J. Pharm. Pharmacol. 1990, 42: 175–180
Received May 29, 1989

Modulation of the $GABA_A$ Receptor by Barbiturates and Pregnane Steroids: Differential Effects of the Influence of Assay Temperature

J. L. W. YAU, D. J. K. BALFOUR AND I. H. STEVENSON

Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital, University of Dundee, Dundee DD1 9SY, UK

Abstract—The effect of temperature on the modulation of the $GABA_A$ receptor by barbiturates and steroids has been investigated in-vitro using a radioreceptor binding assay. Displaceable [^{3}H]muscimol binding to a crude membrane preparation from rat cerebral cortex was enhanced by the endogenous steroid metabolite, 5β-pregnan-3α-ol-20-one, by the synthetic steroid, alphaxalone, and by pentobarbitone in a dose-dependent manner. Hydrocortisone and corticosterone had no significant effect on [^{3}H]muscimol binding. Analysis of binding data using a curve-fitting program ('Ligand') showed that both pentobarbitone (1 mM) and 5β-pregnan-3α-ol-20-one (10 μM) increased the apparent number of high affinity binding sites in the membrane but had no effect on the affinity of [^{3}H]muscimol binding (K_d approx. 11 nM). Increasing the assay temperature from 0°C to 35°C decreased [^{3}H]muscimol binding and decreased the enhancement of binding by pentobarbitone but had no effect on 5β-pregnan-3α-ol-20-one enhancement of binding. 5α-Pregnan-3α-ol-20-one increased the apparent rate of association of [^{3}H]muscimol binding to its receptor whereas pentobarbitone had no effect. These different effects on the apparent association rate and the different responses to temperature, suggest that the barbiturate and steroid may interact with the $GABA_A$ receptor through different binding sites.

The γ-aminobutyric acid $GABA_A$ receptor has been shown to be an oligomeric complex composed of at least three subunits with several distinct regulatory sites which together control a chloride channel (Olsen 1982). Activation of this receptor complex by the inhibitory transmitter GABA results in a transient increase in chloride conductance which usually leads to membrane hyperpolarisation and a decrease in neuronal excitability. There is now clear evidence that several anxiolytic-hypnotic drugs, including benzodiazepines and barbiturates, exert their effects by modulating the response to GABA at this receptor (Keane & Biziere 1987; Simmonds & Turner 1987; Martin 1987).

Recently, some reduced metabolites of progesterone and deoxycorticosterone have been reported to exhibit barbiturate-like actions at the $GABA_A$ receptor (Majewska et al 1986; Harrison et al 1987; Gee et al 1987; Lambert et al 1987). In radioligand binding studies barbiturates and steroids were found to enhance the binding of GABA and benzodiazepine receptor agonists to the receptor complex, whereas they inhibited the binding of TBPS (t-butyl-bicyclophosphorothionate), a convulsant GABA antagonist. Electrophysiological studies indicated that these steroids potentiated GABA-evoked responses by increasing the duration of chloride channel open time using a mechanism which was insensitive to the benzodiazepine antagonist, flumazenil (Barker et al 1986; Majewska et al 1986; Turner 1986). These results suggest that the effects of the active steroids are mediated via the $GABA_A$ receptor complex although they do not appear to interact with the benzodiazepine recognition site on the receptor complex. However, despite there being many similarities between the actions of barbiturates and steroids, it is not known if they modulate responses to GABA by acting on a common regulatory site on the receptor complex.

In the present study, the principal objective was to examine the differential effects of assay temperature on the modulation of [^{3}H]muscimol binding to rat brain membranes by barbiturates and pregnane steroids.

Correspondence to: I. H. Stevenson, Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital, Dundee DD1 9SY, UK.

Materials and Methods

Drugs

[^{3}H]Muscimol (12·2 Ci mmol^{-1}) was purchased from Amersham International. γ-Aminobutyric acid (GABA), sodium pentobarbitone, 5β-pregnan-3α-ol-20-one, 5α-pregnan-3α-ol-20-one, hydrocortisone, and corticosterone were purchased from Sigma.

