Br. J. Pharmacol. (1988), **94**, 1257–1269

Modulation of the $GABA_A$ receptor by depressant barbiturates and pregnane steroids

John A. Peters, *Ewen F. Kirkness, Helen Callachan, [1]Jeremy J. Lambert, & *Anthony J. Turner

Neuroscience Research Group, Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland and *Department of Biochemistry, University of Leeds, Leeds LS2 9JT

1 The modulation of the γ-aminobutyric $acid_A$ ($GABA_A$) receptor by reduced metabolites of progesterone and deoxycorticosterone has been compared with that produced by depressant barbiturates in: (a) voltage-clamp recordings from bovine enzymatically isolated chromaffin cells in cell culture, and (b) an assay of the specific binding of [^{3}H]-muscimol to a preparation of porcine brain membranes.

2 The progesterone metabolites 5α- and 5β-pregnan-3α-ol-20-one ($\geqslant$30 nM) reversibly and dose-dependently enhanced the amplitude of membrane currents elicited by locally applied GABA (100 μM), and over the concentration range 30 nM–100 μM stimulated the binding of [^{3}H]-muscimol. In contrast, 5α- and 5β-pregnan-3β-ol-20-one (30 nM–100 μM) had little effect in either assay, indicating a marked stereoselectivity of steroid action.

3 Scatchard analysis of the ligand binding data suggested an apparent increase in the number, rather than the affinity, of detectable [^{3}H]-muscimol binding sites as the principle action of the active steroid isomers.

4 GABA-evoked currents were also potentiated by androsterone (1 μM) and the deoxycorticosterone metabolite 5α-pregnane-3α,21-diol-20-one (100 nM).

5 Secobarbitone (10–100 μM), pentobarbitone (10–300 μM) and phenobarbitone (100–500 μM) reversibly and dose-dependently potentiated the amplitude of GABA-evoked currents in the absence of any change in their reversal potential.

6 At relatively high concentrations ($\geqslant$30 μM) secobarbitone and pentobarbitone directly elicited a membrane current. It is concluded that such currents result from $GABA_A$ receptor-channel activation since they share a common reversal potential with GABA-evoked responses ($\sim$0 mV), are reversibly antagonized by bicuculline (3 μM), and potentiated by either diazepam (1 μM) or 5β-pregnan-3α-ol-20-one (500 nM).

7 Secobarbitone (1 μM-1 mM) dose-dependently enhanced the binding of [^{3}H]-muscimol. In common with the active steroids, an increase in the apparent number of binding sites was responsible for this effect.

8 A saturating concentration (1 mM) of secobarbitone in the ligand binding assay did not suppress the degree of enhancement of control binding produced by 5β-pregnan-3α-ol-20-one (30 nM–100 μM). Similarly the steroid, at a concentration of 100 μM, did not influence the enhancement of [^{3}H]-muscimol binding by secobarbitone (1 μM–1 mM). In all combinations of concentrations tested, the effects of secobarbitone and 5β-pregnan-3α-ol-20-one on [^{3}H]-muscimol binding were additive.

9 In conjunction with previously published observations, the present data indicate close similarities in the GABA-mimetic and potentiating actions of barbiturates and steroids. However, the results obtained with combinations of steroids and barbiturates in the ligand binding assay appear inconsistent with the two classes of compound interacting with a common site to modulate the $GABA_A$ receptor activity.

[1] Author for correspondence.

Introduction

It is a generally held view that γ-aminobutyric acid (GABA) subserves an important inhibitory neurotransmitter function within the vertebrate central nervous system. Through activating $GABA_A$ receptors, GABA evokes an increase in membrane chloride conductance, an effect which usually leads to membrane hyperpolarization and a decrease in neuronal excitability. Such actions of GABA are subject to modulation by a variety of structurally diverse drugs which possess anxiolytic, hypnotic or general anaesthetic properties, the most extensively studied being certain barbiturate and benzodiazepine compounds (Keane & Biziere, 1987; Martin, 1987; Simmonds & Turner, 1987). By binding to distinct, but interacting, regulatory sites upon the $GABA_A$ receptor, some barbiturates and benzodiazepines modulate the operation of the associated chloride-selective ionophore, causing predominantly an enhancement of channel open-time or an increase in opening frequency respectively (Study & Barker, 1981).

Recently, it has been demonstrated that some metabolites of progesterone and deoxycorticosterone enhance the effects of exogenously applied GABA on cultured central neurones and adrenomedullary chromaffin cells (Majewska *et al.*, 1986; Callachan *et al.*, 1987a; Lambert *et al.*, 1987). Furthermore, like barbiturates and benzodiazepines, certain of these steroids potentiate inhibitory synaptic transmission mediated by GABA (Harrison *et al.*, 1987a,b).

Several lines of evidence indicate that the steroids act in a 'barbiturate-like' manner at the $GABA_A$ receptor (Majewska *et al.*, 1986). Thus in radioligand binding assays, both classes of compound enhance the specific binding of $GABA_A$ receptor agonists and benzodiazepine receptor ligands to rat brain membranes (Leeb-Lundberg *et al.*, 1980; Olsen & Snowman, 1982; Whittle & Turner, 1982; Harrison *et al.*, 1987; Kirkness *et al.*, 1987) and allosterically inhibit the binding of the convulsant GABA antagonist t-butylbicyclophosphorothionate (TBPS) (Squires *et al.*, 1983; Ramanjaneyulu & Ticku, 1984; Majewska *et al.*, 1986; Harrison *et al.*, 1987a). Furthermore, in electrophysiological studies the potentiation of GABA-evoked responses produced by either barbiturates or steroids is insensitive to the benzodiazepine receptor antagonist Ro15-1788 (Simmonds, 1985; Callachan *et al.*, 1987a; Cottrell *et al.*, 1987) and appears to be due to a prolongation of GABA channel burst duration (Barker *et al.*, 1987; Callachan *et al.*, 1987a; Lambert *et al.*, 1987). However, despite these similarities, studies evaluating the anaesthetic effect of binary mixtures of the synthetic steroid alphaxalone and depressant barbiturates suggest that they do not act through a common site or mechanism (Richards & White, 1981; Halsey *et al.*, 1986).

In the present study, the actions of barbiturates and steroids were compared in voltage-clamp recordings made from bovine chromaffin cells, which possess $GABA_A$ receptors similar to those of central neurones (Bormann & Clapham, 1985; Cottrell *et al.*, 1985), and in a radioligand binding assay using [^{3}H]-muscimol and a membrane preparation derived from porcine cerebral cortex. Some structural requirements for steroid activity at the $GABA_A$ receptor were also examined. Whilst confirming a number of similarities between the actions of the barbiturates and steroids, the results of the radioligand binding assay suggest that they modulate $GABA_A$ receptor function through different sites. A preliminary account of some of this work has appeared in abstract form (Callachan *et al.*, 1987b; Kirkness *et al.*, 1987).

Methods

Dissociation and culture of chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated and cultured by the method of Fenwick *et al.* (1982) with minor modifications (Cottrell *et al.*, 1987) and used in electrophysiological experiments 1–7 days after plating.

Electrical recordings

Agonist-activated currents were recorded using the 'whole cell' mode of the patch clamp technique, by standard methods (Hamill *et al.*, 1981), and a List Electronics L/M EPC-7 converter headstage and amplifier. To facilitate voltage-clamping, cells were selected which were devoid of processes and 8 to 18 μm in diameter. Whole cell currents were low-pass filtered (Bessel characteristic) at 500 Hz and recorded on magnetic tape with an FM tape recorder (Racal Store 4DS) for subsequent analysis. Cells were continuously superfused (3–5 ml min^{-1}) with a solution containing (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2, $CaCl_2$ 1.0 and HEPES-NaOH 10 (pH 7.2). The pipette solution employed to dialyse the cell interior comprised (in mM): CsCl 140, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 1.1 and HEPES-NaOH (pH 7.2). Cs was employed as the predominant internal cation to suppress the various K conductances of the cell membrane (Marty & Neher, 1985). Drugs were applied locally by pressure ejection (1.4×10^5 Pa) from modified patch pipettes; by diffusion from coarse tipped (~20 μM) micropipettes, or via the

superfusion medium. All experiments were conducted at room temperature (17–21°C).

