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The γ-aminobutyrate/benzodiazepine receptor from pig brain

Enhancement of γ-aminobutyrate-receptor binding by the anaesthetic propanidid

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The binding of [^{3}H]muscimol, a γ-aminobutyrate (GABA) receptor agonist, to a membrane preparation from pig cerebral cortex was enhanced by the anaesthetic propanidid in a concentration-dependent manner. At 0 °C, binding was stimulated to 220% of control values, with 50% stimulation at 60 μM-propanidid. At 37 °C, propanidid caused a more powerful stimulation of [^{3}H]muscimol binding (340% of control values). Propanidid (1 mM) exerted little effect on the affinity of muscimol binding (K_D approx. 10 nM), but increased the apparent number of high-affinity binding sites in the membrane by 2-fold. Enhancement of [^{3}H]muscimol binding was observed only in the presence of Cl^- ions, half-maximal activation being achieved at approx. 40 mM-Cl^-. Picrotoxinin inhibited the stimulation of [^{3}H]muscimol binding by propanidid with an IC_{50} (concentration causing 50% inhibition) value of approx. 25 μM. The enhancement of [^{3}H]muscimol binding by propanidid was not additive with the enhancement produced by secobarbital. Phenobarbital inhibited the effect of propanidid and secobarbital. The GABA receptor was solubilized with Triton X-100 or with Chaps {3-[(3-cholamidopropyl)dimethylammonio]propanesulphonate}. Propanidid and secobarbital did not stimulate the binding of [^{3}H]muscimol after solubilization with Triton X-100. However, the receptor could be solubilized by 5 mM-Chaps with retention of the stimulatory effects of propanidid and secobarbital. Unlike barbiturates, propanidid did not stimulate the binding of [^{3}H]flunitrazepam to membranes. It is suggested that the ability to modulate the [^{3}H]muscimol site of the GABA-receptor complex may be a common and perhaps functional characteristic of general anaesthetics.

INTRODUCTION

The major class of γ-aminobutyrate (GABA) receptors in the mammalian central nervous system ($GABA_A$ receptors) mediate inhibitory synaptic transmission by regulating membrane Cl^- ion channels and are defined by their sensitivity to the agonist muscimol and the antagonist bicuculline (Olsen, 1981; Turner & Whittle, 1983). GABA-mediated neurotransmission is facilitated by benzodiazepines and by barbiturates (Haefely *et al.*, 1979; Study & Barker, 1981) and membrane-binding studies *in vitro* indicate reciprocal allosteric interactions between GABA-receptor ligands, benzodiazepines and barbiturates (Olsen, 1981).

Barbiturates and the anxiolytic pyrazolopyridine compounds such as etazolate enhance GABA and benzodiazepine receptor binding in a Cl^--dependent manner that is blocked by picrotoxinin (Leeb-Lundberg *et al.*, 1980, 1981; Supavilai *et al.*, 1982; Placheta & Karobath, 1980; Willow & Johnston, 1981; Asano & Ogasawara, 1981; Whittle & Turner, 1982; Olsen & Snowman, 1982). The ability of a series of barbiturates to enhance GABA-receptor binding correlates with their pharmacological activity as central-nervous-system depressants (Whittle & Turner, 1982; Asano & Ogasawara, 1982; Olsen & Snowman, 1982), indicating that the barbiturate recognition site on the GABA-receptor complex is likely to play a role in at least some of the pharmacological actions of these drugs. In addition, a non-barbiturate hypnotic drug, (+)-etomidate, also facilitates GABA-mediated neurotransmission (Evans & Hill, 1978) and enhances GABA and benzodiazepine receptor binding (Willow, 1981; Ashton *et al.*, 1981; Thyagarajan *et al.*, 1983).

Propanidid (Fig. 1) is structurally unrelated to depressant barbiturates and (+)-etomidate, but can similarly cause rapid hypnosis and has been routinely used as an alternative induction agent for anaesthesia (Conway & Ellis, 1970). In the present paper it is reported that propanidid can interact with the GABA/benzodiazepine receptor. The characteristics of this interaction have been examined in membrane-bound and solubilized preparations of the receptor complex.