Membrane preparation

A crude preparation of synaptic membranes was prepared from the cerebral cortex of male Sprague-Dawley rats using a procedure similar to that described by Olsen et al (1981). Rats were killed by stunning and cervical dislocation. The cerebral cortex was removed and homogenised in 10 vol ice-cold 0·32 M sucrose. After an initial centrifugation at 1000 g for 10 min, the supernatant was centrifuged at 20 000 g for 20 min to obtain a crude synaptosomal pellet. The pellet was washed twice by resuspension in 10 vol of ice-cold distilled water followed by centrifugation at 48 000 g for 20 min. Finally the pellet was resuspended in 50 mM Tris/citrate buffer (pH 7·1) containing 100 mM KCl and stored frozen (−20°C). When needed for receptor binding assay, the membranes were thawed, washed once in the same buffer containing 100 mM KCl and dialysed for 20 h against 1000 vol of the same buffer.

Receptor binding assay

The binding of [^{3}H]muscimol to the synaptic membrane preparation was measured by a filtration assay using the protocol of Marangos & Crawley (1982). Aliquots of membrane suspension (equivalent to 0·15–0·25 mg protein) were incubated with the radioligand [^{3}H]muscimol (5 nM, 61 nCi mL^{-1} final concentration), in a total vol of 0·5 mL 20 mM potassium phosphate buffer (pH 7·4) containing 100 mM KCl for 20 min at 0°C in the absence or presence of various concentrations of test drug. Non-specific (non-displaceable) binding was measured in the presence of 100 μM GABA. After incubation, samples were diluted with 4 mL of ice-cold assay buffer and immediately filtered on GF/B filters (Whatman) under suction. Filters were washed three times with 4 mL of buffer, dried and counted for radioactivity in 5 mL Beckman Ready Value scintillation fluid. Protein was determined by the method of Lowry et al (1951) with bovine serum albumin as standard.

[^{3}H]Muscimol binding kinetics

For the association rate study, ligands were added to membranes incubated at 0°, 20° or 35°C. Aliquots of 0·5 mL were pipetted onto glass fibre filters and vacuum filtered at desired times followed by three × 4 mL ice-cold assay buffer washes. The membranes were stirred continuously throughout the experiment. For dissociation experiments, ligands were incubated with membranes at the assay temperature for 20 min, GABA (100 μM) was then added to the incubate and aliquots (0·5 mL) were removed at various times for filtration.

Data analysis

The equilibrium dissociation constants (K_d) and receptor densities (B_{max}) were calculated using the 'Ligand' curve-fitting program of Munson & Rodbard (1980). The effects of the modulators and of temperature were analysed using an analysis of variance for repeated measures in which drug treatment and the assay temperature were the independent variables. The K_{app} and K_{diss} values were calculated using the 'Kinetics' computer program of Munson & Rodbard (1980).

Results

When the assays were performed at 35°C, the displaceable binding of [^{3}H]muscimol to a crude preparation of cerebral cortex membranes was enhanced in a dose-dependent manner by 5β-pregnan-3α-ol-20-one, alphaxalone and pentobarbitone (Fig. 1). Neither hydrocortisone nor corticosterone had any significant effect on [^{3}H]muscimol binding. Stock solutions of the steroids were dissolved in ethanol. Binding assays performed in the presence of the final concentration of ethanol in the incubates (5% v/v) showed that it had no significant effect on displaceable [^{3}H] muscimol binding. The highest concentration of the steroids tested (10 μM) stimulated [^{3}H]muscimol binding by some 130% (5β-pregnan-3α-ol-20-one) and 80% (alphaxalone) of control. Pentobarbitone (1mM) gave an enhancement in binding of approximately 155% of control.

Analysis of control [^{3}H]muscimol binding, using 1–100 nM of radioligand, indicated that the data were best fitted to a single site model (Fig. 2a). The presence of 5β-pregnan-3α-ol-20-one (10 μM) had little effect on the affinity of [^{3}H]muscimol binding ($1/K_d$) but significantly (t-test $P<0·01$) increased the apparent total number of high affinity binding sites (B_{max}) in the membrane (Fig. 2a). The K_d and B_{max} values (mean ± s.e.m., n = 4) were, respectively: control, 12·4 ± 1·0 nM, 1·02 ± 0·10 pmol mg^{-1}; 5β-pregnan-3α-ol-20-one (10 μM), 13·7 ± 1·8 nM, 1·79 ± 0·14 pmol mg^{-1}. Pentobarbitone (1mM) also had no effect on K_d but significantly (t-test. $P<0·01$) increased the B_{max} value (Fig. 2b). The K_d and B_{max} values (mean ± s.e.m., n = 4) were respectively: control, 10·9 ± 1·0 nM, 0·91 ± 0·10 pmol mg^{-1}; pentobarbitone (1 mM), 11·7 ± 0·9 nM, 1·45 ± 0·18 pmol mg^{-1}.