Radioligand binding assays

A crude preparation of synaptic membranes was prepared from pig cerebral cortex essentially as described previously (Kirkness & Turner, 1986). Pig brain was obtained fresh from a slaughterhouse and the cortex removed, chopped, frozen and stored at −70°C until use. Cortex was thawed and homogenized in 10 vol of 10 mM HEPES (pH 7.5) 300 mM sucrose containing the proteinase inhibitors EDTA (1 mM), benzamidine (1 mM), phenylmethanesulphonyl fluoride (0.3 mM), bacitracin (100 mg l^{-1}) and trypsin inhibitors type II-S and II-0 (10 mg l^{-1}; Sigma). 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. This pellet was washed twice by resuspension in 20 vol. of ice-cold 10 mM HEPES (pH 7.5)/proteinase inhibitors, followed by centrifugation at 48,000 *g* for 20 min. The resuspended pellet was stored frozen at −20°C for up to 2 months. Before assay of receptor binding, the membranes were thawed, washed once with 20 mM potassium phosphate buffer (pH 7.5)/100 mM KCl and dialysed for 20 h against 500 vol. of the same buffer containing 0.1 mM EDTA and 0.02% (w/v) NaN_3.

The binding of [^{3}H]-muscimol to the synaptic membrane preparation was performed by a filtration assay (Williams & Risley, 1979). Unless stated otherwise, membranes (0.4–0.7 mg) were incubated in a total volume of 0.5 ml of 20 mM potassium phosphate buffer (pH 7.5)/100 mM KCl for 30 min at 0°C in the presence of 5 nM [^{3}H]-muscimol and various concentrations of drugs. Non-specific binding was measured in the presence of 50 μM muscimol. After incubation, samples were diluted with 4 ml of assay buffer and immediately filtered on GF/B filters (Whatman) under suction. Filters were washed twice with 4 ml of assay buffer, dried and counted for radioactivity in 5 ml of Optiphase Safe (LKB). All experimental points were obtained in triplicate. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Data analysis

Data stored on magnetic tape were analysed by several methods. Whole cell currents elicited by locally applied agonists were either played out at reduced tape speed onto a pen recorder and subsequently inspected by eye, or analysed by a computer programme (Dempster, 1988) run on a PDP 11-73 minicomputer. In the latter method, whole cell currents were digitised into 512–1024 points at 100 Hz, inspected on a display screen and edited. Four validated signal records were then averaged, analysed, and for illustration, plotted on a Hewlett Packard 7470A plotter, Records of membrane noise evoked by bath-applied agonists were low-pass filtered (200 Hz, Butterworth characteristic) and digitised into 512 points at a sampling rate of 2.5 ms. The mean current induced by the agonist was then calculated by computer programme (Dempster, 1988) from the average of 16 such 512 point samples. All quantitative results are expressed as the arithmetic mean ± s.e.mean.

Drugs used

The reagents used in the study were: γ-aminobutyric acid (GABA), (+)-bicuculline, muscimol, picrotoxinin, 5α-androstan-3α-ol-17-one, 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, 5β-pregnan-3α-ol-20-one, 5β-pregnan-3β-ol-20-one, 5α-pregnane-3α, 21-diol-20-one, phenobarbitone, sodium pentobarbitone, sodium secobarbitone (quinalbarbitone) (all obtained from Sigma), diazepam (Roche) and [methylene-^{3}H]-muscimol (8–13 Ci mmol^{-1}) (Amersham International). Stock solutions of the steroids and diazepam were prepared in ethanol. The final concentrations of ethanol in the radioligand binding assay and electrophysiological experiments never exceeded 0.5% (vol/vol) and 0.1% (vol/vol) respectively. At such concentrations, ethanol influenced neither the specific binding of [^{3}H]-muscimol measured in the absence of drugs, nor the amplitude of transmembrane currents elicited by GABA.

Results

Potentiation of GABA-evoked currents by pregnane steroids

The structure of the four pregnane steroids examined in detail in this study are indicated in Figure 1 together with traces illustrating their influence upon responses to GABA, recorded under voltage clamp, from a chromaffin cell. 5α-Pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one, each bath-applied at concentrations of 30 and 100 nM, dose-dependently enhanced the amplitude of inward currents evoked by locally applied GABA (100 μM) (Figure 1 and Table 1). Such potentiation was rapid in onset, occurring immediately upon the introduction of the steroids to the bath, and was readily reversed upon washout. There was no significant difference between the degree of potentiation produced by 5α-pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one at either dose tested (*t* test, $P > 0.1$).

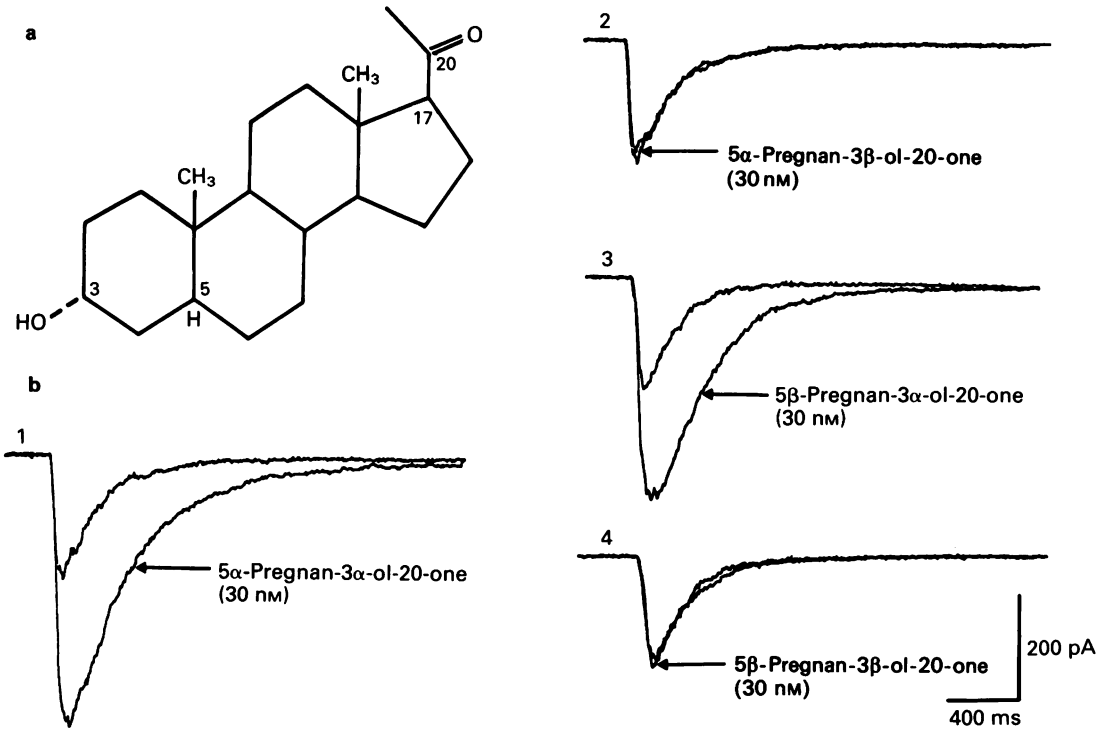

Figure 1 Stereoselective modulation of GABA-evoked responses by pregnane steroids. (a) The structure of the progesterone metabolite 5β-pregnan-3α-ol-20-one. The broken line at C3 indicates that the hydroxyl group projects below the plane of the molecule. In 5β-pregnan-3β-ol-20-one the 3-hydroxyl function lies above the plane of the molecule. The position of the hydrogen at C5 determines whether the A and B rings of the molecule are in the *cis* or *trans* conformation. In 5α-pregnan-3α-ol-20-one the hydrogen moiety projects below the plane of the molecule (*trans* A/B), whereas in 5β-pregnan-3α-ol-20-one the hydrogen lies above the plane (*cis* A/B). (b) Membrane currents elicited by locally applied GABA (100 μM; 0.05 Hz, pressure applied at 1.4×10^5 Pa for 20 ms) in control and in the presence of pregnane steroids. (1 and 2) 5α-Pregnan-3α-ol-20-one (30 nM) enhanced the amplitude and total charge passed during the response to 219.5 and 352.5% of their control values, respectively. The corresponding values of amplitude and charge passed with respect to control on the same cell in the presence of 5α-pregnan-3β-ol-20-one (30 nM) were 89.9 and 97.5%. (3 and 4) Enhancement of response amplitude (200.9%) and total charge passed (331.7%) by 5β-pregnan-3α-ol-20-one (30 nM). In comparison, on the same cell, the response amplitude and total charge passed as a percentage of control in the presence of 5β-pregnan-3β-ol-20-one (30 nM) were 95.2 and 105.3%, respectively. All currents were recorded at a holding potential of −60 mV. The traces illustrated are computer generated averages of 4 responses to GABA (see Methods).

