TITLE

ALLOSTERIC INTERACTIONS OF GABA- AND GLYCINE-GATED CHLORIDE CHANNELS FROM RAT BRAIN

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ABSTRACT

The allosteric interactions of the $GABA_A$ receptor were investigated through the actions of drugs on [^{3}H]-flunitrazepam binding. The effects of varying incubation temperature revealed that pentobarbitone is more efficacious a potentiator at low temperatures than at high. In contrast, the general anaesthetics propofol and alphaxalone did not show a variation in efficacy with temperature. These results argue against a membrane site of action for propofol and alphaxalone. Differences in the dependence of potentiation upon anions were also noted. All three drugs gave increased potentiation with increasing concentrations of anions which can permeate the $GABA_A$ receptor. However, whilst the potency of propofol increased with chloride concentration, its efficacy did not. Pentobarbitone showed an increase in both potency and efficacy but whilst alphaxalone's efficacy increased, its potency did not. These results indicate different sites and modes of interaction for propofol, alphaxalone and pentobarbitone. Interactions of the steroid epipregnanolone with the receptor were also characterised. It antagonised competitively the potentiation produced by its isomers pregnanolone and allopregnanolone whilst having little direct effect upon flunitrazepam binding or on potentiation by pentobarbitone and GABA. Epipregnanolone also antagonised the potentiating effect of alphaxalone but the mode of inhibition was different to that seen against pregnanolone, indicating more than one class of binding site for neurosteroids at the $GABA_A$ receptor. [^{3}H]-flunitrazepam binding to $GABA_A$ receptors immunopurified according to α subunit content was also examined. No potentiation by alphaxalone or pentobarbitone was seen but differences in flunitrazepam affinity and EC_{50} for potentiation by GABA were noted. In an electrophysiological study, the interaction of steroids with the strychnine-sensitive glycine receptor was examined. Certain corticosteroids were shown to be potent enhancers of the actions of glycine and strychnine at this receptor, but steroids which are active on the $GABA_A$ receptor showed no effects.

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Fast synaptic inhibition in the central nervous system is largely mediated by chloride ion channels gated by the inhibitory amino acids glycine and GABA (γ-aminobutyric acid). In the higher areas of the CNS, GABA is the dominant inhibitory transmitter whilst in the spinal cord this function is fulfilled by glycine. Up-regulation of these systems results in an increase in synaptic inhibition which manifests itself in sedation, anaesthesia and muscle relaxation, whilst a decrease in inhibitory function causes hyperexcitability resulting in convulsions, and epileptiform activity. Alterations in inhibitory function are both implicated in disease and exploited in therapeutic strategy (Martin, 1987; Lloyd *et al*,1984; Faingold and Browning, 1987; Buckle *et al*, 1989). Many of the best known and most widely-prescribed hypnotic drugs act at the $GABA_A$ receptor.

Pharmacology of the $GABA_A$ receptor

In addition to gating by the neurotransmitter GABA, the $GABA_A$ receptor is modulated by a wide range of drugs acting at distinct loci (for reviews see Tallman and Gallager, 1985; Stephenson, 1988; Schwartz, 1988). These substances allosterically modulate both the GABA binding site and channel function whilst also mutually interacting with other binding loci on the receptor. This gives rise to an extremely complicated interactional pharmacology but also allows certain aspects of receptor function to be determined indirectly through measurement of the allosteric potentiation or inhibition of ligands binding to one of these modulatory sites. The main classes of modulatory site on the $GABA_A$ receptor are the GABA site (high

affinity agonist ligand muscimol, antagonist bicuculline) (Olsen, McCabe & Wamsley, 1990); the benzodiazepine agonist site (high affinity ligand flunitrazepam, antagonist flumazenil); the benzodiazepine inverse agonist site (high affinity ligand DMCM (dimethoxy-4-ethyl-β-carboline-3-carboxylate), antagonist flumazenil) (Tallman and Gallager, 1985; Martin, 1987); the barbiturate site (ligands pentobarbitone, phenobarbitone) (Leeb-Lundberg, 1980); the convulsant site (ligands picrotoxin, TBOB (t-butyl bicyclo-orthobenzoate), TBPS (t-butyl bicyclophosphorothionate), ?pregnenolone sulphate) (Majewska and Schwartz, 1987; Olsen, Ticku & Miller, 1978; Olsen *et al*, 1990) and the neurosteroid site (agonists alphaxalone, pregnanolone, antagonists epipregnanolone) (Simmonds, 1991; Pettey and Simmonds, 1991; Prince and Simmonds, 1992a). Various other receptor sites have been proposed but not clearly defined. These include sites for ethanol (McQuilkin *et al*, 1989), propofol (Concas *et al*, 1991a & b), chlormethiazole (Harrison and Simmonds, 1983) and the corticosteroids (Majewska, 1987).

Pharmacology of the strychnine-sensitive glycine receptor (ssGR)

The pharmacology of the strychnine sensitive glycine receptor (as distinct from that linked to the NMDA receptor) is much less well developed than that of the $GABA_A$ receptor. The major pharmacological characteristic of the ssGR is, as its name suggests, sensitivity to strychnine. This alkaloid behaves as a competitive antagonist at the glycine site but structural considerations suggest that this may be an allosteric interaction. Recently, it has been suggested that progesterone and certain of its metabolites may act as antagonists of the ssGR (Wu *et al*, 1990). Glycine gated

chloride currents are potentiated by the sedative/anxiolytic chlormethiazole (Gent and Wacey, 1983; Simmonds and Turner, 1987) and by the general anaesthetic propofol (Hales and Lambert, 1991). Interestingly both these substances are also active at the $GABA_A$ receptor (Harrison and Simmonds, 1983; Hales and Lambert, 1991; Concas *et al*, 1991a & b) Barbiturates are only weakly active at the ssGR (Simmonds, 1983).

Molecular Biology of the $GABA_A$ and glycine receptors

Recent years have seen major advances in the cloning of the genes coding for the $GABA_A$ and glycine receptors. Sequence analysis has revealed that there exists a "super family" of ligand gated ion channels consisting of the $GABA_A$ receptor, nicotinic acetylcholine receptor (nAChR) (Cockcroft *et al*, 1990), the $5HT_3$ receptor (McKernan *et al*, 1990) and the ssGR (Betz, 1990) and suggests that these evolved from a common ancestral protein. All of these receptors are multimeric with a molecular mass of approximately 250 kDa. The individual subunits of these receptors have a molecular mass of approximately 50-60 Kda. Hydrophobicity analysis suggests that they possess 4 or 5 membrane spanning segments one of which is amphipathic. It has been postulated that it is these amphipathic domains which together form the ion channel (Olsen and Tobin, 1990; Betz, 1990) (see figure 1.1).

To date, 5 main classes of $GABA_A$ receptor subunit have been cloned with further division of these classes into subtypes. Six alpha subtypes have been isolated (Schofield *et al*, 1987; Levitan *et al*, 1988; Ymer *et al*, 1989a; Pritchett and Seeburg, 1990; Lüddens *et al*, 1990; Khrestchatisky, *et al* 1989), 4 beta subtypes (Ymer *et al* 1989b; Lasham *et al*, 1991), 3 gamma (Ymer *et al*, 1990; Shivers *et al*, 1989);

Wilson-Shaw *et al*, 1991), 1 delta (Shivers *et al*, 1989) and 1 rho (Cutting *et al*, 1991). At present neither the stoichiometry nor subunit composition of the receptor is known. *In-situ* hybridization studies have revealed large variations in distribution (Wisden *et al*, 1992) and recent studies have shown that there exist receptors which possess more than one alpha subtype (Duggan, Pollard and Stephenson, 1991). With probably 5 subunits making up the receptor, the array of possible combinations is, therefore, vast.

Much of our knowledge of the properties of the subunits of the $GABA_A$ receptor has come from transfection studies which have allowed the pharmacology conferred by each subtype to be dissected out. Many important differences have been revealed. Whilst expression of α or β subunits alone will result in the formation of GABA gated homomeric channels (Blair *et al*, 1988), in order for the benzodiazepine pharmacology of the receptor to be expressed, an α, a β and a γ subunit must be coexpressed (Ymer *et al*, 1990). Furthermore, the type of α subunit present determines the type of pharmacology expressed (Doble and Martin, 1992). Recently transfection studies have also revealed subunit dependent differences in the sensitivity of expressed $GABA_A$ receptors to neurosteroids (Shingai, Sutherland and Barnard, 1991; Zaman *et al*, 1992) and to zinc (Smart *et al*, 1991). It is likely therefore that subunit composition is the major determining factor in observed regional and developmental differences seen in $GABA_A$ pharmacology.

Like the $GABA_A$ receptor, the ssGR also exhibits a promiscuity of subunit types. The channel is thought to consist of 2 subunit classes, α and β. Four subtypes of the α

subunit have been isolated. One of these, α2, is abundant in neonatal tissue and may be implicated in the strychnine insensitivity exhibited by neonates (Betz, 1990).

Examination of the allosteric interactions of these receptors is problematic. Whilst a sound theoretical framework for the interaction of antagonistic ligands exists, no such model has been formulated for those of allosteric potentiators. This presents problems when considering whether two drugs possess a common site of action. One of the most frequently used approaches has been to use low concentrations of both compounds and to see if they produce a synergistic or additive effect (eg Kirkness and Turner, 1988; Concas *et al*, 1991a). This has usually been taken to indicate separate sites of action. Whilst synergism clearly shows an allosteric interaction between the two compounds, additivity does not. Conversely, mutual inhibition is often taken to indicate isosteric interactions (eg Majewska *et al*, 1990). However, if the concentrations of the compounds are low enough compared with their EC_{50} values, isosterically acting ligands will show apparent additivity. Also, it is not necessarily the case that allosteric potentiators of receptor function should not allosterically inhibit each others function or binding, or that allosteric inhibitors should not potentiate the binding of other ligands. One example of this is pregnenolone sulphate. At nanomolar concentrations, this steroid inhibits GABA gated chloride flux whilst allosterically potentiating flunitrazepam binding, but allosterically inhibiting the potentiation of flunitrazepam binding by barbiturates (Majewska and Schwartz, 1987).

Aims and Objectives

The complexity of allosteric interactions of the $GABA_A$ receptor obscures analysis using the techniques of mutual facilitation/inhibition. In this thesis I have attempted to examine these interactions via alternative methods, namely the development of specific antagonists for the steroid site and the examination of differences in the temperature and anion sensitivity of the modulations. In addition some of the questions posed by the multiplicity of $GABA_A$ receptors have been addressed and some interactions of the strychnine sensitive glycine receptor examined to determine if these interactions have been conserved during evolution.

Figure 1.1. A) The proposed membrane disposition of the direct ligand gated ion channel subunits. Each of the membrane spanning domains (MSD) (1-4) is thought to consist largely of α helices. The large intracellular loop between MSD3 and MSD4 is the region of highest dissimilarity between the members of this super-family. This region contains several consensus sequences for phosphorylation sites and may therefore be involved in receptor regulation. It is to this region that the subunit specific antibodies used to immunopurify the $GABA_A$ receptor (chapter 7) bind. The "cys loop" is a structural motif of the agonist binding subunits of the DLG super-family. It is found in all cloned $GABA_A$ receptor and strychnine sensitive glycine receptor subunits as well as the subunits of the nACh receptor (Cockcroft *et al*, 1990)

B) Model of the subunit MSD arrangement forming the $GABA_A$ ion channel. MSD2 is amphipathic and is thought to provide the lining of the ion channel pore. The actual arrangement of the MSDs is unclear. Although the stoichiometry of the $GABA_A$ receptor is unknown, spatial considerations suggest there may be 5 subunits per receptor complex.

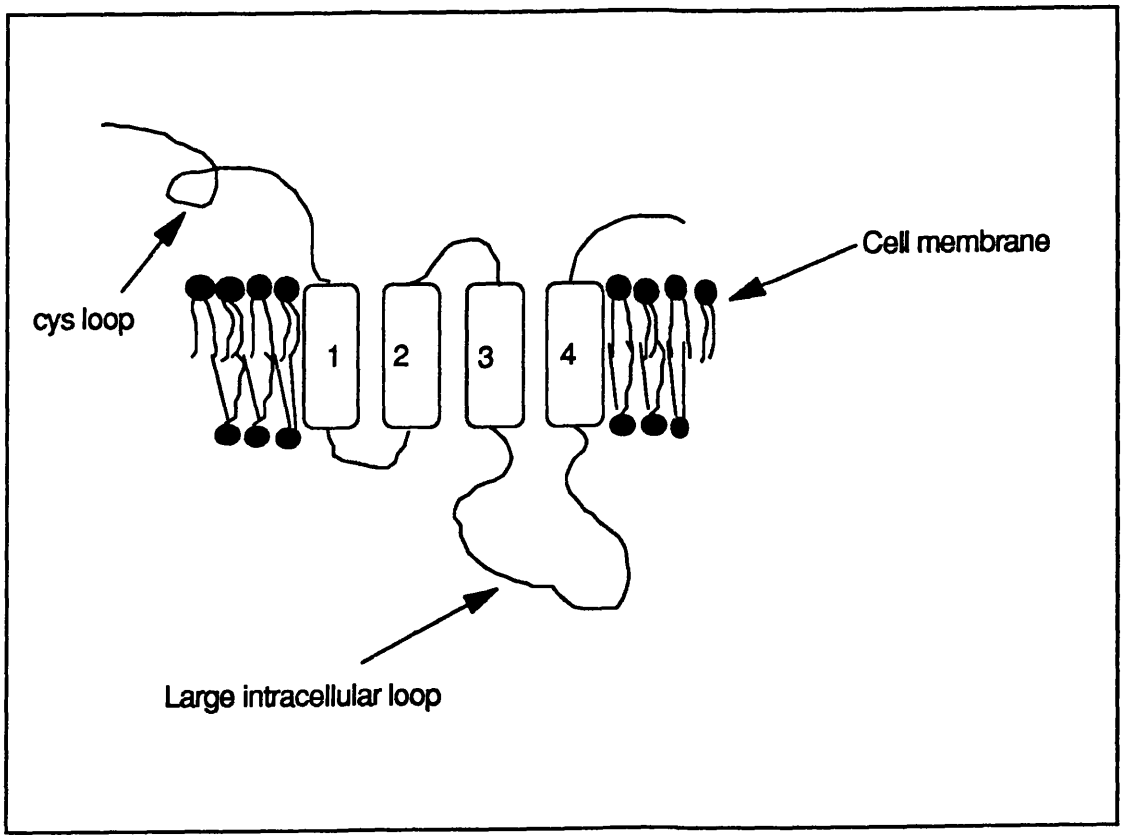


Figure 1.1A

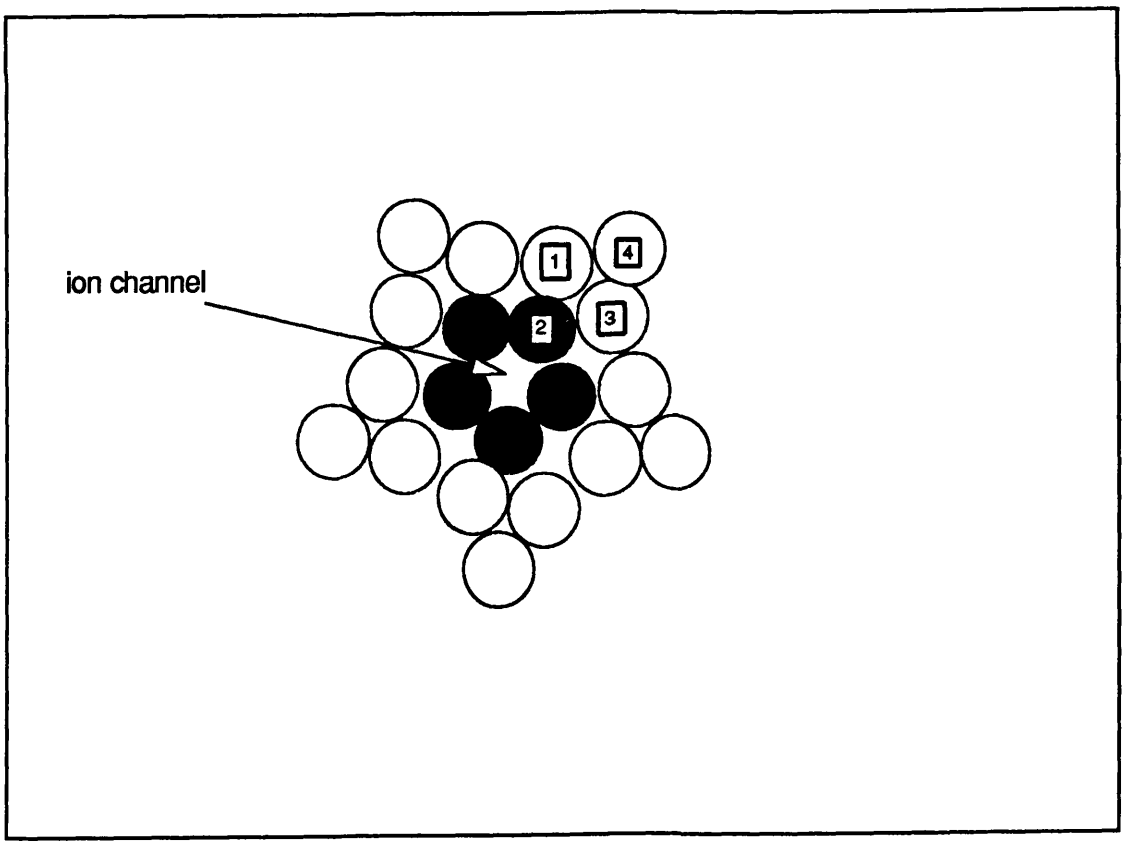


Figure 1.1B

CHAPTER 2

Methods

METHODS

Electrophysiological Studies

Electrophysiological studies were carried out using a two chamber grease gap superfusion system as detailed in figure 2.1. The method used was similar to that detailed by Simmonds (1983) and Turner & Simmonds (1989) but with several modifications. Tissues were superfused with Krebs' solution gassed with 95% O_2, 5% CO_2 and containing 0.1% acetone until a stable base line was obtained. Doses of agonist drugs were added directly to the active compartment of the bath with the superfusion system halted. Mixing was facilitated by bubbling the active chamber with 95% O_2, 5% CO_2. When consistent control responses had been obtained various drugs were added to the superfusion medium and the agonist doses repeated.

[^{3}H] Strychnine Binding to Spinal Cord Membranes

Due to the rather limited pharmacology of the strychnine-sensitive glycine receptor there is only a very restricted choice of ligands available. [^{3}H]-glycine, which has been used for characterisation of the glycine site of the NMDA receptor possesses only very low affinity for the ssGR (Young and Snyder, 1974) and is therefore unsuitable as a binding study ligand. The only high affinity ligand available is the antagonist [^{3}H]-strychnine, which has a Kd in the low nM range and is therefore suitable for use in filtration assays (Young and Snyder, 1974).

Figure 2.1. The "grease gap" two chamber superfusion system described by Simmonds (1983) and Turner and Simmonds (1989). Responses are recorded as the potential difference between the two bath compartments, which are electrically insulated from one another except via the tissue sandwiched between the "grease gap barrier". Superfusion medium flows into the base of the bath and a constant level is maintained by adjustable aspirators in both chambers. Drugs are applied directly to the active chamber, which is the side of the barrier where the tissue nerve terminals are present. In the case of the optic nerve it was found that responses could be obtained from either the retinal or chiasmic end of the isolated tissue, but that better responses were generally obtained when the optic chiasma was placed in the active chamber.

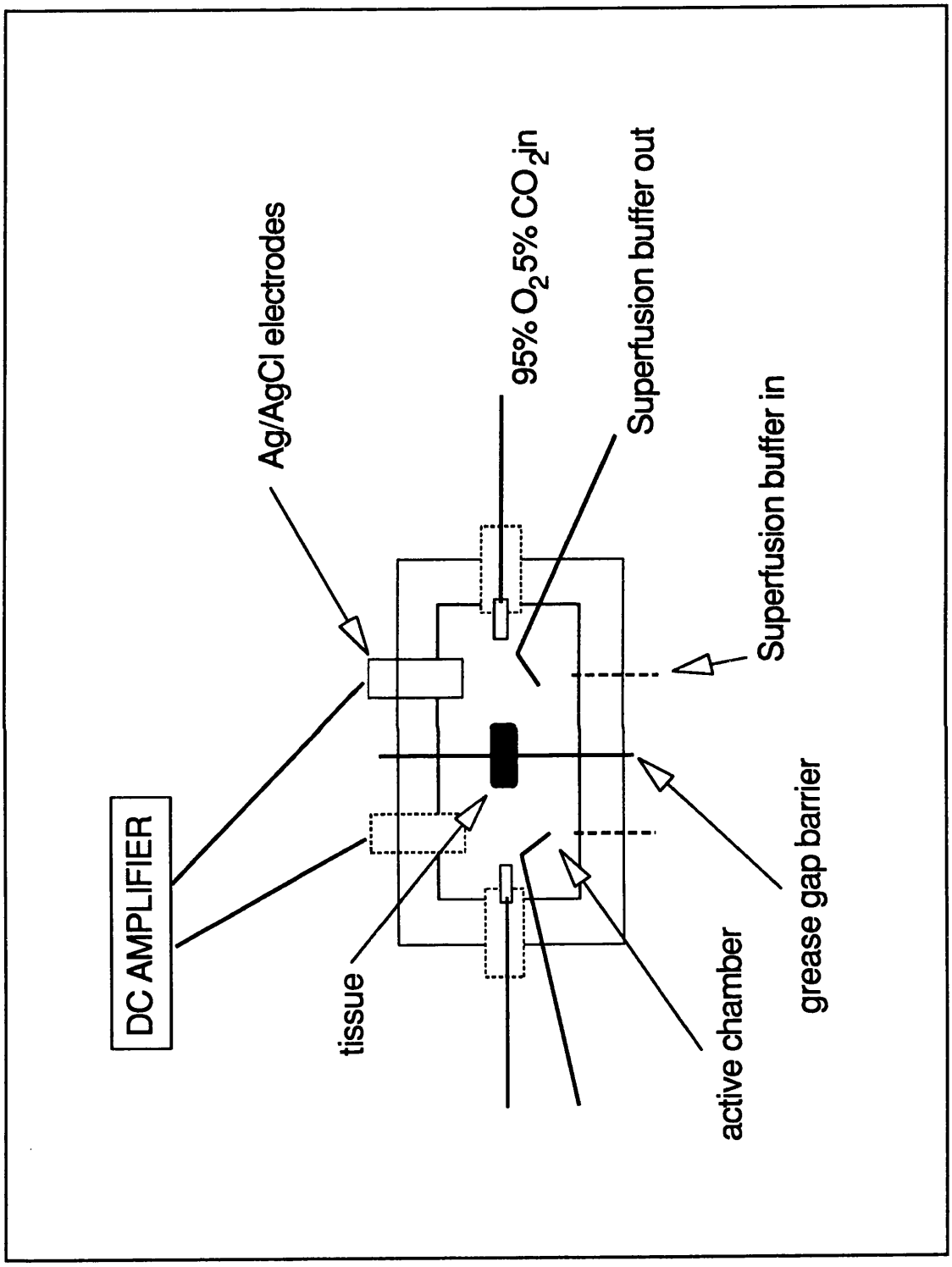

Ag/AgCl electrodes
95% $O_2$5% CO_2in
Superfusion buffer out
Superfusion buffer in
DC AMPLIFIER
tissue
active chamber
grease gap barrier

Figure 2.1

Membrane Preparation

Spinal cord membranes (SCM) were prepared according to the method of Young and Snyder (1974). Male Sprague Dawley rats were sacrificed by decapitation with prior stunning and their brain stems and spinal cords rapidly removed. Compressed air was utilised to facilitate speedy removal of the spinal cord from the spinal column. The tissue was homogenized in 20 volumes of ice-cold 0.32M sucrose solution using a glass/teflon homogenizer and then centrifuged at 1000 x G in order to pellet any debris and unbroken cells. The pellet was discarded and the supernatant centrifuged at 17000 x G for 20 minutes. The supernatant was discarded and the pellet resuspended in 20 volumes ice-cold distilled water using an Ultraturrax apparatus. This suspension was centrifuged for 20 minutes at 8000 x G which resulted in a bilayer pellet. The supernatant and the upper layer of the pellet were carefully removed and were then centrifuged for 20 minutes at 48000 x G. The resultant pellet was stored at -20°C until required (10 days maximum).

[^{3}H] Strychnine Binding

The binding of [^{3}H] strychnine was studied using the method described by Young and Snyder (1974). Aliquots of SCM (0.075-0.2mg total protein) were incubated in a final volume of 2ml phosphate assay buffer (50mM Na^+/K^+ phosphate containing 200mM NaCl pH 7.1) containing various drugs for 10 minutes at 25°C. [^{3}H] strychnine (2nM final concentration) was then added and the mixture incubated for a further 10 minutes. Non-specific binding was determined using 1mM glycine and was in the range 20-25% of total binding for 2nM [^{3}H] strychnine. The binding reaction was

halted by the addition of 2ml ice cold 0.15M NaCl followed by rapid filtration through Whatman GF-C filters using a Brandell Cell Harvester. The filters were washed 3 times with 2ml ice-cold 0.15M NaCl and the radioactivity bound quantified using conventional liquid scintillation techniques. Data were fitted using non-linear regression techniques using INPLOT (Graphpad software) and ENZFITTER (Elsevier Biosoft). 20α-dihydrocortisol (DHC) was initially dissolved in acetone which was present in all assay tubes at a concentration of 0.1%.

$GABA_A$ RECEPTOR BINDING STUDIES

A variety of ligands are available for probing the function of the $GABA_A$ receptor. GABA and its structural analogue muscimol bind with moderately high affinity (10-40nM) and have been extensively used in binding experiments. In many studies it has been demonstrated that there exist two classes of binding site: high (10-40nM) and low (1000nM) affinity. The functional significance of these states is not clear but by analogy with the nAChR, the higher affinity state may correspond to the desensitized form of the receptor (Tallman and Gallager, 1985). Alternatively, it has been suggested that the 2 affinity states may represent distinct populations of the receptor (Olsen, McCabe and Wamsley, 1990). This makes interpretation of binding data rather complicated, especially when it is considered that the effect of modulators such as pentobarbitone and neurosteroids is to increase the prevalence of the high affinity state. As it is only this site which can easily be studied by binding methods, the effect of such modulators is normally seen as an apparent increase in the total number of binding sites (eg Peters *et al*, 1988). Solubilization in harsh detergents such as Triton

X100 and deoxycholate also appears to favour the high affinity state (Sigel and Barnard, 1984).

A variety of ligands for the convulsant site of the $GABA_A$ receptor have also been developed, most notably [^{3}H]-dihydropicrotoxinin, [^{35}S]-TBPS and [^{3}H]-TBOB (Olsen, Ticku and Miller, 1978; Olsen, McCabe and Wamsley, 1990). In many studies it has been shown that "GABA positive" drugs such as barbiturates and steroids cause an allosteric inhibition of convulsant binding. The interpretation of inhibition data is very much easier than that of potentiation therefore these ligands have been widely used in the study of allosteric modulators of GABA function. However, the role of GABA itself is unclear in the allosteric inhibition of TBPS binding (Simmonds, 1991). The isosteric competitive antagonist of GABA, bicuculline, causes an increase in TBPS binding (Majewska, 1987) whereas in well washed membranes it was without effect against [^{3}H]-diazepam binding (Wong and Iversen, 1985). In addition a biphasic effect of barbiturates on TBPS binding has been noted when freeze-thawed membranes were utilised (Turner *et al*, 1989). This implies that the inhibition of TBPS binding by allosteric potentiators of $GABA_A$ function may be dependent upon the presence of GABA. The binding of convulsants to the receptor protein has been suggested to be to a site associated with the ion channel itself (Tallman and Gallager, 1985). Upon solubilization, unless certain measures are taken to prevent receptor denaturation, the binding sites for such compounds are lost (Sigel and Barnard, 1984; Bristow and Martin, 1987).