Pentobarbitone and 5β-pregnan-3α-ol-20-one stimulation of [^{3}H]muscimol binding was examined at 3 different assay temperatures (0°, 20°, 35°C). Increasing the assay temperature resulted in a significant decrease (F temperature (2, 21) = 39; $P<0·001$) in control [^{3}H]muscimol binding from a mean of 0·261 ± 0·022 pmol (mg protein)$^{-1}$ to 0·085 ± 0·01 pmol (mg protein)$^{-1}$ (Fig. 3a). The stimulation of binding by both pentobarbitone (50 μM–1 mM) and 5β-pregnan-3α-ol-one (500 nM–10 μM) was dose-dependent at all three temperatures tested (Fig. 3b, c). The degree of enhancement of binding by pentobarbitone decreased with increasing assay temperature (F drug by temperature (6, 27) = 4·3; $P<0·01$) (Fig. 3b); this was significant at 35°C compared to

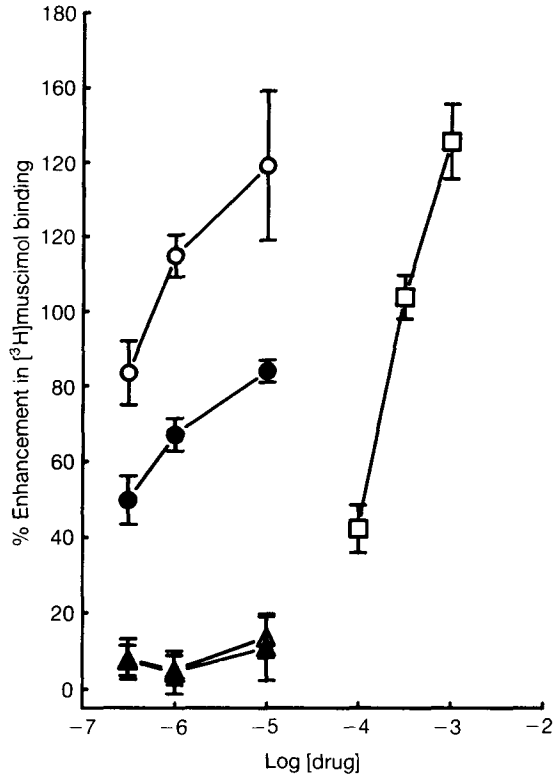

FIG. 1. The effect of 5β-pregnan-3α-ol-20-one (○), alphaxalone (●), hydrocortisone (△), corticosterone (▲) and pentobarbitone (□) on the displaceable binding of [^{3}H]muscimol to crude synaptic membranes. Displaceable [^{3}H]muscimol binding was measured at 35°C using a radioligand concentration of 5 nM. Each point is the mean ± s.e.m. of four experiments. In the absence of additional test drug, the displaceable [^{3}H]muscimol binding was 0·085 ± 0·01 pmol (mg protein)$^{-1}$.

