA, pentobarbital and THMPA. Rats, all Wistar (225–249 g), were obtained from Hilltop Labs (Scottdale, PA). The oophorhysterectomized rats (12–18 days postsurgery) were included because their hormone status approximates that of the target human patient population. The pregnant rats were 17–19 days in gestation when studied. For each rat, the brain was rapidly removed and used as the tissue source as outlined above. Binding data for the flunitrazepam saturation curves (0.1–100 nM) were analyzed using the EBDA and LIGAND programs (Elsevier Biosoft, New York) to obtain the affinity (K_d) and binding capacity (B_{max}). The binding parameters were tested for differences between tissue sources by one-way analysis of variance using SAS (24).

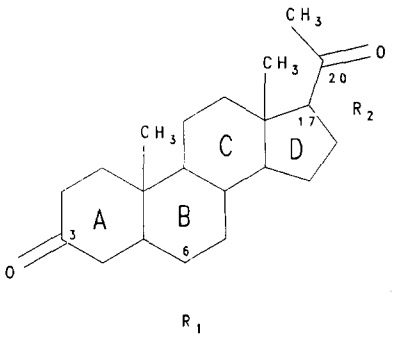
RESULTS

Effect of Progesterone and Its Ring-A Reduced Metabolites

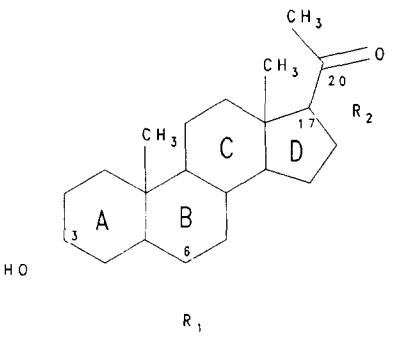
Figure 2 describes the concentration-dependent effects of progesterone and its ring-A reduced metabolites on specific [^{3}H]FNZ binding. As seen in Fig. 2, 3α-OH-5α-DHP at concentrations of 10 nM to 10 μM enhanced specific binding of [^{3}H]FNZ by more than 50% over control in cortical membranes. Both 3α-OH-5α-DHP and 3α-OH-5β-DHP produced similar concentration-dependent effects on specific [^{3}H]FNZ binding. Two 3β-OH compounds tested, 3β-OH-5α-DHP and 3β-OH-5β-DHP, were inactive. The parent compound, progesterone, and the ring-A reduced metabolite, 3,20 pregnanedione, exhibited no concentration-dependent activity on specific [^{3}H]FNZ binding. Interestingly, 3α-OH-5α-DHP failed to enhance specific [^{3}H]FNZ binding in cortical tissue that had been frozen prior to use. 3α-OH-5β-DHP was not tested in frozen tissue.



Progesterone [R_1=H R_2=H]
Medroxyprogesterone Acetate (MPA) [R_1=CH_3 R_2=$OCOCH_3$]



3,20 Pregnanedione [R_1=H R_2=H]
Dihydro-Medroxyprogesterone Acetate (DHMPA) [R_1=CH_3 R_2=$OCOCH_3$]



3α-hydroxy-5α-dihydroprogesterone (3α-OH-5α-DHP) [R_1=H R_2=H]
Tetrahydro-Medroxyprogesterone Acetate (THMPA) [R_1=CH_3 R_2=$OCOCH_3$]

FIG. 1. Metabolic profile of progesterone and the synthetic progestational agent medroxyprogesterone acetate illustrating ring-A reduction with subsequent 3-keto oxidoreduction.