The binding of [^{3}H]-benzodiazepine agonists such as flunitrazepam provides an attractive and robust means of monitoring $GABA_A$ receptor function. Flunitrazepam binding sites are present at high density in the mammalian brain (Tallman and Gallager, 1985; Martin, 1987) and the ligand is available with high specific activity (70-80 Ci/mmol compared with 20 for muscimol). In addition the ligand binds with high specificity and affinity (Kd approximately 3nM, non-specific binding in the region of 5% for the conditions used in this thesis). The benzodiazepine agonist receptor is also extremely robust and survives solubilization with harsh detergents with virtually unchanged affinity and pharmacology (McKernan *et al*, 1991). The site is allosterically linked to the various modulatory sites of the $GABA_A$ receptor which manifest their potentiating effects via an increase in flunitrazepam affinity (Wong and Iversen, 1985; Leeb-Lundberg *et al*, 1980; Simmonds, 1991). This affinity shift, like the potentiating effect upon muscimol, presumably represents a shift from low (4nM) to high (1-2nM) affinity sites. However, as the shift in affinity is over a much smaller range than that seen for muscimol (2-3 fold compared with 2 orders of magnitude), no shift in receptor number is seen. Shifts in affinity are much easier to consider theoretically eg. using van't Hoff plots, than are shifts in receptor number; therefore, in most respects, benzodiazepine ligands are more attractive probes of the $GABA_A$ receptor than GABA and its analogues. [^{3}H]-flunitrazepam has also been shown to bind to the peripheral type benzodiazepine receptor. However, the affinity of these sites is very much lower than that of the central type $GABA_A$-benzodiazepine site (Quast and Mählmann, 1982). At the concentrations of [^{3}H]-flunitrazepam used in the

studies in this thesis it is very unlikely that any binding to peripheral-type receptors would be seen.

Assay conditions for [^{3}H] flunitrazepam binding

As discussed in chapter 5, a distinct anion dependency has been demonstrated for certain interactions at the $GABA_A$ receptor. For this reason it was decided to adopt a high chloride buffer as standard. A concentration of 150-200mM chloride is often utilised in studies examining benzodiazepine interactions (eg Sigel and Barnard, 1984) and for this reason a 50mM Tris-HCl, 150mM NaCl buffer pH 7.4 was adopted as a standard. Where anion dependence was studied, 10mM Tris-citrate was utilised. Citrate ions cannot substitute for chloride in IPSPs and therefore should not interfere with potentiating effects upon allosteric interactions (Araki *et al*, 1961). As Tris buffers show a large temperature dependent shift in pH, for studies on temperature dependence of interactions, a 50mM Na^+/K^+ phosphate buffer containing 200mM NaCl pH 7.1, was utilised. This buffer shows only low temperature dependence.

When these studies were begun, several reports had suggested that the use of physiological temperatures was necessary for full expression of $GABA_A$-receptor interactions (Majewska, 1988; Supavilai and Karobath, 1980; Wong and Iversen, 1985). Many of my early experiments, therefore, utilised an incubation temperature of 37°C. However, as discussed in chapter 5, the potentiation increases observed at 37°C have, in many cases, been reported without taking into account changes in the affinity of the radioligands used in the experiments. Later experiments used an incubation temperature of 4°C in order to maximise flunitrazepam binding. All binding

studies were performed after a preincubation of 10 minutes at 37°C. This was to allow lipophilic modulators to equilibrate with membranes without temperature dependent restrictions on lipid mobility and fluidity.

Variations in tissue preparation procedures

In all early studies, tissue for $GABA_A$ receptor experiments was suspended in 50mM Tris-HCl pH 7.4 containing 150mM NaCl and protease inhibitors, prior to solubilization or freezing. The original purpose of this was to minimise proteolytic degradation of the receptor during solubilization. However, when tissue was required for anion dependency studies, it was frozen in wash buffer (5mM Tris-HCl, 1mM EDTA pH 7.4) in order to minimise contamination by exogenous chloride. The protease inhibitors were omitted. In all subsequent studies, this methodology was adopted.

In certain experiments low levels of endogenous GABA were required. In order to achieve this, a freeze-thaw step was inserted between each centrifugation step. Freeze-thawing has the effect of rupturing membrane vesicles, releasing endogenous neurotransmitters which can then be removed by washing.

Quantification of bound radioactivity

Radioactivity was quantified using liquid scintillation techniques or occasionally using the solid scintillant "Meltilex" together with a betaplate counter. The latter technique involves the melting of a wax like solid scintillant into an array of filter samples. This is then counted directly at approximately 30-35% efficiency. Samples for liquid

scintillation counting were placed in 6ml mini vials and 5ml scintillant added (Emulsifier Safe, Canberra Packard). The samples were vortexed and then left overnight to equilibrate before counting for either 1 or 3 minutes depending on the expected number of counts. Generally, a 3 minute count was utilised for samples where less than 5000 dpm were expected. Counting efficiency was 52% with a background of 20-40 dpm.

Data analysis

All data were analyzed using either the Inplot package (Graphpad software) or Enzfitter (Elsevier Biosoft) using non-linear regression algorithms.

Dose response data were fitted using the following model:

$$B=B_0+B_0\frac{P^h}{EC_{50}^h+P^h}$$

Where B is the specific binding in the presence of a concentration of the potentiator (P), B_0 is the binding in the absence of potentiator, h is the "Hill coefficient" and EC_{50} is the concentration of P which gives 50% of maximal potentiation.

Competition data were analyzed using a simple one site model assuming no cooperativity:

$$B=B_0(1-\frac{I}{I+IC_{50}})$$

Where B is the specific binding in the presence of and B_0 is the specific binding in the absence of a concentration of the inhibitor (I). IC_{50} is the concentration of I which inhibits 50% of specific binding. It is converted to a Ki value by the Cheng-Prussof equation (Cheng and Prussof, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_d}}$$

Where L is the concentration of radioligand used and Kd is the dissociation constant for that ligand.

Saturation data were fitted using a simple one site saturation isotherm:

$$B = B_m \frac{L}{L + K_d}$$

Where B is the specific binding, L is the concentration of radioligand, Kd is the dissociation constant for that ligand and Bm is the binding at saturating concentrations of L. Non-specific binding was subtracted before the data were fitted.

CHAPTER 3

Steroid Antagonism of the $GABA_A$ Receptor

5β-PREGNAN-3β-OL-20-ONE, A SPECIFIC ANTAGONIST AT THE NEUROSTEROID SITE OF THE $GABA_A$ RECEPTOR-COMPLEX.

The text and data that follow have been published:

Prince, R.J. and Simmonds, M.A. (1992) 5β-pregnan-3β-ol-20-one, a specific antagonist of the neurosteroid site of the $GABA_A$ receptor-complex. Neurosci. Lett. 135, 273-275

SUMMARY

Studies were made on the potentiation of [^{3}H]-flunitrazepam binding to rat brain membranes by GABA, pentobarbitone and pregnanolone (5β-pregnan-3α-ol-20-one). Epipregnanolone, the 3β isomer of pregnanolone, inhibited competitively the potentiation by pregnanolone with a Ki of 10.5μM without affecting that of GABA. The potentiation by pentobarbitone was slightly enhanced. Epipregnanolone alone showed only slight potentiation of benzodiazepine binding. These findings demonstrate that epipregnanolone is a specific antagonist of the neurosteroid site of the $GABA_A$ receptor and raise the possibility of a physiological role for 3β-hydroxysteroids in modulating this receptor.

INTRODUCTION

The γ-aminobutyric acid-benzodiazepine receptor complex ($GABA_A$) is a chloride ionophore mediating synaptic inhibition in the CNS and is the locus of action of several important classes of drugs. The receptor has distinct recognition sites for benzodiazepines, for the barbiturates and for convulsants such as picrotoxinin (Kirkness and Turner, 1988; Turner *et al*, 1989). Several classes of steroids are also known to exert modulatory effects at the $GABA_A$ receptor. Certain metabolites of progesterone and deoxycorticosterone such as 5β-pregnan-3α-ol-20-one (pregnanolone), together with synthetic analogues eg. alphaxalone (5α-pregnan-3α-ol-11,20-dione) have been shown to be potent enhancers of responses to GABA (Harrison *et al*, 1987; Turner and Simmonds, 1989). This modulation is similar to that produced by the barbiturates although it occurs through a distinct site of action (Kirkness and Turner, 1988; Turner *et al*, 1989). This site shows a well defined structure-activity relationship with a 3α hydroxy substituent being required for activity and 20-one conferring higher potency than a 20-hydroxy group (Harrison *et al*, 1987, Turner *et al*, 1989).

Other steroids have been shown to be, or proposed as, antagonists at the $GABA_A$ receptor. Pregnenolone sulphate (PS) can directly inhibit muscimol-induced chloride flux in synaptosomes and reduce barbiturate-induced potentiation of benzodiazepine binding, although it directly potentiates flunitrazepam binding in the absence of barbiturate (Majewska and Schwartz, 1987). This steroid also competitively displaces [^{35}S]-TBPS (t-butylbicyclophosphorothionate) binding and therefore may interact with

the picrotoxin-TBPS recognition site (Majewska and Schwartz, 1987). Dehydroepiandrosterone sulphate (DHEAS) shows similar effects to PS except that it does not displace TBPS binding and is, therefore, thought to have a different site of action (Majewska *et al*, 1990). Conversely, certain glucocorticoids have been shown to potentiate TBPS binding in the cerebral cortex, but this has also been argued to reflect an antagonist action at the $GABA_A$ receptor (Majewska, 1987).

Recent studies in our laboratory have shown that the 3β analogue of pregnanolone (epipregnanolone: 5β-pregnan-3β-ol-20-one) is able to antagonise the potentiation of GABA produced by its 3α-ol isomer without directly antagonising GABA responses (Pettey and Simmonds, 1991). In order to determine the mode and site of interaction of epipregnanolone with the $GABA_A$ receptor we have examined its effects upon the enhancement of benzodiazepine binding by pregnanolone, GABA and pentobarbitone.

METHODS

Male Wistar rats (150-200g) were sacrificed by decapitation without anaesthesia or prior stunning and the brains rapidly removed and put on ice. Tissue was either used fresh or frozen at -20°C until required (14 days maximum). Whole brains minus brain stem were homogenised in a glass/teflon homogeniser in 20 volumes of 5mM Tris-HCl buffer containing 1mM EDTA (pH 7.4 at 4°C, wash buffer) and then centrifuged at 1000xG for 10 minutes. The supernatant was centrifuged at 48000xG for 20 minutes and the pellet then resuspended in wash buffer. This wash step was repeated 3 times and the final pellet resuspended in 50mM Tris-HCl buffer pH 7.4 containing 150mM NaCl plus protease inhibitors (chymostatin 5μg/ml; benzethonium

chloride 100µg/ml; soybean trypsin inhibitor 10µg/ml; sodium azide 100µg/ml and bacitracin 100µg/ml) to a final protein concentration of 2-6 mg/ml. The suspension was stored at -20°C, for a maximum of 10 days, until required (McKernan *et al*, 1991). Before use, tissue was thawed and centrifuged at 48000xG for 20 minutes before resuspension into the assay buffer.

Equilibrium binding of [^{3}H] flunitrazepam (FNZ) was studied by the incubation of a 100µl aliquot of membranes in a final volume of 0.5ml of assay buffer (50mM Tris-HCl pH 7.4 at 37°C containing 150mM NaCl). Samples were pre-incubated with drugs at 37°C for 10 minutes in the absence of radioligand. After this period, 1nM [^{3}H] FNZ was added and the samples incubated for a further 60 minutes at 37°C. The binding reaction was terminated by the addition of 2ml ice cold wash buffer followed by rapid filtration through a Brandel Cell Harvester. The filters (Whatman GF-C) were washed with 2 aliquots of 2ml wash buffer before counting by conventional liquid scintillation techniques. Non-specific binding, in the region of 10% of total binding in the absence of drugs, was determined using 10µM FNZ. All steroids were initially dissolved in acetone which was present in the assay at a concentration of 0.2%. All drugs and chemicals were obtained from Sigma. Dose-response curves were analyzed using the non-linear regression programs ENZFITTER (Elsevier Biosoft) and INPLOT (Graphpad) and data were compared using Student's t test.

RESULTS

In the presence of pregnanolone the binding of [^{3}H] FNZ was markedly potentiated, with an EC_{50} for the steroid of 237 ± 27nM (n=10). Co-application of

epipregnanolone gave a rightward parallel shift of the pregnanolone dose response curve indicative of competitive antagonism, with a derived Ki of 10.5μM (see figure 3.1). Epipregnanolone alone did not produce a significant change in FNZ binding at concentrations less than 60μM and at none of the concentrations tested did it significantly alter the maximum potentiating effect of pregnanolone.

In contrast to its effects upon pregnanolone interactions, epipregnanolone did not alter the dose response curve for GABA potentiation of FNZ binding. Neither the slope (control: 1.16 ± 0.026 n=3; plus 60μM epipregnanolone: 1.08 ± 0.14 n=3) nor the EC_{50} (control: 0.34 ± 0.10μM; plus 60μM epipregnanolone: 0.26 ± 0.04μM) for GABA, showed any significant effect of the steroid. The effect of epipregnanolone on pentobarbitone modulation of flunitrazepam binding was also examined. In the absence of the steroid, pentobarbitone gave a very steep dose response curve with a Hill slope of 2.18 ±0.16 and an EC_{50} of 0.41 ±0.04mM (n=8). When 60μM epipregnanolone was co-applied with pentobarbitone the slope of the dose-response curve was reduced to 1.27 ±0.21 ($P < 0.01$ vs pentobarbitone alone, n=4). The EC_{50} for pentobarbitone also appeared to be reduced to a mean of 0.29±0.027mM ($0.05 < P < 0.1$ compared with the value in the absence of steroid)(Figure 3.2). A similar reduction in slope was observed when a low concentration of pregnanolone (0.1μM) was co-applied with pentobarbitone. This gave a mean slope of 1.52±0.18 ($P < 0.05$ compared with no steroid, n=3) with no significant change in EC_{50} for pentobarbitone (0.413±0.04mM). We interpret these data to suggest that the potentiation of subthreshold concentrations of pentobarbitone by epipregnanolone may be due to its weak partial agonist activity.

DISCUSSION

Previous studies, electrophysiological, binding and behavioural, have shown that 3β-hydroxysteroids have little effect at the $GABA_A$ receptor (Atkinson *et al*, 1965; Harrison *et al*, 1987; Peters *et al*, 1988), but that their sulphate esters have mixed agonist/antagonist effects (Majewska and Schwartz, 1987; Majewska *et al*, 1990). A possible explanation, therefore, of the antagonist activity of epipregnanolone would be interaction at PS or DHEAS sites. However, the lack of antagonism of barbiturate potentiation of benzodiazepine binding and lack of direct effects on the GABA recognition site exhibited by epipregnanolone suggests that this is not the case and that epipregnanolone acts at a distinct locus. Structural comparisons suggest that pregnanolone and epipregnanolone act at the same locus on the $GABA_A$ receptor and that epipregnanolone antagonises its 3α isomer through classical competitive blockade of the steroid recognition site.

The metabolism of progesterone in mammalian neuroendocrine tissue is known to lead to the production of 3α-hydroxy pregnanolones and related compounds (Karavolas and Hodges, 1990) and this has led to speculation that these steroids may be endogenous modulators of the $GABA_A$ receptor (Harrison *et al*, 1987). Whether 3β isomers of these compounds are produced in the CNS is uncertain (Karavolas and Hodges, 1990), but these steroids have been detected in the periphery (Holzbauer, 1976; Karavolas and Hodges, 1990). It is, therefore, a possibility that 3β-hydroxy steroids may have a role in the endogenous modulation of this receptor, although the circulating concentration of the steroid is likely to be lower than its Ki value.

In conclusion, this study has demonstrated that epipregnanolone is a specific competitive antagonist at the neurosteroid recognition site of the $GABA_A$ receptor. This should serve as a useful first step in the development of more potent antagonists. It is possible that this and other 3β-ol pregnane steroids, previously thought inactive at this site (Harrison *et al*, 1987; Morrow *et al*; 1990; Peters *et al*, 1988), may have a physiological role in the modulation of the $GABA_A$ receptor.

Figure 3.1: Competitive antagonism of pregnanolone by epipregnanolone. The pregnanolone dose response curve for the potentiation of 1nM [^{3}H] flunitrazepam binding (●) is shifted rightward in a parallel fashion by 60μM epipregnanolone (◆). Data represent the mean ± SEM of 10 control and 4 epipregnanolone experiments.

Inset: The Ki for epipregnanolone was derived from the relationship EC^*_{50}/EC_{50} = (1+I/Ki) where EC^*_{50}/EC_{50} represents the pregnanolone EC_{50} ratio for flunitrazepam binding in the presence and absence of epipregnanolone, I is the concentration of epipregnanolone and Ki is the inhibition constant for epipregnanolone. Data points represent the mean ± SEM of 4-6 experiments compared with the grouped control values.

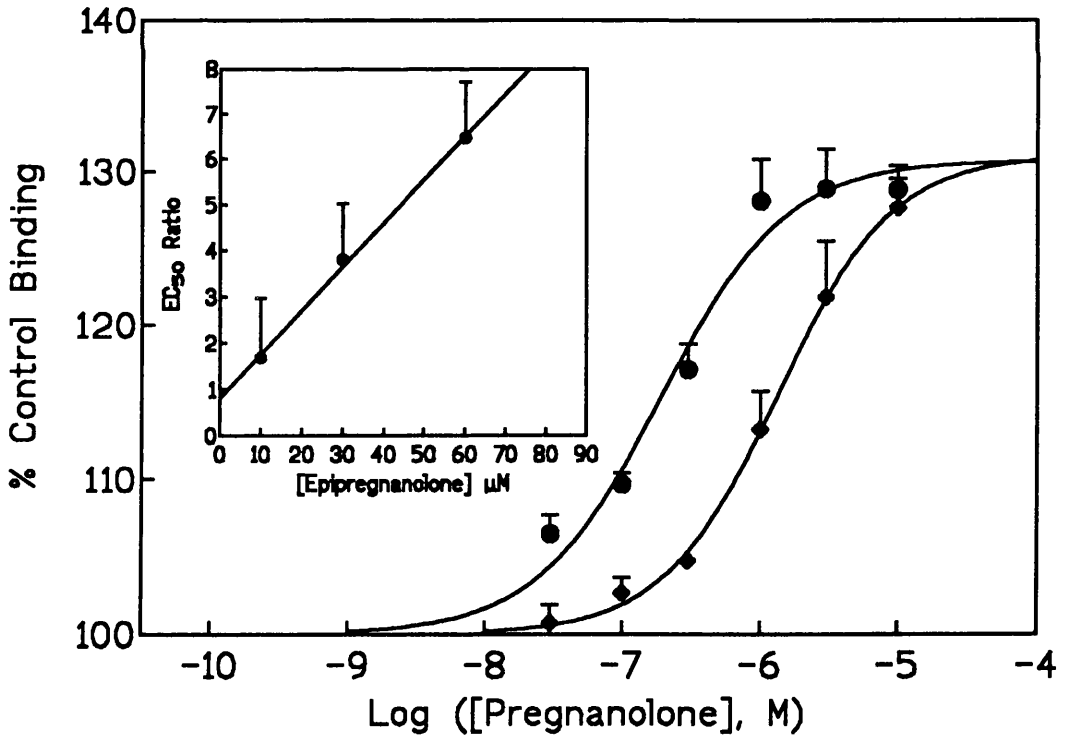
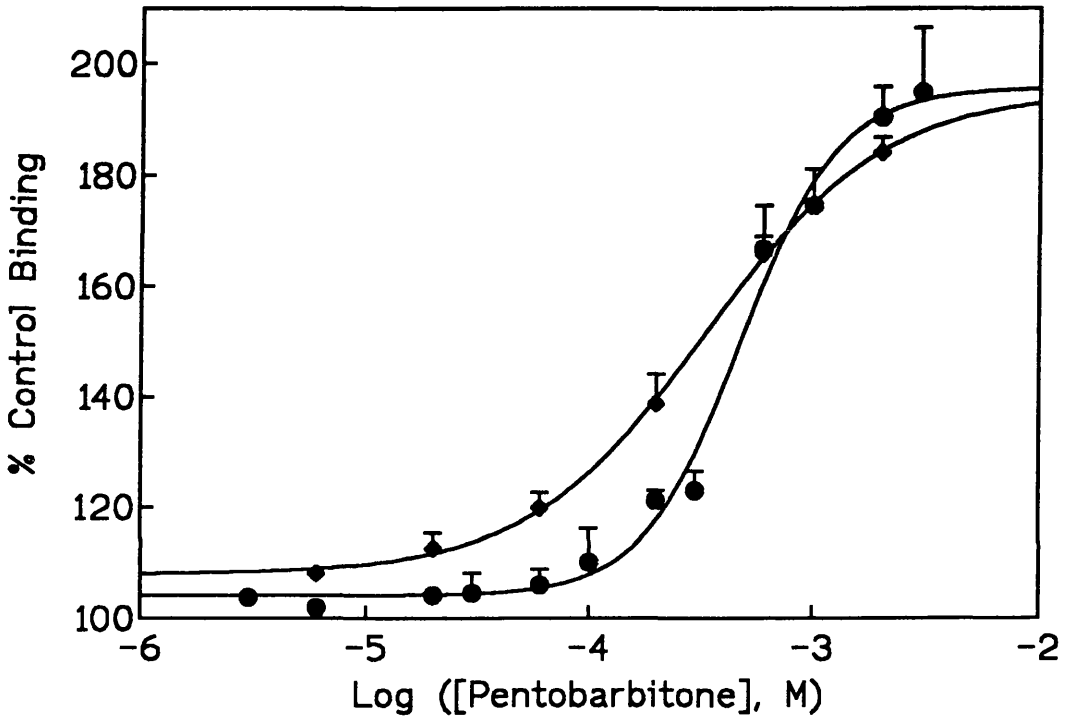


Figure 3.2: Effect of 60μM epipregnanolone on pentobarbitone potentiation of 1nM [^{3}H] flunitrazepam binding. Data are the mean ± SEM of 8 and 4 experiments for control (●) and plus epipregnanolone (♦) respectively.



DIFFERENTIAL ANTAGONISM BY EPIPREGNANOLONE OF ALPHAXALONE AND PREGNANOLONE POTENTIATION SUGGEST MORE THAN ONE CLASS OF BINDING SITE FOR STEROIDS AT $GABA_A$ RECEPTORS.

The text and data which follow have been submitted to Neuropharmacology

SUMMARY

In rat brain membranes, the 3α-hydroxy pregnane steroids, pregnanolone, allopregnanolone, alphaxalone and 5β-alphaxalone potentiated 1nM [^{3}H]-flunitrazepam binding at the $GABA_A$ receptor with maximal potentiations of 140-150% of control. The potencies of the 5α isomers were greater than the 5β, and the presence of an 11-keto group conferred lower potency. The potentiation produced by these steroids was antagonised by the 3β-OH isomers epipregnanolone, isopregnanolone and betaxalone (60μM). The dose-effect curves for pregnanolone and allopregnanolone were shifted to the right with no reduction in the maximal potentiation. In contrast, the maximal effect of alphaxalone and 5β-alphaxalone was reduced with no change in EC_{50}. Alphaxalone (1μM) caused an increase in the binding of [^{3}H]-flunitrazepam in the presence of maximal concentrations of pregnanolone or allopregnanolone. These results suggest multiple sites of action for neurosteroids in the brain.

INTRODUCTION

The $GABA_A$ (γ-aminobutyric acid (A)) receptor is a multimeric protein complex mediating fast synaptic inhibition in the CNS (Schwartz, 1988; Stephenson, 1988). The interaction of GABA with its receptor results in the opening of the anion channel which forms an integral component of the complex, with a concomitant increase in the chloride permeability of the cell membrane. The receptor is the target of action of a number of pharmacologically important classes of drugs such as the benzodiazepines (Tallman and Gallager, 1985) and barbiturates (Leeb-Lundberg, Snowman, and Olsen, 1980). It has also been demonstrated that the $GABA_A$ receptor is modulated by certain metabolites of progesterone and deoxycorticosterone such as pregnanolone (5β-pregnan-3α-ol-20-one), as well as synthetic analogues such as the anaesthetic steroid alphaxalone (5α-pregnan-3α-ol-11,20-dione) (Simmonds, 1991). The enzymes required for the synthesis of such steroids are present in neuronal tissues, therefore these are putative endogenous modulators of the $GABA_A$ receptor complex (Baulieu and Robel, 1990). Recently it has been shown that brain and plasma levels of certain $GABA_A$-active pregnane steroids rise during swim-stress thus providing further hints as to a physiological role for these metabolites (Purdy, Morrow, Moore, and Paul, 1991).

Like the barbiturates, these steroids, termed neurosteroids, potentiate the action of GABA at its receptor by prolonging the mean life time of the channel open state (Simmonds, 1991; Paul and Purdy, 1992). Also in common with the barbiturates, neurosteroids potentiate the binding of benzodiazepines to the $GABA_A$ receptor (Prince and Simmonds, 1992a), and inhibit the binding of the convulsant TBPS

(t-butylbicyclophosphorthionate) (Harrison, Majewska, Harrington and Barker, 1987). The site of action of such steroids is, however, distinct from that of the barbiturates (Peters, Kirkness, Callachan, Lambert and Turner, 1988).

This modulation shows a distinct structure activity relationship with a 3α-OH moiety being a requirement for activity and a 20 keto group being favoured over an hydroxyl (Harrison *et al*, 1987; Gee, Bolger, Brinton, Coirini, and McEwen, 1988). Also, in general, the 5α isomers show greater potency than the 5β (Simmonds, 1991). Recently, we have demonstrated that the 3β isomer of pregnanolone, epipregnanolone (5β-pregnan-3β-ol-20-one), is a competitive antagonist of the potentiation of [^{3}H]-flunitrazepam (FNZ) binding by pregnanolone (Prince and Simmonds, 1992a) and antagonises pregnanolone's potentiation of GABA-induced chloride currents in cultured cells (Pettey and Simmonds, 1991). We have now extended this study and examined the interaction of epipregnanolone with other 3α-OH steroids acting at the $GABA_A$ receptor in order to determine if antagonism by epipregnanolone is a general characteristic of these modulators, and have determined the antagonistic potency of the 3β-OH pregnane steroids isopregnanolone (5α-pregnan-3β-ol-20-one) and betaxalone (5α-pregnan-3β-ol-11,20-dione) at this receptor

METHODS

The binding of [^{3}H]-FNZ to rat whole brain membranes was determined as previously described (Prince and Simmonds, 1992a). Briefly, male Wistar rats (150-200g) were killed by decapitation without prior stunning and their brains homogenised in 20 volumes of ice cold wash buffer (5mM Tris-HCl, 1mM EDTA pH 7.4). The homogenate was centrifuged for 10 minutes at 1000 x G in order to pellet any debris and unbroken cells. The supernatant from this step was centrifuged for 20 minutes at 48000 x G. The resultant pellet was resuspended in 20 volumes ice cold wash buffer. The tissue was washed three further times by centrifugation at 48000 x G followed by resuspension. The final pellet was resuspended in wash buffer and then frozen at -20°C until required (14 days maximum). On the day of the assay the tissue was thawed and then centrifuged at 48000 x G for 20 minutes. The pellet was resuspended in assay buffer (50mM Tris-HCl containing 150mM NaCl pH 7.4 at 4°C) to a final protein concentration of approximately 2mg/ml as determined by the Biorad Bradford assay.

Aliquots (100µl) of membranes were preincubated with drugs in a final volume of 0.5ml assay buffer for 10 minutes at 37°C before the addition of [^{3}H]-FNZ to a final concentration of 1nM. The samples were then incubated for 60 minutes at 4°C. The binding reaction was terminated by addition of 2ml ice cold wash buffer followed by rapid filtration through Whatman GF-C filters using a Brandel Cell Harvester. The filters were washed twice with 2ml ice cold wash buffer and the bound activity quantified using conventional liquid scintillation techniques. Non-specific binding,

which was in the region 5-10% of total binding, was determined using 10μM cold FNZ. Steroids were initially dissolved in acetone which was present in all samples at a concentration of 0.2%. All drugs and chemicals were obtained from Sigma with the exception of 5α-alphaxalone, 5β alphaxalone and betaxalone which were gifts from Glaxo, [^{3}H]-FNZ (78-84 Ci/mmol) which was obtained from Amersham and NaCl which was obtained from BDH (Analar grade). Data were fitted using the Inplot package (Graphpad Software). Unless otherwise stated, statistical comparisons are by Student's t-test.

RESULTS

The neurosteroids pregnanolone, allopregnanolone (5α-pregnan-3α-ol-20-one), 5α-alphaxalone and 5β-alphaxalone (5β-pregnan-3α-ol-11,20-dione) produced a marked potentiation of [^{3}H]-flunitrazepam binding with EC_{50} values in the high nM range. The 3β isomers of pregnanolone (epipregnanolone) and allopregnanolone (isopregnanolone) produced only small potentiations (figure 3.3). In contrast, the 3β isomer of alphaxalone, betaxalone, produced a noticeable potentiation in some experiments, but was not as efficacious or as potent as the 3α steroids (tables 3.1 and 3.2). As previously described, the structure activity relationship for potentiation of the $GABA_A$ receptor shows a strong requirement for a 3α hydroxy moiety with the 5α configuration being favoured over 5β, and hydrogens at position 11 being favoured over a keto group.