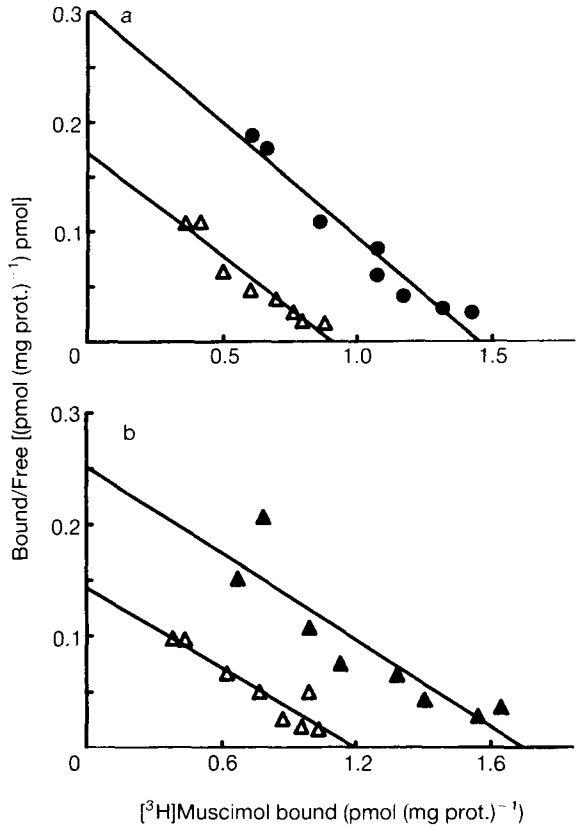

FIG. 2. Typical Scatchard plots of [^{3}H]muscimol binding to rat synaptic membranes in the absence (△) and presence of 10 μM 5 β-pregnan-3α-ol-20-one (●) and 1 mM pentobarbitone (▲). The concentration of [^{3}H]muscimol used ranged from 1 to 100 nM. Displaceable [^{3}H]muscimol binding was measured in the presence of 100 μM GABA. Each point is the mean of duplicate assays carried out at 0°C.

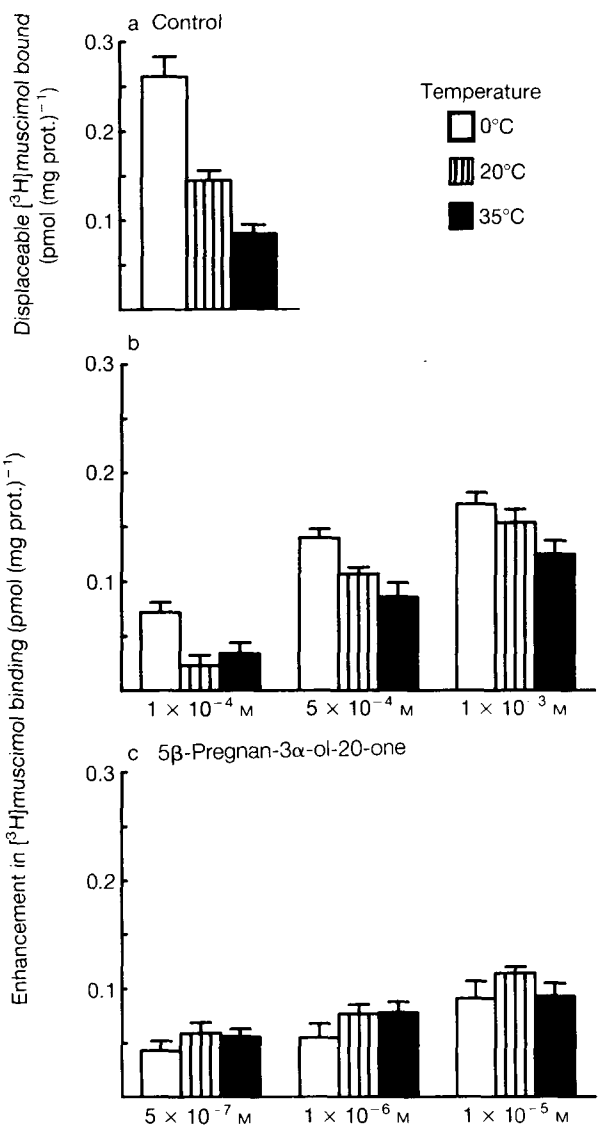

FIG. 3. (a) Control [^{3}H]muscimol binding at 0°, 20° and 35°C using a radioligand concentration of 5 nM (b) and (c) the enhancement of binding by pentobarbitone (0·1, 0·5, 1·0 mM) and 5β-pregnan-3α-ol-20-one (0·5, 1·0, 10 μM), respectively, at the three temperatures tested. Results are the mean ± s.e.m. of four experiments.

0°C (Duncan's test, $P < 0{\cdot}01$). 5β-Pregnan-3α-ol-20-one enhancement of binding was, however, not significantly affected by the assay temperature (Fig. 3c).

An examination of [^{3}H]muscimol binding kinetics at the three assay temperatures, indicated that the decrease in absolute binding with increasing assay temperature resulted principally from an increase in the initial dissociation of [^{3}H]muscimol from its receptor (Fig. 4a). The dissociation rates (mean values from 3 experiments) were 0·58, 1·40 and 1·62 min^{-1} for 0°, 20° and 35°C, respectively. At 20°C, the inclusion of either pentobarbitone (1mM) or 5α-pregnan-3α-ol-20-one (10 μM) in the assay had no effect on the initial dissociation rate of [^{3}H]muscimol binding (Fig. 5a). The dissociation rates (mean values from four experiments) were 1·3 and 1·52 min^{-1}, respectively.

