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TEMPERATURE AND ANION DEPENDENCE OF ALLOSTERIC INTERACTIONS AT THE γ-AMINOBUTYRIC ACID–BENZODIAZEPINE RECEPTOR

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Abstract—The temperature dependence of [^{3}H]flunitrazepam ([^{3}H]FNZ) 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 FNZ 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 FNZ binding and, therefore, is more efficacious at low temperatures. The EC_{50} values of all three modulators increased with temperature indicating that their interactions with the receptor may be enthalpy driven. The EC_{50} values of all three modulators were also anion dependent, showing a decrease in the presence of γ-aminobutyric acid ($GABA_A$)-channel permeant anions. The efficacies of alphaxalone and pentobarbitone, but not that of 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.

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 [1, 2]. The gating of the channel by GABA has been shown to be enhanced by a variety of sedative and hypnotic drugs including the benzodiazepines [3] barbiturates [4] and certain pregnane steroids termed the neurosteroids [5], and this has been proposed to be the major mechanism of action of these drugs. 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) [3]. The potentiation of [^{3}H]benzodiazepine binding by modulators such as barbiturates [3] 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.

In addition, the binding of benzodiazepines to the $GABA_A$ receptor exhibits a well characterized temperature and anion dependence [6–8]. Due to the exothermic nature of the binding reaction at temperatures of 10° 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 K_{eq} and reciprocal absolute temperature as predicted by the van't Hoff equation:

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

Temperature is known to exert a strong influence on the state of fluidity of biological membranes [9]. Perturbation of membrane structure has been advanced as a possible mechanism of anaesthetic action [10] and it has been demonstrated that the $GABA_A$ potentiators alphaxalone (5α-pregnan-3α-ol-11,20-dione) [11] together with the barbiturates [9] 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]FNZ binding by alphaxalone, pentobarbitone and propofol.

MATERIALS AND METHODS

Crude rat brain synaptic membranes were prepared as described previously [12]. Briefly, male Wistar rats (150–220 g) were killed 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 homogenized in 20 vol. ice-cold wash buffer (5 mM Tris–HCl containing 1 mM EDTA, pH 7.4 at 4°) using a glass/Teflon homogenizer. This homogenate was centrifuged at 1000 *g* for 10 min to pellet unbroken cells and debris.

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† Abbreviations: GABA, γ-aminobutyric acid; FNZ, flunitrazepam.

‡ Prince and Simmonds, submitted to *Brain Research*.

The supernatant was carefully removed and centrifuged at 48,000 *g* for 20 min. The resultant pellet was resuspended and centrifuged a further three times. The final pellet was resuspended in wash buffer and then frozen at −20° until required (14 days maximum). On the day of the experiment, the tissue was thawed and centrifuged for 20 min at 48,000 *g* before resuspension in assay buffer [either 50 mM Na^+/K^+ phosphate containing 200 mM NaCl, pH 7.1 (buffer 1); or 10 mM Tris-citrate containing various concentrations of salts, pH 7.4 at 4° (buffer 2)].

The binding of [^{3}H]FNZ was determined by the incubation of 100 μL aliquots of tissue containing 0.1–0.3 mg total protein (Biorad protein assay), for 10 min at 37° in the absence of radioligand, in a total volume of 0.5 mL assay buffer containing various concentrations of drug as required. [^{3}H]FNZ was then added to a final concentration of 1 nM and the samples incubated for a further 60 min at the required assay temperature. The binding reaction was halted by the addition of 2 mL ice-cold wash buffer followed by rapid filtration through Whatman GF-C filters using a Brandel Cell Harvester. The filters were washed twice with 2 mL aliquots of wash buffer before quantification of bound radioactivity by conventional liquid scintillation techniques. Non-specific binding was determined using 10 μM cold FNZ and was generally in the region of 5–10% of total bound. Data were fitted using the Inplot package (Graphpad Software) and are expressed as means ± 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 FNZ at various temperatures was measured in buffer 1 by means of a competition assay, taking K_{eq} as $1/K_i$. K_i was derived from IC_{50} values by means of the Cheng–Prusoff equation [13]. The van't Hoff plot under control conditions yielded a linear relationship for temperatures from 0 to 47°. Other workers have observed a deviation from this relationship at temperatures of less than 10° [6, 7]. To allow comparison with previous investigations we have restricted our analyses to temperatures of 10° and greater. This yielded a value for $\Delta H°$ of −38.1 ± 4.35 kJ/mol and for $\Delta S°$ of 27.8 ± 14.43 J/°K/mol.