In the presence of 60μM epipregnanolone, or 60μM isopregnanolone, the EC_{50} values for pregnanolone and allopregnanolone increased significantly whilst the maximal potentiation was not significantly altered. This is illustrated in figure 3.4. Betaxalone also caused an apparent shift in the dose response curve for allopregnanolone, but this was smaller than with epi- or iso-pregnanolone and was not significant at the 95% confidence interval. In contrast, the EC_{50} values for alphaxalone and 5β-alphaxalone were not significantly altered by the presence of 60μM epipregnanolone but the maximal potentiation was significantly reduced as shown in figure 3.5. The maximal response to alphaxalone was also significantly reduced by the presence of 60μM isopregnanolone but betaxalone was without effect on either the maximal response or

EC_{50} of alphaxalone. In the light of these differences in the interactions of 3β steroids with the pregnanolones and the alphaxalones, we examined combinations of alphaxalone with pregnanolone or allopregnanolone. In the presence of 1μM alphaxalone, the maximal potentiation produced by pregnanolone or allopregnanolone was significantly increased (figure 3.6). This increase tended to be larger when alphaxalone was added to pregnanolone than when alphaxalone was added to allopregnanolone. These data are summarised in tables 3.1 and 3.2.

Table 3.1. The EC_{50} and Emax for pregnanolone and allopregnanolone under various conditions. The data are the mean ± SEM of (n) experiments. ΔEmax represents the mean ± SEM difference in maximal potentiation relative to the 3α steroid alone. Epipregnanolone (EP), isopregnanolone (IP) and betaxalone (BX) were present at 60μM. Alphaxalone (AX) was present at a concentration of 1μM. The Emax values are expressed as a percentage of the control binding in the absence of steroids.

	EC_{50} (μM)	Emax (%)	ΔEmax (%)
Pregnanolone	0.488± 0.08	147.68± 1.67 (5)	-
Pregnanolone + EP	1.96± 0.61*	153.88± 1.81 (3)	6.0± 3.46
Pregnanolone + AX	2.67± 0.78*	170.2± 8.13 (5)	22.5± 8.3**
Allopregnanolone	0.126± 0.01	144.52± 2.18 (9)	-
Allopregnanolone +EP	0.793± 0.23*	141.46± 4.15 (4)	-4.1± 5.2
Allopregnanolone +IP	0.82± 0.35*	139.9± 12.46 (3)	-6.8± 8.9
Allopregnanolone +BX	0.196± 0.04	147.69± 7.85 (4)	1.82± 5.94
Allopregnanolone +AX	0.349± 0.09*	148.11± 3.44 (4)	7.81± 2.3**

* Significantly different from EC_{50} value for 3α steroid alone by Student's t-test ($P<0.05$)

** Significantly greater than zero by Student's paired t-test ($P<0.05$)

Table 3.2. The effects of 3β-OH steroids on dose-effect curves for alphaxalone and 5β-alphaxalone. The data are the mean ± SEM of (n) experiments and Emax is expressed as percent control binding in the absence of steroids and ΔEmax represents the mean ± SEM difference in the maximal potentiation compared with the value for the 3α steroid alone. The data for betaxalone potentiation is taken from the 3 experiments which gave significant potentiation. Epipregnanolone (EP), isopregnanolone (IP) and betaxalone (BX) were all used at a concentration of 60μM.

	EC_{50} (μM)	Emax (%)	ΔEmax (%)
Alphaxalone	1.28± 0.22	142.6± 1.7 (9)	-
Alphaxalone + EP	2.1± 0.85	130.2± 4.5 (4)	-12.1± 2.7*
Alphaxalone + IP	1.21± 0.25	133.1± 0.6 (3)	-11.2± 1.7*
Alphaxalone + BX	1.04± 0.43	149.1± 16.2 (4)	1.93± 17.5
5β-alphaxalone	3.64± 0.59	151.2± 5.6 (3)	-
5β-alphaxalone + EP	3.04± 0.95	127± 3.4 (3)	-24.3± 5.4*
Betaxalone	5.12± 2.3	127± 3.04 (3/6)	-

* Significantly different to zero by Students paired t-test ($P<0.05$)

Figure 3.3. Effect of pregnanolone and its 3β-isomer, epipregnanolone, on the binding of 1nM [^{3}H]-flunitrazepam. Data are the mean ± SEM of 5 (pregnanolone: open symbols) and 3 (epipregnanolone: closed symbols)

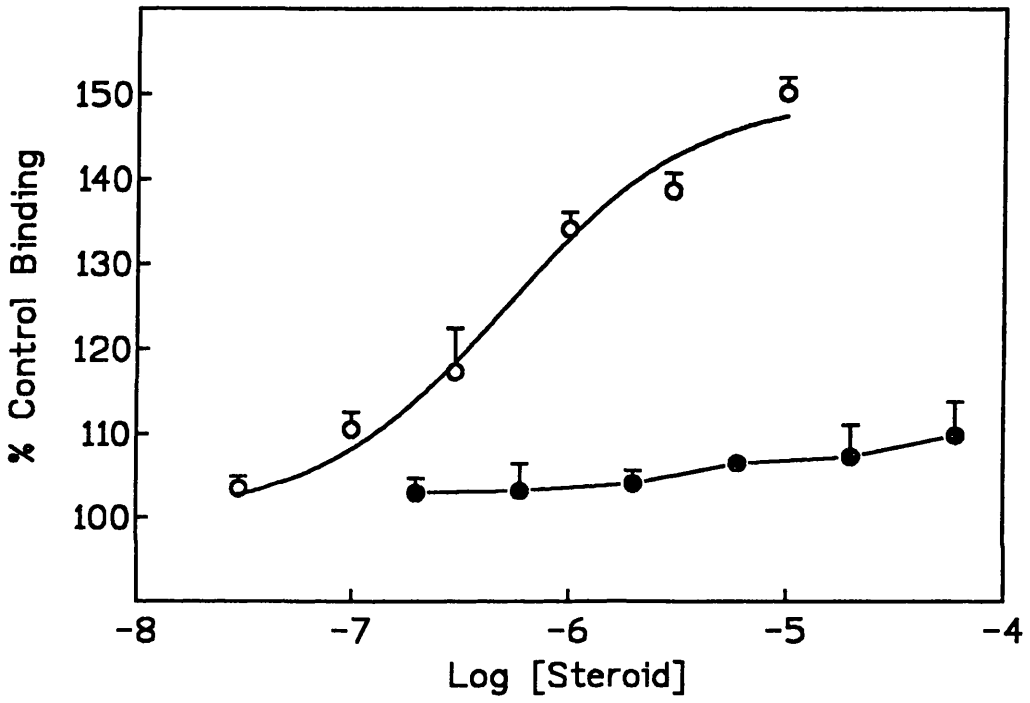


Figure 3.4. The effect of epipregnanolone on the potentiation of 1nM [^{3}H]-flunitrazepam binding by allopregnanolone. Control values are represented by open symbols. The closed symbols are in the presence of 60μM epipregnanolone. The data are the mean ± SEM of 9 and 4 experiments respectively and are expressed relative to the binding in the absence of steroids.

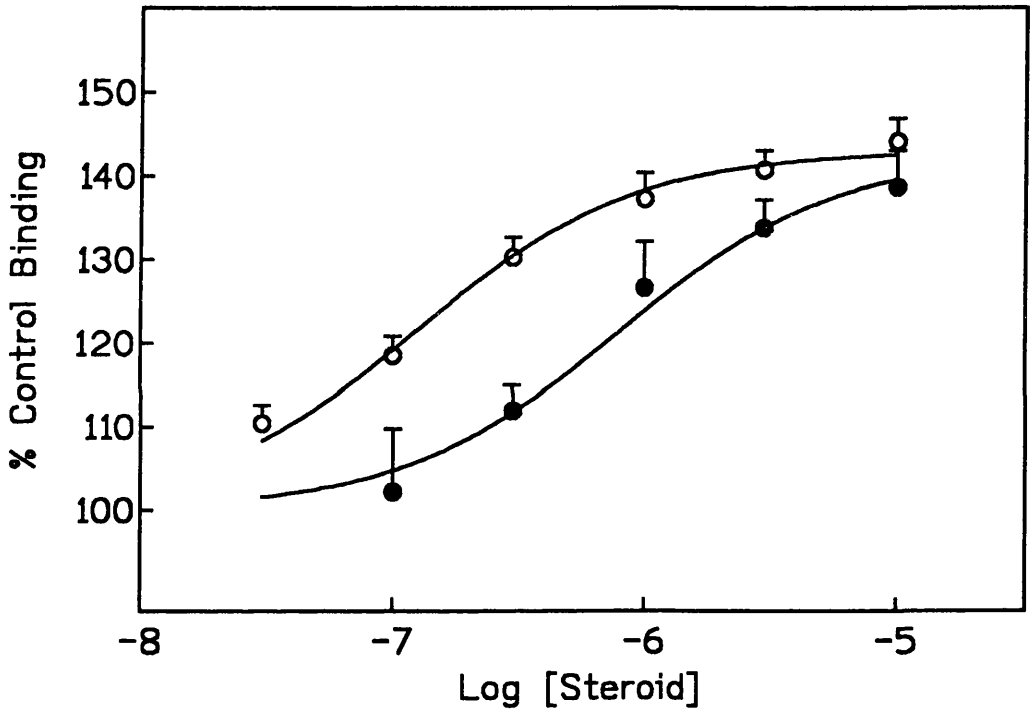


Figure 3.5. The effect of epipregnanolone on the potentiation of 1nM [^{3}H]-flunitrazepam binding by alphaxalone. Data are the mean ± SEM of 9 (control) and 4 (+ 60μM epipregnanolone) experiments. The control values are represented by open symbols. Data are expressed relative to the binding in the absence of steroids.

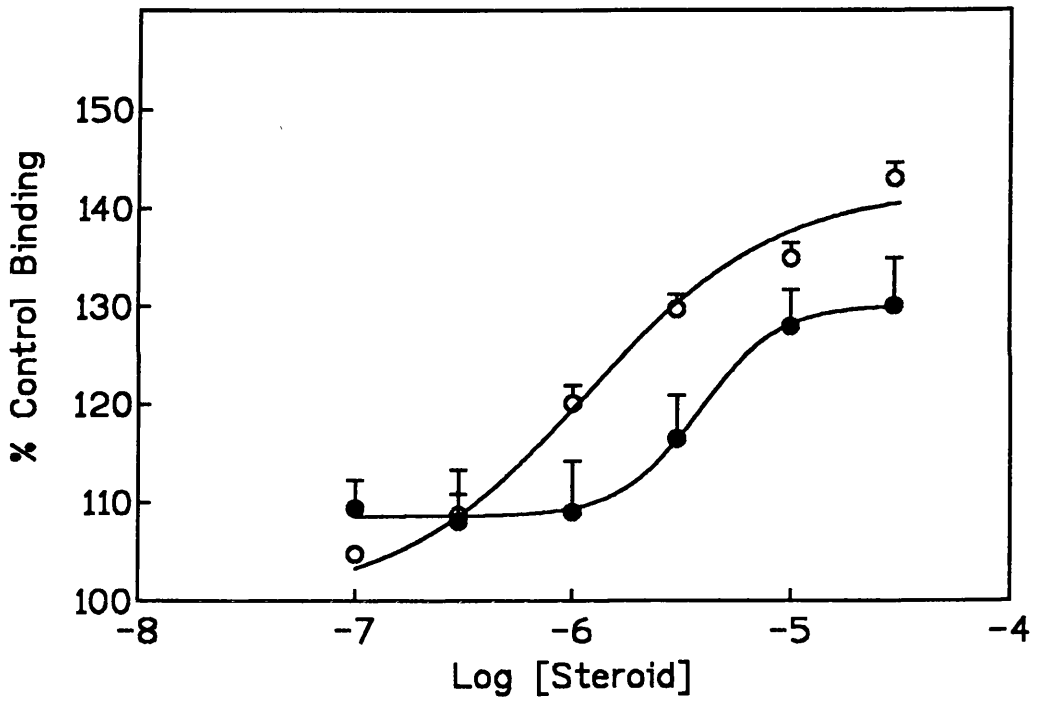
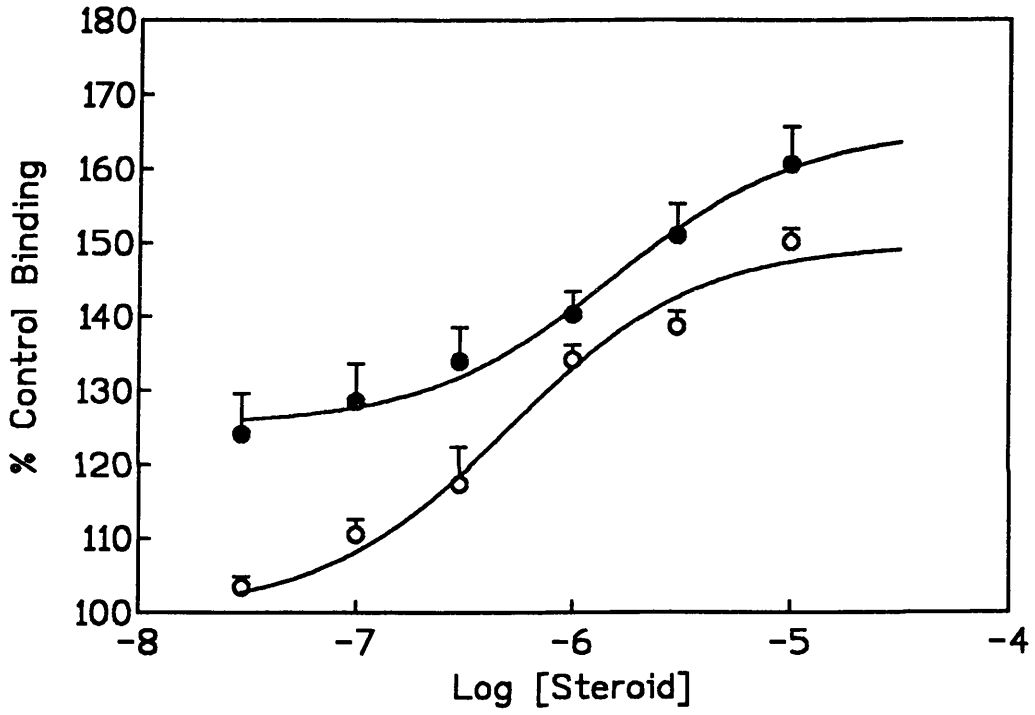


Figure 3.6. The effect of 1μM alphaxalone on the potentiation of 1nM [^{3}H]-flunitrazepam binding by pregnanolone. The data are the mean ± SEM of 5 paired experiments expressed relative to the binding in the absence of steroids. The open symbols represent the control values.



DISCUSSION

In our previous study we demonstrated that epipregnanolone is a competitive antagonist of its 3α isomer, pregnanolone (Prince and Simmonds, 1992a). We have now demonstrated that allopregnanolone, the 5α isomer of pregnanolone is antagonised in a similar manner. However, the interactions of the 11-keto derivatives of pregnanolone and allopregnanolone, 5β-alphaxalone and alphaxalone respectively, with the $GABA_A$ receptor appear to be antagonised by epipregnanolone through a different mechanism.

Various explanations can be suggested for the reduction in the maximal response to alphaxalone caused by epipregnanolone. The simplest of these is to postulate two sites of action for alphaxalone, one of which is sensitive to antagonism and the other which is not. The monophasic dose response curve for alphaxalone suggests that if these sites exist, they would have approximately the same affinity for the steroid. An alternative explanation, which we consider less likely, is that the alphaxalone dose response curve consists of two poorly resolved components of differing EC_{50}, both of which are shifted to the right by the presence of epipregnanolone.

In order to determine how the site(s) of action of alphaxalone relate to those of pregnanolone and allopregnanolone, we examined the effect of a fixed concentration of alphaxalone upon dose response curves to the pregnanolones. Interestingly, whilst the effects of the two steroids were not simply additive, the fitted maximal response to both of the pregnanolones was increased by the presence of alphaxalone. This suggests that there is some component of alphaxalone's action which is not shared by

the pregnanolones. Examination of the maximal responses to the alphaxalones and the pregnanolones alone revealed no significant differences. This suggests that pregnanolone does not simply act at a subset of alphaxalone sites. In order to accommodate these observations it is, therefore necessary to postulate more than one set of sites of action for the pregnanolones. Taken together, these results suggest that there exist at least three sites of action for the neurosteroids: a population of epipregnanolone insensitive receptors which are selective for the alphaxalones; a population of epipregnanolone sensitive, pregnanolone selective receptors and an additional set of receptors which are non-selective for the pregnanolones or alphaxalones and which are antagonised by epipregnanolone. The possible nature of these sites is discussed below.

The observed agonistic action of betaxalone in certain experiments prompted us to examine the effects of isopregnanolone on $GABA_A$ receptor/steroid interactions. Unlike betaxalone, isopregnanolone showed no direct potentiation of [^{3}H]-flunitrazepam binding indicating that the 11-keto moiety and not the 5α conformation of betaxalone may be the determining factor in its apparent partial-agonist action. The approximate doubling of the EC_{50} for allopregnanolone by 60μM betaxalone corresponds to a Ki of around 60μM. This is far higher than the EC_{50} for potentiation observed in experiments where betaxalone increased [^{3}H]-flunitrazepam binding. This suggests that if it does act as a partial agonist, this action is not via the same site of action as its antagonistic actions. The antagonistic potency of isopregnanolone upon potentiation by allopregnanolone was approximately equal to that of epipregnanolone,

whilst that of betaxalone was much lower. This corresponds to the observed potency of the 3α isomers for potentiation.

The molecular biology of the $GABA_A$ receptor has proved to be extremely complex. To date, 14 different subunits have been cloned each showing a distinct distribution and conferring differing properties on expressed receptors (Sigel, Baur, Trube, Möhler and Malherbe, 1990; Wisden, Laurie, Monyer and Seeburg, 1992). At present, neither the subunit composition nor stoichiometry of any distinct population of $GABA_A$ receptors is known. However, expression studies have revealed composition dependent differences in responsiveness to GABA, to benzodiazepines, to barbiturates (Sigel *et al*, 1990), to agonistic neurosteroids (Shingai, Sutherland and Barnard, 1991) and to the antagonistic steroid pregnenolone sulphate (Zaman, Shingai, Harvey, Darlison and Barnard, 1992). It is likely, therefore, that the pharmacology of native receptors is also highly subunit dependent. Differential responsiveness to steroids in various brain regions support this (Gee *et al* 1988; Gee and Lan, 1991), and suggest that the present results may be due to composition-dependent differences in sensitivity to the pregnanolones and to alphaxalone. Other pieces of evidence also point to multiple sites of action. Substantial differences have been noted in the effects of various steroids upon dose response curves to muscimol and on picrotoxin Schild plots in the cuneate nucleus (Turner and Simmonds, 1989). Biphasic dose response curves to pregnanolone have also been noted in chloride flux experiments (Morrow, Pace, Purdy and Paul, 1990). In addition to heterogeneity of sensitivity between complexes, it is also possible that $GABA_A$ receptors contain more than one site of neurosteroid action within the complex. If, as has been suggested, steroids bind to the axial clefts between the

receptor subunits in direct-ligand-gated receptors (Jones and McNamee, 1988), the hetero-oligomeric structure of the complex allows for multiple, distinct sites of action, the molecular character of which would be determined by the subunit composition and the arrangement of subunits within the complex.

In conclusion, we have demonstrated a differential interaction of 3β-OH pregnane steroids with $GABA_A$ receptors potentiated by pregnanolone and allopregnanolone; and those potentiated by alphaxalone and 5β-alphaxalone. This may reflect more than one site of action for 3α-OH pregnane steroids at this receptor. In addition, it has been shown that the steroids isopregnanolone and betaxalone are also competitive antagonists of 3α-OH pregnanolones and that the structure activity relationship for antagonism is similar to that for potentiation.

CHAPTER 4

Propofol potentiates the binding of [^{3}H]-flunitrazepam to the $GABA_A$ receptor complex

The text and data that follow have been accepted by Brain Research

SUMMARY

Propofol (2,6-diisopropylphenol) robustly stimulated the binding of 1nM [^{3}H]-flunitrazepam (FNZ) to rat brain membranes with an EC_{50} of 146μM in chloride free buffer and 23μM in buffer containing 200mM NaCl. NaCl showed an EC_{50} of 40mM for its ability to increase the potency of propofol. The ability of a range of anions to potentiate propofol's interactions with the $GABA_A$/benzodiazepine receptor was closely correlated with their permeabilities at this ion channel. Propofol, at a concentration of 300μM, decreased the EC_{50} for the potentiation of FNZ binding by NaCl from 39mM to 13mM with no change in the maximal potentiation. At a concentration of 30μM, propofol significantly decreased the EC_{50} for potentiation of FNZ binding by the neurosteroid alphaxalone whilst increasing that for potentiation by pentobarbitone. We conclude that propofol is a potent barbiturate-like potentiator of [^{3}H]-flunitrazepam binding.

INTRODUCTION

The potentiation of flunitrazepam binding by allosteric agonists of the $GABA_A$ receptor is a pharmacologically well defined phenomenon. Benzodiazepine binding is stimulated via an increase in receptor affinity by GABA (gamma-aminobutyric acid) (Skolnick *et al*, 1981; Wong and Iversen, 1985) and its analogues, barbiturates (Skolnick, Paul and Barker, 1980) and certain pregnane steroids such as alphaxalone (5α-pregnan-3α-ol-11,20-dione) and the pregnanolone series (Majewska *et al*, 1986; Simmonds, 1991). In general, the dose response relationship for benzodiazepine potentiation is closely correlated with that for other parameters of $GABA_A$ receptor function such as inhibition of [^{35}S]-TBPS binding, potentiation of [^{3}H]-muscimol binding and potentiation of chloride flux (Wong and Iversen, 1985; Leeb-Lundberg *et al*, 1980; Majewska *et al*, 1986; Olsen *et al*, 1986). Recently, the novel general anaesthetic, propofol (2,6-diisopropylphenol), which is a potent enhancer of GABA responses in electrophysiological studies (Hales and Lambert, 1991), has been shown to enhance [^{3}H]-muscimol binding and decrease [^{35}S]-TBPS binding (Concas *et al*, 1991a). This suggests that the mode of action of propofol is through an increase in synaptic inhibition mediated by the $GABA_A$ receptor. However, unlike the steroids and barbiturates, propofol has been reported to show no apparent interaction with the benzodiazepine binding site. At concentrations of up to 300μM propofol failed to significantly increase [^{3}H]-FNZ binding whilst in the same study enhancing [^{3}H]-muscimol binding (Concas *et al*, 1991a). This situation is rather akin to the problems which were experienced in the late 1970's in demonstrating that barbiturates modulated benzodiazepine binding. It was found then that interactions between the

benzodiazepine and barbiturate sites of the $GABA_A$ receptor are dependent upon the presence of certain anions, notably chloride (Leeb-Lundberg *et al*, 1980; Skolnick *et al*, 1981; Skolnick, Paul and Barker, 1980). With this in mind, we have undertaken a study of the interaction of propofol with the $GABA_A$-benzodiazepine receptor and the dependency of this interaction upon various ions.

MATERIALS AND METHODS

Male Wistar rats (180-200g) were sacrificed by decapitation with prior stunning. Whole brains, minus brainstem, were rapidly dissected out and either stored at -20°C until required (14 days maximum) or used immediately. Crude synaptic membranes were prepared as described previously (Prince and Simmonds, 1992a). The tissue was homogenised in 20 volumes of ice cold wash buffer (5mM Tris-HCl 1mM EDTA pH 7.4) using a glass/teflon homogeniser. This homogenate was then centrifuged for 10 minutes at 1000 x G to pellet any blood vessels and debris. The supernatant was carefully removed and the pellet discarded. After centrifugation for 20 minutes at 48000 x G, the tissue was resuspended in wash buffer. This step was repeated 3 times and the final homogenate frozen at -20°C until required (14 days maximum). Where tissue with low endogenous GABA levels was required, a freeze/thaw step was incorporated between each centrifugation.

On the day of the assay, tissue was thawed and centrifuged for 20 minutes at 48000 x G. The pellet was resuspended in assay buffer (10mM Tris-citrate pH 7.4). Aliquots of tissue containing 0.1-0.3mg total protein were preincubated in a total volume of 0.5ml containing various concentrations of drugs and salts for 10 minutes at 37°C. [^{3}H]-FNZ (1.0nM final concentration) was then added and the samples incubated for 60 minutes at 4°C. The binding reaction was halted by the addition of 2.5ml ice cold wash buffer and rapid filtration through Whatman GF-C filters using a Brandel Cell Harvester. The filters were washed twice with 2.5ml ice cold wash buffer and the bound activity quantified using conventional liquid scintillation counting. Non-specific

binding was determined using 10μM FNZ and was in the region of 5-10% total binding. Similar results were obtained using 10μM flumazenil as the displacing agent. Protein determinations were performed using the Biorad Bradford Assay. Data was analyzed using Inplot software (Graphpad). Data are expressed as mean ± SEM (n) and, unless otherwise stated, are compared using Student's t-test.

Propofol and alphaxalone were dissolved initially in acetone which was present in all tubes at a concentration of 0.1%, and which did not itself affect the binding of [^{3}H]-FNZ. Distilled water was then added to the acetone solutions and the mixture briefly sonicated until a homogeneous suspension or solution was obtained. All drugs and chemicals were obtained from Sigma with the exception of NaCl (BDH). Alphaxalone was a gift from Glaxo and propofol was donated by ICI.

RESULTS

In Tris-citrate buffer containing no added salts, propofol gave a dose dependent enhancement of [^{3}H]-FNZ binding with an EC_{50} of 146.9± 29.4μM and a mean maximal potentiation of 160.4± 9.46% of basal binding (n=4). In the presence of 200mM sodium chloride, the dose response curve for propofol was shifted significantly to the left by -0.87± 0.1 log units, to give an EC_{50} of 23.81± 6.75μM ($P<0.05$). The mean maximal potentiation was not significantly different at 151.3± 8.83% of control (n=3). Likewise, 0.3mM propofol gave a significant shift in the dose response curve for NaCl potentiation of [^{3}H]-FNZ binding with mean EC_{50} values of 38.8± 7.03mM (6) in the absence of, and 12.80± 3.01mM (4) in the presence of 0.3mM propofol ($P<0.05$). The maximal potentiation obtained with NaCl compared with salt-free buffer (141± 7.85% of control) was not significantly altered by the presence of 0.3mM propofol (138.4± 7.1% of control). These interactions are illustrated in figure 4.1.

The EC_{50} for propofol's potentiation of [^{3}H]-FNZ binding was also determined at a range of different (0.6-200mM) NaCl concentrations (figure 4.2). The EC_{50} for the potentiating effect of NaCl derived from these data was 40.45mM. In buffer containing sodium chloride, at concentrations of propofol higher than 0.3mM, a decrease in potentiation was often observed, leading to a bell shaped dose response curve (eg figure 4.1). In salt-free buffer this decrease was not apparent.

A range of salts at concentrations of 200mM were compared for their ability to potentiate propofol's action at the $GABA_A$ receptor. These data are presented in table 4.1.

In order to investigate whether this potentiation was due to direct effects upon the flunitrazepam site or via potentiation of endogenous GABA, propofol interactions were also studied using extensively freeze-thawed tissue in assay buffer containing 200mM NaCl. Neither the EC_{50} value obtained in this series of experiments (26.12± 9.66μM (3)) nor the maximal potentiation (144.37± 4.82% basal binding (3)) were significantly different from that above, indicating that propofol acts directly to potentiate FNZ binding.

The mechanism of propofol's potentiation of [^{3}H]-FNZ binding was examined in a series of displacement experiments. In the presence of 200mM NaCl, FNZ gave a Ki of 2.3± 0.3nM (4) for displacement of [^{3}H]-FNZ. This was significantly decreased to 1.35± 0.18nM (4) in the presence of 0.3mM propofol ($P<0.05$ by Student's paired t-test). These data are illustrated in figure 4.3. The Bmax for FNZ, calculated from the Ki and total binding, was not significantly altered by the presence of propofol (control: 3.14± 1.17 pmol/mg protein (4); +0.3mM propofol: 3.39± 0.95 pmol/mg protein (4)). Propofol, therefore, in common with GABA (Wong and Iversen, 1985), pentobarbitone (Leeb-Lundberg *et al*, 1980) and the neurosteroids (Simmonds, 1991), potentiates benzodiazepine binding by an increase in affinity rather than the number of binding sites.