The apparent association rate was also found to increase with assay temperature (Fig. 4b); the mean values from three experiments were 0·85, 1·51 and 2·26 min^{-1} for 0°, 20° and 35°C, respectively. As a consequence of this increased association rate, equilibrium binding was reached more rapidly at the higher assay temperatures. The inclusion of pentobarbitone (1 mM) in the assay at 20°C had no effect on the apparent association rate whereas the presence of 5α-pregnan-3α-ol-20-one (10 μM) increased the apparent association rate (Fig. 5b). The association rates (mean values from 4 to 5 experiments) were 1·69 and 2·18 min^{-1} respectively. The endogenous steroid metabolite, 5α-pregnan-3α-ol-20-one has been shown to be equipotent to its 5 β-pregnane isomer at enhancing [^{3}H]muscimol binding (Peters et al 1988). 5α-Pregnan-3α-ol-20-one was examined because of the limited amount of the 5β-pregnane steroid available for testing.

Discussion

Several similarities between the actions of the active steroids and those of the hypnotic barbiturates in parallel radioligand binding assays have been reported. For example both barbiturates and steroids have been shown to displace the

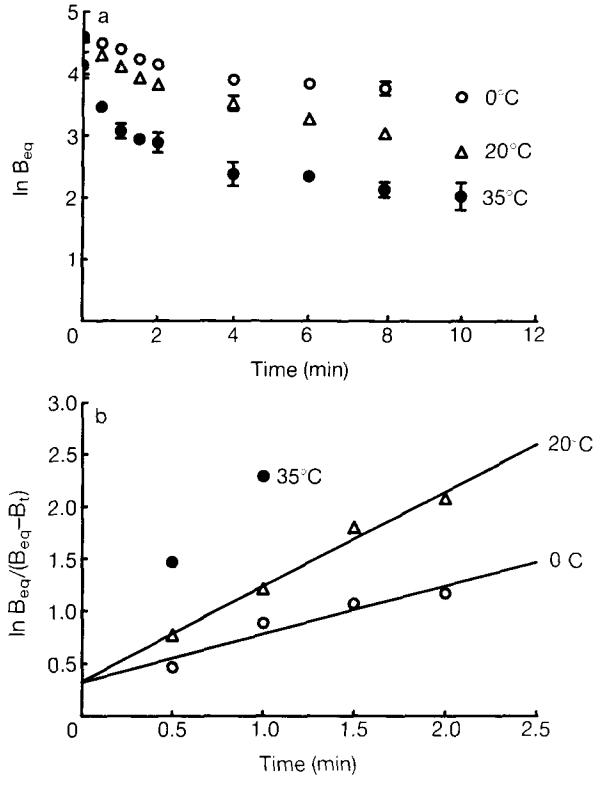

FIG. 4. [^{3}H]Muscimol binding kinetics at 0°, 20° and 35°C. (a) Semilog plots of the dissociation data. Dissociation was induced by adding GABA (100 μM final concentration) at equilibrium. (b) Semilog plots of the association data. Radioligand (5 nM final concentration) was added into constantly stirred membranes at each of the assay temperatures and 0·5 mL aliquots were removed for filtration at indicated time intervals. B_{eq} = specific ligand bound at equilibrium. B_t = amount of specific bound [^{3}H]muscimol at time t.

binding of [^{35}S]TBPS ([^{35}S]t-butylbicyclophosphorothionate) and to enhance [^{3}H]flunitrazepam and [^{3}H]muscimol binding (Ramanjaneyulu & Ticku 1984; Richter & Yamamura 1985; Harrison et al 1987). In the present study, the endogenous steroid metabolite, 5β-pregnan-3α-ol-20-one and the synthetic steroid, alphaxalone (500 nM–10 μM) showed a dose-dependent enhancement in [^{3}H]muscimol binding to rat brain membranes in a manner similar to the barbiturate, pentobarbitone (50 μM–1 mM). Both steroids were more potent than pentobarbitone in the stimulation of binding; 5β-pregnan-3α-ol-20-one being approximately 100 times more potent. The kinetics of muscimol binding to brain membranes is usually described as biphasic, with the binding data analysed in terms of a high- and a low-affinity population of binding sites (Olsen 1981). However, over the relatively narrow range of muscimol concentrations used here, analysis of the data from four independent experiments showed that the binding data were best fitted to a single site model. Curve-fitting analysis indicated that, with both the barbiturate and the steroid, the stimulation resulted principally from an increase in the number of detectable high affinity binding sites in the membrane. This is in agreement with other workers who have also reported that pentobarbitone, 5β-pregnan-3α-ol-20-one and alphaxalone enhance [^{3}H]muscimol binding by this mechanism (Olsen & Snowman 1982; Harrison & Simmonds 1984; Kirkness et al 1987). These findings suggest a possible common site or mechanism of interaction of barbiturates and steroids with the $GABA_A$ receptor complex.