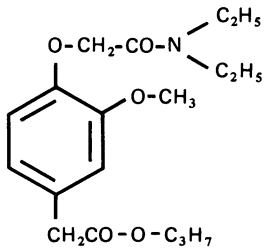

Fig. 1. The chemical structure of propanidid [3-methoxy-4-(*NN*-diethylcarbamoylmethoxy)phenylacetic acid n-propyl ester]

Abbreviations used: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonate; CSM, crude synaptic membranes; GABA, γ-aminobutyrate; PEG, poly(ethylene glycol); IC_{50}, concentration causing 50% inhibition.

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EXPERIMENTAL

Materials

[*methylene*-^{3}H]Muscimol (20.6 Ci/mmol) was purchased from New England Nuclear. Propanidid was a gift from Bayer U.K. Ltd. Muscimol, secobarbital, picrotoxinin and Chaps were from Sigma. All other chemicals were obtained from BDH, Poole, Dorset, U.K.

Preparation of crude synaptic membranes

A crude preparation of synaptic membranes (CSM) was prepared from pig cerebral cortex essentially as described previously (Whittle & Turner, 1982). Pig brain was obtained fresh from a slaughterhouse, and the cortex removed, chopped, frozen and stored at −70 °C until use. Cortex was thawed and homogenized in 10 vol. of 10 mM-Hepes buffer (pH 7.5)/1 mM-EDTA/300 mM-sucrose. After an initial centrifugation at 1000 *g* for 10 min, the supernatant was centrifuged at 20000 *g* for 20 min to obtain a crude synaptosomal pellet. This pellet was washed twice by resuspension in 20 vol. of ice-cold distilled water, followed by centrifugation at 48000 *g* for 20 min. The pellet was then washed once with 20 mM-potassium phosphate buffer, pH 7.5, containing 100 mM-KCl (referred to as 'buffer'), resuspended in buffer and stored frozen at −20 °C for up to 2 months. Before assay of receptor binding, the membranes were thawed, washed once with buffer and dialysed for 20 h against 500 vol. of buffer containing 0.1 mM-EDTA and 0.02% (w/v) NaN_3.

Preparation of solubilized receptor

Frozen CSM were thawed, washed once with buffer and resuspended at a protein concentration of 2–3 mg/ml in buffer containing Triton X-100 or Chaps at the indicated concentrations. The suspension was briefly hand-homogenized and incubated with agitation for 30 min at 4 °C, followed by centrifugation at 140000 *g* for 60 min. The supernatant was dialysed for 12 h before the binding assay against 500 vol. of buffer containing 0.1 mM-EDTA, 0.02% (w/v) NaN_3 and 0.2% Triton X-100 or 5 mM-Chaps as appropriate.

Binding assays

The binding of [^{3}H]muscimol to CSM was performed by a centrifugation assay (Beaumont *et al.*, 1978). Unless stated otherwise, CSM (0.5–0.8 mg) were incubated in a total volume of 1 ml of buffer for 30 min at 0 °C in the presence of 4 nM-[^{3}H]muscimol and various concentrations of drugs. Non-specific binding was measured in the presence of 50 μM-muscimol. After incubation, samples were centrifuged at 48000 *g* for 10 min and the pellets rinsed superficially twice with ice-cold buffer. Rinsed pellets were solubilized overnight in 0.3 ml of NCS Tissue Solubilizer (Amersham) and counted for radioactivity in 5 ml of scintillation fluid [0.5% PPO (2,5-diphenyloxazole) in toluene].

Binding of [^{3}H]muscimol to the solubilized preparation was assayed by a γ-globulin/PEG precipitation/filtration method (Gavish *et al.*, 1979). Solubilized extract (0.3–0.5 mg) was incubated as described above with 10 nM-[^{3}H]muscimol in buffer containing 0.1% (w/v) Triton X-100 or 5 mM-Chaps as appropriate. After incubation, 3% (w/v) γ-globulin (75 μl), followed by 36% (w/v) PEG (425 μl), both in assay medium, were added. Samples were filtered on GF/B glass-fibre filters (Whatman) under suction and washed twice with 5 ml of 10% (w/v) PEG in buffer. Filters were dried and counted in 5 ml of scintillation fluid {0.5% (w/v) PPO, 0.05% (w/v) POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in a toluene/Triton X-100 (1:1. v/v) mixture}. All experimental points were obtained in triplicate. Stock solutions