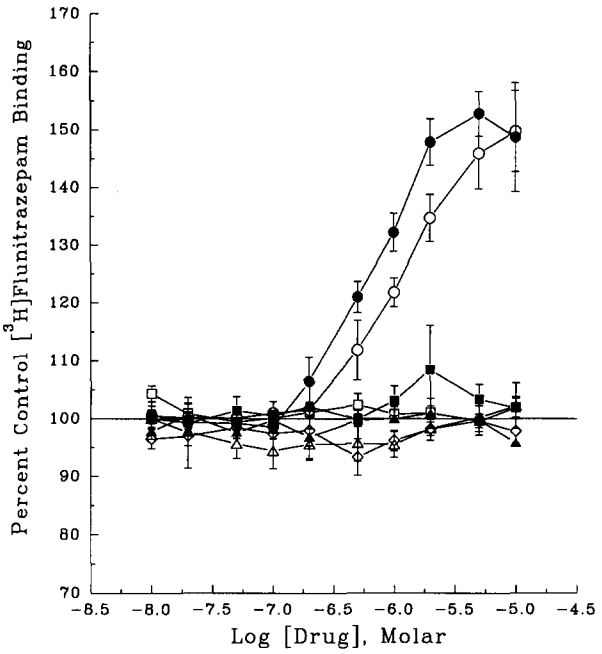


FIG. 2. Effect of progesterone and its ring-A reduced metabolites on specific [^{3}H]flunitrazepam (FNZ) binding to cortical membranes from male rats: 3α-OH-5α-DHP (○), 3α-OH-5β-DHP (●), 3β-OH-5α-DHP (□), 3β-OH-5β-DHP (■), 3,20 pregnanedione (△), progesterone (▲), and 3α-OH-5α-DHP (frozen tissue) (◇). The results are presented as mean ± SE from at least three animals. Fresh cortical tissue was used in all studies except where noted.

Effect of MPA and its Ring-A Reduced Metabolites

Figure 3 depicts the concentration-dependent relationship of MPA, DHMPA, and THMPA on specific [^{3}H]FNZ binding. Neither DHMPA nor THMPA exhibited any concentration-dependent effect while MPA exhibited a concentration-dependent partial inhibition of specific [^{3}H]FNZ binding to a maximum of 40% with half-maximal inhibition observed at 1 μM.

Effect of Tissue Storage

In our initial studies utilizing frozen cortical tissue, we were unable to detect enhancement of specific [^{3}H]FNZ binding by 3α-OH-5α-DHP. In contrast, the frozen tissue did demonstrate clear concentration-dependent effects of GABA, bicuculline, pentobarbital, numerous benzodiazepine agonists, and Ro 15-1788 on specific [^{3}H]FNZ binding (data not shown). The results for these drugs were reproducible and similar to previously published reports (5,19,22,26–28). Neither the addition of GABA (300 nM) nor variations in assay temperature (0 or 37°C) altered the lack of effect of 3α-OH-5α-DHP on specific [^{3}H]FNZ binding in thoroughly washed, previously frozen membranes. However, subsequent studies using fresh tissue demonstrated the 3α-OH-5α-DHP enhancement of specific [^{3}H]FNZ binding (Fig. 2).

Effect of Gender and Hormonal Status

Figure 4 depicts the concentration-dependent relationship of five test drugs on specific [^{3}H]FNZ binding in brain homogenates from male, oophorhysterectomized female, and pregnant female rats. The concentration-dependent effects on specific [^{3}H]FNZ binding for each test drug were similar in all three tissue sources: 3α-OH-5α-DHP demonstrated enhancement (EC_{50} ~ 1 μM), flunitrazepam demonstrated full inhibition (IC_{50} ~ 5 nM), MPA demonstrated moderate inhibition (IC_{50} ~ 1 μM), pentobarbital demonstrated enhancement (EC_{50} ~ 500 μM), and THMPA had no effect. Analysis of the flunitrazepam binding parameter estimates revealed no significant differences in the binding parameters between tissue sources (Table 1).

DISCUSSION

These results demonstrate differences between natural and synthetic progestational agents for their effect on specific [^{3}H]FNZ binding. Ring-A reduced metabolites of progesterone but not of MPA enhance benzodiazepine binding to the GBRC using [^{3}H]FNZ as a marker. These results are surprising based upon the similarity in MPA's metabolic profile to progesterone and corresponding structure-activity requirements, as well as the preliminary clinical findings (18). Our results show that neither DHMPA nor THMPA affect specific [^{3}H]FNZ binding and thus suggest that, by this mechanism, these ring-A reduced metabolites are not responsible for the decrease in seizure frequency described by Mattson and colleagues (18). However, this does not preclude the interaction of these compounds at the GBRC to reduce seizure frequency by some as yet unknown mechanism. The lack of effect of THMPA on specific [^{3}H]FNZ binding suggests the addition of the C6 α-methyl and the C17 acetoxy group may inhibit the interaction of this steroid with the stereoselective steroid recognition site on the GBRC.