In contrast to the 3α-ol isomers, pregnane steroids in which the 3-hydroxyl group is in the β-position lack potency in potentiating GABA-evoked currents. Thus low concentrations (30–100 nM) of 5α-pregnan-3β-ol-20-one and 5β-pregnan-3β-ol-20-one had no effect upon the amplitude of responses to locally applied GABA (100 μM) evoked from cells demonstrably sensitive to the 3α-ol-isomers (Figure 1). Furthermore, far greater concentrations (1–10 μM) of the 3β-ol-isomers produced relatively little potentiation of GABA-evoked responses (Table 1). The hormone progesterone (10 μM) from which the pregnane isomers are derived by reductive metabolism had no effect on GABA-evoked currents ($n = 7$) confirming earlier findings (Callachan *et al.*, 1987a).

The deoxycorticosterone metabolite 5α-pregnane-3α,21-diol-20-one and the sex hormone androsterone (5α-androstan-3α-ol-17-one) have previously been shown to potentiate responses to exogenously applied GABA recorded from rodent central neurones (Simmonds *et al.*, 1984; Harrison *et al.*, 1987a). In the present study, 5α-pregnan-3α,21-diol-20-one (100 nM) and androsterone (1 μM) enhanced the amplitude of GABA-evoked currents to 201.6 ± 16.9% ($n = 9$) and 202.2 ± 12.8% ($n = 11$) of their control values, respectively, a degree of poten-

Table 1 Stereoselective potentiation of GABA-evoked currents by steroids

Steroid	*Concentration* (μM)	*Amplitude of GABA-evoked current as a percentage of control*
5α-Pregnan-3α-ol-20-one	0.03	204.8 ± 12.6 (8)
	0.10	292.3 ± 26.7 (9)
5β-Pregnan-3α-ol-20-one	0.03	207.5 ± 14.3 (6)
	0.10	254.0 ± 28.9 (9)
5α-Pregnan-3β-ol-20-one	1.00	102.7 ± 2.3 (4)
	10.00	110.6 ± 8.7 (3)
5β-Pregnan-3β-ol-20-one	1.00	113.9 ± 5.1 (6)
	10.00	116.7 ± 4.8 (5)
5α-Pregnane-3α,21-diol-20-one	0.10	201.6 ± 16.9 (9)
5α-Androstan-3α-ol-17-one	1.00	200.2 ± 12.8 (11)

Results shown are the mean ± s.e.mean of n (number in parentheses) observations.

tiation similar to that observed with either 5α- or 5β-pregnan-3α-ol-20-one at a lower concentration (30 nM) (Table 1).

Potentiation of GABA-evoked currents by depressant barbiturates

Pentobarbitone, within the concentration range 10–300 μM, dose-dependently enhanced the amplitude of transmembrane currents evoked by locally applied GABA (100 μM). A qualitatively similar potentiation of GABA-evoked responses occurred with bath-applied secobarbitone (10–100 μM) or phenobarbitone (100–500 μM) (Figure 2a, b). The dose-effect curves presented in Figure 2b indicate that the 3 barbiturates potentiate the amplitude of GABA-evoked currents with the order of potency: secobarbitone > pentobarbitone > phenobarbitone. By comparing equieffective concentrations of the various modulators, secobarbitone and pentobarbitone were estimated respectively to be 130 and 400 fold less potent than the steroid 5β-pregnan-3α-ol-20-one in potentiating responses to GABA. As the dose-effect curves for these 3 drugs are approximately parallel (Figure 2b), such estimates should be independent of the level of potentiation at which the comparison is performed. Since the range of phenobarbitone concentrations examined was limited, no attempt was made to quantify its relative potency. The potentiating effect of the barbiturate compounds was not associated with any change in the reversal potential of the GABA response, this being illustrated for pentobarbitone in Figure 2c. Similarly, the potentiation of GABA-evoked currents due to 5β-pregnan-3α-ol-20-one does not involve any shift in their reversal potential (Callachan *et al.*, 1987a).

Direct agonist action of barbiturates

Secobarbitone and pentobarbitone, when bath-applied at concentrations greater than those necessary for substantial potentiation of GABA-evoked currents, directly activated a membrane conductance in bovine chromaffin cells. Representative records of the inward currents evoked by pentobarbitone and secobarbitone, each applied to a chromaffin cell at a concentration of 100 μM are illustrated in Figure 3a,b. Although the relative potencies of these two barbiturates were not assessed in detail, secobarbitone (100 μM) consistently evoked a larger current response (−22.4 ± 10.9 pA, $n = 4$) than an equimolar concentration of pentobarbitone (−3.3 ± 1.2 pA, $n = 4$) in the same sample of cells. Phenobarbitone (100 μM) was ineffective when applied to cells demonstrably sensitive to secobarbitone (100 μM). The agonist actions of these barbiturates thus appear to parallel their potencies as potentiating agents.

Relatively high concentrations (1 mM) of bath-applied pentobarbitone or secobarbitone elicited a complex response consisting of an initial peak inward current which gradually declined to a plateau level. During washout of the barbiturate, a transient increase in inward current was often observed. This phenomenon was particularly pronounced when pentobarbitone (5 mM) was rapidly applied to cells by the microperfusion technique, as shown in Figure 3c. Indeed, under such conditions, the current observed during wash off was of a greater amplitude than the initial response to pentobarbitone. Similar currents in response to pentobarbitone have been recorded from frog sensory neurones and may possibly be attributed to blockade of the $GABA_A$ receptor-linked chloride ion channel by pentobarbitone at high concentrations, a notion supported by