In the presence of 100 μM alphaxalone the slope of the line was not significantly different yielding a $\Delta H°$ of −38.4 ± 3.40 kJ/mol. The derived $\Delta S°$ from this line was 33.9 ± 11.23 J/°K/mol. 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 contribution of the entropy component to the total binding energy is in agreement with other workers [6–8].

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 300 μM propofol yielded a $\Delta H°$ of −37.3 ± 5.3 kJ/mol and $\Delta S°$ of 35.68 ± 17.63 J/°K/mol. 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 1 mM pentobarbitone caused a significant increase in the enthalpy of the binding reaction to −57.04 ± 5.83 kJ/mol. Pentobarbitone potentiation is therefore greater at low than at high temperatures. These data are illustrated in Fig. 1 and summarized in Table 1.

Dose–response curves for alphaxalone, propofol and pentobarbitone were compared at 4° and 37° (also in buffer 1). All three showed a significant increase in EC_{50} at 37° compared with 4°. These data are summarized in Table 2.

At 4°, in the absence of added salts (buffer 2), pentobarbitone and alphaxalone showed only a small potentiation of FNZ binding. Addition of 200 mM NaCl caused a significant decrease in the EC_{50} value for pentobarbitone and 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 Fig. 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 200 mM 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. These data are summarized in Table 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 ± 6.52 mM [7]. In the presence of 1 mM pentobarbitone, the dose–response curve for NaCl was significantly shifted to the left with an EC_{50} value of 19.71 ± 1.94 mM (4) ($P < 0.05$ by Student's paired *t*-test). Alphaxalone at 10 μM also caused a lowering of the EC_{50} value for NaCl to 20.9 ± 3.64 mM (5) but this was not significant at the 95% confidence level ($0.05 < P < 0.1$). These data are illustrated in Fig. 3. In our previous study* we have shown that propofol has a similar effect to pentobarbitone, 300 μM propofol causing the NaCl dose–response curve to shift leftwards with an EC_{50} value for NaCl of 12.80 ± 3.01 mM (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

* Prince and Simmonds, submitted to *Brain Research*.