Even though the ring-A reduced metabolites of MPA did

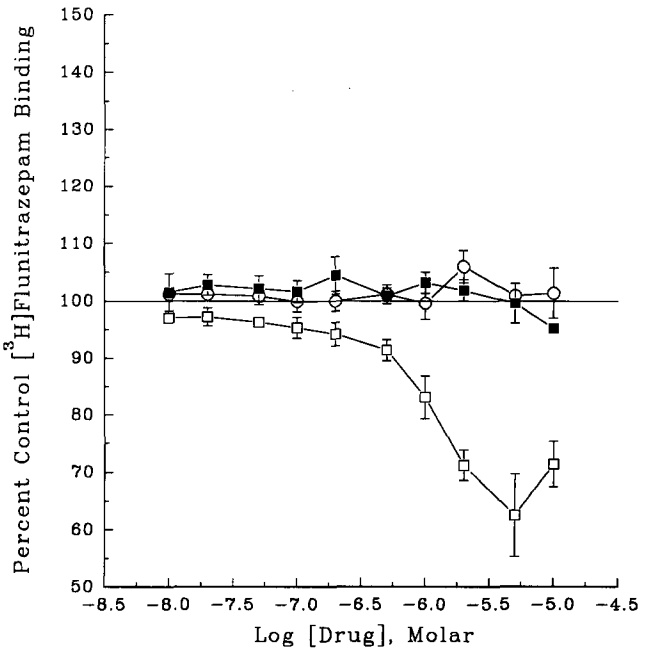


FIG. 3. Effect of medroxyprogesterone acetate (MPA) (□), dihydroMPA (DHMPA) (■), and tetrahydroMPA (THMPA) (○) on specific [^{3}H]flunitrazepam (FNZ) binding. The results are presented as mean ± SE from at least three animals. Fresh cortical tissue from male rats was utilized throughout.