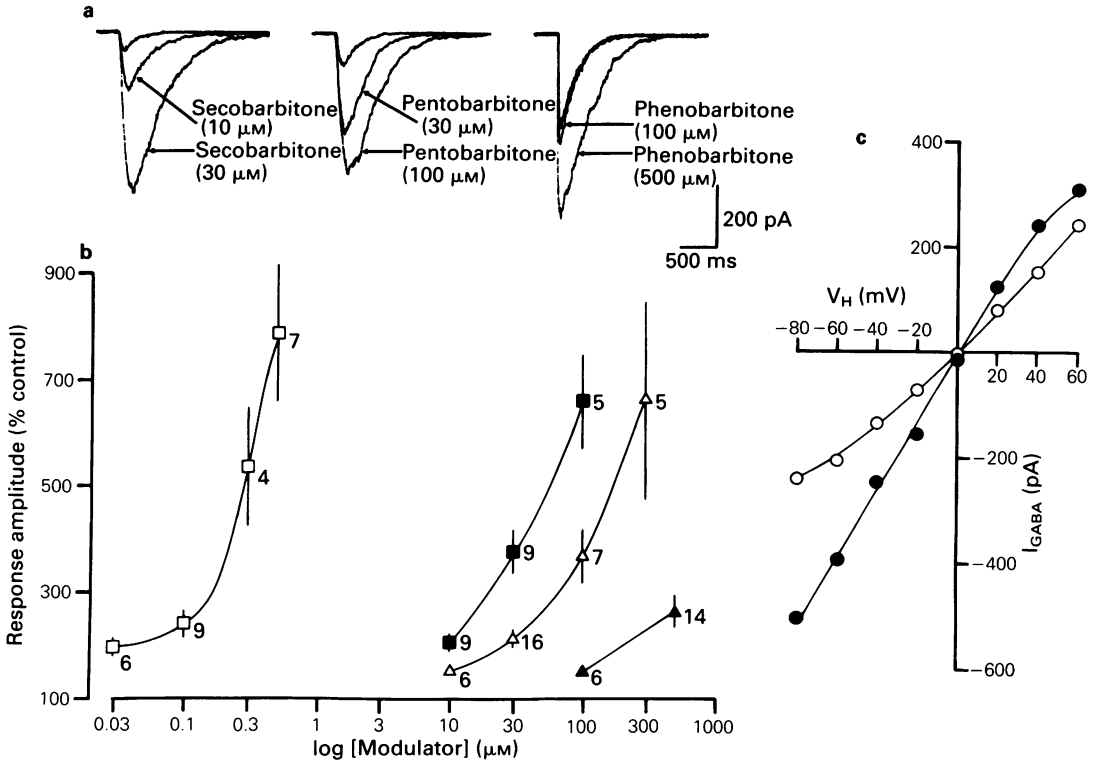

Figure 2 Enhancement of GABA-evoked responses by depressant barbiturates. (a) Dose-dependent potentiation of responses to locally applied GABA (100 μM) by secobarbitone (10 and 30 μM), pentobarbitone (30 and 100 μM) and phenobarbitone (100 and 500 μM). The results illustrated are from 3 different cells voltage-clamped at a holding potential of −60 mV. (b) Graph comparing the potencies of secobarbitone (■), pentobarbitone (△), phenobarbitone (▲) and the pregnane steroid 5β-pregnan-3α-ol-20-one (□) as potentiators of GABA-induced currents. The amplitude of the GABA-evoked current, expressed as a percentage of its control value, is plotted against the log of the concentration of the modulating drug in the bathing medium. Data points are the mean values of the number of observations indicated adjacent to each symbol. Vertical lines indicate the s.e.mean. (c) Relationships between response amplitude (I_{GABA}) and holding potential (V_H) for GABA (100 μM) applied locally in the absence (○) and presence (●) of pentobarbitone (30 μM). The curves, fitted to the data points by eye, yield interpolated reversal potentials of 1.0 and 2.0 mV in the absence and presence of pentobarbitone, respectively.

the observation that such currents are suppressed by bicuculline (Akaike *et al.*, 1987).

The membrane currents evoked by the barbiturates were more conveniently studied using the technique of local, pressure application. Figure 4a illustrates current responses to locally applied pentobarbitone (1 mM), recorded over a range of holding potentials (−60 to 60 mV). From such experiments the reversal potential of the pentobarbitone-induced response was estimated, by interpolation, to be +1.0 ± 0.5 mV ($n = 4$), a value similar to that found for GABA (+3.0 mV, Cottrell *et al.*, 1985) when the internal and external concentrations of chloride ions are essentially equal. Additionally currents elicited by pentobarbitone were reversibly suppressed by the $GABA_A$ receptor antagonist bicuculline (3 μM; Figure 4b) and potentiated to 190 ± 12% ($n = 6$) of their control value by diazepam (1 μM; Figure 4b). In common with responses to GABA, currents evoked by pentobarbitone were greatly enhanced in amplitude by the pregnane steroid 5β-pregnan-3α-ol-20-one. At a concentration of 500 nM, this steroid potentiated currents elicited by GABA (100 μM) and pentobarbitone (1 mM) to 789 ± 129% ($n = 7$) and 1156 ± 199% ($n = 6$) of their control values, respectively (Figure 4c,d). Collectively, these data reinforce the prevalent notion that certain barbiturates, at relatively high doses, may activate the chloride ion channel associated with the $GABA_A$ receptor, perhaps by their association with an allosteric modulatory site (Barker & Ransom, 1978; Higashi & Nishi, 1982; Owen *et al.*, 1986; Akaike *et al.*, 1987).

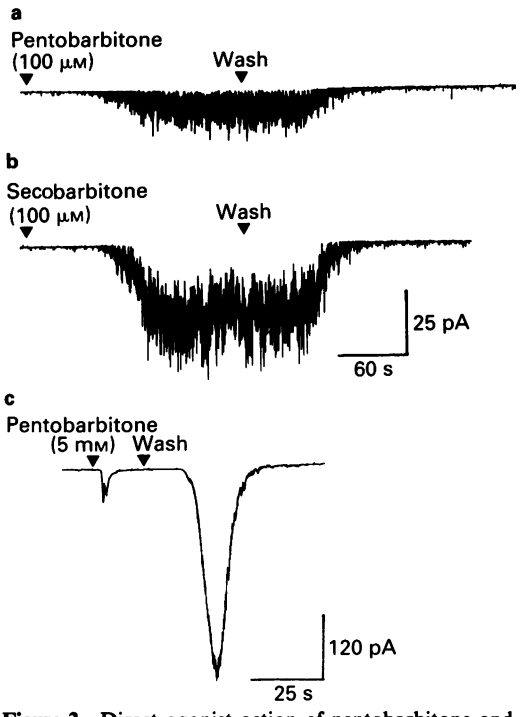

Figure 3 Direct agonist action of pentobarbitone and secobarbitone. Bath application of (a) pentobarbitone (100 μM) and (b) secobarbitone (100 μM) evoked a large increase in membrane noise corresponding to a mean inward current of −6.4 pA and −28.4 pA, respectively. Agonist-induced currents were quantified by a computer programme as described in Methods. The delay in the onset of barbiturate-evoked currents is due to the dead space of the perfusion system. (c) Trace illustrating a response to pentobarbitone (5 mM) applied by the microperfusion technique. Note that a current occurs upon termination of agonist application. All currents were recorded at a holding potential of −60 mV.

Pentobarbitone (10^{-4}–10^{-3} M) has been shown to depolarize directly cat primary afferent neurones, an effect partly due to a suppression of potassium conductance in some cells (Higashi & Nishi, 1982). In the present experiments the direct effects of barbiturates were investigated using a caesium-based pipette solution to suppress the various potassium conductances of the chromaffin cell (Marty & Neher, 1985) and, hence, the possibility that barbiturates exert an influence on chromaffin cell potassium channels remains to be determined.

Enhancement of [^{3}H]-muscimol binding by pregnane steroids

The specific binding of [^{3}H]-muscimol to a crude preparation of cerebral cortex membranes was enhanced, in a dose-dependent manner, by both 5α-pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one (30 nM–100 μM). The stimulation of binding observed with the highest concentration of steroid tested (100 μM), was approximately 55% of control for either of the 3α-ol isomers (Figure 5a). Through out the concentration range examined, 5α-pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one were approximately equipotent in stimulating [^{3}H]-muscimol binding, reinforcing the observation that the 3α-ol-isomers produce a similar degree of potentiation of the GABA-evoked current. In further corroboration of the results obtained with electrophysiological techniques, 5α-pregnan-3β-ol-20-one and 5β-pregnan-3β-ol-20-one (30 nM–100 μM) were only weakly active in the ligand binding assay, producing only a 10–15% enhancement of [^{3}H]-muscimol binding at a concentration of 100 μM (Figure 5a). The effect of the active 3α-ol isomers was dependent upon the presence of chloride ions in the assay. Hence the [^{3}H]-muscimol binding activity of membranes prepared and assayed in the absence of KCl was insensitive to 5α-pregnan-3α-ol-20-one or 5β-pregnan-3α-ol-20-one (10 μM). Addition to the assay medium of KCl (100 mM) or NaCl (100 mM), but not K_2SO_4 (50 mM), restored the ability of the 3α-ol isomers to stimulate control binding. The GABA$_A$ antagonist picrotoxinin (10 μM) reduced the stimulation of control binding by 3 μM 5β-pregnan-3α-ol-20-one from 137.0 ± 5.0% to 110.0 ± 4.0% (n = 4). Scatchard analysis of control [^{3}H]-muscimol binding indicated a single class of high affinity binding sites (Figure 5b). At concentrations of 3 μM, 5α-pregnan-3α-ol-20-one or 5β-pregnan-3α-ol-20-one had little effect on the affinity of [^{3}H]-muscimol binding but significantly increased the apparent total number of high-affinity binding sites in the membrane (Figure 5b).