The interaction of propofol with pentobarbitone and alphaxalone on the potentiation of flunitrazepam binding was also investigated in a short series of experiments utilising a 50mM Tris-HCl buffer pH 7.4 at 4°C containing 150mM NaCl, by determining the effect of a fixed concentration (30μM) of propofol upon the dose response curves to these modulators. Alphaxalone and pentobarbitone both showed robust potentiation of FNZ binding. The EC_{50} value for pentobarbitone was significantly increased in the presence of propofol whilst that for alphaxalone showed a significant decrease (table 4.2). The maximal potentiations obtained with alphaxalone and with pentobarbitone were not significantly altered by the presence of 30μM propofol compared with values in the absence of propofol (table 4.2). However, in two out of the three experiments where propofol was added to pentobarbitone, the ability of concentrations of pentobarbitone above 1mM to enhance flunitrazepam binding was inhibited, giving rise to a bell shaped dose response curve for pentobarbitone as shown in figure 4.4.

Figure 4.1. Interactions of propofol and NaCl. The enhancement of 1nM [^{3}H]-flunitrazepam binding by propofol was measured in 10mM Tris-citrate buffer (open squares) and in 10mM Tris-citrate containing 200mM NaCl (closed squares). Note the bell shaped dose response curve in the presence of NaCl. The enhancement of [^{3}H]-flunitrazepam binding by NaCl in the absence (open circles) and presence (closed circles) of 300μM propofol. Data are the mean ± SEM of triplicate values from representative experiments.

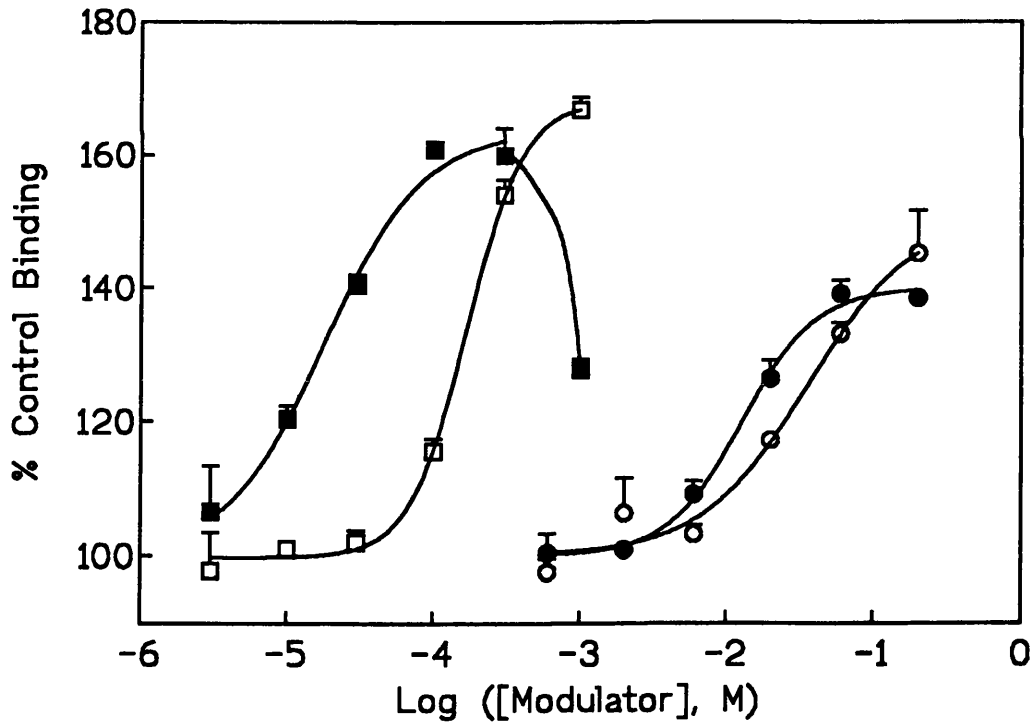


Figure 4.2. The sodium chloride-induced decrease in the EC_{50} for propofol potentiation of [^{3}H]-flunitrazepam binding. Data are the mean ± SEM of 3 experiments. NaCl concentration is mol.dm^{-3}

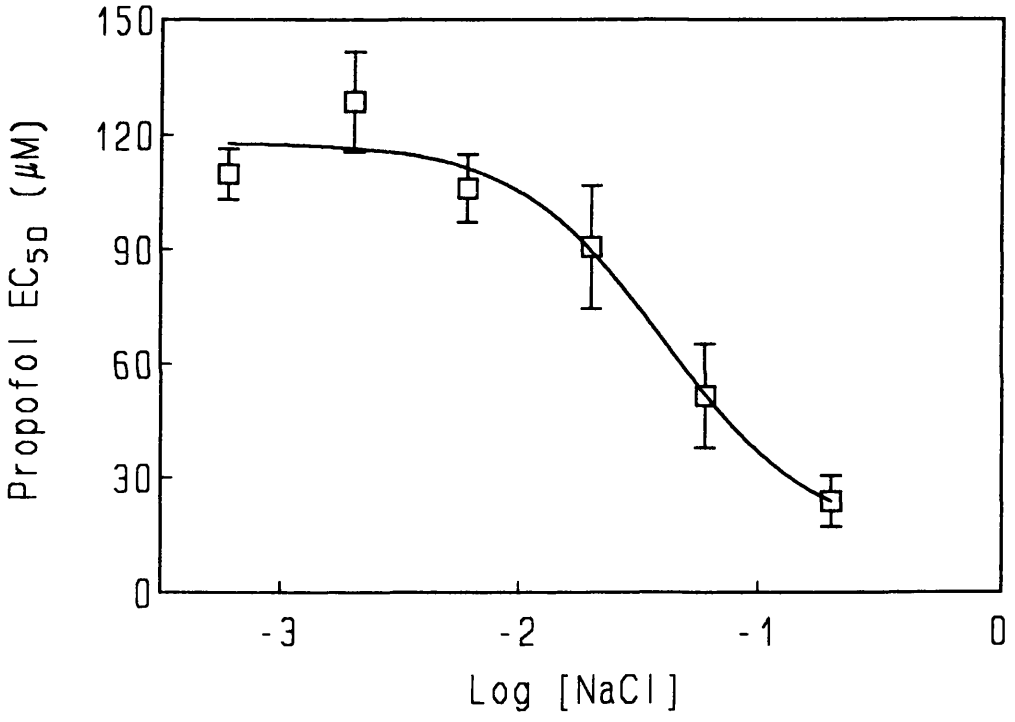


Figure 4.3. The enhancement by propofol of [^{3}H]-flunitrazepam affinity. The competition curve for flunitrazepam displacement of 1nM [^{3}H]-flunitrazepam is shifted leftward approximately 0.3 log units by the presence of 300μM propofol. Data are the mean ± SEM of triplicate values from a representative experiment and are expressed relative to specific binding in the absence of displacing agent. Where error bars are not shown, the SEM is smaller than the data symbol.

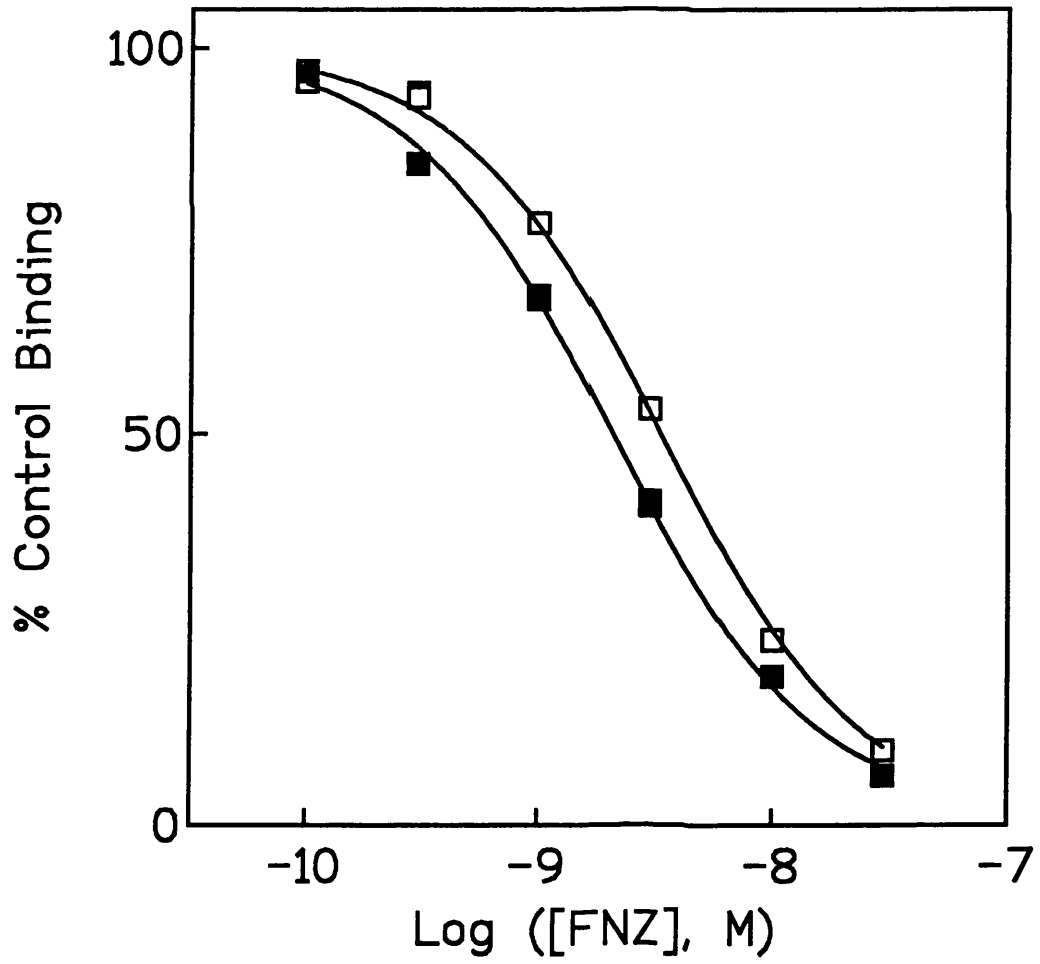


Figure 4.4. The effect of 30μM propofol upon dose-effect curves for alphaxalone (squares) and pentobarbitone (circles). Open symbols represent the control curves and closed symbols the curves in the presence of propofol. Note the bell shaped dose-effect curve for pentobarbitone in the presence of propofol. Data are expressed relative to control binding in the absence of drugs and are the mean ± SEM of triplicate values from representative experiments. Where no error bars are shown, the SEM is smaller than the data symbol.

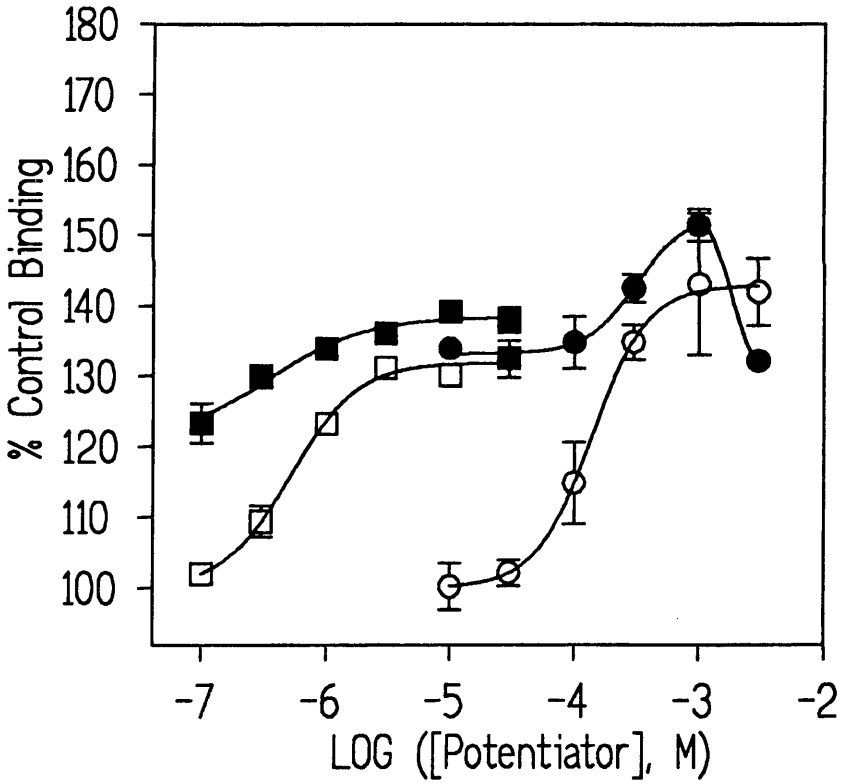


Table 4.1. The EC_{50} values for propofol enhancement of the binding of 1nM [^{3}H]-flunitrazepam in the presence of 10mM Tris-citrate containing various salts at a concentration of 200mM. Data are the mean ± SEM of (n) experiments.

Salt	EC_{50} (μM)
Control (no added salts)	146.9± 29.4 (4)
NaCl	23.81± 6.75 (3)[a]
KCl	28.34± 3.09 (3)[b]
KI	9.34± 1.77 (3)[a,c]
NaF	67.04± 13.33 (3)[b,c]
$CaCl_2$	17.32± 5.19 (3)[a]
$NaNO_3$	13.84± 3.26 (4)[a]
NaOOCH	38.71± 9.19 (4)[a]
NaCl (extensively freeze-thawed tissue)	26.12± 9.66 (3)[a]

a) $P< 0.01$ vs control values by Student's t-test

b) $P< 0.05$ vs control values by Student's t-test

c) $P< 0.05$ vs control+ 200mM NaCl values by Student's t-test

Table 4.2. The effect of 30μM propofol upon dose-effect parameters for pentobarbitone and alphaxalone enhancement of 1nM [^{3}H]-flunitrazepam binding in 50mM Tris-HCl buffer containing 150mM NaCl pH 7.4. The data are expressed as mean ± SEM of (n) experiments. Maximal potentiation (Emax) is given with reference to the binding of flunitrazepam in the absence of drugs.

	EC_{50} (μM)	Emax (%)
Alphaxalone	0.69± 0.06	136.5± 3.4 (4)
Alphaxalone + propofol	0.29± 0.05[a]	140.9± 6.2 (4)
Pentobarbitone	138± 10	154.8± 7.1 (3)
Pentobarbitone + propofol	250± 33[a]	156.5± 5.2 (3)

[a]significantly different to absence of propofol by Student's paired t-test ($P<0.05$)

DISCUSSION

This study has demonstrated that propofol is capable of potentiating the binding of flunitrazepam and that this potentiation occurs via an increase in receptor affinity rather than receptor number. This mechanism is similar to that of both the barbiturates (Leeb-Lundberg *et al*, 1980) and the steroids (Simmonds, 1991) and correlates with data obtained in other studies showing that propofol is a potent enhancer of both binding and electrophysiological parameters of the $GABA_A$ receptor (Hales and Lambert, 1991; Concas *et al*, 1991a). It has previously been demonstrated that circulating concentration of propofol during anesthesia is approximately 35μM (Gepts *et al*, 1985). This is in excellent agreement with the EC_{50} for propofol potentiation of flunitrazepam binding at physiological levels of chloride (120mM) (as deduced from figure 4.2), thereby underlining the importance of the $GABA_A$ receptor in propofol's mechanism of action. The rank order of potency for flunitrazepam binding potentiation is the same as that observed for muscimol binding: alphaxalone > propofol > pentobarbitone[4].

Like that by the barbiturates (Leeb-Lundberg *et al*, 1980), potentiation by propofol appears to be strongly dependent upon the presence of chloride ions with nearly a 7 fold increase in the potency of propofol when chloride is present at 200mM. However, the efficacy of propofol potentiation, in contrast to that of pentobarbitone (Leeb-Lundberg *et al*, 1980) is unaffected by the concentration of salt present. Although the concentrations of propofol utilised by Concas *et al* (1991a) under chloride free conditions were above the EC_{50} for propofol found in the present study under similar

conditions, it is possible that the use of a Tris-citrate buffer without added salts is the cause of the lack of potentiation of [^{3}H]-FNZ observed in their study. This finding emphasises the need for the use of high chloride buffers when examining the allosteric interactions of this site.

The effects of the various ions tested for their ability to enhance propofol potency are very closely related to their permeability at the GABA$_A$ receptor. Bormann *et al* (1987) found the permeability sequence for anions at this receptor to be $I^- > NO_3^- > Cl^- > CHOO^- >> F^-$, which is the same as for potentiation of propofol interactions found in the present study, the permeability being significantly linearly correlated with effects upon propofol ($r = -0.904$, $P < 0.05$). Similar results have been observed for pentobarbitone (Leeb-Lundberg *et al*, 1980) and, in common with our results for propofol, no significant cation-dependent effect was seen. However, in contrast to observations on pentobarbitone interactions (Leeb-Lundberg *et al*, 1980), ions which are essentially impermeable such as F^- and SO_4^{2-} (Araaki, Ito and Oscarson, 1961), also have a significant potentiating effect upon the potency of propofol. This indicates that in addition to the specific effect of ions which can substitute for chloride at the channel, there is also a non-specific chaotropic effect..

In order to clarify whether the interaction of propofol with the GABA$_A$ receptor were due to interactions at either the steroid or barbiturate recognition site, we examined the effects of a submaximal concentration of propofol upon the dose response curves for pentobarbitone and alphaxalone enhancement of [^{3}H]-FNZ binding. As is consistent with the results obtained by Conas *et al* ((1991a), propofol significantly

increased the potency of alphaxalone. This presumably represents an allosteric enhancement of alphaxalone binding by propofol and suggests that these drugs do not interact at the same site. The maximal potentiation seen, was not altered by the presence of propofol. This suggests that there exists a maximal affinity state for the benzodiazepine site, beyond which potentiation is not possible. In contrast, the EC_{50} for pentobarbitone potentiation was significantly increased by the presence of propofol. Such an increase in EC_{50} would be predicted if the two compounds were acting at the same site and behaving in a competitive manner. This increase in EC_{50} might also be explained by the facilitation of the inhibitory component of the propofol dose response curve by pentobarbitone. However, this would predict a lowering of the maximal potentiation obtained with pentobarbitone, which was not observed. In a recent electrophysiological study, propofol was shown to have a similar action to pentobarbitone, prolonging the burst duration of the $GABA_A$ receptor linked channel. In addition, whilst pregnanolone was equipotent in enhancing GABA and pentobarbitone evoked currents, propofol produced a much larger enhancement of GABA currents than pentobarbitone currents (Hales and Lambert, 1991). Taken together, these results suggest that propofol may act at the same site as the barbiturates. However, the efficacy of propofol, unlike that of pentobarbitone, is independent of the presence of permeant ions. In addition, recent experiments in our laboratory have demonstrated that the effect of propofol upon the temperature dependency of [^{3}H]-flunitrazepam binding differs to that of pentobarbitone (unpublished results). An allosteric mechanism for the effect of propofol upon the dose-effect curve for pentobarbitone is, therefore, a distinct possibility. Studies of the

binding of [^{3}H]-propofol should provide a clear answer as to the site of interaction of this compound (Concas *et al*, 1991b).

In conclusion, we have demonstrated an anion dependent, direct interaction between propofol and the benzodiazepine binding site of the $GABA_A$ receptor. Whilst this interaction shows many similarities to that of barbiturates with the benzodiazepine site, no firm conclusion can be drawn as to the site of propofol interactions with the $GABA_A$ receptor.

CHAPTER 5

Temperature and anion dependence of allosteric interactions at the γ-aminobutyric acid-benzodiazepine receptor.

The text and data that follow have been accepted by Biochemical Pharmacology

Abstract

The temperature dependence of [^{3}H]-flunitrazepam binding to rat brain membranes was examined in the presence of the anaesthetics, pentobarbitone, alphaxalone and propofol. Van't Hoff plots showed the binding of flunitrazepam to be largely enthalpy driven. Alphaxalone and propofol increased the entropy of the binding reaction but not the enthalpy and therefore did not show temperature dependence in their efficacy. In contrast, pentobarbitone increased the enthalpy of flunitrazepam binding and therefore, is more efficacious at low temperatures. The EC_{50}s of all three modulators increased with temperature indicating that their interactions with the receptor may be enthalpy driven. The EC_{50}s of all three modulators were also anion dependent, showing a decrease in the presence of $GABA_A$-channel permeant anions. The efficacies of alphaxalone and pentobarbitone, but not propofol, also increased with increasing chloride ion concentration. The results indicate that all three modulators interact with the $GABA_A$ receptor at distinct recognition sites.

INTRODUCTION

The $GABA_A$ (γ-aminobutyric acid) receptor is a multimeric protein comprising an integral chloride ion channel gated by the inhibitory amino acid GABA together with several classes of modulatory site (Schwartz, 1988; Stephenson, 1988). The gating of the channel by GABA has been shown to be enhanced by a variety of sedative and hypnotic drugs including the benzodiazepines (Tallman and Gallager, 1985), barbiturates (Leeb-Lundberg *et al*, 1980), and certain pregnane steroids termed the neurosteroids (Simmonds, 1991), and it has been proposed that this is their major mechanism of action. As well as direct modulation of the GABA binding site, the action of drugs such as the barbiturates and neurosteroids can also be studied via their allosteric enhancement of the binding of benzodiazepines such as flunitrazepam (FNZ) (Tallman and Gallager, 1985). The potentiation of [^{3}H]-benzodiazepine binding by modulators such as barbiturates (Leeb-Lundberg *et al*, 1980), and the novel general anaesthetic propofol (2,6-diisopropylphenol) has been shown to be highly dependent upon the presence of certain anions, the facilitatory action of which is correlated with their permeability at the $GABA_A$ receptor ion channel (Prince and Simmonds, 1992b).

In addition, the binding of benzodiazepines to the $GABA_A$ receptor exhibits a well characterised temperature and anion dependence (Doble, 1983; Quast *et al*, 1982; Kochman and Hirsch, 1982). Due to the exothermic nature of the binding reaction at temperatures of 10°C and above, an increase in temperature causes a decrease in the affinity of the receptor for its ligand and gives a straight line relationship between log Keq and reciprocal absolute temperature as predicted by the van't Hoff equation:

$$\ln Keq = \frac{-\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R}$$

Temperature is known to exert a strong influence on the state of fluidity of biological membranes (Harris and Schroeder, 1982). Perturbation of membrane structure has been advanced as a possible mechanism of anaesthetic action (Seeman, 1972) and it has been demonstrated that the $GABA_A$ potentiator alphaxalone (5α-pregnan-3α-ol-11,20-dione) (Makriyannis *et al*, 1990) together with the barbiturates (Harris and Schroeder, 1982) have such an effect upon lipid bilayers. If such effects of alphaxalone and pentobarbitone are relevant to their modulation of the $GABA_A$ receptor, it might well be expected that they would alter the temperature dependence of FNZ binding. We have investigated this possibility and have also compared the effects of certain anions upon modulation of [^{3}H]-flunitrazepam binding by alphaxalone, pentobarbitone and propofol.

MATERIALS AND METHODS

Crude rat-brain synaptic membranes were prepared as previously described (Prince and Simmonds, 1992a). Briefly, male Wistar rats (150-220g) were sacrificed by decapitation with prior stunning and their brains rapidly removed and placed on ice. Tissue was either used fresh or frozen until required (14 days maximum). Whole brains minus brain stem were homogenised in 20 volumes ice cold wash buffer (5mM Tris-HCl containing 1mM EDTA, pH 7.4 at 4°C) using a glass/Teflon homogeniser. This homogenate was centrifuged at 1000 x G for 10 minutes to pellet unbroken cells and debris. The supernatant was carefully removed and centrifuged at 48000 x G for 20 minutes. The resultant pellet was resuspended and centrifuged a further three times. The final pellet was resuspended in wash buffer and then frozen at -20°C until required (14 days maximum). On the day of the experiment, the tissue was thawed and centrifuged for 20 minutes at 48000 x G before resuspension in assay buffer (either 50mM Na^+/K^+ phosphate containing 200mM NaCl, pH 7.1 (buffer 1); or 10mM Tris-citrate containing various concentrations of salts, pH 7.4 at 4°C (buffer 2)).

The binding of [^{3}H]-flunitrazepam was determined by the incubation of 100µl aliquots of tissue containing 0.1-0.3mg total protein (Biorad protein assay), for 10 minutes at 37°C in the absence of radioligand, in a total volume of 0.5ml assay buffer containing various concentrations of drugs as required. [^{3}H]-FNZ was then added to a final concentration of 1nM and the samples incubated for a further 60 minutes at the required assay temperature. The binding reaction was halted by the addition of 2ml ice cold wash buffer followed by rapid filtration through Whatman GF-C filters using

a Brandel Cell Harvester. The filters were washed twice with 2ml aliquots of wash buffer before quantification of bound radioactivity by conventional liquid scintillation techniques. Non-specific binding was determined using 10μM cold FNZ and was generally in the region 5-10% of total bound. Data were fitted using the Inplot package (Graphpad Software) and are expressed as mean± SEM (n). Statistical comparisons are by Student's t-test.

Alphaxalone and propofol were initially dissolved in acetone which was present in all assay tubes at a concentration of 0.1%. All drugs and chemicals were obtained from Sigma with the exceptions of NaCl (BDH Analar grade), Alphaxalone which was a gift from Glaxo, and propofol which was donated by ICI.

RESULTS

The affinity of flunitrazepam at various temperatures was measured in buffer 1 by means of a competition assay, taking Keq as 1/Ki. Ki was derived from IC_{50} values by means of the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The van't Hoff plot under control conditions yielded a linear relationship for temperatures from 0-47°C. Other workers have observed a deviation from this relationship at temperatures of less than 10°C (Quast *et al*, 1982; Doble, 1983). To allow comparison with previous investigations we have restricted our analyses to temperatures of 10°C and greater. This yielded values for $\Delta H°$ of -38.1± 4.35 $kJ.mol^{-1}$ and for $\Delta S°$ of 27.8± 14.43 $J.K^{-1}.mol^{-1}$.

In the presence of 100μM alphaxalone the slope of the line was not significantly different yielding a $\Delta H°$ of -38.4± 3.40 $kJ.mol^{-1}$. The derived $\Delta S°$ from this line was 33.9± 11.23 $J.K^{-1}.mol^{-1}$. Due to the high error associated with extrapolating back to zero on a reciprocal scale, it is difficult to make meaningful comparisons of $\Delta S°$ values. However, the small-medium contribution of the entropy component to the total binding energy is in agreement with other workers (Quast *et al*, 1982; Kochman and Hirsch, 1982; Doble, 1983).

The potentiation of [^{3}H]-FNZ binding by propofol showed a similar temperature dependency profile to that by alphaxalone. The van't Hoff plot for FNZ binding in the presence of 300μM propofol yielded a $\Delta H°$ of -37.3± 5.3 $kJ.mol^{-1}$ and $\Delta S°$ of 35.68± 17.63 $J.K^{-1}.mol^{-1}$. The slope of the line was not significantly different from the control value.

In contrast to the results for alphaxalone and propofol, the potentiation of FNZ binding by pentobarbitone shows a distinct temperature dependency. Inclusion of 1mM pentobarbitone caused a significant increase in the enthalpy of the binding reaction to -57.04± 5.83 $kJ.mol^{-1}$. Pentobarbitone potentiation is therefore greater at low temperatures than at high. These data are illustrated in figure 5.1 and summarised in table 5.1.

Dose response curves for alphaxalone, propofol and pentobarbitone were compared at 4°C and 37°C (also in buffer 1). All three showed a significant increase in EC_{50} at 37°C compared with 4°C. These data are summarised in table 5.2.

At 4°C, in the absence of added salts (buffer 2), pentobarbitone and alphaxalone showed only a small potentiation of flunitrazepam binding. Addition of 200mM NaCl caused a significant decrease in the EC_{50} value for pentobarbitone. There also appeared to be a decrease in the EC_{50} for the steroid in the presence of NaCl but this was not significant at a confidence level of 95%. The effects of NaCl are shown in figure 5.2. In the presence of NaF, the EC_{50} value for pentobarbitone was not significantly different to that in the absence of added salts. No significant potentiation by alphaxalone was obtained in the presence of NaF. In contrast, KI at 200mM significantly decreased the EC_{50} values for both pentobarbitone and alphaxalone compared with both salt-free and NaCl values. We have previously reported similar data for propofol interactions at the $GABA_A$ receptor (Prince and Simmonds, 1992b). These data are summarised in table 5.3.

The effect of constant concentrations of the modulatory drugs upon dose response curves to NaCl were also examined. In the absence of added drugs in buffer 2, NaCl showed a dose dependent potentiation of [^{3}H]-FNZ binding with an EC_{50} of 36.14± 6.52mM (7). In the presence of 1mM pentobarbitone, the dose response curve for NaCl was significantly shifted to the left with an EC_{50} value of 19.71± 1.94mM (4) ($P < 0.05$ by Student's paired t-test). Alphaxalone at 10μM also caused a lowering of the EC_{50} value for NaCl to 20.9± 3.64mM (5) but this was not significant at the 95% confidence level ($0.05 < P < 0.1$). These data are illustrated in figure 5.3. In our previous study (Prince and Simmonds, 1992b) we have shown that propofol has a similar effect to pentobarbitone, 300μM propofol causing the NaCl dose response curve to shift leftwards to an EC_{50} value for NaCl of 12.80± 3.01mM (5) ($P < 0.05$ Student's paired t-test).