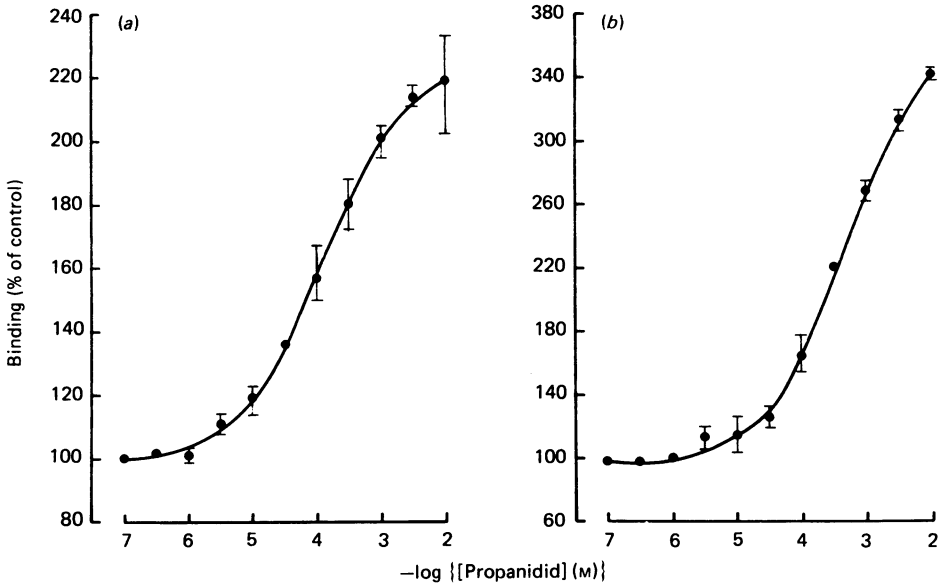

Fig. 2. Effect of propanidid concentration on [^{3}H]muscimol binding to CSM at (*a*) 0 °C and (*b*) 37 °C

The binding of [^{3}H]muscimol to well-washed, frozen and thawed CSM was measured by the centrifugation assay as described in the Experimental section. Binding assays were performed at 0 °C or 37 °C in buffer using 4 nM-[^{3}H]muscimol. Each point represents the mean and range of three separate determinations.

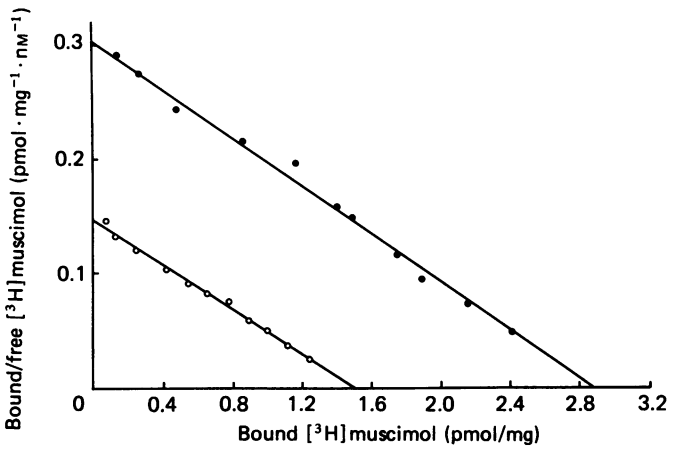

Fig. 3. Scatchard analysis of [³H]muscimol binding to CSM in the absence (○) and presence (●) of 1 mM-propanidid

The concentration of [³H]muscimol was varied from 0.5 to 10 nM at seven concentration points, using 10 μM muscimol at each point to determine the background binding; then the concentration of muscimol was varied by using [³H]muscimol at 10 nM. Results are typical for three separate experiments. The mean value (and range) of the binding coefficients (K_D, $B_{max.}$) were respectively: control, 10.6 nM (10.3–10.9), 1.8 pmol/mg (1.5–1.9); 1 mM-propanidid, 9.6 nM (8.9–10.1), 3.5 pmol/mg (2.9–3.9).