Enhancement of [^{3}H]-muscimol binding by barbiturate/steroid combinations

Depressant barbiturates and pregnane steroids exert strikingly similar actions on the GABA$_A$ receptor in electrophysiological and ligand binding assays. In order to examine the possibility that these two classes of compounds act through a common regulatory site on the receptor, the effect of their combined action on [^{3}H]-muscimol binding was determined. Enhancement by secobarbitone alone was detectable at 1–10 μM and was maximal (65% enhancement) at a concentration of 1 mM (Figure 6a). It is notable that the lowest concentration at which enhancement could be observed with secobarbitone was 10–100 fold greater than for 5β-pregnan-3α-ol-20-one; a difference in potency comparable to that observed in the electrophysiological studies. The magnitude of

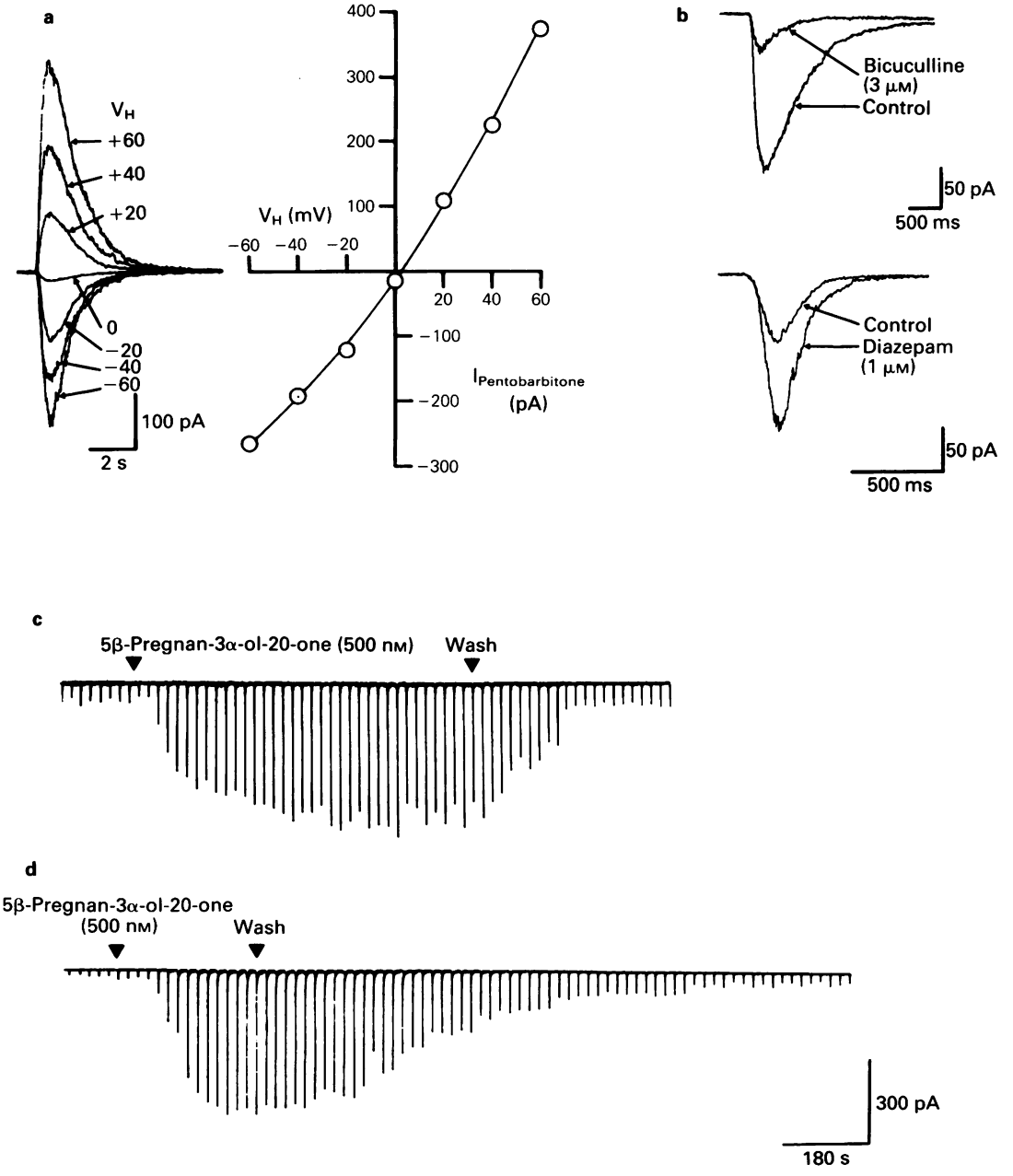

Figure 4 The direct agonist action of pentobarbitone and its modulation by compounds acting at $GABA_A$ receptors. (a) Traces illustrating the currents evoked by locally applied pentobarbitone (1 mM) at holding potentials (V_H) ranging from −60 to +60 mV. The relationship between holding potential and response amplitude is also shown graphically, and indicates that the pentobarbitone-induced current has a reversal potential of +2.0 mV in this example. (b) Upper panel: traces illustrating the blockade of the current induced by locally applied pentobarbitone (1 mM) by bicuculline (3 μM). Lower panel: enhancement by diazepam (1 μM) of the response to locally applied pentobarbitone (1 mM). All currents were recorded at a holding potential of −60 mV. (c) Reversible enhancement of responses to locally applied GABA (100 μM) by 5β-pregnan-3α-ol-20-one (500 nM). In (d) a similar effect of 5β-pregnan-3α-ol-20-one upon membrane currents induced by locally applied pentobarbitone (1 mM) is illustrated. Currents evoked by agonists were recorded at a holding potential of −60 mV.

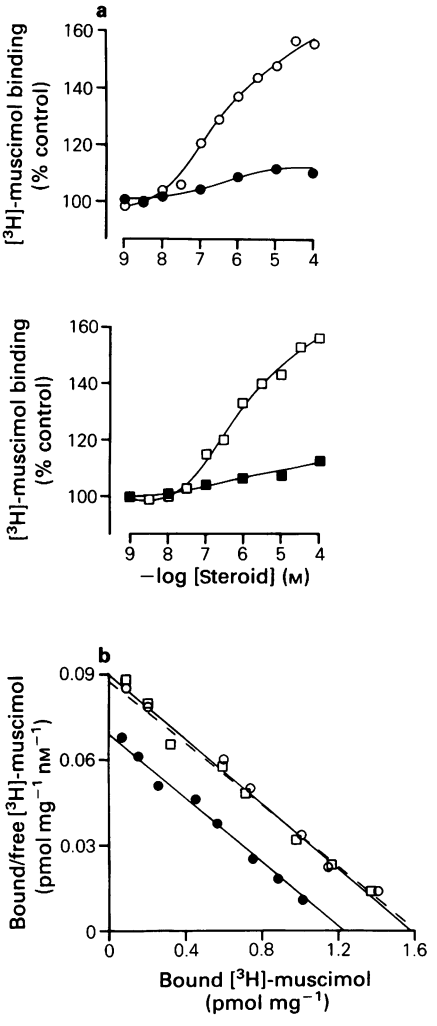

Figure 5 The influence of pregnane steroids upon the specific binding of [^{3}H]-muscimol. (a) Binding of [^{3}H]-muscimol to crude synaptic membranes was measured in the presence of 5α-pregnan-3α-ol-20-one (○), 5α-pregnan-3β-ol-20-one (●), 5β-pregnan-3α-ol-20-one (□) and 5β-pregnan-3β-ol-20-one (■). Each point represents the mean of three separate observations which, in all cases, varied by less than 5% from the mean. (b) Scatchard analysis of [^{3}H]-muscimol binding to membranes in the absence of steroid (●) and in the presence of 3 μM 5α-pregnan-3α-ol-20-one (○) or 3 μM 5β-pregnan-3α-ol-20-one (□). The concentration of [^{3}H]-muscimol was varied from 1.0 to 100 nM using 50 μM muscimol at each point to determine background binding. The mean value (and range) of the binding coefficients (K_D, B_{max}) were, respectively: control, 18.1 nM (17.9–18.3), 1.2 pmol mg^{-1} (1.1–1.3); 3 μM 5α-pregnan-3α-ol-20-one, 17.9 nM (17.1–18.4), 1.6 pmol mg^{-1} (1.5–1.7); 3 μM 5β-pregnan-3α-ol-20-one, 17.7 nM (17.2–18.0), 1.6 pmol mg^{-1} (1.4–1.7).