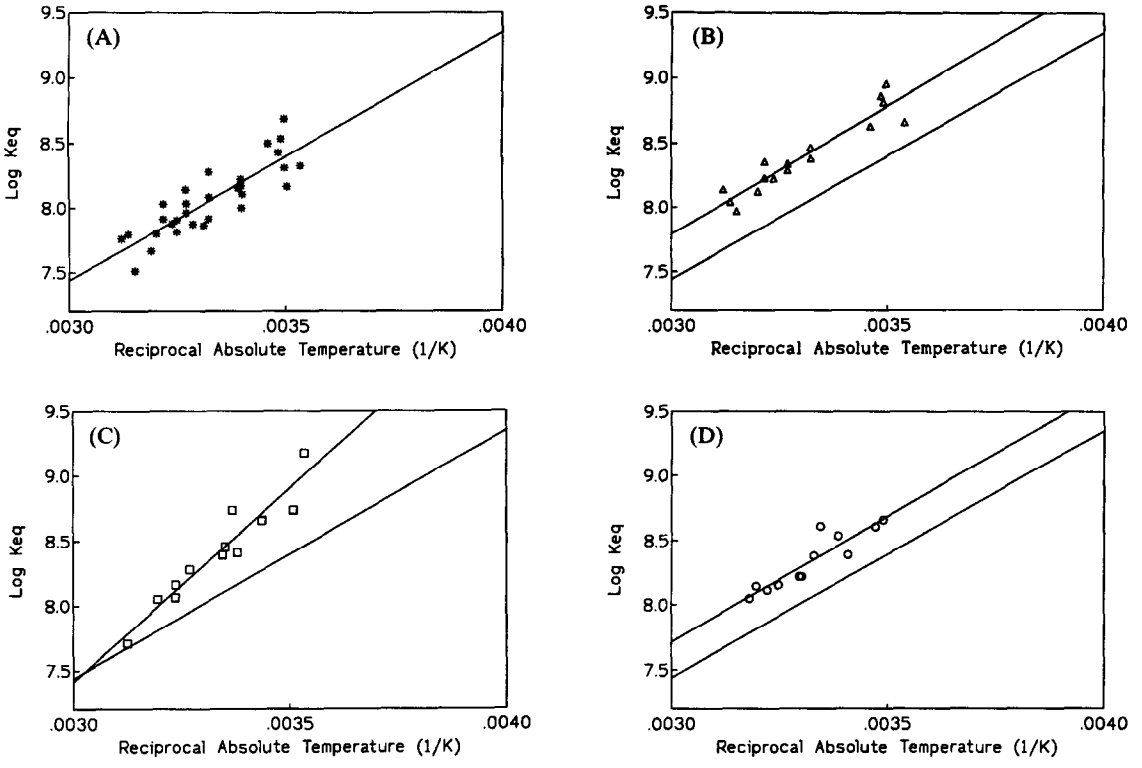

Fig. 1. van't Hoff plots for the binding of [^{3}H]FNZ. (A) control; (B) + 100 μM alphaxalone; (C) + 1 mM pentobarbitone and (D) + 300 μM propofol. K_{eq} values are derived from competition data with K_{eq} taken as $1/K_i$. K_i was derived from IC_{50} values by the Cheng–Prusoff equation. In (B), (C) and (D) the derived control line is included for comparison.

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 9.4 mM in the presence of 1 mM pentobarbitone and 13.7 mM in the presence of 10 μM alphaxalone. These interactions are illustrated in Fig. 4.

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 [8]. However, the value of $\Delta H°$ derived from our present data is somewhat higher than that obtained by other workers [6, 7]. 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

Table 1. Thermodynamic parameters for the binding of [^{3}H]FNZ in 50 mM Na^+/K^+ buffer pH 7.1 containing 200 mM NaCl, in the presence of various modulators

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

The data are derived from linear regression and are expressed as means ± SEM. The slopes were compared using Student's t-test.
* Significantly different from control ($P < 0.05$).

Table 2. EC_{50} values at 4° and 37° for pentobarbitone, propofol and alphaxalone

	EC_{50} (μM) at 4°	EC_{50} (μM) at 37°
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)*

Data are the means ± SEM of (N) experiments.
* Significantly different to 4° by Student's t-test ($P < 0.05$).

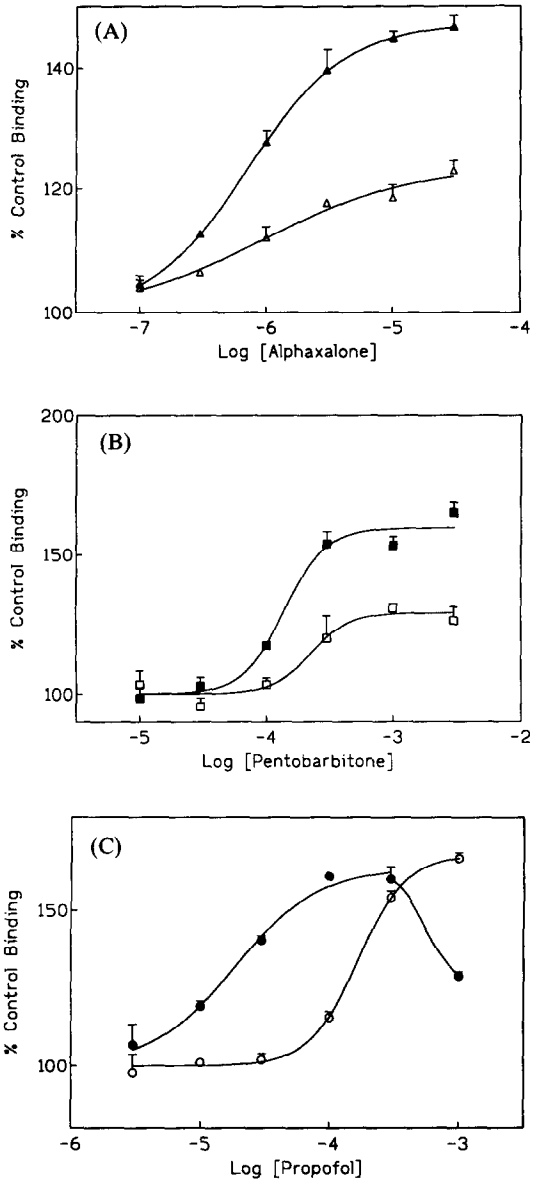

Fig. 2. Dose–response curves for (A) alphaxalone, (B) pentobarbitone and (C) propofol in 10 mM Tris-citrate buffer (open symbols) and in 10 mM Tris-citrate + 200 mM NaCl (filled symbols). Data are the means ± SEM of triplicate values from representative experiments.