of picrotoxinin and propanidid were prepared in ethanol, and dilutions of propanidid were dispersed by sonication. The final concentration of ethanol in the assay never exceeded 0.5% and did not affect basal specific [³H]muscimol binding measured in the absence of drugs. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Stimulation of [³H]muscimol binding to CSM by propanidid

The binding of [³H]muscimol to an extensively washed membrane preparation from pig cerebral cortex was enhanced by propanidid (10 μM–10 mM) in a concentration-dependent manner (Fig. 2). Binding at 0 °C was stimulated by a maximum of 120% (220% of control) with 50% stimulation at 60 μM-propanidid (Fig. 2*a*). At 37 °C, propanidid caused a more powerful stimulation of up to 340% of control binding (Fig. 2*b*). In the control situation, the proportion of total binding represented by specific binding was reduced from 92% to 75% at the higher temperature. For this reason, all subsequent experiments were performed at 0 °C.

Scatchard analysis of control [³H]muscimol binding to CSM indicated a single class of high-affinity binding sites (Fig. 3). Although the possible existence of a second, very-low-affinity class of binding sites was suggested when the ligand concentration was raised above 0.1 μM, the binding coefficients describing this site were difficult to quantify accurately owing to the larger errors involved when measuring low-affinity binding (results not shown). Propanidid had little effect on the affinity of [³H]muscimol binding, but significantly increased the apparent total number of high-affinity binding sites in the membrane.

Cl^--dependence and picrotoxinin sensitivity are two major characteristics of barbiturate-activated GABA receptor binding (Whittle & Turner, 1982; Olsen & Snowman, 1982). Enhancement of [³H]muscimol binding by propanidid was also observed only in the presence of Cl^- ions (Table 1). The action of Cl^- was concentration-dependent and saturable, with a half-maximal effect at approx. 40 mM. Fig. 4 shows that picrotoxinin inhibited propanidid-stimulation of [³H]muscimol binding in a concentration-dependent manner, with an IC_{50} value of approx. 25 μM and abolition of enhancement at 500 μM. In agreement with the findings of other authors, both Cl^- and picrotoxinin (> 10 μM) were observed to reduce basal levels of ligand binding to the GABA receptor (Olsen & Snowman, 1982; Supavilai *et al.*, 1982).

In contrast with the anaesthetic barbiturates secobarbital and pentobarbital, which stimulate GABA receptor binding powerfully, the effect of phenobarbital is reported to be much weaker (Asano & Ogasawara, 1982) or absent (Whittle & Turner, 1982; Olsen & Snowman, 1982). Phenobarbital can, however, antagonize pentobarbital-induced activation (Whittle & Turner, 1982; Skerritt & Johnston, 1983*a*). Here, secobarbital and phenobarbital were examined for an effect on binding enhanced by propanidid. [³H]Muscimol binding was enhanced by 100 μM-propanidid (158% of control), 1 mM-secobarbital (212%) and 1 mM-phenobarbital (119%), acting individually. In combination, stimulation by propanidid and secobarbital was not additive (217%), whereas phenobarbital reduced the effect of propanidid alone (129%). Addition of phenobarbital also

Table 1. Cl^--dependence of propanidid enhancement of [³H]muscimol binding to CSM

CSM were prepared as described in the Experimental section, but in the absence of KCl. Binding assays were performed in 20 mM-potassium phosphate, pH 7.5, with several concentrations of KCl in the absence and presence of 1 mM-propanidid. Results represent the mean (and range) of three separate determinations.

[KCl] (mM)	Specific binding (pmol·mg⁻¹)		Binding as % of control
	Control	Propanidid	
0	0.93 (0.84–1.03)	0.99 (0.87–1.15)	106
10	0.92 (0.84–1.00)	1.11 (1.04–1.17)	121
20	0.79 (0.76–0.85)	1.18 (1.06–1.26)	149
50	0.74 (0.71–0.78)	1.30 (1.16–1.39)	176
100	0.66 (0.59–0.72)	1.34 (1.16–1.48)	203
200	0.58 (0.52–0.64)	1.28 (1.13–1.38)	221