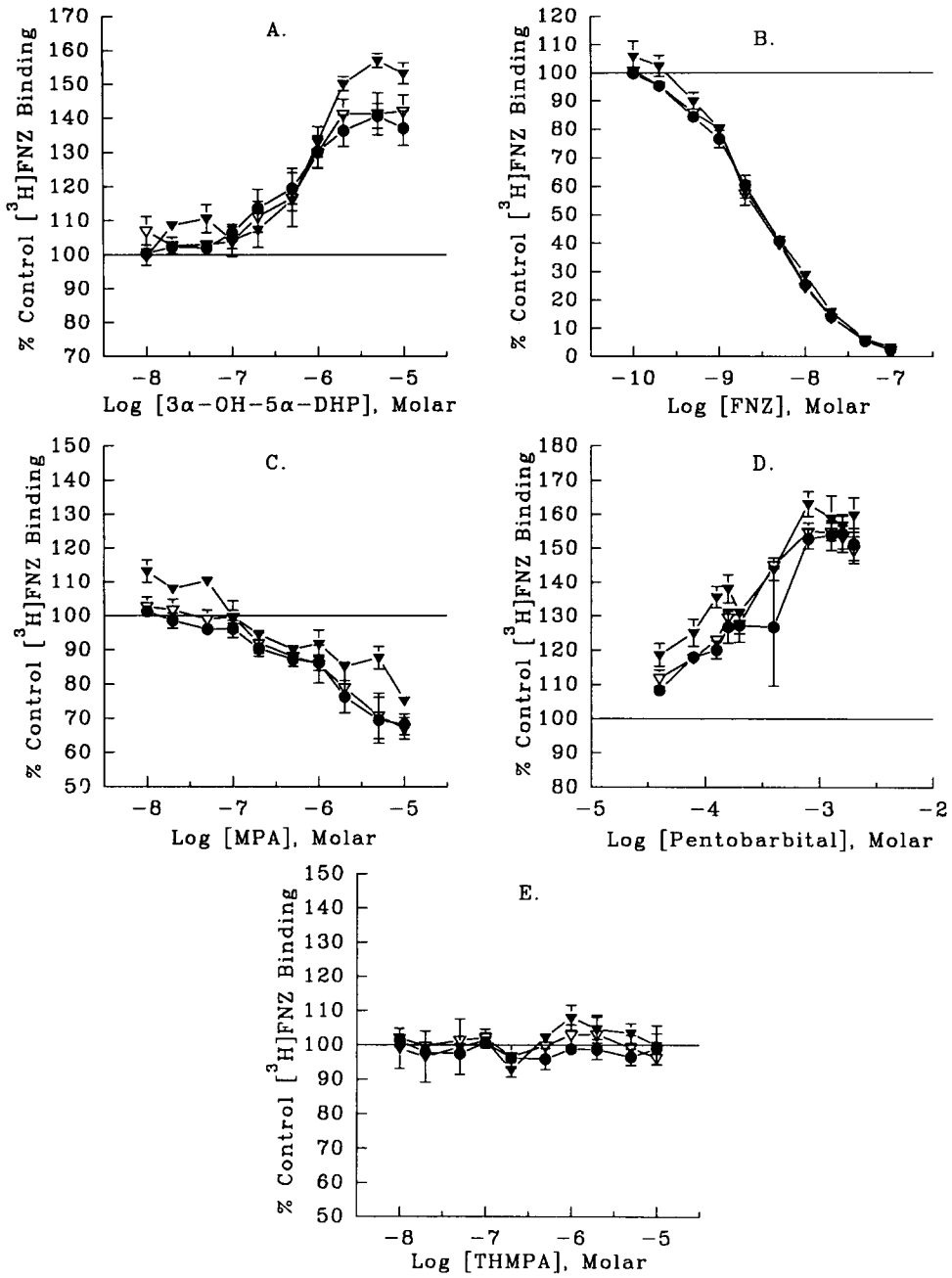


FIG. 4. Effect of gender and hormonal status on known and potential modulators of specific [^{3}H]flunitrazepam (FNZ) binding: (A), 3α-OH-5α-DHP; (B), flunitrazepam; (C), medroxyprogesterone acetate (MPA); (D), pentobarbital; (E) tetrahydroMPA (THMPA). Male rats (▽), oophorhysterectomized female rats (●), and pregnant female rats (▼). The results are presented as mean ± SE from four animals.

not modulate specific [^{3}H]FNZ binding, we saw an effect of MPA. The moderate inhibition of specific [^{3}H]FNZ binding by MPA does not appear to be due to an interaction at the steroid recognition site on the GBRC based upon three observations. First, MPA does not fit the structure-activity requirements; second, MPA inhibition was evident in frozen cortical tissue (data not shown) whereas enhancement of [^{3}H]FNZ binding by 3α-OH-5α-DHP was not (Fig. 2); third, the addition of MPA [10 μM] to a 3α-OH-5α-DHP concentration-response curve did not alter the enhancement of specific [^{3}H]FNZ binding by 3α-OH-5α-DHP (data not shown). MPA probably does not act at the benzodiazepine site on the GBRC as partial inhibition is not consistent with competitive interaction. The moderate inhibition cannot be explained by a solvent effect because inhibition was not observed with other test drugs dissolved in similar amounts of alcohol. Thus, this con-

TABLE 1

BINDING AFFINITIES AND CAPACITIES IN THREE DIFFERENT TISSUE SOURCES

Tissue	K_d (nM)	B_{max} (pmol/mg protein)
Male	2.153 ± 0.764	1.320 ± 0.498
Oophorhysterectomy	2.668 ± 0.595	1.755 ± 0.531
Pregnant	2.776 ± 0.348	1.156 ± 0.131
p value	0.3306	0.1781

Data are presented as mean (n = 4) ± SD.

centration-dependent inhibition is unexplained. The high concentration at which this moderate inhibition occurs in comparison to physiologically achieved plasma concentrations after MPA administration would suggest little or no clinical consequences.