The potentiation due to the modulator was also examined by comparing the binding with the value in the absence of drug at the same concentration of NaCl. Whilst pentobarbitone and alphaxalone gave progressively greater potentiation as the concentration of NaCl was increased, the potentiation by propofol was not significantly altered. Dose response curves constructed from these data yielded EC_{50} values for NaCl of 9.4mM in the presence of 1mM pentobarbitone and 13.7mM in the presence of 10μM alphaxalone. These interactions are illustrated in figure 5.4.

Table 5.1. Thermodynamic parameters for the binding of [^{3}H]-flunitrazepam binding in 50mM Na^+/K^+ buffer pH 7.1 containing 200mM NaCl, in the presence of various modulators. The data are derived from linear regression and are expressed ± SEM. The slopes were compared using Student's t-test.

	$\Delta H°$ kJ.mol^{-1}	$\Delta S°$ J.K^{-1}.mol^{-1}
Control	-38.07± 4.35	27.81± 14.43
+1mM Pentobarbitone	-57.04± 5.83*	-29.24± 19.44
+100µM Alphaxalone	-38.36± 3.4	33.86± 11.32
+300µM Propofol	-37.31± 5.30	35.68± 17.63

* Significantly different from control ($P< 0.05$)

Table 5.2. EC_{50} values at 4°C and 37°C for pentobarbitone, propofol and alphaxalone.

Data are expressed as mean ± SEM (n).

	EC_{50} (μM) 4°C	EC_{50} (μM) 37°C
Propofol	12.72± 6.42 (3)	59.29± 12.26 (3)*
Pentobarbitone	201± 35 (8)	347± 41 (6)*
Alphaxalone	1.36± 0.25 (6)	2.998± 0.53 (5)*

*Significantly different to 4°C by Student's t-test ($P< 0.05$)

Table 5.3. EC_{50} values at 4°C for pentobarbitone (PB), alphaxalone (alphax.) and propofol in the presence of 10mM Tris-citrate pH 7.4, and Tris-citrate + various salts. Data are the mean ± SEM of (n) experiments.

EC_{50} (μM)	Control Tris-Citrate	+200mM NaCl	+200mM NaF	+200mM KI
Propofol†	147± 29 (4)	23.8± 6.7 (3)*	67± 13.3 (3)*	9.4± 1.8 (3)**
PB	490± 98 (6)	157± 34 (4)*	424± 90 (3)	74.6± 8.4 (3)**
Alphax.	1.38± 0.2 (3)	0.93± 0.19 (3)	ND	0.43± 0.13 (4)*

*Significantly different from control ($P<0.05$)

**Significantly different from control and from NaCl values ($P<0.05$)

ND: lack of potentiation precluded determination

†Data taken from Prince and Simmonds (1992b)

Figure 5.1. Van't Hoff plots for the binding of [^{3}H]-flunitrazepam A) control; B) + 100μM alphaxalone; C) + 1mM pentobarbitone and D) + 300μM propofol. Keq values are derived from competition data with Keq taken as 1/Ki. Ki was derived from IC50 values by the Cheng-Prusoff equation. In B, C and D the derived control line is included for comparison.

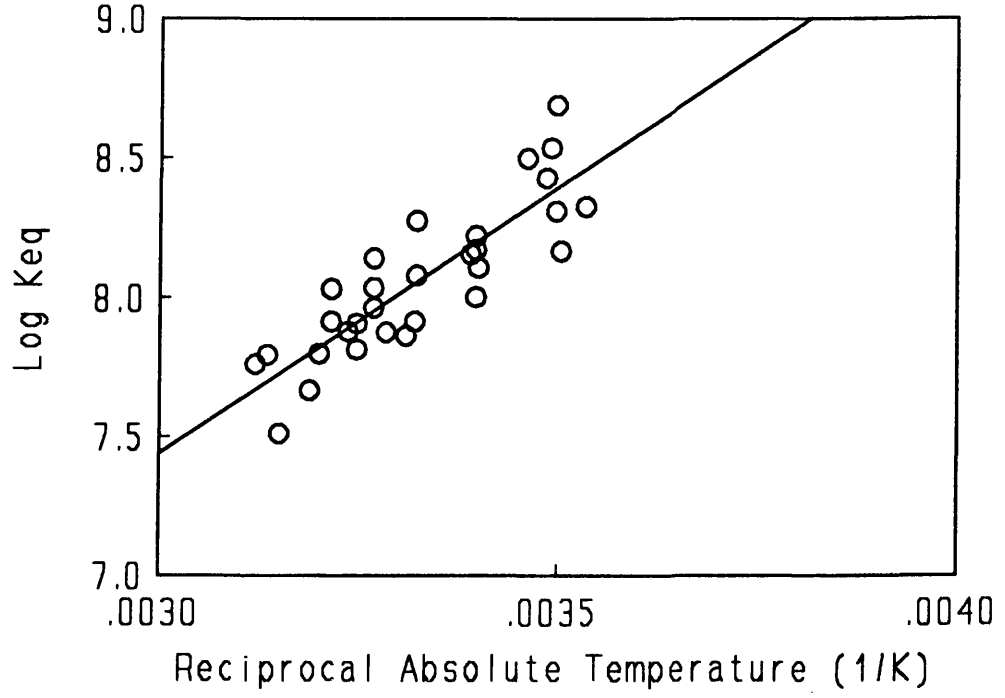


Fig. 5.1A

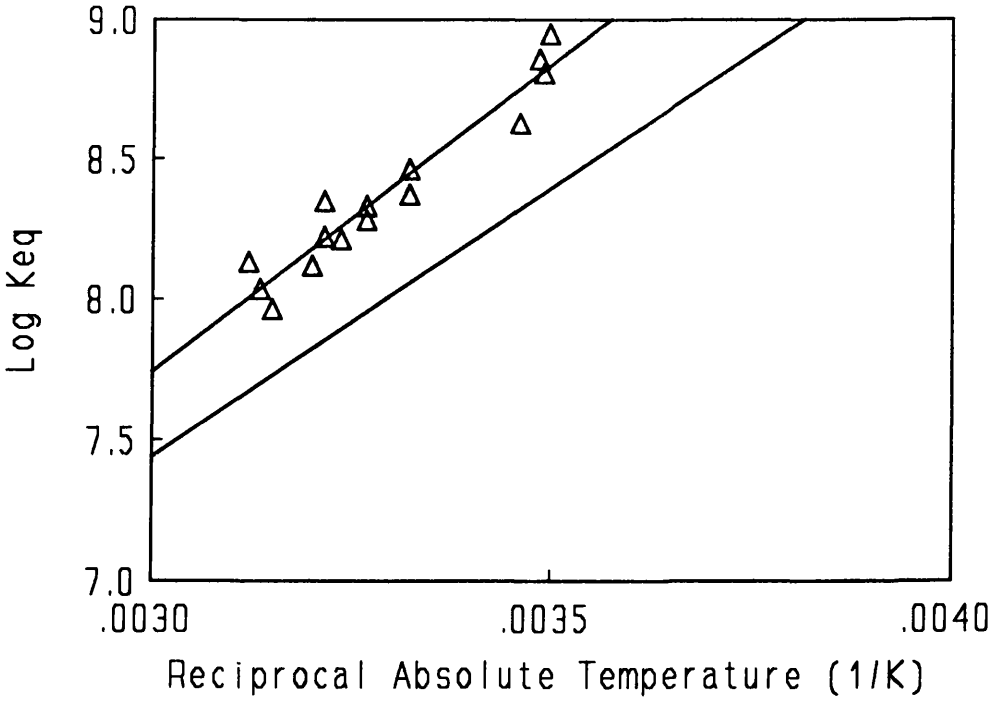


Fig. 5.1B

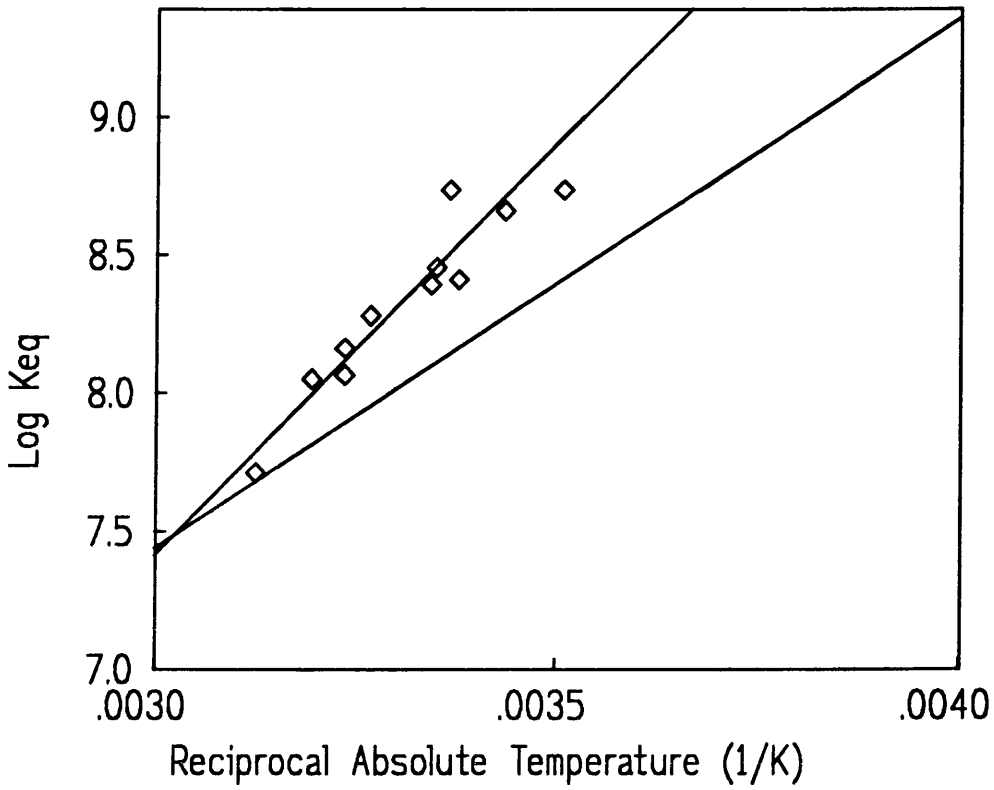


Fig. 5.1C

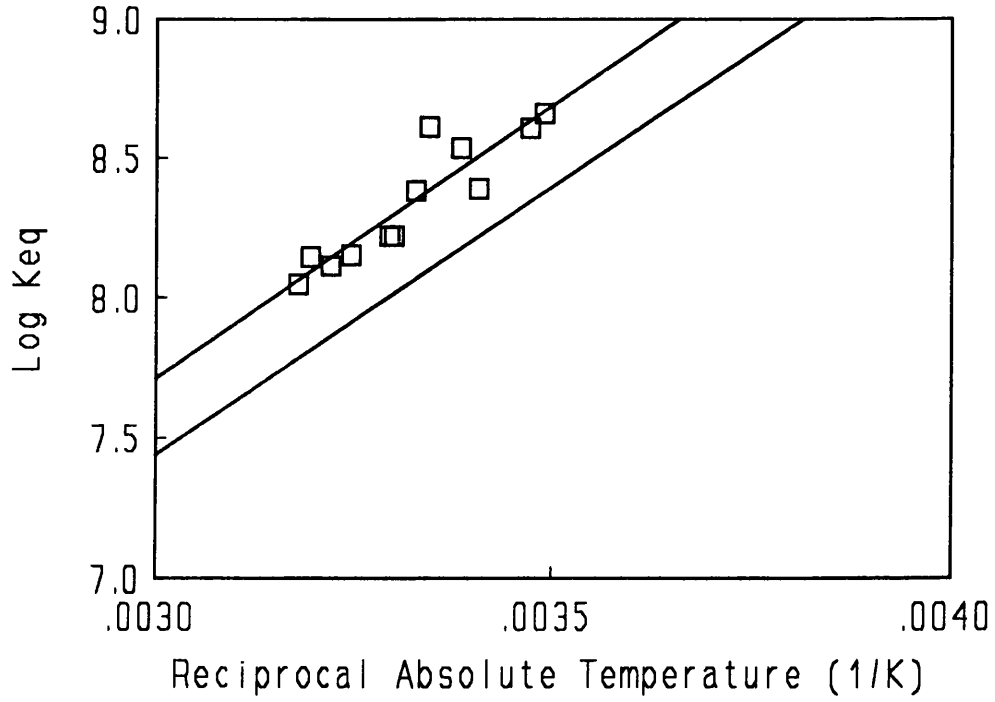


Fig. 5.1D

Figure 5.2. Dose response curves for A) alphaxalone, B) pentobarbitone and C) propofol in 10mM Tris-citrate buffer (open symbols) and in 10mM Tris-citrate + 200mM NaCl (filled symbols). The data are the mean ± SEM of triplicate values from representative experiments.

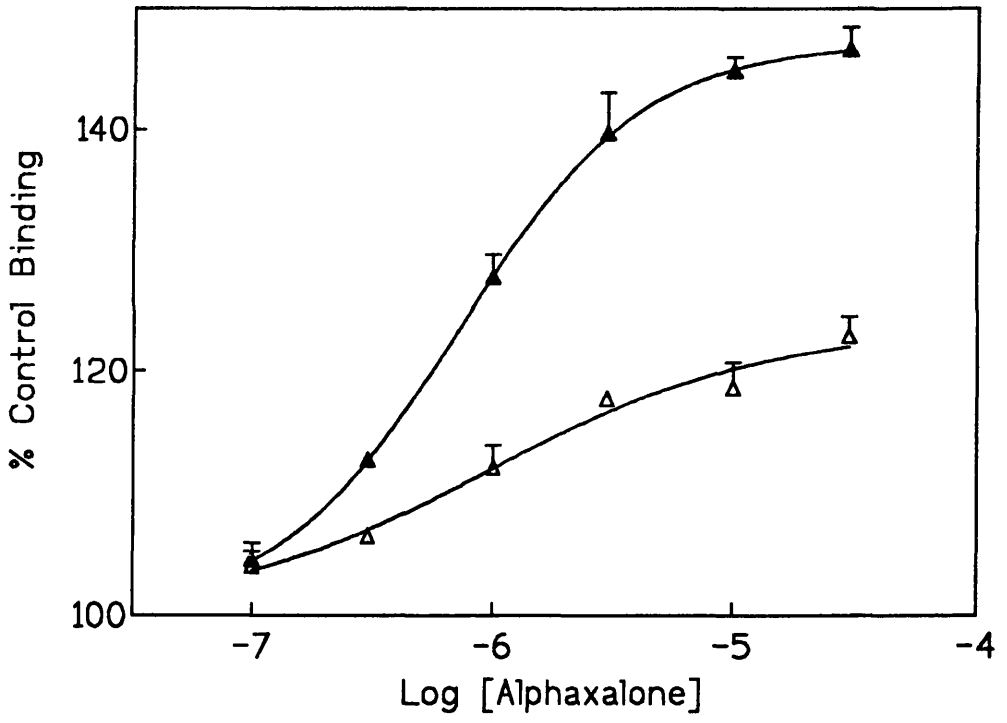


Fig. 5.2A

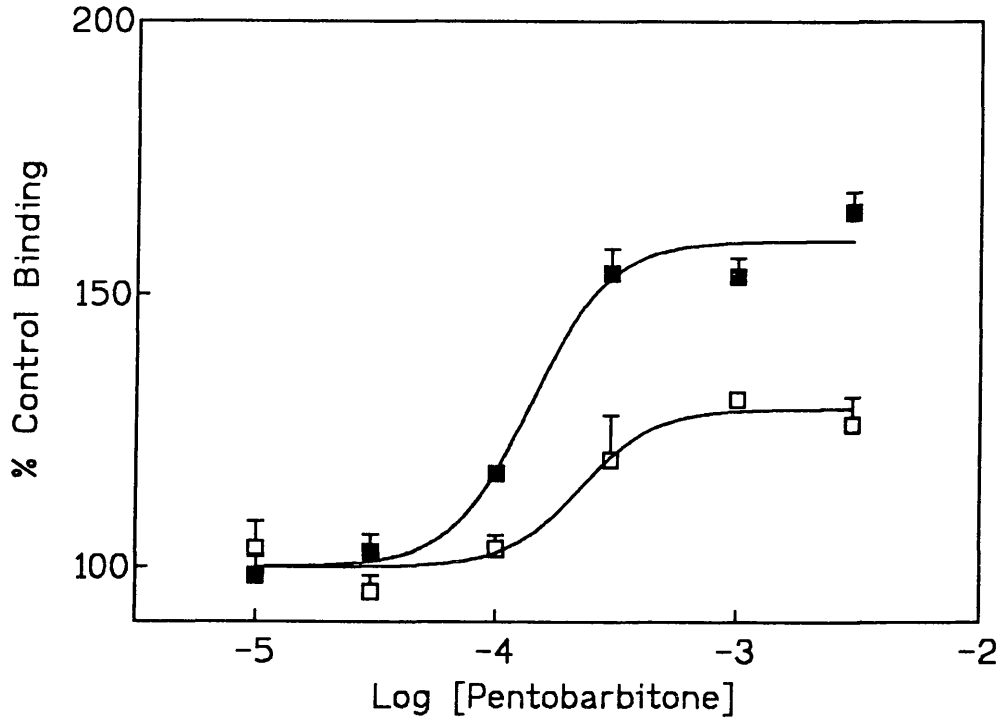


Fig. 5.2B

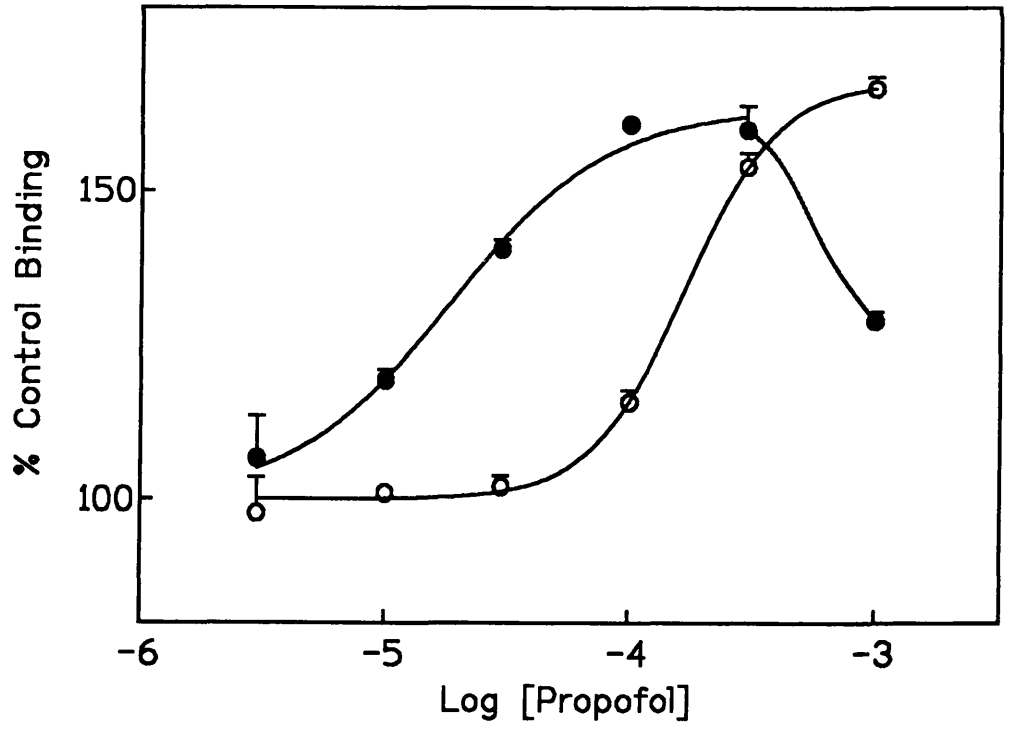


Fig. 5.2C

Figure 5.3. The potentiation of 1nM [^{3}H]-flunitrazepam binding by NaCl in 10mM Tris-citrate buffer in the presence and absence of various drugs. Data are the mean ± SEM of triplicate values from a representative experiment and are expressed relative to the basal binding in the absence of added salts or drugs. ◇, control; □, +1mM pentobarbitone; △,+10μM alphaxalone; ○, +300μM propofol.

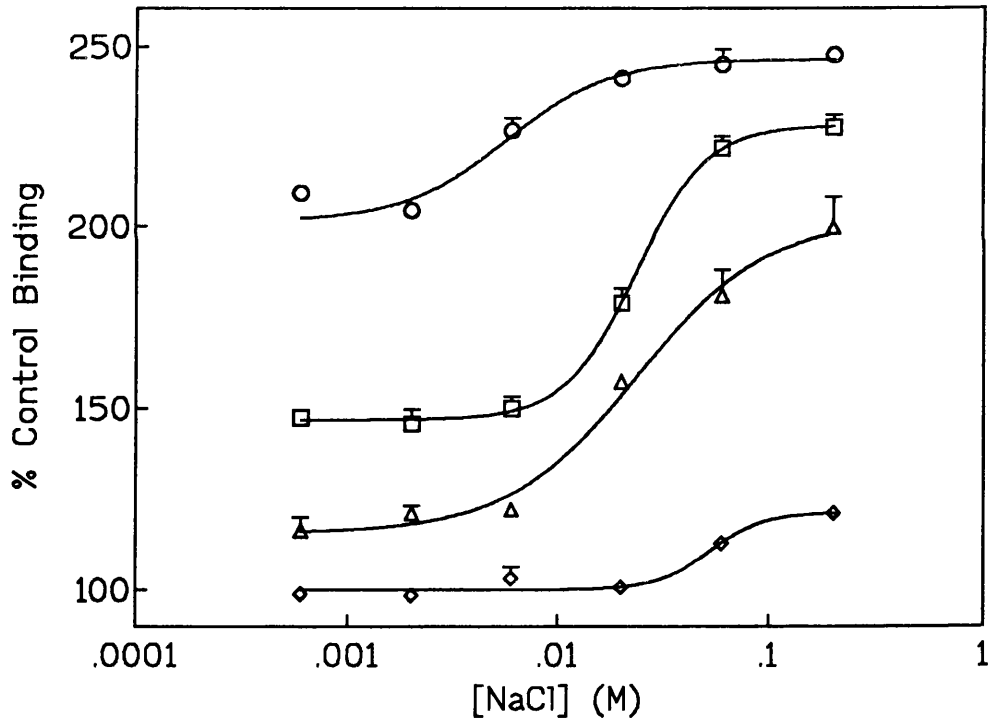
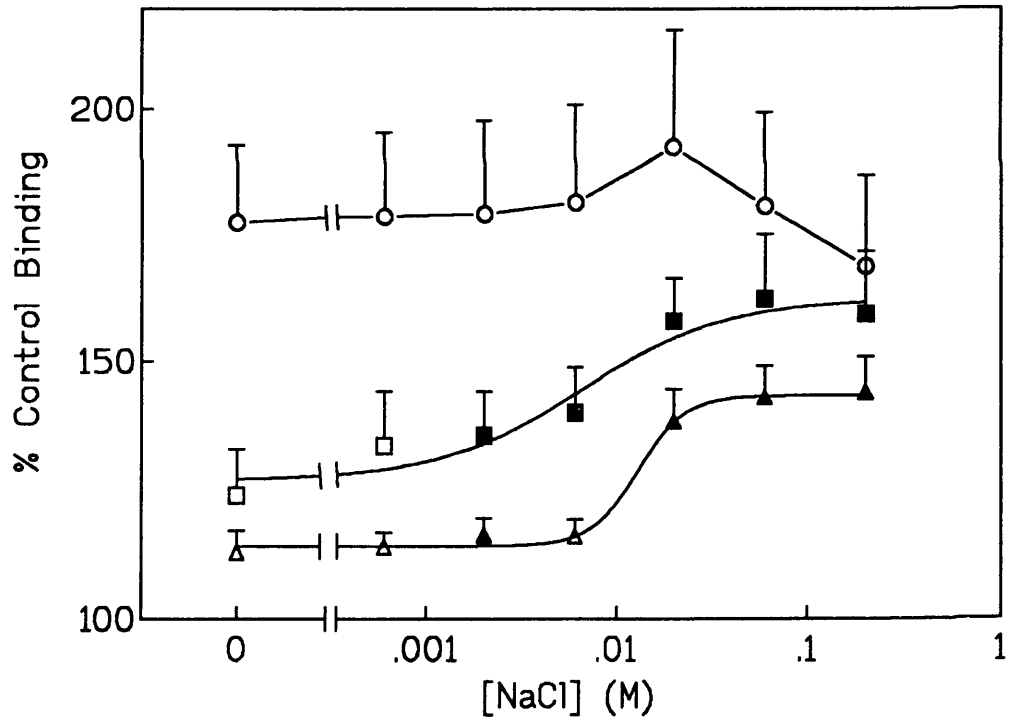


Figure 5.4. The potentiation of 1nM [^{3}H]-flunitrazepam binding by 1mM pentobarbitone (squares); 10μM alphaxalone (triangles) and 300μM propofol (circles) in the presence of varying concentrations of NaCl. Closed symbols indicate values which are significantly different from the potentiation at zero NaCl by Student's t-test ($P< 0.05$). The data are presented as the mean of 3-4 experiments ± SEM and are expressed relative to the binding in the absence of drugs.



DISCUSSION

The van't Hoff plot for the binding of [^{3}H]-FNZ indicates that the association of this ligand with its receptor is largely enthalpy driven with only a small entropy component. The calculated parameters are very similar to those obtained by Kochman and Hirsch (1982). However, the value of $\Delta H°$ derived from our present data is somewhat higher than that obtained by Quast *et al* (1982) and Doble (1983). This may be due to our failure to observe the entropy driven component of the [^{3}H]-FNZ van't Hoff plot which these authors observed at lower temperatures. This would have the effect of skewing the derived $\Delta H°$ to a more positive value.

In the presence of alphaxalone or propofol, the enthalpy component of the binding energy does not change significantly suggesting that an increase in entropy is responsible for the actions of these modulators. The effect of this is that the efficacy of propofol and alphaxalone is temperature insensitive. Previous studies have suggested that certain modulators of $GABA_A$ ligand binding are more efficacious at higher temperatures (Quast and Brenner, 1983; Wong and Iversen, 1985; McQuilkin and Harris, 1989; Majewska, 1988). However, many of these studies have utilised a fixed concentration of ligand without taking into account the change in the affinity of that ligand with temperature. In contrast to alphaxalone and propofol, the effect of pentobarbitone appears to be via an increase in the contribution of the enthalpic component to the binding energy. This means that pentobarbitone is less efficacious at high temperatures than at low and suggests that the mechanism of action of pentobarbitone is different to those of alphaxalone and propofol. A similar difference

in the temperature dependency of pentobarbitone and neurosteroid effects has been observed for the potentiation of muscimol (Yau *et al*, 1990).

The present results tend to rule out the possibility of membrane fluidization as a mechanism of action for both propofol and alphaxalone in modulating flunitrazepam binding. If fluidity changes were the mode of action of these drugs then it might be expected that temperature-induced fluidization would decrease their efficacy. The results for pentobarbitone, however, are consistent with such a mechanism although other data raise doubts about this interpretation. Thus, whilst it has been demonstrated that the membrane perturbing effect of pentobarbitone is dependent upon the initial fluidity state of the membranes used, the peturbation being lower at higher temperatures (Harris and Schroeder, 1982), no discrimination was found between convulsant and anticonvulsant barbiturates, or between barbiturate enantiomers which differ in pharmacological potency. In addition, it has been demonstrated that membrane fluidization by A_2C (2-[2-methoxyethoxy]-ethyl 8-[cis-2-n-octylcyclopropyl]-octanoate) does not enhance muscimol-stimulated $^{36}Cl^-$ flux nor does it produce anaesthesia (Buck *et al*, 1989). Conversely, the effect of pentobarbitone in causing an enthalpy-induced change in slope of the van't Hoff plot for FNZ binding is similar to that noted for chloride ions, which are clearly not lipid soluble perturbants (Doble, 1983). Therefore the temperature dependence of pentobarbitone's interactions cannot be interpreted unequivocally in terms of membrane perturbation.

Previous studies have demonstrated different sites of action of barbiturates and neurosteroids (Turner *et al*, 1989), and propofol and the neurosteroids (Concas *et al*,

1991a). However, in our previous study (Prince and Simmonds, 1992b) we were unable to rule out the possibility that propofol and pentobarbitone act at the same site. The present results suggest that a common site of action for these two compounds is unlikely and that our previously observed reduction of pentobarbitone potency by propofol is allosteric rather than isosteric.