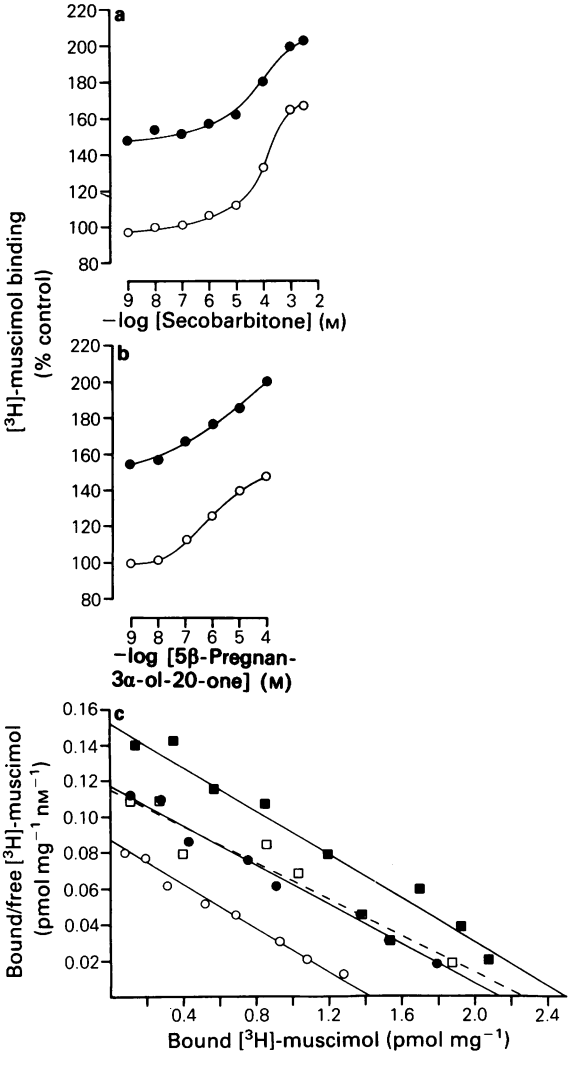

Figure 6 The influence of 5β-pregnan-3α-ol-20-one and secobarbitone upon specific [^{3}H]-muscimol binding. Binding of [^{3}H]-muscimol to crude synaptic membranes was measured; (a) over a range of secobarbitone concentrations in the absence (○) and presence (●) of 100 μM 5β-pregnan-3α-ol-20-one and (b) over a range of 5β-pregnan-3α-ol-20-one concentrations in the absence (○) and presence (●) of 1 mM secobarbitone. Each point represents the mean of four separate observations which, in all cases, varied by less than 10% from the mean. (c) Scatchard analysis of [^{3}H]-muscimol binding to membranes in the absence of modulators (○), and in the presence of 100 μM 5β-pregnan-3α-ol-20-one (●), 1 mM secobarbitone (□), or 100 μM 5β-pregnan-3α-ol-20-one and 1 mM secobarbitone (■). The concentration of [^{3}H]-muscimol was varied from 1.0 to 100 nM using 50 μM muscimol at each point to determine background binding. The binding coefficients are listed in Table 2.

Table 2 Binding coefficients determined by Scatchard analysis of [^{3}H]-muscimol binding as described in the legend to Figure 6

	K_D (nM)	B_{max} (pmol mg^{-1})	B_{max} (% control)
Control	18.7 (16.4–22.0)	1.42 (1.22–1.59)	
5β-Pregnan-3α-ol-20-one 100 μM	19.8 (18.2–20.8)	2.12 (2.07–2.14)	149
Secobarbitone 1 mM	20.1 (19.5–20.9)	2.24 (2.08–2.32)	158
5β-Pregnan-3α-ol-20-one 100 μM plus secobarbitone 1 mM	16.9 (16.4–17.8)	2.65 (2.49–2.82)	187

The data represent the mean and range of values from three separate observations.

the enhancements produced by a range of secobarbitone concentrations was little affected by the inclusion of 5β-pregnan-3α-ol-20-one (100 μM) in the assay (Figure 6a). Similarly, the extent to which a range of 5β-pregnan-3α-ol-20-one concentrations stimulated control binding was not altered significantly by the presence of a saturating concentration (1 mM) of secobarbitone (Figure 6b). In all combinations of concentrations examined, the enhancements by the barbiturate and the steroid were additive. Using mixtures of secobarbitone, 5β-pregnan-3α-ol-20-one and another active steroid, alphaxalone (Harrison & Simmonds, 1984), it was found that only combinations of barbiturate and steroid produced enhancing effects which were additive. [^{3}H]-muscimol binding was enhanced by 1 mM secobarbitone (64 $\pm$ 4%; $n = 3$), 10 μM 5β-pregnan-3α-ol-20-one (36 $\pm$ 2%; $n = 3$) and 10 μM alphaxalone (34 $\pm$ 3%; $n = 3$) acting individually. In combination, stimulation by secobarbitone and alphaxalone was strictly additive (98 $\pm$ 6%; $n = 3$), whereas a combination of the two steroids was no more effective than either agent alone (37 $\pm$ 6%; $n = 3$).

Scatchard analysis of [^{3}H]-muscimol binding in the presence of secobarbitone and 5β-pregnan-3α-ol-20-one, indicated that both compounds, acting either alone or together, stimulated binding principally by increasing the number of detectable high-affinity binding sites in the membrane (Figure 6c, Table 2). The [^{3}H]-muscimol binding affinity, as indicated by the K_D values, was little affected by either agent either alone or in combination.

Discussion

The results of the present study indicate that modulation of the $GABA_A$ receptor by pregnane steroids is a highly stereoselective phenomenon. Whilst 5α- and 5β-pregnan-3α-ol-20-one were potent in stimulating [^{3}H]-muscimol binding and enhancing the membrane currents evoked by GABA, their corresponding 3β-ol-isomers were virtually devoid of activity. It has been demonstrated previously that the steroid anaesthetic alphaxalone, which differs from 5α-pregnan-3α-ol-20-one in its possession of a ketone group at C11, is effective in potentiating responses to GABA (Harrison & Simmonds, 1984; Barker *et al.*, 1987; Cottrell *et al.*, 1987). In accord with the relative inactivity of the 3β-ol pregnane steroids examined here, the β-hydroxy isomer of alphaxalone (betaxalone) is ineffective in potentiating responses to GABA (Harrison & Simmonds, 1984; Barker *et al.*, 1987) and at relatively high concentrations (10–100 μM) acts as an antagonist (Cottrell *et al.*, 1987). Electrophysiological and radioligand binding experiments performed on cultured rodent central neurones and rat brain synaptosomal membranes, respectively, by Harrison *et al.* (1987a) also show the α configuration of the C3 hydroxyl group to be an essential structural feature and, additionally, demonstrate the requirement for a saturated pregnane skeleton and the presence of a ketone group at C20 for steroid modulation of the $GABA_A$ receptor.

The strict structural requirements for activity suggest an interaction of the steroid with a specific protein binding site. However, in the case of these highly lipophilic steroids, other interpretations are also plausible. Although the isomeric pairs alphaxalone/betaxalone and 5α-pregnan-3α-ol-20-one/5α-pregnan-3β-ol-20-one do not vary significantly in their lipid solubility or molecular volume, they do differ in the degree of disorder which they induce in model membranes, the isomers which are potent in potentiating GABA being the most effective in this respect (Lawrence & Gill, 1975). Hence it is conceivable that the stereoselective effects displayed by the steroids derive from a perturbation of the lipid structures adjacent to the $GABA_A$ receptor. Alternatively, an interaction between the steroids and a hydrophobic pocket of the $GABA_A$ receptor complex itself may be postulated (Lambert & Peters, 1988). In this respect it is known that a range of general anaesthetics competitively inhibit the activity of the purified protein firefly luciferase, and such an

action is well correlated with their anaesthetic potency (Franks & Lieb, 1987). However, although some progesterone metabolites share this ability to inhibit a luciferase enzyme, their effect lacks appropriate stereoselectivity and does not correlate with their potency as anaesthetics (Banks & Peace, 1985). We are presently internally perfusing bovine chromaffin cells with the active steroids in an attempt to elucidate their site of action.

Whatever the physical nature of the 'site' with which the steroids interact may be, there are close similarities between their actions and those of some barbiturates upon the $GABA_A$ receptor. Both classes of compound potentiate membrane currents elicited by exogenously applied GABA and prolong the decay of inhibitory postsynaptic currents mediated by GABA in cultured neurones of hippocampal origin (Segal & Barker, 1984; Harrison *et al.*, 1987b). A prolongation of the GABA channel burst duration by barbiturates and steroids is likely to underlie such effects (Study & Barker 1981; Barker *et al.*, 1987; Callachan *et al.*, 1987a; Lambert *et al.*, 1987).