Before we tested the effect of synthetic progestational agents on the GBRC, we first demonstrated effects of known modulators, including 3α-OH-5α-DHP, on specific [^{3}H]FNZ binding. The enhancement of specific [^{3}H]FNZ binding by 3α-OH-5α-DHP closely parallels that reported by Majewska et al. (16). Our results reaffirm the stereospecificity involved in the steroid-GBRC interaction because both 3β-OH-5α-DHP and 3β-OH-5β-DHP showed no activity (11). Progesterone has been shown to be devoid of activity at the GBRC by other investigators as well (4,11,20). Likewise, our results show no activity from 3,20 pregnanedione, a ring-A reduced metabolite of progesterone with a C3 ketone that does not fit the structure-activity requirements (11). In contrast, two reports have demonstrated limited 3,20 pregnanedione activity, using markers other than specific [^{3}H]FNZ binding (4,14).

An important tissue storage requirement became apparent in our assay development when we were unable to demonstrate any effect of 3α-OH-5α-DHP on specific [^{3}H]FNZ binding using previously frozen, thoroughly washed rat cortex. Interestingly, of the four reports in the literature that study the steroidal-GBRC interaction using [^{3}H]FNZ as a marker (10,11,16,34) three state that freshly obtained brain tissue was used. Although the use of fresh rat brain tissue has not previously been alluded to as a requirement for studying this interaction, in the single report in which frozen membranes were used Sutanto et al. demonstrated a far smaller enhancement of [^{3}H]FNZ binding by 3α-OH-5α-DHP than reported by others who used fresh tissue. The use of frozen membranes may account for the smaller effect. It is worth noting that other investigators have used frozen membranes to study the interaction between steroids and the GBRC with ligands and/or measures other than [^{3}H]FNZ (35). Our data support the evidence that the GBRC can be frozen and stored for extended periods without loss of activity for test drugs other than 3α-OH-5α-DHP. We demonstrated modulation of the GBRC by GABA, bicuculline, several benzodiazepine agonists, and Ro 15-1788 (data not shown). There are reports in the literature that examine the effects of freezing on [^{3}H]benzodiazepine binding. Roca et al. found that freezing tends to decrease enhancement when using [^{3}H]FNZ as a probe for the GBRC (23). Also, Skolnick et al., using [^{3}H]diazepam, reported that frozen membranes are less sensitive to the effects of barbiturates when compared to nonfrozen membranes (31). These investigators demonstrate a decrease in enhancement using previously frozen tissue, unlike the total lack of effect observed for 3α-OH-5α-DHP in our studies. The finding that 3α-OH-5α-DHP enhances specific [^{3}H]FNZ binding only in freshly obtained rat cortical tissue may not only be a methodological requirement: It may also reveal further insight into the presently unknown exact mechanism or site of action of steroid modulation on the GBRC.

We initially speculated that the gender and hormonal status of the animal might alter the response to added progestational agents in the [^{3}H]FNZ binding assay. However, the addition of 3α-OH-5α-DHP to cortical tissue enhances specific [^{3}H]FNZ binding to a similar extent in tissue obtained from animals in which progesterone serum concentrations are expected to be high (pregnant rats) and low (male and oophorhysterectomized female rats). Neither the binding curves (Fig. 4) nor the binding parameters (Table 1) were different between tissue sources. These results are similar to those reported by Wilson, who found no marked differences in binding parameters between male, female, and ovariectomized rats using [^{3}H]FNZ binding (38). It is interesting to note that Wilson described a correlation between high circulating progesterone levels in females and heightened seizure thresholds induced by bicuculline. This would suggest that the influence of gender and hormonal status is different depending upon the technique used to study the steroidal-GBRC interaction.

In summary, ring-A reduced metabolites of progesterone, but not of MPA, enhance benzodiazepine binding to the GBRC using [^{3}H]FNZ as a marker. The clinical implications of these in vitro results are currently under investigation. Tissue storage, but not tissue source, is important in studying the in vitro steroidal-[^{3}H]flunitrazepam interaction. Further study of this finding may reveal more insight into the presently unknown exact mechanism of action of the steroidal-GBRC interaction.