The temperature dependence of the association of alphaxalone, propofol and pentobarbitone, as demonstrated by the increase in EC_{50} from 4°C to 37°C, indicates that the binding of these modulators with the $GABA_A$ receptor has a significant enthalpic component. This suggests that the binding of these drugs is not simply a hydrophobic association with the receptor with a concomitant increase in entropy. As discussed by Quast *et al* (1982), this may reflect an initial hydrophobic association with entropy gain followed by an entropy decrease brought about by conformational changes in the receptor or in the membrane which expose previously hidden domains and force an ordering of the solvent. The enthalpic component must arise by the formation of favourable interactions which may be between the receptor and the ligand, the receptor and the modulator, receptor subunits, the receptor and the membrane or the modulator and the membrane or indeed a combination of these.

As has been demonstrated in other studies (Leeb-Lundberg *et al*, 1980), both the EC_{50} and maximal potentiation (Emax) for pentobarbitone are dependent upon the concentration and type of anion present. In the present study similar results have been obtained for alphaxalone but in the case of the neurosteroid, the effect of increased chloride ion concentration appears to cause a much more marked shift in the Emax

than in the EC_{50}. However, the decrease in EC_{50} in the presence of iodide and the lack of potentiation in the presence of NaF, suggest that a similar spectrum of facilitatory anions exist for alphaxalone potentiation as for pentobarbitone. Propofol, as we have previously demonstrated (Prince and Simmonds, 1992b), also exhibits a barbiturate like anion dependency for EC_{50} but also shows a non-specific decrease in EC_{50} in the presence of impermeable salts. In addition to this, the maximal potentiation obtained with propofol is not significantly different in the presence or absence of chloride. However, at concentrations of propofol > 300μM, a chloride dependent decrease in potentiation was observed leading to a bell shaped dose response curve. A wide variety of $GABA_A$ receptor ligands have now been demonstrated to display anion dependency of their interactions with the receptor. The binding of benzodiazepine agonists and inverse agonists (Evonuik and Skolnick, 1988), and of the convulsant TBPS (t-butylbicyclophosphorothionate) (Marvizon and Skolnick, 1988), as well as the interactions of alphaxalone, pentobarbitone and propofol demonstrated in this study, are all modulated by anions whose effects are correlated with their permeabilities at the $GABA_A$ linked chloride channel. This suggests that anion dependency may be a general characteristic of the allosteric interactions of this receptor.

In conclusion, we have demonstrated a difference in the temperature dependency of flunitrazepam binding in the presence of pentobarbitone compared with that in the presence of alphaxalone or propofol. This, taken together with differences in the anion dependency of these drugs suggests distinct *sites* of action for each drug and indicates

that pentobarbitone has a different *mode* of interaction with the $GABA_A$ receptor to that of alphaxalone or propofol.

CHAPTER 6

Steroid modulation of the strychnine sensitive glycine receptor

The text and data that follow have been published:

Prince, R.J. and Simmonds, M.A. (1992) Steroid modulation of the strychnine-sensitive glycine receptor. Neuropharmacol. 31, 201-205

SUMMARY

Electrophysiological responses to glycine (0.25-5mM) were obtained on preparations of rat optic nerve. The glycine log dose-response curve was shifted to the left by a factor of 2 by 1μM 20α-dihydrocortisol and by a factor of about 1.5 by 1μM α-cortol and 10μM hydrocortisone. A similar effect was obtained with 100μM chlormethiazole, but the GABA potentiating steroid alphaxalone (1μM) was ineffective on glycine responses. 20α-dihydrocortisol and chlormethiazole also appeared to increase the antagonistic potency of strychnine against glycine. These observations suggest that the active steroids and chlormethiazole increase the functional interaction of both glycine and strychnine with the glycine-gated chloride channel.

INTRODUCTION

Modulation of the $GABA_A$ receptor by certain steroids is now a pharmacologically well-established phenomenon showing a distinct structure activity relationship (Harrison and Simmonds, 1984; Turner and Simmonds, 1989; Morrow, Pace, Purdy and Paul, 1990). Progesterone metabolites such as the pregnanolones have been demonstrated to exert potentiating effects upon the $GABA_A$ receptor, similar to those of the barbiturates but acting at a distinct site (Turner, Ransom, Yang and Olsen, 1989) whereas corticosteroids such as hydrocortisone do not potentiate GABA but have been shown to potentiate the binding of TBPS (t-butylbicyclophosphorothionate), a GABA antagonist (Majewska, 1987). Recently, antagonistic effects of certain progesterone metabolites have been noted on the strychnine-sensitive glycine receptor (Wu, Gibbs, and Farb, 1990). In the light of the considerable homology shown in primary sequence and probable tertiary structure between the subunits of the $GABA_A$ receptor and those of the strychnine-sensitive glycine receptor (Grenninglogh, Rienitz, Schmitt, Methfessel, Zensen, Beyreuther, Gundelfinger and Betz, 1987; Schofield, Darlison, Fujita, Rodriguez, Burt, Stephenson, Rhee, Ramachandran, Glencourse, Reale, Seeburg and Barnard, 1987), we have undertaken an electro-physiological examination of the effects of some corticosteroids on the glycine receptor.

MATERIALS AND METHODS

Male Wistar rats (150-200g) were sacrificed by decapitation with prior stunning. Optic nerves were rapidly dissected out and a 5-10mm section including the optic chiasma was placed in a two chamber "grease-gap" perfusion system (Simmonds, 1983; Turner and Simmonds, 1989). The optic chiasma was placed on the "active" side of the apparatus (that to which depolarising drugs were applied). The potential difference across the grease-gap was measured using a pair of silver/silver chloride electrodes connected to a DC amplifier and a chart recorder. Tissues were superfused with Krebs solution gassed with 95% O_2, 5% CO_2 and containing 0.1% acetone (steroid vehicle), until a stable base line was obtained (approximately 1 hour). Doses of glycine (0.25-5mM) were then applied by direct addition of 5 - 10µl of concentrated stock solutions to the active compartment (volume 1.8ml) with the superfusion system halted. Mixing was facilitated by bubbling the active chamber with 95% O_2, 5% CO_2. The response was allowed to develop for 3 minutes after which time superfusion was restarted and the glycine washed out. Halting the flow did not, of itself, cause a consistent D.C. shift of the base line. A 12 minute recovery time was allowed between doses. When consistent control responses had been obtained, various drugs were added to the superfusion medium and the glycine doses repeated. Steroid effects began to develop after 15 minutes and the full effect was generally seen within 30 minutes. The parallel shift of the dose response curve elicited by the various modulators was measured and is expressed as the log dose ratio for equivalent size responses, which were measured at their peak amplitude. In the experiments with low concentrations of strychnine, which produced a non-parallel shift to the left, the percent increase in the

response to a dose of 2.5mM glycine was measured. All results are expressed as mean ± SEM (n).

All drugs were obtained from Sigma, with the exception of alphaxalone which was a gift from Glaxo, and chlormethiazole which was donated by Astra. Steroids were initially dissolved in acetone and then diluted to give an acetone concentration of 0.1% in the perfusion medium.

A short series of experiments was also performed on the rat cuneate nucleus slice, with the method described by Turner and Simmonds (1989), to determine the effect of 20α-dihydrocortisol on responses of the $GABA_A$ receptor to muscimol.

RESULTS

Potentiation of glycine responses

The corticosteroids 20α-dihydrocortisol (4-pregnene-11β,17α,20α,21-tetrol-3-one; DHC) and α-cortol (5β-pregnane-3α,11β,17α,20α,21-pentol) at a concentration of 1μM, caused marked potentiations of glycine evoked depolarisations, giving log shifts of -0.31± 0.05 (8) ($P < 0.01$) and -0.164± 0.06 (7) ($P< 0.05$) respectively (eg. figure 6.1). Hydrocortisone (4-pregnene-11β,17α,21-triol-3,20-dione) also caused a potentiating effect at 10μM giving a log shift of -0.18± 0.05 (5) ($P< 0.05$). In 2 out of 3 experiments, progesterone at 10μM showed a potentiating effect on glycine responses with a mean log shift of -0.13±0.07 (3). The established glycine modulator, chlormethiazole, (Gent and Wacey, 1983) also showed a potentiation of depolarisations but 100μM was required to give a log shift of -0.26± 0.06 (5) ($P< 0.05$). In contrast,

the GABA modulating steroid alphaxalone, was without effect at 1μM, a concentration at which it caused clear potentiation at the $GABA_A$ receptor. Table 6.1 is a summary of these data.

In the experiments on muscimol responses in the cuneate nucleus preparation, 1μM DHC had no effect with a log. shift of the muscimol dose-response curve of -0.06± 0.04 (7). In two of the experiments, subsequent exposure to 1μM alphaxalone caused log. shifts of -0.18 and -0.35, which were similar to those previously reported for this steroid (Turner and Simmonds, 1989).

Interaction with strychnine

The major pharmacological feature of the glycine gated chloride channel is the antagonism of glycine by the alkaloid strychnine. In this study 1μM strychnine gave a clear rightward shift of the glycine dose response curve with a log shift of 0.234± 0.017 (5) ($P< 0.01$). It was expected that the application of DHC to strychnine inhibited tissues would result in the relief of this inhibition through the previously described potentiation of glycine. However, it was found that the co-application of strychnine and DHC resulted in a significant ($P < 0.05$) further shift to the right compared with strychnine alone (total log shift of 0.35± 0.04 (6) eg. figure 6.2a).

At low concentrations strychnine, has been demonstrated to have a small *potentiating* effect upon responses to high doses of glycine (Simmonds, 1983; Long, Evans and Krijzer, 1989). A similar effect was seen in the present study, with 0.1μM strychnine potentiating responses to 2.5mM glycine in 11 out 15 experiments (mean potentiation

10.5± 4.1% (15) (P< 0.05)). In 10 of these experiments, 1μM DHC was added to the strychnine and this reduced the responses by 3.9± 1.5% (P< 0.05).

To determine whether chlormethiazole would also interact with strychnine, 100μM chlormethiazole was added to 1μM strychnine. There was no further shift of the glycine dose response curve (-0.04± 0.04 (4)) in either direction.

Effect of corticosteroids on fade of glycinergic responses

In addition to the potentiation of the responses to glycine already noted, a further phenomenon occurred in the presence of corticosteroids. This was the appearance of a marked decay of the glycine response from its peak before the commencement of the washout phase. This was particularly noticeable with hydrocortisone as illustrated in figure 6.3. The degree of fade tended to increase with the dose of glycine, even after the plateau in the dose response curve had been reached. This effect also sometimes occurred in the absence of steroid at near maximal concentrations of glycine. However, for similar sized responses in the absence and presence of steroid, fade was generally much more pronounced when steroid was present.

Table 6.1. Log shifts of the dose response curve for glycine

Modulator	Mean ± SEM
20α-Dihydrocortisol (1μM)	-0.31 ± 0.05 (8)*
α-Cortol (1μM)	-0.16 ± 0.06 (7)*
Hydrocortisone (0.1μM)	-0.08 ± 0.04 (3)
Hydrocortisone (1μM)	-0.05 ± 0.11 (6)
Hydrocortisone (10μM)	-0.18 ± 0.05 (5)*
Alphaxalone (1μM)	-0.04 ± 0.04 (4)
Chlormethiazole (100μM)	-0.26 ± 0.06 (5)*
Strychnine (1μM)	0.23 ± 0.02 (5)*
Strychnine (1μM) + DHC (1μM)	0.35 ± 0.04 (6)**

*significantly different from control ($P<0.05$)

**significantly different from strychnine alone ($P<0.05$)

Figure 6.1: (A) Potentiation of responses to glycine by 1μM 20α-dihydrocortisol. Sample responses to 1.1 and 2.5mM glycine for control (CON) and in the presence of DHC from a typical experiment. Applications of glycine (3 minutes) are represented by the solid black bars, during which time the superfusion was halted. (B) Typical dose response curve to glycine in the presence (+) and absence (O) of 1μM DHC. Data are taken from the same experiment as (A).

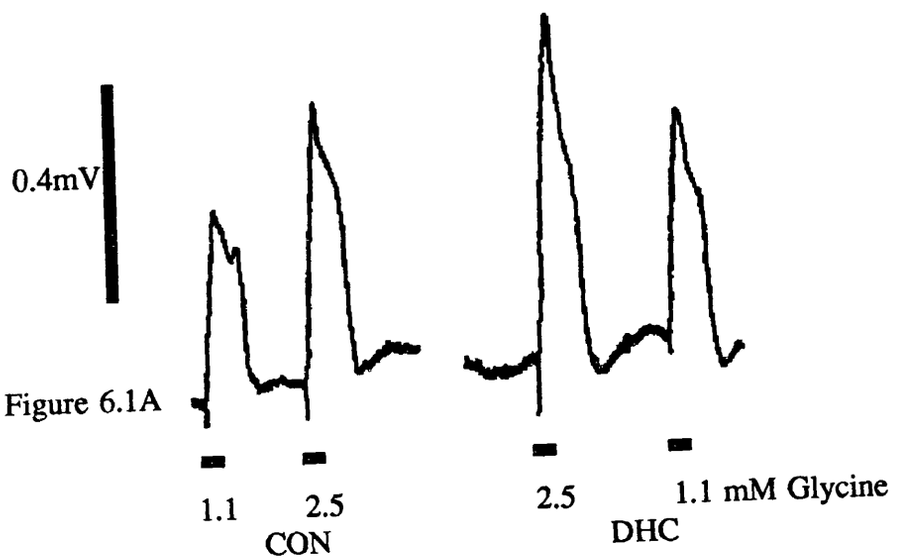


Figure 6.1A

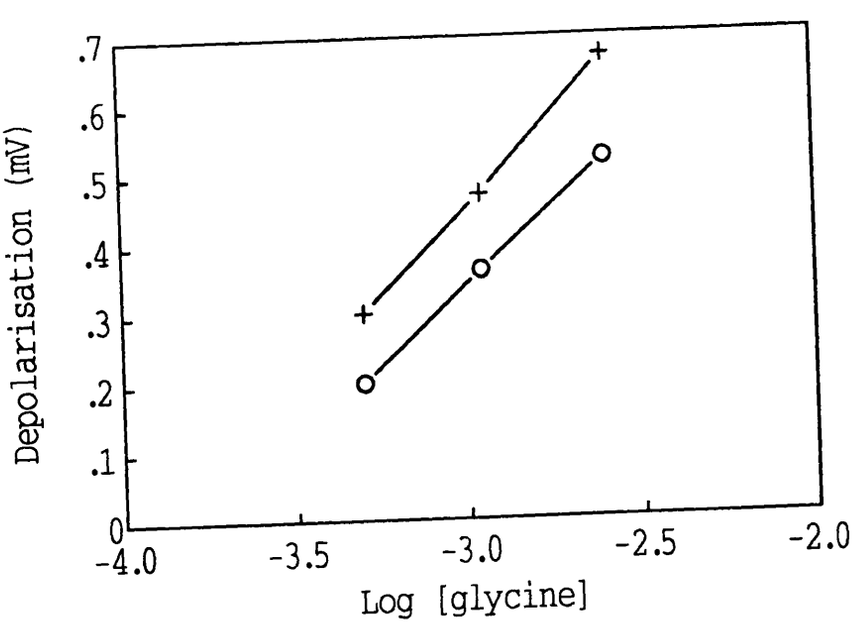
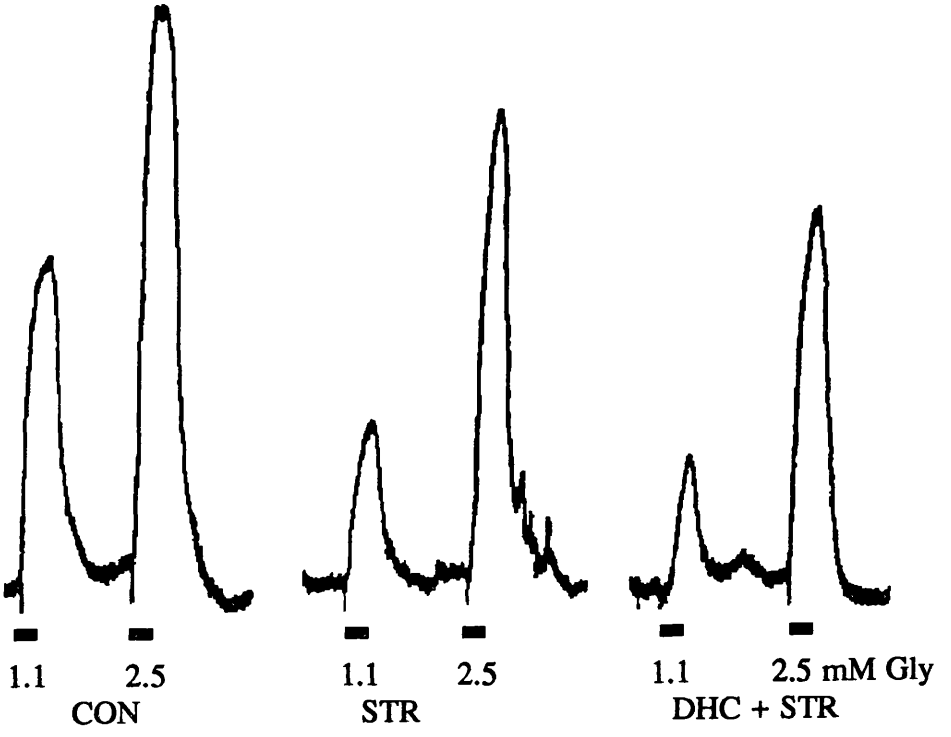


Figure 6.1B

Figure 6.2: (A) Antagonism of responses to glycine by 1μM strychnine (STR) and the effect of co-application of 1μM DHC (DHC) compared with control responses (CON); and (B) the interaction of 1μM DHC (DHC) with 0.1μM strychnine (STR). Applications of 1.1 and 2.5mM glycine (3 minutes) are represented by the solid black bars, during which time the superfusion was halted.

Fig.
6.2A



0.4mV

Fig.
6.2B

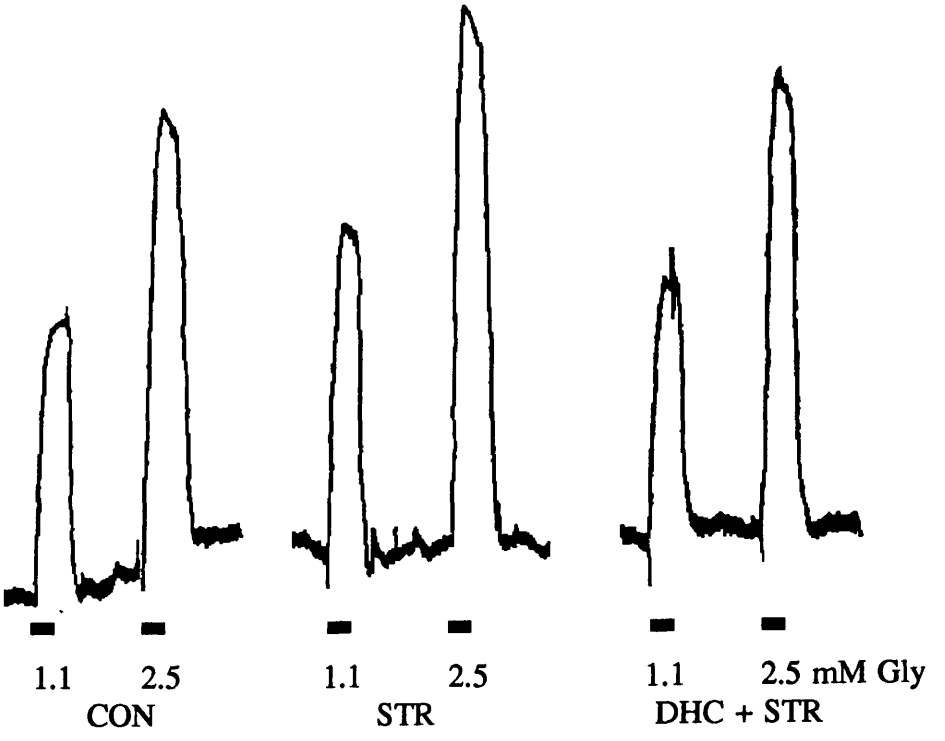
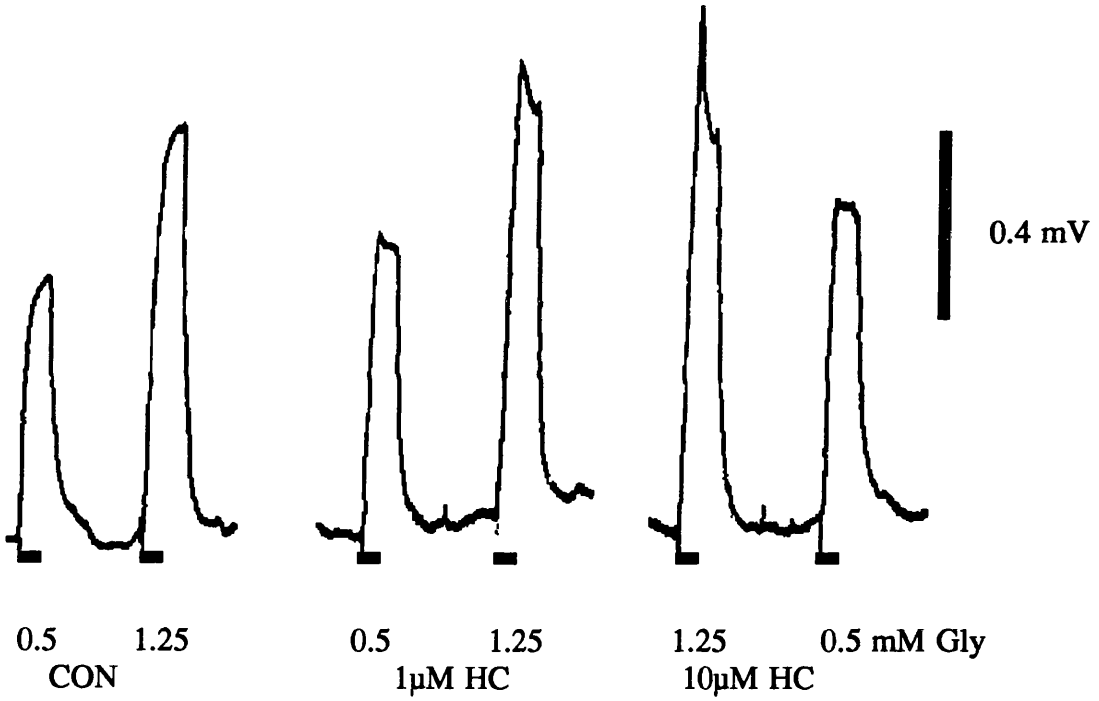


Figure 6.3: Increase in amplitude and decay of responses to 0.5 and 1.25mM glycine in the presence of 1 and 10μM hydrocortisone (HC) compared with control (CON). Applications of glycine (3 minutes) are represented by the solid black bars, during which time the superfusion was halted.



DISCUSSION

The results show a clear potentiation of responses to glycine at the strychnine-sensitive glycine receptor by certain corticosteroids. Previous to this study only chlormethiazole had been shown to enhance glycine responses (Gent and Wacey, 1983; Harrison and Simmonds, 1983). The structure activity relationship for this effect of the steroids is clearly different from that for potentiation of the $GABA_A$ receptor. Thus, alphaxalone is amongst the most potent GABA potentiators but in the present experiments was inactive on glycine, whereas DHC was inactive on the $GABA_A$ receptor in the cuneate nucleus slice, but clearly potentiated glycine. Whereas, for GABA potentiation, a 3α-hydroxy substituent and a 20-one substituent confer higher potency than a 20-hydroxy (Harrison, Majewska, Harrington and Barker, 1987), for glycine potentiation hydroxyls at C20 and C21 appear to be favourable. The polarities of alphaxalone and DHC would indicate that they might orientate themselves in the membrane differently: both would lie between the fatty acid tails of the phospholipids but alphaxalone with the A-ring and DHC with the D-ring, towards the polar surface. Interestingly, one of the steroids shown here to potentiate glycine responses, hydrocortisone, has been demonstrated to have antagonistic effects on GABA receptors (Majewska, 1987). A differential modulation of the two receptors by progesterone metabolites in cultured chick spinal neurones has also been demonstrated (Wu *et al*, 1990). However, the latter study indicates that progesterone is antagonistic towards glycine responses. This is contrary to our own finding that progesterone (1μM) gave a small enhancement of glycine induced depolarisations. Whether this is due to the much higher doses utilised by Wu *et al* (up to 100μM) or due to differences in the

tissue utilised (recent studies have indicated heterogeneity of glycine gated chloride channels (Betz, 1990)) is not known.

High doses of steroids such as alphaxalone have been shown to be capable of an antagonistic effect upon GABA responses and this has been interpreted as being due to an enhancement of desensitisation of the receptor (Simmonds and Pettey, 1990). The fade of the glycine responses noted in the present study, which was increased by the addition of steroid, may be due to a similar mechanism. An alternative explanation would be that the steroid is potentiating glycine uptake. However, this is unlikely as the fade continued to increase after the dose response curve plateaued.

Steroid actions upon the $GABA_A$ protein have been shown to be mediated through direct, cell membrane linked effects upon the receptor rather than via the cell nucleus (Belelli, Lan and Gee, 1990). As the optic nerve preparation used in this study does not contain neuronal cell bodies, this suggests a direct interaction of steroids with the glycine receptor also. The interaction of DHC and strychnine seems to indicate that not only does the steroid potentiate the action of glycine but also that of the competitive antagonist strychnine. An enhanced potency of strychnine might also be expected to convert the potentiation of glycine by low doses of strychnine to an antagonism of glycine, such as is normally seen with higher doses of strychnine and this was indeed observed. Likewise, an enhanced potency of strychnine by chlormethiazole might also explain the apparent lack of a glycine potentiating effect of chlormethiazole on responses already antagonised by strychnine. In this case, glycine potentiation by chlormethiazole and enhanced antagonism by strychnine may

have had equal and opposite effects. When these types of interaction are added to the possibility of increased desensitization of responses, it becomes difficult to predict the direction and magnitude of the shift in the glycine dose-response relationship which would occur with any particular combination of strychnine and steroid. Overall, however, we interpret these interactions as the steroid or chlormethiazole facilitating the functional interaction of both strychnine and glycine with the glycine-gated chloride channel.

EXPERIMENTS ON THE BINDING OF [^{3}H]-STRYCHNINE

A short series of experiments was also performed in an attempt to correlate binding data with that obtained from electrophysiological experiments. The binding of [^{3}H]-strychnine to rat spinal cord and brain stem membranes was examined as described previously (chapter 2).

RESULTS

In the absence of DHC, strychnine bound to spinal cord/brain stem membranes (SCM) with a Ki of 4.39± 0.51nM (7). The binding of strychnine was not significantly affected by the presence of DHC at concentrations of 0.01 - 1μM (figure 6.4). In addition, no significant difference was seen in the affinity of strychnine in the presence of 0.01μM DHC (Ki: 5.27± 1.56nM (3)) nor 1.0μM DHC (Ki:4.97± 0.9nM (3)). Glycine displaced strychnine with a Ki of 11.35± 0.92μM (15). Addition of DHC resulted in a significant log-shift in the displacement curve of -0.16± 0.04 (6) ($P< 0.05$ by Student's paired t-test) at 0.01μM DHC and in log-shifts of -0.273± 0.125 (4) and -0.07± 0.05 (5) for 0.1μM and 1.0μM DHC respectively, neither of which were significant at a confidence interval of 95%.

Figure 6.4. Action of DHC upon binding of 2nM [^{3}H]-strychnine to rat spinal cord and brain stem membranes. Data are expressed relative to the binding in the absence of steroid and are the mean ± SEM of 3-6 experiments.