Further similarities between the actions of barbiturates and steroids are apparent in the results of radioligand binding experiments, where representatives of each class of compound have been shown to displace the binding of [^{35}S]-TBPS and to enhance [^{3}H]-flunitrazepam and [^{3}H]-muscimol binding (Harrison & Simmonds, 1984; Simmonds *et al.*, 1984; Harrison *et al.*, 1987a; Kirkness *et al.*, 1987). In addition, the types of [^{35}S]-TBPS binding inhibition caused by the depressant steroid, 5α-pregnan-3α-ol-20-one (mixed) and the putative excitatory steroid, pregnenolone sulphate (competitive) are similar to that described for depressant and convulsant barbiturates, respectively (Ticku *et al.*, 1985; Trifiletti *et al.*, 1985; Majewska & Schwartz, 1987). Thus there is considerable experimental evidence which is consistent with the notion that barbiturates and steroids may exert their effects upon the $GABA_A$ receptor through a common site.

In the present study this possibility was evaluated by examining secobarbitone and 5β-pregnan-3α-ol-20-one, either alone or in combination, for their effects on [^{3}H]-muscimol binding to a crude membrane preparation. Over the concentration ranges examined, each compound increased binding, in a dose-dependent manner, by a maximum of 50–70%. Scatchard analysis indicated that, in both cases, the stimulation resulted principally from an increase in the number of detectable high-affinity binding sites in the membrane. Enhancement of [^{3}H]-muscimol binding by the barbiturate, pentobarbitone and the steroid, alphaxalone has also been shown to occur by this mechanism (Olsen & Snowman, 1982, Harrison & Simmonds, 1984). Although enhancement by secobarbitone appears to reach a maximum value at the highest concentrations examined (Figure 6), the saturating concentration of 5β-pregnan-3α-ol-20-one could not be established owing to its limited solubility. The extent by which secobarbitone stimulated control binding was unaffected by the presence of 100 μM 5β-pregnan-3α-ol-20-one, i.e. the magnitudes of the enhancements caused by each compound were additive. Similarly, a saturating concentration of secobarbitone did not affect the degree of stimulation by the steroid. In contrast, the stimulatory effects of combinations of pentobarbitone and secobarbitone or alphaxalone and 5β-pregnan-3α-ol-20-one were not additive. Collectively, these results suggest that, under the conditions of the radioligand binding assay, secobarbitone and 5β-pregnan-3α-ol-20-one stimulate [^{3}H]-muscimol binding by interacting with distinct modulatory sites.

A previous study, describing stimulation of GABA receptor binding by the hypnotic agent etomidate, also indicated that binding enhancement by this compound was additive with that produced by the barbiturate, pentobarbitone (Thyagarajan *et al.*, 1983). Although additive effects of etomidate and pentobarbitone were not detected by other workers (Quast & Brenner, 1983), this discrepancy may result from the binding assay conditions which varied considerably between the two investigations. Studies aimed at clarifying the nature of the interactions between the $GABA_A$ receptor and combinations of barbiturates, steroids and other depressant agents are currently in progress. Several observations made in electrophysiological studies are consistent with the barbiturates and steroids interacting with distinct sites to modulate $GABA_A$ receptor function. It has previously been demonstrated that the direct 'agonist' action of alphaxalone, 5β-pregnan-3α-ol-20-one or 5α-pregnan-3α-ol-20-one is potentiated by phenobarbitone (Cottrell *et al.*, 1987; Callachan *et al.*, 1987a; Lambert & Peters, 1988), and in the present study 5β-pregnan-3α-ol-20-one was found to enhance greatly the amplitude of membrane currents elicited by pentobarbitone. However, conclusive electrophysiological evidence for separate sites of interaction will require a quantitative assessment of the effects of a range of barbiturate and steroid concentrations upon GABA-evoked responses.

It has been shown previously that at concentrations (>300 nM) greater than those required for a demonstrable potentiation of GABA-evoked responses, the steroids 5β-pregnan-3α-ol-20-one and alphaxalone directly activate a transmembrane current in cultured chromaffin cells (Cottrell *et al.*, 1987; Callachan *et al.*, 1987; Lambert *et al.*, 1987). Such currents are antagonized by bicuculline, potentiated by diazepam, and from measurements of their reversal potential appear to be mediated by chloride ions. The present study demonstrates that currents

elicited by high concentrations of the barbiturates secobarbitone and pentobarbitone share all of these features, confirming data obtained in studies of the postsynaptic actions of pentobarbitone in other systems (e.g. Owen *et al.*, 1986; Akaike *et al.*, 1987). In addition to the barbiturates and steroids, $GABA_A$ agonist actions have been found for the hypnotic anticonvulsant chlormethiazole (Hales & Lambert, 1988a) and the general anaesthetics propofol (Hales & Lambert 1988b) and propanidid (Lambert & Peters, unpublished observations). It seems unlikely that such chemically diverse structures bind to the GABA recognition site to activate the associated chloride ion channel, but alternatively they may act to perturb the membrane around the GABA ion channel and in some way alter the rate constants governing ion channel opening and closing.

In conclusion, the present study emphasizes the similarities between the depressant barbiturates and steroids in both radioligand and electrophysiological experiments, but interaction studies suggest such sites may be distinct. Whatever the nature of the barbiturate/steroid binding site(s), the demonstration of the potent modulation of $GABA_A$ receptors by endogenous steroids offers the future prospect of the development of new steroidal general anaesthetics and anticonvulsants, and a better understanding of the influence of the endocrine system on central nervous system function.

This work was supported by grants from the Medical Research Council, U.K., Tenovus Tayside and a Dundee University Support Grant. J. Dempster, who developed the computer software, was supported by a Wellcome Trust Research Grant, E.F.K. is an Emma and Leslie Reid Research Fellow of the University of Leeds.

References

AKAIKE, N., MARUYAMA, T. & TOKUTOMI, N. (1987). Kinetic properties of pentobarbitone-gated chloride current in frog sensory neurones. *J. Physiol.*, **394**, 85–98.

BANKS, P. & PEACE, C.B. (1985). Enzyme inhibition by steroid anaesthetic agents derived from progesterone. *Br. J. Anaesth.*, **57**, 512–514.

BARKER, J.L., HARRISON, N.L., LANGE, G.D. & OWEN, D.G. (1987). Potentiation of γ-aminobutyric-acid-activated chloride conductance by a steroid anaesthetic in cultured rat spinal neurones. *J. Physiol.*, **386**, 485–501.

BARKER, J.L. & RANSOM, B.R. (1978). Pentobarbitone pharmacology of mammalian central neurones grown in tissue culture. *J. Physiol.*, **280**, 355–372.

BORMANN, J. & CLAPHAM, D.E. (1985). γ-Amino-butyric acid receptor channels in adrenal chromaffin cells: a patch-clamp study. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 2168–2172.

CALLACHAN, H., COTTRELL, G.A., HATHER, N.Y., LAMBERT, J.J., NOONEY, J.M. & PETERS, J.A. (1987a). Modulation of the $GABA_A$ receptor by progesterone metabolites. *Proc. R. Soc. Lond.*, **B231**, 359–369.

CALLACHAN, H., LAMBERT, J.J. & PETERS, J.A. (1987b). Modulation of the $GABA_A$ receptor by barbiturates and steroids. *Neurosci. Letts. Suppl.*, **29**, S21.

COTTRELL, G.A., LAMBERT, J.J. & PETERS, J.A. (1985). Chloride currents activated by GABA in cultured bovine chromaffin cells. *J. Physiol.*, **365**, 90P.

COTTRELL, G.A., LAMBERT, J.J. & PETERS, J.A. (1987). Modulation of $GABA_A$ receptor activity by alphaxalone. *Br. J. Pharmacol.*, **90**, 491–500.

DEMPSTER, J. (1988). In *Microcomputers in Physiology: A Practical Approach*, ed. Fraser, P.J. Oxford: IRL Press (in press).