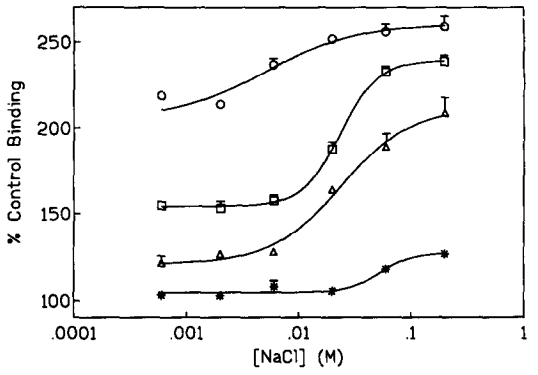

Fig. 3. The potentiation of 1 nM [^{3}H]FNZ binding by NaCl in 10 mM Tris-citrate buffer in the presence and absence of various drugs. Data are the means ± SEM of triplicate values from a representative experiment and are expressed relative to the basal binding in the absence of added salt or drug (*) Control; (□) +1 mM pentobarbitone; (△) +10 μM alphaxalone; (○) +300 μM propofol.

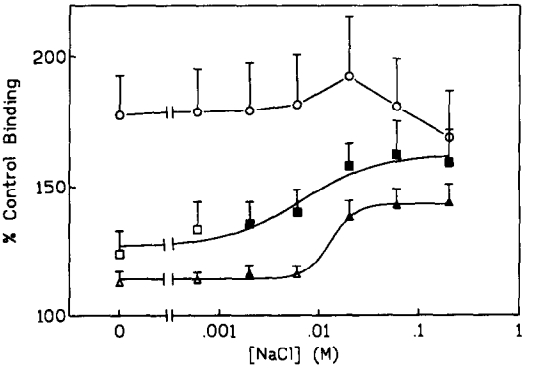

Fig. 4. The potentiation of 1 nM [^{3}H]FNZ binding by 1 mM pentobarbitone (squares); 10 μM alphaxalone (triangles) and 300 μM propofol (circles) in the presence of varying concentrations of NaCl. Closed symbols indicate values which are significantly different from the control (zero NaCl) by Student's *t*-test ($P < 0.05$). Data are the means of 3–4 experiments ± SEM.

Table 3. EC_{50} values at 4° for pentobarbitone, alphaxalone and propofol in the presence of 10 mM Tris-citrate pH 7.4, and Tris-citrate + various salts

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

Data are the means ± SEM of (N) experiments.
* 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 (submitted to *Brain Res.*).

propofol and alphaxalone is temperature insensitive. Previous studies have suggested that certain modulators of $GABA_A$ ligand binding are more efficacious at higher temperatures [14–17]. However, many of these studies have utilized a fixed concentration of ligand and have failed to take 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 [18].

The present results tend to rule out the possibility of membrane fluidization as a mechanism of action for both propofol and alphaxalone in modulating FNZ 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 potentiation being lower at higher temperatures [9], 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 2-[2-methoxyethoxy]-ethyl 8-[*cis*-2-*n*-octylcyclopropyl]-octanoate does not enhance muscimol-stimulated $^{36}Cl^-$ flux or produce anaesthesia [19]. 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 [6]. 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 [20], and propofol and the neurosteroids [21]. However, in our previous study* 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° to 37°, indicates that the association of these modulators with the $GABA_A$ receptor also 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.* [7], 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 [4], both the EC_{50} and maximal potentiation (E_{max}) 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 E_{max} 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 exists for alphaxalone potentiation as for pentobarbitone. Propofol, as we have demonstrated previously,* 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 $> 300\ \mu M$, 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 [22], and of the convulsant *t*-butylbicyclo-phosphorothionate [23], 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 FNZ 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.

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