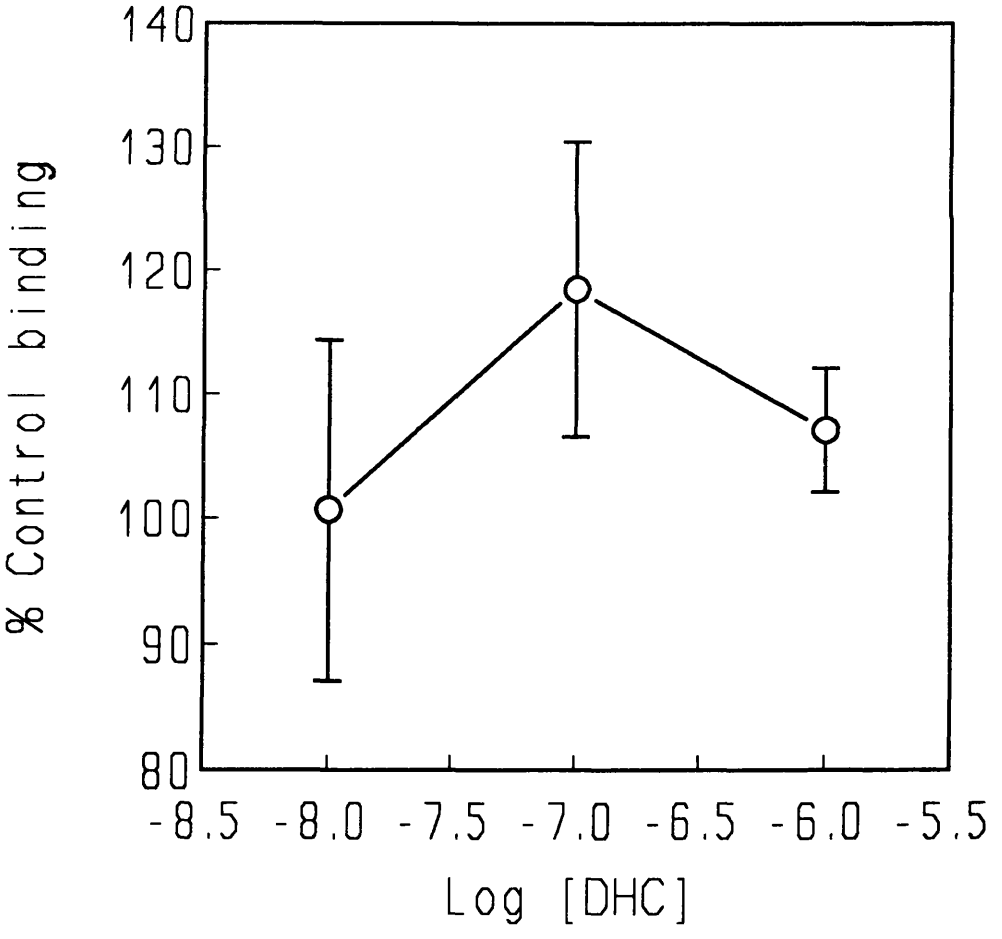


Figure 6.4

DISCUSSION

Whilst the electrophysiological data presented in this chapter clearly show an effect of certain corticosteroids upon the strychnine-sensitive glycine receptor (ssGR), the data from binding studies is more difficult to interpret. No significant effect of DHC, the most potent of the steroids in the electrophysiological study, was seen upon the binding of [^{3}H]-strychnine and only a small effect upon the affinity of glycine was noted, this being significant only at the lowest concentration of DHC tested (10nM). As the membrane preparation used is likely to contain a high concentration of nerve terminals, there is probably a high concentration of endogenous glycine present. If the affinity of strychnine and glycine were similarly affected by the addition of DHC, the increase in affinity of strychnine would be masked by the simultaneous increase in affinity of glycine, a competitive antagonist. This mechanism, however, depends upon the relative concentration of glycine in the incubation mixture.

$$B = Bm.\frac{L}{L + Kd\left(1 + \frac{I}{Ki}\right)}$$

As can be seen from the above equation, where B is bound activity and Bm is maximal binding, if glycine (I) is present at its Ki value, the apparent affinity of strychnine will change by only 25%, when the Ki for glycine and true Kd for strychnine (L) are decreased by a factor of 2. Exogenous glycine would, in these circumstances, also show an increase in affinity, but this too would be smaller than

in the absence of endogenous glycine. This possibility, together with that of concentration dependent differences in effect, make these data very difficult to interpret. However, another possibility is that there is a difference in the steroid sensitivity of the spinal cord and optic nerve receptors. Subunit dependent differences in modulation by pregnane steroids have been noted for the $GABA_A$ receptor (Zaman *et al*, 1992; Shingai *et al*, 1991). Given the degree of homology between the $GABA_A$ receptor and the ssGR (Betz, 1990), it is conceivable that subunit composition differences may be responsible for the different pharmacologies observed.

CHAPTER 7

Characterisation of Immunopurified $GABA_A$ receptors

ALLOSTERIC INTERACTIONS OF IMMUNOPURIFIED $GABA_A$ RECEPTORS

The text and data that follow have been submitted to The Journal of Receptor Research

SUMMARY

$GABA_A$ (γ-aminobutyric acid) receptors were immunopurified from deoxycholate solubilised rat brain membranes using antibodies specific for α1, α2 and α3 or γ subunit types. Modulation of [^{3}H] flunitrazepam binding to the immunopurified receptors by GABA, alphaxalone and pentobarbitone was compared to that in membranes. At 4°C, little difference was seen in affinity between the immunopurified receptors and that of the membrane bound. However, the affinity at 37°C for flunitrazepam was two-fold lower in α1 and α3 containing receptors compared with those of α2 and membrane bound receptors. Immunopurified receptors showed robust potentiation of benzodiazepine binding by GABA. The EC_{50} for GABA was approximately ten-fold higher in membranes than in immunopurified receptors. The maximal degree of potentiation was in both cases approximately the same, indicating that this was not simply due to the presence of endogenous GABA in the membrane preparation. Pentobarbitone, alphaxalone and chloride gave a marked potentiation of flunitrazepam binding in membranes but were without effect in receptors immunopurified with γ subunit specific antiserum. Though the immunopurification procedure results in the loss of allosteric interactions with barbiturates and with alphaxalone, immunopurified receptors reveal subunit-composition dependent differences in native GABA receptors and may therefore be a useful tool in examining some aspects of these receptor interactions.

INTRODUCTION

The subunits of the $GABA_A$ receptor have been a target of intense research in recent years. Cloning studies have shown that the receptor is composed of several distinct subunit groups which have been divided into 4 main classes (α, β, γ, δ) based on sequence homologies. Within these classes there exists further sequence divergence and to date 6α (Schofield *et al*, 1987; Levitan *et al*, 1988; Ymer *et al*, 1989a; Pritchett & Seeburg, 1990; Lüddens *et al*, 1990; Khrestchatisky *et al*, 1989), 4β (Ymer *et al*, 1989b; Lasham *et al*, 1991), 3 γ (Ymer *et al*, 1990; Shivers *et al*, 1989; Wilson-Shaw *et al*, 1991) and 1 δ (Shivers *et al*, 1989) sequences have been cloned. The *in vivo* composition and stoichiometry of the $GABA_A$ receptor, however, remains largely unknown.

The use of *in situ* hybridisation techniques has revealed considerable divergence in the distribution of the various subunit mRNAs (Wisden *et al*, 1988; Lolait *et al*, 1989; Lüddens *et al*, 1990; Malherbe *et al*, 1990), which suggests that subunit composition may play a role in the *in vivo* differences in $GABA_A$ pharmacology seen in various brain regions. The composition dependence of $GABA_A$ pharmacology has been extensively studied using transfection techniques. Co-transfection of various subunit combinations has shown that although singly expressed subunits are capable of forming GABA-gated ion channels which are blocked by picrotoxin and potentiated by barbiturates (Blair *et al*, 1988), further subunits are required in order for the full pharmacological profile of the receptor to be expressed. Importantly, for benzodiazepine sensitivity to be expressed, either a γ1 or γ2 subunit is required in

addition to an α and a β (Pritchett *et al*, 1989a; Ymer *et al*, 1990). It has further been shown that the benzodiazepine pharmacology is largely dependent upon the α subunit present (Pritchett *et al*, 1989b; Pritchett & Seeburg, 1990; von Blankenfeld *et al*, 1990). Although certain subunit combinations have been shown to possess the pharmacological properties of *in vivo* receptors, and that the mRNAs of these subunits appear to be co-localised (Sigel *et al*, 1990), there is as yet no clear evidence that these subunit combinations exist *in vivo*. Another approach therefore has been to examine the stoichiometry and composition of the $GABA_A$ receptor using subunit specific antibodies (Duggan and Stephenson, 1990). Recently we have described the use of high-titre specific antibodies raised against cytoplasmic loop sections of α subunits to immunopurify $GABA_A$ receptors on the basis of their α subunit content (McKernan *et al*, 1991). In this paper we examine the interactional pharmacology of immunopurified $GABA_A$ receptors.

METHODS AND MATERIALS

Membrane preparation

Male Sprague Dawley rats (130-200g) were sacrificed by decapitation with prior stunning and their brains rapidly removed and placed on ice. Tissue was either used fresh or frozen at -20°C until required. Crude synaptosomal membranes were prepared as described previously (McKernan *et al*, 1991). Briefly, whole brains were homogenised in 20 volumes ice cold wash buffer (5mM Tris-HCl, 1mM EDTA, pH 7.4 at 4°C) and were then centrifuged at 1000 x G for 10 minutes. The supernatant was carefully removed and recentrifuged at 48000 x G for 20 minutes. The resultant pellet was resuspended in 20 volumes ice cold wash buffer and then centrifuged at 48000 x G for 20 minutes. This step was repeated twice more to facilitate the removal of endogenous GABA and divalent cations (protease activators). The final pellet was resuspended in Tris buffered saline (TBS: 10mM Tris-HCl, 150mM NaCl pH 7.4 at 4°C) containing protease inhibitors (chymostatin 5µg/ml, benzethonium chloride 100µg/ml, soybean trypsin inhibitor 10µg/ml, sodium azide 100µg/ml and bacitracin 100µg/ml) to a final protein concentration of approximately 5mg/ml. This suspension was either solubilised immediately or stored at -20°C until required. Membranes used in binding studies were always frozen and then thawed before use, and then centrifuged at 48000 x G before resuspension into assay buffer (50mM Tris-HCl, 150mM NaCl pH 7.4 at 37°C or 4°C; or 10mM Tris-citrate containing various concentrations of NaCl, pH 7.4 at 4°C).

Solubilization procedure

Solubilization was achieved by the addition of 1/10th volume of 5% sodium deoxycholate (DOC) to give a final concentration of 0.5% DOC. After solubilization, the preparation was centrifuged at 100000 x G for 30 minutes to pellet any insoluble protein. The supernatant was carefully removed and utilised in immunopurification.

Immunopurification Procedure

Subunit specific antibodies, prepared as described previously (McKernan *et al*, 1991), were coupled to protein-A Sepharose beads by incubation at 4°C for 1 hour. To 50µl beads suspended in 2 volumes H_2O were added 10µl antiserum and the volume made up to 10ml with TBS. After incubation the coupled beads were washed twice by centrifugation and resuspension in 10ml TBS containing 0.1% Tween. To the washed, pelleted beads were then added 10ml DOC solubilised membranes. Immunopurification was achieved by overnight incubation at 4°C followed by washing twice with 10ml TBS/Tween before resuspension in 10ml assay buffer containing 0.1% Tween.

[^{3}H] Flunitrazepam Binding Assay

Equilibrium binding of [^{3}H]FNZ was studied by incubation of 100µl aliquots of membranes or immunopurified receptor in a final volume of 0.5ml assay buffer containing 0.5-150nM [^{3}H]FNZ, (2, 5 or 10nM in the displacement and potentiation experiments), modulators (pentobarbitone 0.01-10mM, alphaxalone 0.01-100µM, GABA 0.01-100µM) and displacers (FNZ 0.1-300nM) as required, for 60 minutes at

4 or 37°C. Non-specific binding was determined using 10μM FNZ. The binding reaction was terminated by the addition of 2ml ice cold wash buffer followed by rapid filtration through Whatman GF-C filters, using a Brandel Cell Harvester. The filters were washed twice with 2ml ice cold wash buffer and the bound radioactivity quantified using either conventional liquid scintillation counting or where crude membranes were used, with solid "Meltilex" scintillant using a Pharmacia Betaplate counter. Kd values were calculated from saturation data for α3 containing receptors at 37°C but all other data were derived from competition curves using the Cheng-Prussof equation (Cheng and Prussof, 1973). Where Ki values are given, saturation studies yielded similar results. All data were fitted using the non-linear regression program ENZFITTER (Biosoft).

Materials

[^{3}H]FNZ was purchased from Amersham. Alphaxalone was a gift from Glaxo. GABA, sodium pentobarbitone, flunitrazepam, sodium azide, DOC, CHAPS, bacitracin, soybean trypsin inhibitor (type I-S), and chymostatin were purchased from Sigma. All other chemicals were purchased from BDH (Analar grade).

RESULTS

Allosteric interactions

At 37°C, GABA, Alphaxalone and pentobarbitone robustly potentiated the binding of 5nM [^{3}H]-FNZ to membranes. (Figures 7.1 and 7.2). In DOC solubilised immunopurified receptor, purified for α1, α2 or α3, robust potentiation by GABA was observed. The EC_{50} for GABA potentiation of [^{3}H]-FNZ binding to α2 containing receptors was significantly higher ($P<0.05$, by Student's t-test) than those for α1 and α3 containing receptors. The EC_{50} for GABA potentiation in membranes was significantly higher than those of all three types of immunopurified receptor. These allosteric interactions are summarised in table 7.1. Using antisera against the γ subunit (non-specific for γ1 and γ2) yielded receptors which were not potentiated by steroid or barbiturate (if anything, alphaxalone showed a tendency to inhibit [^{3}H]FNZ binding) but which were potentiated by GABA.

Chloride Dependency

Certain anions, most notably chloride, have been shown to be potent enhancers of benzodiazepine binding in membrane preparations (Evoniuk and Skolnick, 1988). We therefore examined the chloride dependency of flunitrazepam binding utilising a Tris-citrate buffer containing 0.6-200mM NaCl at 4°C. [^{3}H]-FNZ (2nM) binding to membranes was robustly potentiated by sodium chloride with a best-fit EC_{50} of 41.05± 8.2mM (5). In contrast, [^{3}H]-FNZ binding to immunopurified γ containing receptors showed no significant chloride dependence. This is shown in figure 7.3.

Flunitrazepam affinity

At 37°C, receptors containing α2 subunits showed significantly higher affinity for FNZ than those containing α1 or α3 subunits ($P<0.05$, by Student's t-test) and approximately the same affinity as in membranes. Significant shifts of the binding or displacement curves were obtained in all cases in the presence of 100μM GABA ($P<0.05$, by Student's t-test). These affinities are given in table 7.2. At 4°C, similar shifts in affinity were seen when 100μM GABA was added. However, no significant difference in affinity was seen between the immunopurified receptors and membranes. Preliminary experiments (RMM, unpublished results) have revealed that GABA decreases the affinity of DMCM (dimethoxy-4-ethyl-β-carboline-3-carboxylate) for receptors containing α1, α2 and α3. The allosteric interaction of GABA with both benzodiazepine agonists and inverse-agonists are, therefore, preserved in immunopurified receptors.

Figure 7.1. The potentiation of 5nM [^{3}H]-flunitrazepam binding by GABA in immunopurified receptors containing α1 (solid squares) and receptors from rat brain membranes (open squares), at 37°C. The data points are the mean ± SEM of representative experiments performed in triplicate.

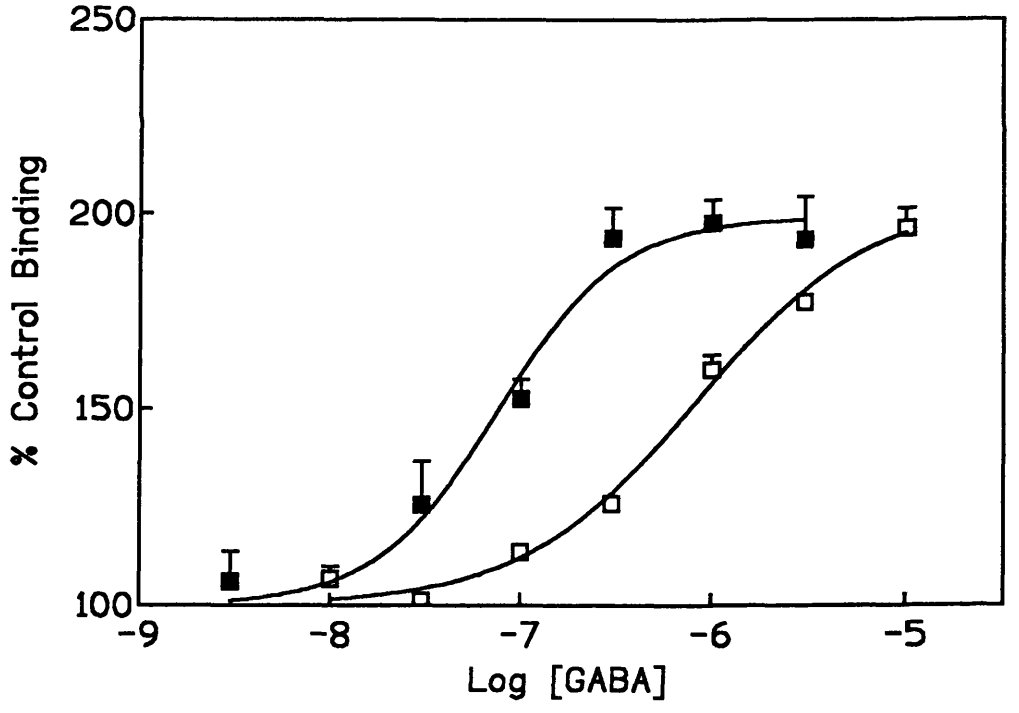


Figure 7.2. The effect of pentobarbitone (open symbols) and alphaxalone (solid symbols) upon 5nM [^{3}H]-flunitrazepam binding at 37°C in rat brain membranes (squares) and in receptors purified using antibodies non-specific for the γ1 and γ2 subunits. Data points represent the mean ± SEM of 3-5 experiments performed in triplicate.

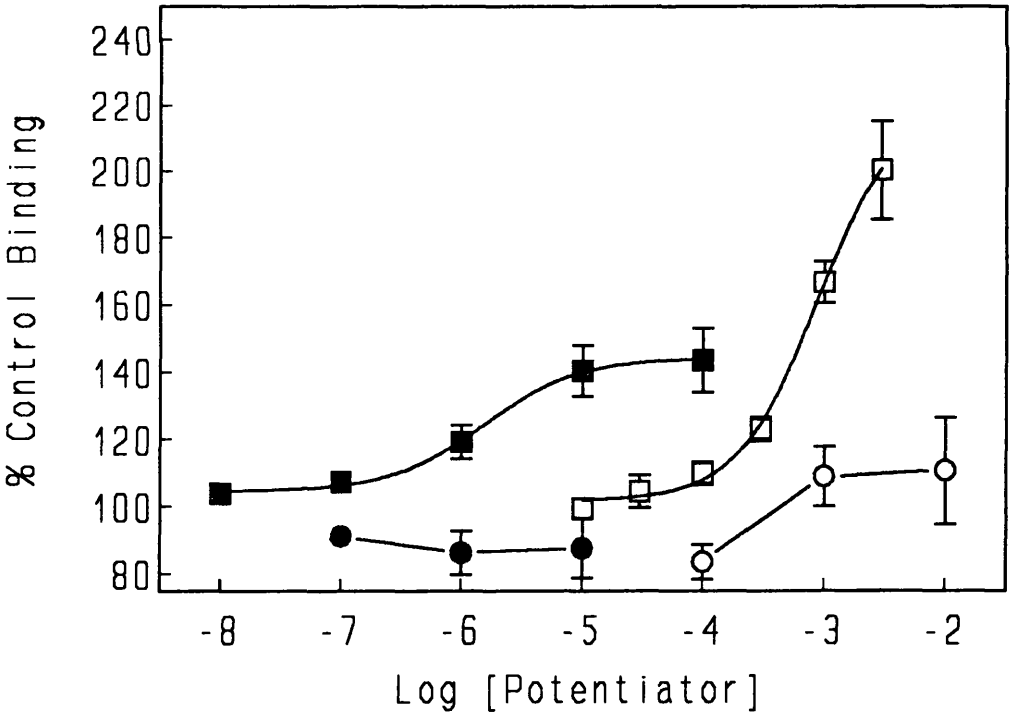


Figure 7.3. Chloride dependency of 2nM [^{3}H]-flunitrazepam binding at 4°C to rat brain membranes (circles) and receptors purified using anti-γ antibodies. The data are taken from a representative experiment and are the mean ± SEM of triplicate determinations.

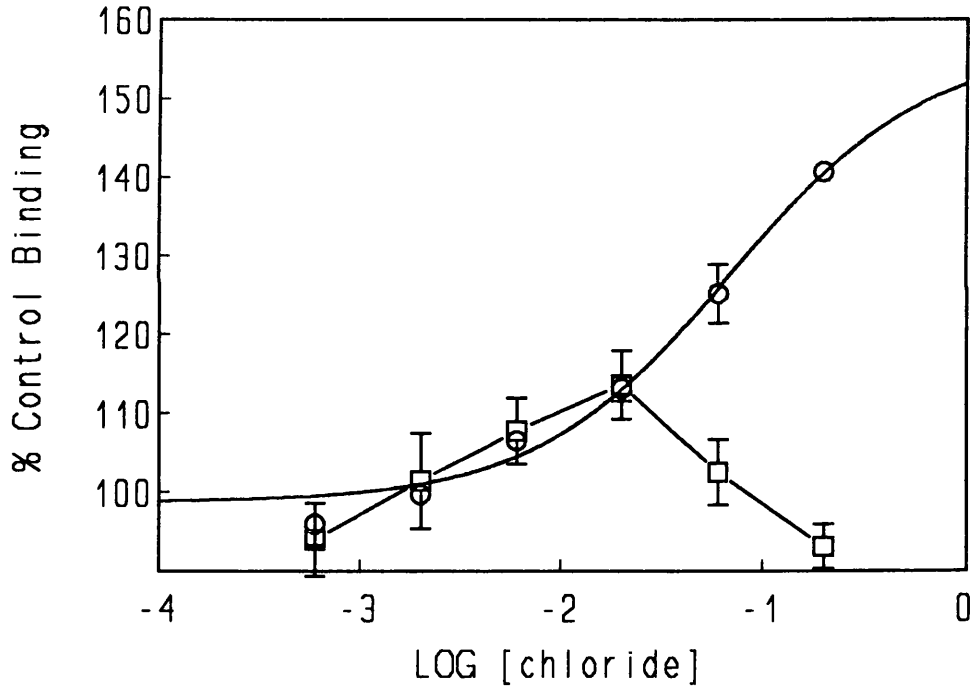


Table 7.1. EC_{50} values (μM) for the potentiation of 5nM [^{3}H]-flunitrazepam binding to rat brain membranes and immunopurified receptors at 37°C in the presence of 150mM NaCl. Data are the mean ± SEM of (n) experiments.

	GABA	Alphaxalone	Pentobarbitone
Membranes	0.90 ± 0.15 (4)	1.36 ± 0.2 (4)	375 ± 100 (4)
α1	0.069 ± 0.008 (4)**	-	-
α2	0.136 ± 0.033 (3)*	-	-
α3	0.052 ± 0.01 (5)**	-	-

* significantly different to membranes ($P<0.05$) by Student's t-test

** significantly different to membranes and to α2 ($P<0.05$) by Student's t-test

Table 7.2. The affinity of [^{3}H]-flunitrazepam for immunopurified receptors and rat brain membranes at 4 and 37°C in the absence and presence of 100μM GABA. Data are Ki values (nM) except where marked and are the mean ± SEM of (n) experiments.

	4°C		37°C	
	Control	+100μM GABA	Control	+100μM GABA
Membranes	3.5± 0.43 (5)	1.4± 0.16 (3)	17.2± 3.0 (7)	7.7± 1.0 (4)
α1	4.0± 0.68 (3)	2.1± 0.52 (3)	31.7± 3.3 (5)*	12.4± 2.3 (5)
α2	3.2± 0.45 (4)	1.3± 0.21 (4)	19.1± 4.6 (6)	6.9± 1.7 (6)
α3	4.0± 0.73 (4)	2.0± 0.39 (3)	32.9± 4.3 (5)*	14.0± 1.8 (4)

*Significantly different from membrane-bound and α2 containing receptors ($P<0.05$) by Student's t-test

DISCUSSION

The present results demonstrate that immunopurified $GABA_A$ receptors exhibit a subunit-dependent difference in allosteric interactions between GABA and flunitrazepam. In addition, the affinity of flunitrazepam appears to be influenced by the subunit composition of the receptor, in a temperature dependent manner.

The sensitivity of various combinations of subunits to GABA has been extensively examined by cloning techniques. Expressing combinations of the type $\alpha x\beta 1$ in oocytes, Levitan *et al* (1988) reported EC_{50}s for GABA of 12μM, 1.3μM and 42μM for α1, α2 and α3 respectively. This is in contrast to our results. However, the α/β subunit combinations do not represent fully functional $GABA_A$ receptors as most native complexes contain a γ subunit, which is required for the expression of benzodiazepine interactions with the receptor (Ymer *et al*, 1990). By the use of flunitrazepam binding, we have excluded all non-γ-containing complexes from the present study, therefore it is difficult to make comparisons with such data. The use of the β1 subunit, which shows the lowest expression of the β class in rat brain (Wisden *et al*, 1992) also makes correlation of these results with the present study difficult. A recent study by Sigel *et al* (1990) has shown that the substitution of the β1 subunit for the β2 subtype suppresses the allosteric interactions between diazepam and GABA. As no significant difference is seen in GABA shift between the immunopurified and membrane bound receptors, a possible interpretation of our data is that the β1 subunit does not contribute significantly to GABA-benzodiazepine receptors containing α1, α2 or α3 subunits. In contrast to our present data, Sigel *et al* (1990) observed

differences in the GABA sensitivity of α1βxγ2 and α3βxγ2 subunit combinations. However, the values of K_A for GABA varied over 2 orders of magnitude depending on whether β1 or β2 was utilised and on whether other α and β subunits were co-expressed. The lower GABA sensitivity of α2 containing receptors correlates with the lower affinity of this population for [^{3}H]-muscimol observed in our previous study (McKernan *et al*, 1991) and also with the results of a recent study which demonstrated that hippocampal membranes (in which α2 is the most abundant subunit overall (Wisden *et al*, 1988)) show 2-3 fold higher EC_{50} for GABA potentiation of flunitrazepam binding than membranes from other brain areas (Ruano *et al*, 1992). The lower affinity of membrane bound receptors for GABA compared with the immunopurified receptors could possibly be due to the presence of endogenous GABA in the membrane preparation, which is removed during the immunopurification procedure. However, several lines of argument suggest that this is not the case. Little difference is seen in the affinities of receptors for FNZ at 4°C whereas if significant concentrations of GABA were present one would expect the affinity of the membrane bound receptors to be higher than the immunopurified. Also, the maximal log shift caused by GABA is approximately the same for all classes of immunopurified receptors and for membranes. If endogenous GABA were responsible for the differences seen, then one would expect the immunopurified receptor GABA shift to be larger than in membranes. In addition, α1 and α3 containing receptors contribute significantly to the membrane population and therefore one would expect the affinities of these receptors to be closer to those of the membrane bound, when a maximal concentration of GABA is present. Whilst it is possible that some other endogenous

modulatory substance has been removed by the immunopurification procedure, a more likely explanation is that the receptor structure has been perturbed by the detergent disruption of the membrane. The effects, or rather lack of them, of pentobarbitone, chloride and alphaxalone on γ subunit containing receptors support this argument. These receptors represent all GABA-linked benzodiazepine sites, therefore it can be assumed that these interactions are lost during the purification procedure. In contrast to DOC solubilised receptors, CHAPS and CHAPS + lipids solubilised receptors have been shown to exhibit modulation by barbiturates (Bristow and Martin, 1987). However, in our hands CHAPS has shown itself to be inferior in yield to DOC in terms of percent solubilization and also does not give such high yield during the immunopurification procedure (R.M.M. unpublished results). This may due to a relatively harsh detergent being required in order to achieve full denaturation, and therefore immunorecognition of the intracellular loop of the subunits. Olsen *et al* (1986) found that whilst receptors solubilized in Triton X100 showed no potentiation by barbiturates, these interactions were not irreversibly lost and could be recovered upon exchange into CHAPS. However, substitution of CHAPS for Tween when washing and resuspending immunopurified receptors did not result in the recovery of barbiturate potentiation (results not shown). This suggests either that DOC solubilization results in irreversible loss of these interactions or that the binding of the antibody to the intracellular loop locks the receptor into a denatured state.

Little difference was seen in our previous study (McKernan *et al*, 1991) in the affinity of the various immunopurified receptors for [^{3}H]-FNZ at 4°C. The present results agree with this finding but show that at 37°C, there is a significant difference between

the affinities of α2 containing receptors and those containing α1 or α3. α1 and α3 containing receptors also show significantly lower affinity than membranes at 37°C but not at 4°C. The binding of [^{3}H]-FNZ to membranes preparations in rat brain has been demonstrated to be highly temperature dependent due to the exothermic nature of the binding reaction (Doble, 1983). The present results indicate that there may be subunit dependent differences in the temperature sensitivity of the $GABA_A$ receptor.