REFERENCES

1. Aufrere, M. B.; Benson, H. Progesterone: An overview and recent advances. J. Pharm. Sci. 65:783-800; 1976.
2. Barker, J. L.; Harrison, N. L.; Lange, G. D.; Majewska, M. D.; Owen, D. G. Voltage-clamp studies of the potentiation of GABA-activated chloride conductance by the steroid anaesthetic alphaxalone and a reduced metabolite of progesterone in cultured rat CNS neurones. J. Physiol. 377:83P; 1986.
3. Belelli, D.; Lan, N. C.; Gee, K. W. Anticonvulsant steroids and the GABA/benzodiazepine receptor-chloride ionophore complex. NeuroSci. Biobehav. Rev. 14:315-322; 1990.
4. Callachan, H.; Cottrell, G. A.; Hather, N. Y.; Lambert, J. J.; Nooney, J. M.; Peters, J. A. Modulation of the $GABA_A$ receptor by progesterone metabolites. Proc. Roy. Soc. Lond. 231:359-369; 1987.
5. Clow, A.; Glover, V.; Sandler, M. Triazolam, an anomalous benzodiazepine receptor ligand: In vitro characterization of alprazolam and triazolam binding. J. Neurochem. 45:621-625; 1985.
6. Costa, E. The allosteric modulation of $GABA_A$ receptors: Seventeen years of research. Neuropsychopharmacology 4:225-235; 1991.
7. Cottrell, G. A.; Lambert, J. J.; Peters, J. A. Modulation of $GABA_A$ receptor activity by alphaxalone. Br. J. Pharmacol. 90: 491-500; 1987.
8. de Kloet, E. R.; Joëls, M. Brain corticosteroid receptors and