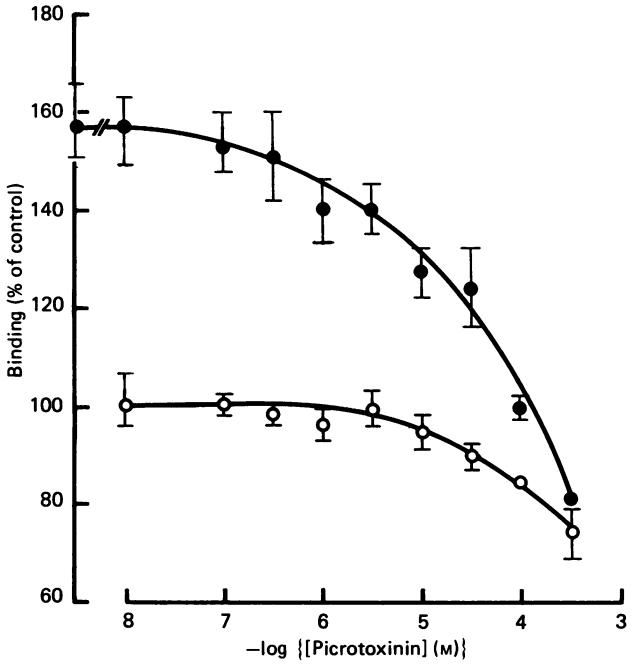

Fig. 4. Picrotoxinin sensitivity of propanidid-enhanced [^{3}H]muscimol binding to CSM

The effect of picrotoxinin on [^{3}H]muscimol binding was measured in the absence (○) and presence (●) of 500 μM-propanidid. Results represent the means and ranges from three separate experiments.

reduced stimulation by secobarbital (172%). Data are the means of three observations which, in all cases, varied by less than 5% from the mean.

Enhancement of solubilized [^{3}H]muscimol binding activity by propanidid

Solubilization of [^{3}H]muscimol binding activity that retains its sensitivity to barbiturates has been achieved by using the mild zwitterionic detergent Chaps (Stephenson & Olsen, 1982). Table 2 lists some characteristics of [^{3}H]muscimol binding activity when solubilized from CSM with 1% Triton X-100 or various concentrations of Chaps. Without prior dialysis, it was difficult to measure specific binding to extracts owing to the presence of endogenous GABA released during solubilization (Greenlee & Olsen, 1979). Dialysis increased baseline specific binding activity by over 400% at 10 nM-[^{3}H]muscimol. Treatment of membranes with Triton X-100 caused an overall activation of baseline binding so that, although the detergent solubilized 70% of the original binding activity, this represented only 37% of the total recovered activity. Secobarbital and propanidid did not stimulate binding to either the Triton X-100-solubilized fraction or the residual membranes. The quantity of binding sites solubilized by Chaps was dependent on the concentration used for initial extraction. Solubilization with 50 mM-Chaps, the highest concentration examined, released 28% of the original membrane-bound activity. The total recovered binding activity was generally in the range 55–65% of original membrane-bound activity, i.e. Chaps caused no activation of baseline binding, in contrast with that observed after Triton X-100 treatment. Enhancement of binding by propanidid was greatest after solubilization with low concentrations of Chaps (< 10 mM). Maximum stimulation was approximately one-third of that observed for the effect on membranes. The high concentrations of Chaps used for solubilization similarly reduced the subsequent stimulation of binding by secobarbital. In addition, it was noted that activation by secobarbital (1 mM) and GABA (0.1 mM) of [^{3}H]diazepam binding to the Chaps-solubilized extracts was also maximal after treatment with low concentrations of Chaps (results not shown). Although the [^{3}H]muscimol binding activity solubilized with 5 mM-Chaps (8% initial membrane-bound) was substantially less than that released at higher concentrations, this activity showed maximum stimulation by propanidid and was therefore used in subsequent experiments.

The ability of propanidid and secobarbital to stimulate binding to the Chaps-solubilized extracts was very unstable to storage at −20° or 4 °C, with maximum enhancement reduced by 90% within 48 h. Baseline [^{3}H]muscimol binding activity remained stable under the same conditions. Fig. 5 shows the concentration-

Table 2. Effects of secobarbital and propanidid on [^{3}H]muscimol binding activity solubilized with 1% Triton X-100 or various concentrations of Chaps

Solubilized binding activity was extracted from CSM and measured by using 10 nM-[^{3}H]muscimol in a precipitation/filtration assay as described in the Experimental section. Binding to CSM and detergent-treated CSM was measured by the same filtration assay, but omitting precipitation. Triton X-100-treated CSM are the insoluble membrane fragments remaining after solubilization with 1% Triton X-100. The data are from one experiment which was representative of two to five separate experiments.