In conclusion, we have demonstrated temperature-sensitive, subunit-dependent differences in the affinities of immunopurified $GABA_A$ receptors for flunitrazepam and for GABA modulation of benzodiazepine binding. Whilst the interactions of these receptors with barbiturates, chloride and steroids are lost, immunopurified receptors should prove a useful tool in the elucidation of certain aspects of the molecular pharmacology of the $GABA_A$ complex.

Additional work in support of this chapter.

This results which follow were published as part of a multi-author paper: McKernan, R.M., Quirk, K., Prince, R., Cox, P.A., Gillard, N.P., Ragan, C.I., and Whiting, P., $GABA_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties, Neuron 7 (1991) 667-676

Initial studies on the pharmacology of the immunopurified receptors were carried out as detailed above with the exception of the assay buffer utilised: this was 10mM Tris-HCl containing 1mM EDTA pH 7.4 at 4°C. [^{3}H]-Muscimol (2-100nM) binding was carried out as described for flunitrazepam binding with the exception that non-specific binding was defined using 100μM GABA as a displacing ligand. [^{3}H]-Ro15-1788 binding (0.05-10nM) was carried out as described for flunitrazepam binding utilising 10μM flunitrazepam as the displacing ligand for determination of non-specific binding. All experiments described in this section were carried out at 4°C.

RESULTS

The Kd and Bmax for the ligands described above were determined by saturation analysis for receptors immunopurified with α1, α2, α3 and α5 subtype specific antibodies. The ratio of the Bmax's obtained for flunitrazepam and muscimol was expressed as a ratio of that obtained for Ro15-1788 (flumazenil). These results are given in tables 7.3 and 7.4.

Table 7.3. The affinities (Kd, nM) for [^{3}H]-muscimol, [^{3}H]-Ro15-1788 and [^{3}H]-flunitrazepam of receptor populations immunopurified on the basis of α subunit composition. The data are the mean ± SEM of 4-7 experiments.

	[^{3}H]-Ro15-1788	[^{3}H]-flunitrazepam	[^{3}H]-muscimol
α1	2.1 ± 0.8	4.5 ± 4	10.0 ± 3
α2	3.8 ± 0.5	4.0 ± 1.4	18.0 ± 2.2*
α3	2.7 ± 1.4	3.7 ± 1.7	9.2 ± 1.4
α5	2.1 ± 0.1	4.5 ± 0.6	9.7 ± 1.5

*Significantly different from other subunits by Student's t test ($P<0.05$)

Table 7.4. The ratio of binding sites for [^{3}H]-flunitrazepam and [^{3}H]-muscimol relative to those for [^{3}H]-Ro15-1788 in receptors immunopurifed on the basis of α subunit composition. The data are the mean ± SEM of 4-7 paired experiments.

	[^{3}H]-flunitrazepam	[^{3}H]-muscimol
α1	1.15 ± 0.1	2.4 ± 0.5*
α2	1.21 ± 0.24	3.4 ± 0.5*
α3	1.4 ± 0.5	6.3 ± 1.3*
α5	1.2 ± 0.27	4.1 ± 0.9*

* significantly different from unity by Students t test ($P<0.05$)

DISCUSSION

The results presented above indicate that the pharmacology of solubilized immunopurified $GABA_A$ receptors is broadly similar to that of native receptors and of expressed in transfected cells. Thus the affinities of the benzodiazepine ligands flunitrazepam and flumazenil do not appear to vary with the type of α subunit present. This is consistent with results obtained by Pritchett and Seeburg (1990) using $\alpha x\beta 3\gamma 2$ combinations in transfected cells. The affinities of the immunopurified receptors are in the same range as reported for native membranes and for an $\alpha 1\beta 2\gamma 2$ combination expressed in transfection studies by Lüddens *et al* (1990). The affinity of muscimol is significantly lower for $\alpha 2$ containing receptors than for those containing $\alpha 1$, $\alpha 3$ or $\alpha 5$. The significance of this in relation to recent studies on regional heterogeneity of $GABA_A$ receptors has been discussed above.

In order to determine if there exists a significant population of $GABA_A$ receptors which do no contain benzodiazepine binding sites, the ratio of muscimol to benzodiazepine Bmax was examined in the various immunopurified complexes. Whilst the ratio of flunitrazepam to flumazenil binding remained largely constant and was not significantly different from 1 for any of the subtypes, the ratio of muscimol to flumazenil binding varied considerably. For $\alpha 1$ containing receptors the ratio of muscimol binding was approximately 2, which has been suggested as the stoichiometry of GABA to benzodiazepine sites in classical $GABA_A$/benzodiazepine receptors (see Stephenson, 1988 for review). However, in $\alpha 3$ containing receptors, the ratio of binding was approximately 6 indicating that there exists a considerable

population of receptors containing this α subunit which do not possess benzodiazepine sites. It has been demonstrated that high affinity benzodiazepine binding is only seen in receptors containing an α subunit in combination with a β and a γ2 (Ymer *et al*, 1990; Shivers *et al*, 1989). These non-benzodiazepine receptors may therefore correspond to receptors which lack β subunits or do not possess a γ2 subunit. It is possible that some of these receptors contain γ1 or δ subunits which do not confer high-affinity benzodiazepine binding.

CHAPTER 8

General Discussion

SITES OF ACTION

Protein versus Lipid

The lipophilicity of general anaesthetics such as the barbiturates and the neurosteroids has prompted many to suggest that these diverse compounds interact with the nervous system primarily through perturbation of the lipid bilayer of nerve cell membranes, leading to conformation changes in membrane bound proteins which result in anaesthesia (for review see Seeman, 1972). In support of this has been cited the striking correlation between potency of action at the $GABA_A$ receptor and oil/water partition coefficients for compounds such as the neurosteroids and barbiturates (Simmonds and Turner, 1987). In addition it has been demonstrated that these substances are indeed capable of perturbing membrane structure (Makriyannis *et al*, 1990; Harris and Schroeder, 1982). However, whilst this theory is superficially attractive, there exists a stronger body of evidence which favours a specific protein site of action for such sedative drugs.

a) Increasing temperature is a well characterised method of perturbing membrane structure resulting in an increase in fluidity (Buck *et al*, 1989). As discussed in chapter 5, a large shift in temperature would be expected to interfere with the potentiation of [^{3}H]-FNZ binding by alphaxalone and propofol if the mechanism of action of these anaesthetics involved a component of membrane fluidization. This has been shown not to be the case. In fact it has been demonstrated that anaesthetics produce rather small changes in membrane order compared with that produced by temperature, and that shifts in temperature do not result in anaesthetic-like actions (Franks and Lieb, 1987).

The results obtained in this thesis for the temperature dependency of pentobarbitone potentiation are not inconsistent with a lipid perturbation mechanism of action. However, these results can also be explained by a thermodynamic shift in the nature of benzodiazepine binding.

b) Strict structure activity relationships have been defined for the neurosteroids (Harrison *et al*, 1987; Simmonds, 1991). These are reminiscent of enzyme/substrate specificity relationships. This analogy has been further extended in this thesis by the characterisation of a specific antagonist of pregnanolone's actions at the $GABA_A$ receptor. However, it can be argued that this specificity reflects a structure-activity relationship for membrane perturbation rather than interaction with a protein. It has been demonstrated that 3α hydroxy steroids such as pregnanolone are capable of perturbing membrane structure whilst their 3β analogues stabilise membrane structure (Makriyannis *et al*, 1990). Thus the agonist/antagonist relationship of these steroids is not incompatible with lipid theories. However, in studies on the membrane fluidisation properties of barbiturates it was shown that membrane perturbation was directly related to hydrophobicity, but that pharmacological activity was not discriminated. Thus, membrane perturbation could distinguish neither enantiomeric pairs which differ in potency nor distinguish between convulsant and anticonvulsant barbiturates (Harris and Schroeder, 1982). It has been suggested that the dependence of anaesthetic potency upon hydrophobicity is due to the binding of anaesthetics to a hydrophobic site on a protein receptor. When ascending a series of alkanols, a point is reached above which anaesthetic potency is not increased despite increasing hydrophobicity. This is interpreted as the alkanols binding to a site on the protein of

finite molecular proportions (Franks and Lieb, 1987). If such sites are present on $GABA_A$ and glycine receptors and are responsible for the binding of steroids, barbiturates and other anaesthetics, then there must exist more than one type of site and each site must be capable of discrimination of structure on the basis of more than hydrophobicity, as these substances show different sites, and as demonstrated in chapter 5, different modes of interaction. In fact there is considerable evidence for this. The interaction of steroids with the glycine receptor, as described in chapter 6 and in other studies shows a clearly different structure-activity relationship to that with the $GABA_A$ receptor. The glycine receptor and the $GABA_A$ receptors are both modulated by the anaesthetics propofol and chlormethiazole, but the interaction of the barbiturates is much stronger with the $GABA_A$ receptor than the glycine receptor (Hales and Lambert, 1991; Simmonds and Turner, 1987). In addition multiple subtypes of $GABA_A$/steroid and $GABA_A$/barbiturate receptors have recently been demonstrated in expression studies (Polenzani *et al*, 1991; Zaman *et al*, 1992; Shingai *et al*, 1991). This is discussed in more detail below. A study on a wide variety of aquatic species has revealed that whilst sensitivity to alcohol anaesthesia was found in all organisms examined, only vertebrate species were sensitive to anaesthesia by steroid (Oliver *et al*, 1991). Whilst differences in receptor structure might result in differential responses to membrane perturbation, resulting in receptors which are more or less sensitive to anaesthetic action, it is difficult to perceive how the lipid theories of action can account for selective sensitivity to certain anaesthetics.

d) Recently the phenomena of membrane perturbation and anaesthesia have been elegantly separated. Buck *et al* (1989) have shown that anaesthetic concentrations of

benzyl-alcohol stimulated muscimol-induced chloride flux, but had little or no effect on membrane order. Conversely the membrane fluidising agent, A_2C (2-[2-methoxyethoxy]-ethyl-8-[cis-2-n-octylcyclopropyl]-octanoate), did not produce anaesthesia nor did it increase chloride flux at concentrations where it gave a clear change in membrane order. At higher concentrations of benzyl alcohol, an increase in membrane fluidity was seen, but this was accompanied by an inhibition of chloride flux. At higher concentrations, A_2C too, reduced chloride flux.

e) It has also been reported that steroid and barbiturate modulation of $GABA_A$ receptors can be retained in a purified, solubilized preparation (Olsen, 1990; Bristow and Martin, 1987). Whilst it is considered unlikely that the most tightly bound lipids would be removed from the receptor complex, this protein is likely to be largely devoid of associated membrane structure.

Nature of the binding sites

Whilst the consensus of evidence favours a protein site of action, in addressing the question of the sites of interaction of such drugs as the barbiturates and the neurosteroids it will undoubtedly be of great importance to examine closely the nature of the interactions between proteins and lipids in cell membranes. Studies on the nicotinic acetylcholine receptor, a member of the direct-ligand gated receptor super-family, have shown that there is a strong interaction between the receptor and phospholipids and sterols, which results in an annular layer of lipids around the protein (Jones and McNamee, 1988). This interaction is selective, with phosphatidic acid being more strongly bound than dioleoylphosphatidylcholine, which in turn is

more strongly bound than cholesterol. In addition to this annular layer, it has been shown that there exists an additional class of binding sites which preferentially bind cholesterol. Jones and McNamee have proposed that these sites might lie in the "axial clefts" between the receptor subunits, acting as "molecular lubricants" between the rough intersections of the subunits (see figure 8.1). It is interesting to speculate whether it is to analogous sites that the neurosteroids bind at the $GABA_A$ and glycine receptors, displacing cholesterol. This is an attractive hypothesis in the light of the data presented in this thesis which show that 3β hydroxy steroids act as weak partial agonists at the steroid site(s) of the $GABA_A$ receptor. Cholesterol also possesses a 3β hydroxy group and whilst its structure is sufficiently different to those of the prototypical neurosteroids such as alphaxalone that it is likely to possess only low affinity, its high concentration in cell membranes makes it an attractive candidate as an endogenous ligand for these receptors. Another interesting possibility which is raised by this theory is that the action of certain steroidal detergents such as deoxycholate, in altering the allosteric interactions of the receptor may be linked to them displacing essential lipids from the surface of the receptor. In fresh CHAPS-solubilized $GABA_A$ receptors it is possible to demonstrate allosteric potentiation by barbiturates but this is lost with time (Sigel and Barnard, 1984) and as shown in this thesis, steroid and barbiturate interactions are completely lost from deoxycholate-solubilized $GABA_A$ receptor upon overnight storage. The use of milder detergents together with the incorporation of phospholipids and cholesterol results in these interactions being more stably retained (Bristow and Martin, 1987). It has been shown that in solubilized nACh receptors, cholate and cholesterol compete for binding

sites on the receptor (Jones and McNamee, 1988). It is also possible to speculate that barbiturates act in a similar fashion to that proposed for the neurosteroids displacing some distinct membrane component closely associated with the receptor.

One site or many?

The presence of multiple subunit types for the $GABA_A$, glycine and other direct ligand gated receptors is now clear (Wisden *et al*, 1992; Betz, 1990; Cockcroft *et al*, 1990). The large number of subunit types for the $GABA_A$ receptor makes a bewildering array of subunit combinations available. The evidence presented in this thesis shows that there exist in the brain, receptors possessing binding sites of differing affinity for flunitrazepam together with differing affinities for both the high and low affinity sites for GABA (it is assumed that the GABA high affinity site is that labelled by muscimol whilst the that which modulates benzodiazepine binding corresponds to the low affinity state), and also that there exist receptors of differing subunit compositions which contain GABA sites but no benzodiazepine sites. Until recently, receptors insensitive to barbiturates and steroids had not been demonstrated. However, the expression of novel GABA receptors from the retina which are insensitive to barbiturate modulation raises the possibility that similar receptors may exist which are insensitive to the neurosteroids (Polenzani, Woodward and Miledi, 1991). Olsen (1990) has reported that the $GABA_A$ receptors in the cerebellar granule cells are insensitive to steroids in autoradiographic binding studies. However, this may be due to there being a high concentration of GABA in this region. In addition, it has been shown through transfection studies, that certain subunit combinations are less sensitive

to steroids than others (Shingai *et al*, 1991; Zaman *et al*, 1992). Exactly how these studies relate to the native receptor is not clear but indicates that differentially sensitive complexes may exist. In terms of regional heterogeneity, it has recently been shown by Gee and Lan (1991) that $GABA_A$ receptors in the spinal cord have a different structure activity relationship for steroid modulation to those in the cortex. The study presented in this thesis also demonstrates that there may exist multiple classes of steroid modulatory site, which differ in their sensitivity to pregnanolone, alphaxalone and 3β-hydroxy pregnanolones (Chapter 3).

The nature of these multiple sites is not clear and the interpretation depends largely on the exact location of the steroid binding site on the receptor. If as suggested above, the neurosteroids displace cholesterol from the non-annular binding sites on the receptor, it is likely that there will exist multiple, non-equivalent binding sites on the same receptor complex. The exact molecular nature of these sites would depend upon the stoichiometry and the juxtaposition of the receptor subunits. For example, in a receptor containing 2α1 subunits, 2β2 and 1γ2, in the arrangement αβγβα, there would exist five distinct binding domains: α-β; β-γ; γ-β; β-α and α-α (with - representing the axial cleft). In the arrangement βαγαβ there would also be 5 distinct domains but these would then be: β-α; α-γ; γ-α; α-β and β-β. As well as providing multiple sites of action for the pregnane steroids, this proposal allows for potential sites for the other putative neurosteroid classes ie. DHEAS and pregnenolone sulphate (Majewska and Schwartz, 1987; Majewska *et al*, 1990).

Endogenous ligands

The examination of responses to steroids, barbiturates and other modulators of the $GABA_A$ receptor has prompted speculation that there exist endogenous ligands corresponding to these exogenous ones (Simmonds, 1991; Majewska and Schwartz, 1987). It has been demonstrated that there exist in the brain all the enzymes required to synthesise $GABA_A$ active steroids from cholesterol (Karavolas and Hodges, 1990; Baulieu and Robel, 1990). The major pathways for the synthesis of pregnanolone and pregnenolone sulphate are shown in figure 8.2. It has also recently been suggested that there may be a difference in the metabolism of steroids in rat and man with the rat producing mainly 5α metabolites and man producing 5β (Paul and Purdy, 1992). It would be interesting to examine the relative potencies of the two isomers in human brain tissue - it is possible to speculate that in contrast to the rat, the human receptor would have higher affinity for the 5β isomer over the 5α. As discussed in chapter 3, the metabolism of 3β steroids in the brain is not clearly defined. This may reflect in part, the consideration until recently that these steroids are largely inactive. With the demonstration that the 3β isomers are antagonists of neuroactive steroids, the physiological role of the 3β steroids must receive wider consideration. These steroids are certainly produced in the periphery and therefore may reach the brain via the circulatory system. The circulating concentration of such steroids is likely to be rather low compared with the Ki of epipregnanolone in inhibiting pregnanolone potentiation of [^{3}H]-FNZ, but this certainly may be one means whereby modulation may take place. In addition, the possible identity of cholesterol as the endogenous ligand of the

$GABA_A$ and glycine neurosteroid sites (as discussed above in more detail) cannot be ruled out.

With regard to the barbiturates and propofol the situation is not so clear. No endogenous substances have been proposed as possible ligands for these sites and their existence as modulatory loci may be entirely due to evolutionary chance. The neurosteroid pregnenolone sulphate has been proposed as an endogenous ligand for the convulsant site (Majewska and Schwartz, 1987).

Future perspectives

With the clear importance of membrane domains for the function of direct ligand gated ion channels, it is likely that the composition of the lipid bilayer heavily influences the function of such proteins. As well as providing a reservoir of potential endogenous ligands, the membrane clearly has a major structural role and manipulation of its composition in terms of lipid type and phospholipid/cholesterol ratio may provide clues as to its interaction with receptors. There are several reasons to believe that this may be the case. For example, in solubilization studies on the $GABA_A$ receptor it has been demonstrated that cholesterol and phospholipids are necessary for the functions of the receptor to be maintained (Bristow and Martin, 1987). In addition, cholesterol has been shown to be necessary for the function of the nicotinic acetylcholine receptor (Fong and McNamee, 1986) and $GABA_A$ receptors have also been shown to be modulated by phosphatidylserine (Hammond and Martin, 1987). As well as incorporation of various lipids in solubilization procedures it may also be possible to manipulate lipid composition using non-specific lipid transfer

proteins (Crain and Zilversmit, 1980) which shuttle lipids down concentration gradients from liposomes to membranes.

The striking homologies seen in the membrane spanning regions of the direct ligand gated receptors (Cockcroft *et al*, 1990) may also provide a means by which the sites of action of barbiturates and steroids may be addressed. These homologies suggest that the striking differences seen in steroid pharmacology may be due to relatively minor differences in amino acid sequence. By the creation of hybrid receptors containing various portions of steroid-responsive vertebrate $GABA_A$ receptors and steroid-insensitive invertebrate $GABA_A$ receptors or indeed vertebrate glycine receptors, it should be possible to localize the regions of the receptor which are important for steroid ligand interactions. This may then allow the molecular modelling of the steroid binding site. Already generalised models of the agonist binding site of the direct ligand gated receptors have been constructed (Cockcroft *et al*, 1990), and models of the three dimensional structure of the membrane spanning domains of the nACh receptor which incorporate the non-annular binding sites for cholesterol are underway (G.G. Lunt, personal communication). Such models should provide the basis for detailed investigations of the interaction of steroids with other DLG receptors such as the $GABA_A$ receptor.

Figure 8.1. A model for the interaction of lipids with direct ligand gated ion channels (adapted from Jones and McNamee (1988)). The subunits of the DLG receptors may interact with lipids at 2 distinct sites. A) cholesterol binds preferentially to "non-annular" sites which may be located at the protein/protein junctions of the subunits. In receptors which are hetero-oligomers these sites will not all be equivalent. B) phospholipids and C) cholesterol bind to the annular sites around the protein surface. These sites appear to bind phospholipids in preference to cholesterol. For clarity, the transmembrane domains of the receptor subunits are illustrated on only one subunit.

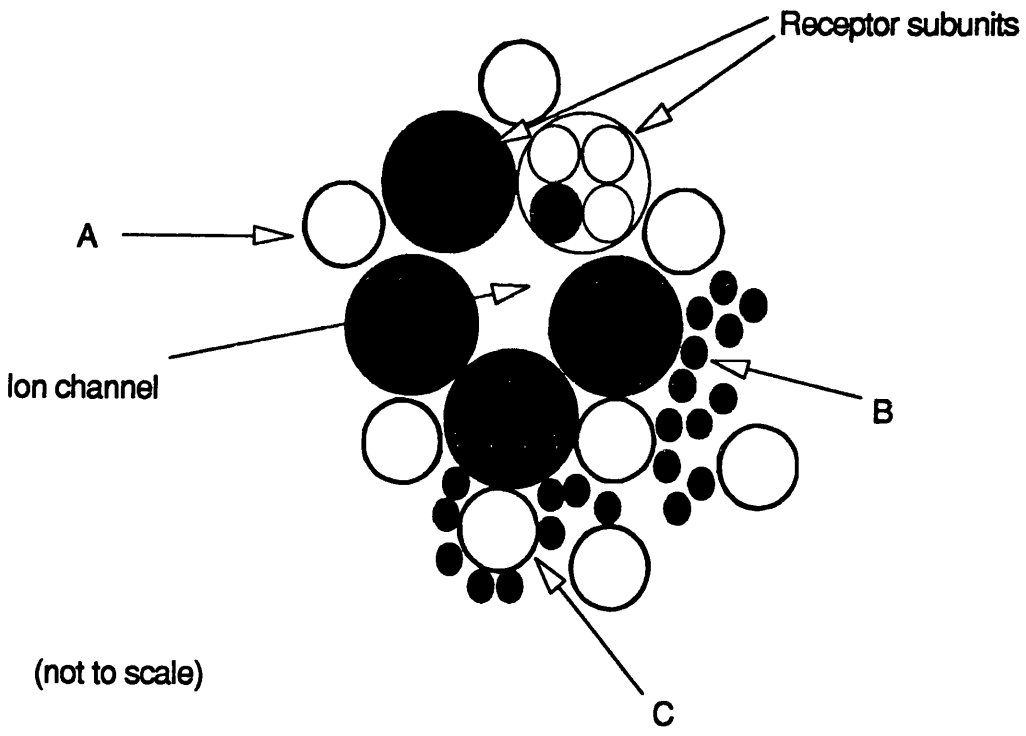

Receptor subunits
A
Ion channel
B
C
(not to scale)

Figure 8.2. The major metabolic pathways leading to the formation of pregnanolone and pregnenolone sulphate in the rat brain. The various enzymes involved have been localised in neuronal tissue by immunohistochemical means. A) Cytochrome P450; B) 3β-hydroxysteroid dehydrogenase; C) 3β-hydroxysteroid sulphotransferase; D) Δ4 steroid 5β hydrogenase; E) 3α-hydroxysteroid oxidoreductase. The conversion of cholesterol sulphate to pregnenolone sulphate is presumed to occur but has not been definitively demonstrated (Baulieu and Robel, 1990).

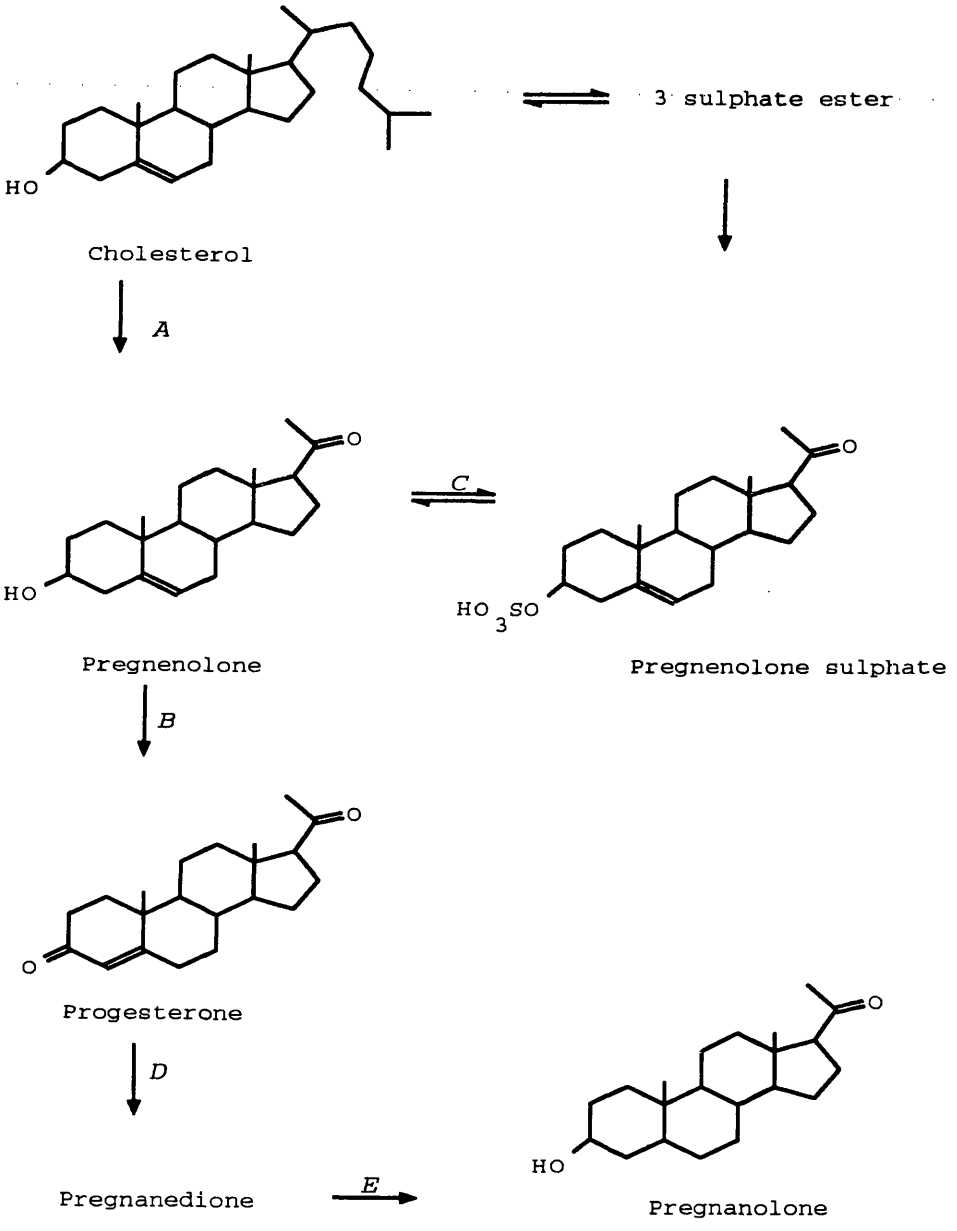

HO
3 sulphate ester
Cholesterol
A
O
HO
C
HO 3SO
Pregnenolone
Pregnenolone sulphate
B
O
O
Progesterone
D
Pregnanedione
E
HO
O
Pregnanolone

Figure 8.2

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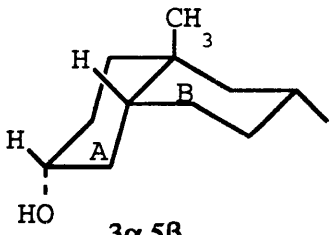
Appendix I

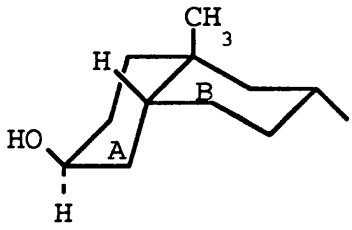
A) The conformation of the A and B rings of 3α, 3β, 5α and 5β steroids.

B) The ring numbering system for steroids. The positions of greatest importance are 3, 5, 11, 17, 20 and 21. An unsaturated bond between carbons 4 and 5 is designated 4-ene or Δ4; and between carbons 5 and 10, 5-ene or Δ5.

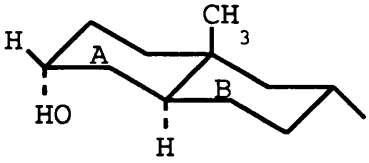
A



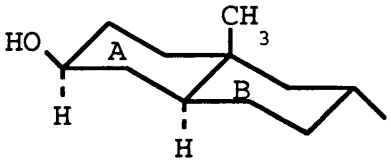
3α 5β



3β 5β

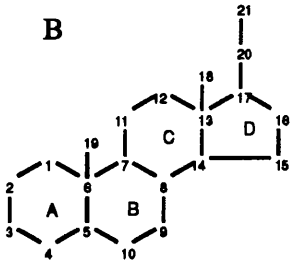


3α5α



3β5α

B



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