The unsaturated steroids, hydrocortisone and corticosterone both showed little effect on the stimulation of [^{3}H]muscimol binding, indicating that not all steroids can interact with the $GABA_A$ receptor. Harrison et al (1987) have reported

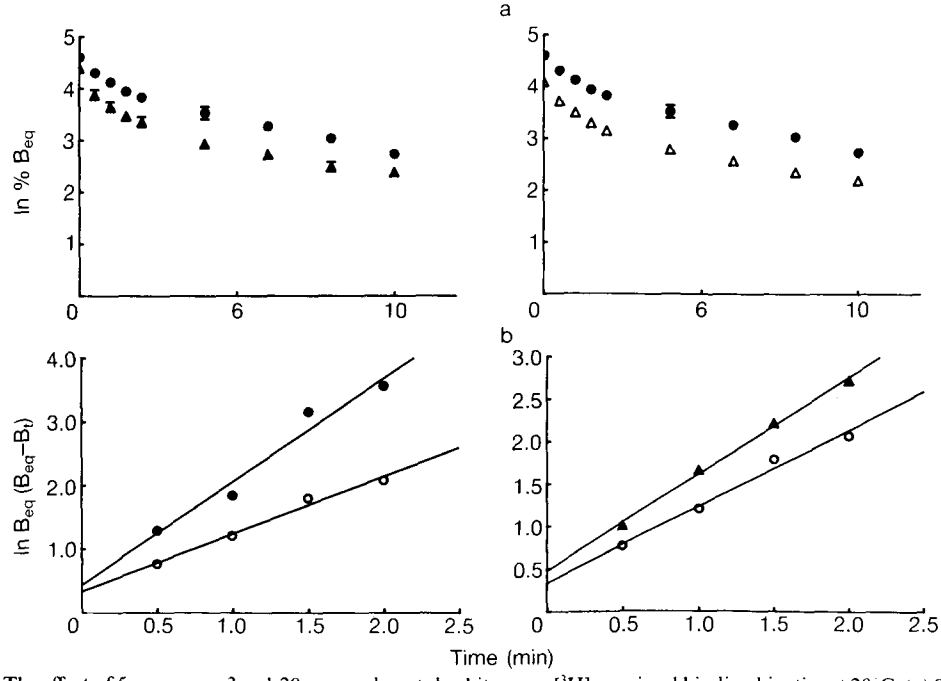

FIG. 5. The effect of 5α-pregnan-3α-ol-20-one and pentobarbitone on [^{3}H]muscimol binding kinetics at 20 C. (a) Semilog plots of the dissociation data in the absence (●) and presence of 5α-pregnan-3α-ol-20-one (▲) and pentobarbitone (△). (b) Semilog plots of the association data in the absence (○) and presence of 5α-pregnan-3α-ol-20-one (●) and pentobarbitone (▲).

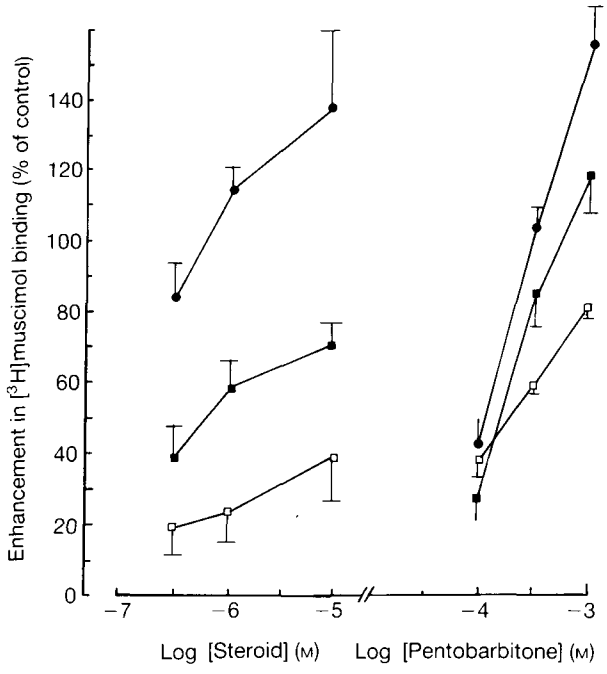

FIG. 6. The enhancement of [^{3}H]muscimol binding by 5 β-pregnan-3α-ol-20-one and pentobarbitone expressed as a percentage of control. The assays were carried out at 0° (□), 20° (■) and 35°C (●).