FENWICK, E.M., MARTY, A. & NEHER, E. (1982). A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *J. Physiol.*, **331**, 577–597.

FRANKS, N.P. & LIEB, W.R. (1987). What is the molecular nature of general anaesthetic target sites? *Trends Pharmacol. Sci.*, **8**, 169–174.

HALES, T.G. & LAMBERT, J.J. (1988a). The action of chlormethiazole on the $GABA_A$ receptor of bovine chromaffin cells in culture. *J. Physiol.*, **398**, 14P.

HALES, T.G. & LAMBERT, J.J. (1988b). Modulation of the $GABA_A$ receptor by propofol. *Br. J. Pharmacol.*, **93**, 84P.

HALSEY, M.J., WARDLEY-SMITH, B. & WOOD, S. (1986). Pressure reversal of alphaxalone/alphadalone and methohexitone in tadpoles: evidence for different molecular sites for general anaesthesia. *Br. J. Pharmacol.*, **89**, 299–305.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high resolution current recordings from cells and cell free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HARRISON, N.L., MAJEWSKA, M.D., HARRINGTON, J.W. & BARKER, J.L. (1987a). Structure activity relationships for steroid interaction with the γ-amino-butyric $acid_A$ receptor complex. *J. Pharmacol. Exp. Ther.*, **241**, 346–353.

HARRISON, N.L. & SIMMONDS, M.A. (1984). Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res.*, **323**, 287–292.

HARRISON, N.L., VICINI, S. & BARKER, J.L. (1987b). A steroid anaesthetic prolongs inhibitory postsynaptic currents in cultured rat hippocampal neurons. *J. Neurosci.*, **7**, 604–609.

HIGASHI, H. & NISHI, S. (1982). Effect of barbiturates on the GABA-receptor of cat primary afferent neurones. *J. Physiol.*, **332**, 299–314.

KEANE, P.E. & BIZIERE, K. (1987). The effect of general anaesthetics on GABAergic synaptic transmission. *Life Sci.*, **41**, 1437–1448.

KIRKNESS, E.F., LAMBERT, J.J., PETERS, J.A. & TURNER, A.J. (1987). Stereoselective modulation of the $GABA_A$ receptor by pregnane steroids. *Br. J. Pharmacol.*, **91**, 484P.

KIRKNESS, E.F. & TURNER, A.J. (1986). The gamma-aminobutyrate/benzodiazepine receptor from pig brain.

Enhancement of gamma-amino-butyrate-receptor binding by the anaesthetic propanidid. *Biochem. J.*, **233**, 259–264.

LAMBERT, J.J. & PETERS, J.A. (1988). Steroidal modulation of the $GABA_A$-benzodiazepine receptor complex: an electrophysiological investigation. In *The Allosteric Modulation of Amino Acid Receptors and Its Therapeutic Implications.* ed. Costa, E. & Barnard, E. New York: Raven Press (in press).

LAMBERT, J.J., PETERS, J.A. & COTTRELL, G.A. (1987). Actions of synthetic and endogenous steroids on the $GABA_A$ receptor. *Trends Pharmacol. Sci.*, **8**, 224–227.

LAWRENCE, D.K. & GILL, E.W. (1975). Structurally specific effects of some steroid anaesthetics on spin-labelled liposomes. *Mol. Pharmacol.*, **11**, 280–286.

LEEB-LUNDBERG, F., SNOWMAN, A. & OLSEN, R.W. (1980). Barbiturate receptor sites are coupled to benzodiazepine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7468–7472.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MAJEWSKA, M.D., HARRISON, N.L., SCHWARTZ, R.D., BARKER, J.L. & PAUL, S.M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science*, **232**, 1004–1007.

MAJEWSKA, M.D. & SCHWARTZ, R.D. (1987). Pregnenolone-sulfate: an endogenous antagonist of the γ-aminobutyric acid receptor complex in brain? *Brain Res.*, **404**, 355–360.

MARTIN, I.L. (1987). The benzodiazepines and their receptors: 25 years of progress. *Neuropharmacology*, **26**, 957–970.

MARTY, A. & NEHER, E. (1985). Potassium channels in cultured bovine chromaffin cells. *J. Physiol.*, **367**, 117–141.

OLSEN, R.W. & SNOWMAN, A.M. (1982). Chloride-dependent enhancement by barbiturates of γ-aminobutyric acid receptor binding. *J. Neurosci.*, **2**, 1812–1823.

OWEN, D.G., BARKER, J.L., SEGAL, M. & STUDY, R.E. (1986). Postsynaptic actions of pentobarbital in cultured mouse spinal neurones and rat hippocampal neurones. In *Molecular and Cellular Mechanisms of Anaesthetics.* ed. Roth, S.W. & Miller, K.W. pp. 27–41. New York, London: Plenum Press.

QUAST, U. & BRENNER, O. (1983). Modulation of [^{3}H] muscimol binding in rat cerebellar and cerebral cortical membranes by picrotoxin, pentobarbitone and etomidate. *J. Neurochem.*, **41**, 418–425.

RAMANJANEYULU, R. & TICKU, M.K. (1984). Binding characteristics and interaction of depressant drugs with ^{35}[S]t-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. *J. Neurochem.*, **42**, 221–229.

RICHARDS, C.D. & WHITE, A.E. (1981). Additive and non-additive effects of mixtures of short-acting intravenous anaesthetic agents and their significance for theories of anaesthesia. *Br. J. Pharmacol.*, **74**, 161–170.

SEGAL, M. & BARKER, J.L. (1984). Rat hippocampal neurons in culture: Voltage clamp analysis of inhibitory synaptic connections. *J. Neurophysiol.*, **52**, 469–487.

SIMMONDS, M.A. (1985). Antagonism of flurazepam and other effects of Ro 15-1788, PK 8165 and Ro 5-4864 on the GABA-A receptor complex in rat cuneate nucleus. *Eur. J. Pharmacol.*, **117**, 51–60.

SIMMONDS, M.A. & TURNER, J.P. (1987). Potentiators of responses to activation of γ-aminobutyric acid ($GABA_A$) receptors. *Neuropharmacology*, **26**, 923–930.

SIMMONDS, M.A., TURNER, J.P. & HARRISON, N.L. (1984). Interactions of steroids with the GABA-A receptor complex. *Neuropharmacology*, **23**, 877–878.

SQUIRES, R.F., CASIDA, J.E., RICHARDSON, M. & SAEDERUP, E. (1983). [^{35}S]t-Butylbicyclophosphorothionate binds with high affinity to brain specific sites coupled to γ-aminobutyric acid-A and ion recognition sites. *Mol. Pharmacol.*, **23**, 326–336.

STUDY, R.E. & BARKER, J.L. (1981). Diazepam and (−) pentobarbital: Fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid responses in cultured central neurones. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7180–7184.

THYAGARAJAN, R., RAMANJANEYULU, R. & TICKU, M.K. (1983). Enhancement of diazepam and γ-aminobutyric acid binding by (+) etomidate and pentobarbital. *J. Neurochem.*, **41**, 578–585.

TICKU, M.K., RASTOGI, S.K. & THYAGARAJAN, R. (1985). Separate site(s) of action of optical isomers of l-methyl-5-phenyl-5-propylbarbituric acid with opposite pharmacological activities at the GABA receptor complex. *Eur. J. Pharmacol.*, **112**, 1–9.

TRIFILETTI, R.R., SNOWMAN, A.M. & SNYDER, S.H. (1985). Barbiturate recognition site on the GABA/benzodiazepine receptor complex is distinct from the picrotoxinin/TBPS recognition site. *Eur. J. Pharmacol.*, **106**, 441–447.

WHITTLE, S.R. & TURNER, A.J. (1982). Differential effects of sedative and anti-convulsant barbiturates on specific [^{3}H] GABA binding to membrane preparations from rat brain cortex. *Biochem. Pharmacol.*, **31**, 2891–2895.

WILLIAMS, M. & RISLEY, E.A. (1979). Characterisation of the binding of [^{3}H] muscimol, a potent gamma-aminobutyric acid agonist, to rat brain synaptosomal membranes using a filtration assay. *J. Neurochem.*, **32**, 713–718.

(Received February 2, 1988
Revised March 21, 1988
Accepted April 5, 1988)

Keywords: $GABA_A$ receptors, Pregnane steroids, Barbiturates.