Treatment	Protein (mg)	Specific [^{3}H]muscimol binding: Control (pmol·mg^{-1})	1 mM-Secobarbital (% of control)	1 mM-Propanidid (% of control)
CSM	50.0	0.787	205	209
1% Triton X-100 extract	25.2	1.130	102	96
1% Triton X-100-treated CSM	20.3	2.340	94	89
Chaps extract				
2 mM	4.5	0.202	153	133
5 mM	13.6	0.246	192	136
10 mM	19.6	0.310	156	117
20 mM	24.5	0.347	138	115
50 mM	29.6	0.382	122	107

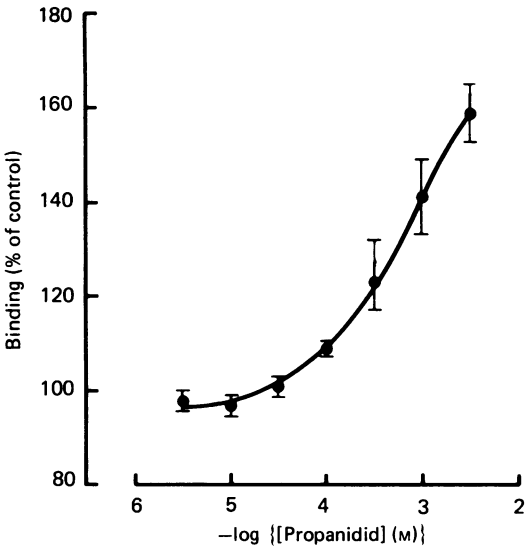

Fig. 5. Enhancement by propanidid of [^{3}H]muscimol binding to a 5 mM-Chaps-solubilized extract

Each point represents the mean and range for determinations from three independent preparations.

dependent enhancement by propanidid (100 μM–5 mM) of binding to the receptor extract solubilized with 5 mM-Chaps. Binding was stimulated by a maximum of 59%.

DISCUSSION

The binding of GABA to washed brain membrane preparations is enhanced by barbiturates and etomidate with a specificity and stereospecificity that correlates with their pharmacological activity as central-nervous-system depressants (Whittle & Turner, 1982; Olsen & Snowman, 1982; Thyagarajan *et al.*, 1983). Enhancement is optimal in the presence of Cl^- ions, is sensitive to picrotoxinin and results from an increase in the apparent number of GABA-binding sites. In the present study we have examined the ability of an unrelated hypnotic, propanidid, to interact with the GABA-binding site of membrane-bound and solubilized preparations of the GABA/benzodiazepine-receptor complex. Propanidid caused a concentration-dependent enhancement of [^{3}H]muscimol binding to well-washed membranes with 50% stimulation over control binding at 60 μM and a maximum stimulation at 0 °C of 120%. In these respects, propanidid is as potent as the anaesthetic barbiturates secobarbital and pentobarbital (Whittle & Turner, 1982; Olsen & Snowman, 1982). Raising the binding temperature from 0 °C to 37 °C has been reported either to increase pentobarbital enhancement of [^{3}H]muscimol binding (Quast & Brenner, 1983) or to have little effect (Olsen & Snowman, 1982). In the present study, the maximum stimulation by propanidid was doubled at the higher temperature, although the threshold concentration required for an effect remained unchanged.