neurotransmission. In: Fuxe, K.; Agnati, L. F., eds. Volume transmission in the brain: Novel mechanisms for neural transmission. New York: Raven Press; 1991:213-224.
9. Gee, K. W. Steroid modulation of the GABA/benzodiazepine receptor-linked chloride ionophore. Mol. Neurobiol. 2:291-317; 1988.
10. Gee, K. W.; Bolger, M. B.; Brinton, R. E.; Coirini, H.; Mcewen, B. S. Steroid modulation of the chloride ionophore in rat brain: Structure-activity requirements, regional dependence and mechanism of action. J. Pharmacol. Exp. Ther. 246:803-812; 1988.
11. Harrison, N. L.; Majewska, M. D.; Harrington, J. W.; Barker, J. L. Structure-activity relationships for steroid interaction with the γ-aminobutyric acid$_A$ receptor complex. J. Pharmacol. Exp. Ther. 241:346-353; 1987.
12. Healing, C.; Hilton, T.; Simmonds, M. Alphaxalone reveals $GABA_A$ receptor-mediated inhibition in guinea-pig ileum. J. Pharm. Pharmacol. 41:410-411; 1989.
13. Lambert, J. J.; Peters, J. A.; Cottrell, G. A. Actions of synthetic and endogenous steroids on the $GABA_A$ receptor. Trends Pharmacol. Sci. 8:224-7; 1987.
14. Lopez-Colome, A. M.; Mccarthy, M.; Beyer, C. Enhancement of [^{3}H]muscimol binding to brain synaptic membranes by progesterone and related pregnanes. Eur. J. Pharmacol. 176:297-303; 1990.
15. Majewska, M. D. Steroids and brain activity. Biochem. Pharmacol. 36:3781-3788; 1987.
16. Majewska, M. D.; Harrison N. L.; Schwartz, R. D.; Barker, J. L.; Paul, S. M. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science 232:1004-1107; 1986.
17. Martin, F.; Järvenpää, P.; Kosunen, K.; Somers, C.; Lindstrom, B.; Adlercreutz, H. Ring-A reduction of medroxyprogesterone acetate [17α-acetoxy-6α-methyl-4 pregnene-3,20-dione (MPA)] in biological systems. J. Steroid Biochem. 12:491-497; 1980.
18. Mattson, R. H.; Cramer, J. A.; Caldwell, B. V.; Siconolfi, B. C. Treatment of seizures with medroxyprogesterone acetate: Preliminary report. Neurology 34:1255-1258; 1984.
19. Mehta, A. K.; Ticku, M. K. Characteristics of flunitrazepam binding to intact primary cultured spinal cord neurons and its modulation by GABAergic drugs. J. Neurochem. 49:1491-1497; 1987.
20. Peters, J. A.; Kirkness, E. F.; Callachan, H.; Lambert, J. L.; Turner, A. J. Modulation of the $GABA_A$ receptor by depressant barbiturates and pregnane steroids. Br. J. Pharmacol. 94:1257-1269; 1988.
21. Purdy, R. H.; Morrow, A. L.; Blinn, J. R.; Paul, S. M. Synthesis, metabolism, and pharmacological activity of 3α-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. J. Med. Chem. 33:1572-1581; 1990.
22. Richter, J. A.; Yamamura, H. I. Effects of pentobarbital on *t*-[^{35}S]butylbicyclophosphorothionate and [^{3}H]flunitrazepam binding to membrane-bound and solubilized preparations from rat forebrain. J. Pharmacol. Exp. Ther. 233:125-133; 1985.
23. Roca, D. J.; Rozenberg, I.; Farrant, M.; Farb, D. H. Chronic agonist exposure induces down-regulation and allosteric uncoupling of the γ-aminobutyric acid/benzodiazepine receptor complex. Mol. Pharmacol. 37:37-43; 1990.
24. SAS. SAS user's guide: Basics. 5th ed. Cary, NC: SAS Institute; 1985.
25. Schumacher, M. Rapid membrane effects of steroid hormones: An emerging concept in neuroendocrinology. Trends Neurosci. 13:359-362; 1990.
26. Sethy, V. H.; Collins, R. J.; Daniels, E. G. Determination of biological activity of adinazolam and its metabolites. J. Pharm. Pharmacol. 36:546-548; 1984.
27. Sethy, V. H.; Harris, D. W. Determination of biological activity of alprazolam, triazolam and their metabolites. J. Pharm. Pharmacol. 34:115-116; 1982.
28. Sethy, V. H.; Russell, R. R.; Daenzer C. L. Interaction of triazolobenzodiazepines with benzodiazepine receptors. J. Pharm. Pharmacol. 35:524-526; 1983.
29. Seyle, H. Anesthetic effect of steroid hormones. Proc. Soc. Exp. Biol. Med. 46:116-121; 1941.
30. Sivilotti, L.; Nistri, A. GABA receptor mechanisms in the central nervous system. Prog. Neurobiol. 36:35-92; 1991.
31. Skolnick, P.; Rice, K. C.; Barker, J. L.; Paul, S. M. Interaction of barbiturates with the benzodiazepine receptors in the central nervous system. Brain Res. 233:143-156; 1982.
32. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Provenzano, M. D,; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85; 1985.
33. Stephenson, F. A. Understanding the $GABA_A$ receptor: A chemically gated ion channel. Biochem. J. 249:21-32; 1988.
34. Sutanto, W.; Handelmann, G.; de Bree, F.; de Kloet, E. R. Multifaceted interaction of corticosteroids with the intracellular receptors and with membrane $GABA_A$ receptor complex in the rat brain. J. Neuroendocrinol. 1:243-247; 1989.
35. Turner, D. M.; Ransom, R. W.; Yang, J. S.; Olsen, R. W. Steroid anesthetics and naturally occurring analogs modulate the γ-aminobutyric acid receptor complex at a site distinct from barbiturates. J. Pharmacol. Exp. Ther. 248:960-966; 1989.
36. Vincens, M.; Shu, C.; Moguilewsky, M.; Philibert, D. A progesterone metabolite enhances the activity of the $GABA_A$ receptor complex at the pituitary level. Eur. J. Pharmacol. 168:15-21; 1989.
37. Wiebe, J. P.; Deline, C.; Buckingham, K. D.; Dave, V.; Stothers, J. B. Synthesis of the allylic gonadal steroids, 3α-hydroxy-4-pregnen-20-one and 3α-hydroxy-4-androsten-17-one, and of 3α-hydroxy-5α-pregnan-20-one. Steroids 45:39-51; 1985.
38. Wilson, M. A. Influences of gender, gonadectomy, and estrous cycle on GABA/BZ receptors and benzodiazepine responses in rats. Brain Res. Bull. 29:165-172; 1992.
39. Zorumski, C. F.; Isenberg, K. E. Insights into the structure and function of GABA-benzodiazepine receptors: Ion channels and psychiatry. Am. J. Psychiatry 148:162-173; 1991.