that for steroids to be active in this system they must have a 5α- or 5β-reduced pregnane skeleton with a hydroxyl at C3 in the α-position and a ketone group at C20. This high degree of stereochemical and structural specificity suggest that steroids may act selectively at a recognition site on the receptor complex. However, until an interaction between steroids and a lipid-free solubilized preparation of the GABA$_A$ receptor complex can be demonstrated, the possible involvement of lipids in this steroid action cannot be excluded.

In the present study, the effect of increasing the assay temperature from 0° to 35°C on the modulation of [^{3}H]muscimol binding by pentobarbitone and 5β-pregnan-3α-ol-20-one in rat brain membranes was examined. The results when expressed in terms of absolute muscimol binding in pmol (mg protein)$^{-1}$, indicate a significant reduction in the stimulation of binding by pentobarbitone at 35°C compared with that at 0°C whereas there appears to be no significant effect of temperature on the steroid modulation of binding. There also appears to be a dose-related temperature effect for the responses to pentobarbitone in which the decreased enhancement of binding seen at the higher assay temperature becomes less apparent with increasing pentobarbitone concentrations. For example, the enhancement of binding (pmol (mg protein)$^{-1}$ was reduced by approximately 53, 38 and 27% for pentobarbitone concentrations of 50, 100 μM and 1 mM, respectively when assayed at 35°C compared with 0°C (see Fig. 3b). The kinetic studies indicated that the steroid and pentobarbitone had no effect on the dissociation rate whereas the apparent association rate for [^{3}H]muscimol binding to the receptor was increased in the presence of the steroid but not in the presence of pentobarbitone. This difference in response to assay temperature together with the difference in effect on association rate, suggests that the mechanisms which mediate the effects of the two classes of drug on the GABA$_A$ receptor complex may be different.

Other workers have also examined the effect of anaesthetic drugs on radioligand binding to the GABA$_A$ receptor complex at different assay temperatures. For example, propanidid produced a more powerful stimulation of [^{3}H]muscimol binding to a membrane preparation from pig cerebral cortex when assayed at 37°C compared with 0°C (Kirkness & Turner 1986). The increase in [^{3}H]muscimol binding to GABA$_A$ receptors evoked by pentobarbitone can also be enhanced by increasing the assay temperature (Quast & Brenner 1983) although this is not always found to be the case (Olsen & Snowman 1982). In agreement with these earlier studies, the results presented here have shown that, when expressed as a percentage of control, the responses to pentobarbitone and 5β-pregnan-3α-20-one were also increased if the assay temperature was increased, the effect on the response to the steroid being more marked than that on the response to the barbiturate (see Fig. 6). However, increasing the assay temperature was found to be associated with a decrease in displaceable [^{3}H]muscimol binding to the membranes when it was measured in the absence of test drug. This effect, which was also reported by Kirkness & Turner (1986), seemed to be caused by a temperature-related increase in the initial dissociation rate of [^{3}H]muscimol from the receptor. Therefore, expressing the results in this manner does not take into account the change in baseline [^{3}H]muscimol binding. As control [^{3}H]muscimol binding decreases with increasing assay temperature, expressing the same increase in absolute binding over a lower control binding results in a greater percentage increase, thus giving the false impression of a greater enhancement of binding at higher assay temperatures. This was found to be so with the steroid at all concentrations tested whereas with pentobarbitone this effect is less marked and becomes apparent only at the higher concentrations.

In conclusion, therefore, the study has shown that modulation of the GABA$_A$ receptor by pentobarbitone and 5β-pregnan-3α-ol-20-one appears to be influenced differentially by the temperature used for the assay. The data appear to be consistent with other reports (Kirkness & Turner 1988; Peters et al 1988) which suggest that these drugs act through different regulatory sites on the receptor complex, although further work needs to be done to confirm this conclusion.

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