The kinetics of muscimol binding to brain membranes, like those of GABA binding, is usually described as biphasic, with binding data analysed in terms of a high- and a low-affinity population of binding sites (Olsen, 1981). Selective binding of muscimol to one subclass of GABA-binding sites in well-washed membranes has also been reported (Skerritt & Johnston, 1983*b*). The absolute values for the dissociation constants characterizing the two populations of binding sites can vary over orders of magnitude depending on the method of membrane preparation. Pentobarbital enhances [^{3}H]GABA and [^{3}H]muscimol binding principally by increasing the number of high-affinity binding sites (Whittle & Turner, 1982; Olsen & Snowman, 1982; Supavilai *et al.*, 1982), possibly by increasing the affinity of previously undetectable, very-low-affinity binding sites (Olsen & Snowman, 1982). Propanidid was also found to enhance [^{3}H]muscimol binding by increasing the apparent number of high-affinity binding sites. The existence of very-low-affinity binding sites for [^{3}H]muscimol was suggested at ligand concentrations greater than 0.1 μM, but estimates of the binding coefficients describing these sites were highly variable and did not provide any additional information regarding the mechanism of propanidid enhancement. Enhancement of binding by propanidid was dependent on the presence of Cl^-. In common with the Cl^--dependent stimulation of GABA-receptor binding by pentobarbital (Olsen & Snowman, 1982; Supavilai *et al.*, 1982), enhancement by propanidid results principally from a protection against the inhibitory actions of Cl^-. Picrotoxinin inhibition of barbiturate enhancement has been interpreted in terms of a barbiturate/picrotoxinin site on the GABA/benzodiazepine-receptor complex (Olsen, 1981), although recent evidence suggests that recognition sites for depressant barbiturates and picrotoxinin may be distinct, although closely associated (Maksay & Ticku, 1985; Trifiletti *et al.*, 1985). Picrotoxinin can also block the enhancing effect of propanidid at concentrations that only slightly inhibit baseline [^{3}H]muscimol binding. This would suggest that propanidid and the barbiturates may interact with the GABA-binding site via the same, or related, sites. This notion is supported by additional evidence showing that stimulation of binding by secobarbital and propanidid is not additive, and that phenobarbital, a non-depressant barbiturate that blocks enhancement of binding by pentobarbital (Whittle & Turner, 1982), also inhibits stimulation by propanidid. Unlike barbiturates, however, preliminary studies have indicated that propanidid does not cause a stimulation of [^{3}H]flunitrazepam binding to membranes, but slightly inhibits binding at high concentrations (E. F. Kirkness & A. J. Turner, unpublished work). The reason for this difference in ability to interact with the benzodiazepine receptor is presently unknown.

To characterize further the interaction of propanidid with GABA-receptor binding, we have solubilized and purified the GABA/benzodiazepine-receptor complex from pig brain [the following paper (Kirkness & Turner, 1986)]. In agreement with other reports, treatment of membranes with Triton X-100 was found to stimulate baseline GABA-receptor binding (Enna & Snyder, 1977; Chiu & Rosenberg, 1979), whereas the solubilized and residual membrane-binding activities were unresponsive to both secobarbital and propanidid. The ability of pentobarbital to stimulate the binding of GABA to membranes is greatly reduced after treatment of the membranes with low concentrations of Triton X-100 (Asano & Ogasawara, 1981; Willow & Johnston, 1981; Olsen & Snowman, 1982). By using the detergent Chaps, GABA-receptor-binding activity has been solubilized in a form that can be stimulated by pentobarbital (Stephenson & Olsen, 1982). We have found that optimal

enhancement of binding by both secobarbital and propanidid occurred after extraction of membranes with low concentrations of Chaps (< 10 mM). Enhancement of solubilized benzodiazepine binding activity in the presence of GABA was also found to be sensitive to the concentration of Chaps used for solubilization, with a maximal effect after extraction with low concentrations (Mernoff *et al.*, 1983; E. F. Kirkness & A. J. Turner, unpublished work). The concentration of Chaps used for solubilizing the GABA/benzodiazepine-receptor complex would appear to be critical for the efficient maintenance of the various interactions between the different recognition sites in the solubilized form. Propanidid stimulated [^{3}H]muscimol binding to a 5 mM-Chaps extract in a dose-dependent manner. The response, however, was much reduced compared with the situation in intact membranes, and rapidly decayed under storage conditions that did not affect baseline [^{3}H]muscimol binding. A factor possibly contributing to the decreased response could be a reduced concentration of available drug, owing to its solubility in detergent micelles (Stephenson & Olsen, 1982). This may explain why secobarbital enhances solubilized binding activity to a greater extent than the more lipophilic propanidid (Table 2). The ability of propanidid to enhance [^{3}H]muscimol binding to a purified preparation of the GABA/benzodiazepine receptor complex was retained after solubilization and purification in the presence of 5 mM-Chaps (see the following paper, Kirkness & Turner, 1986).

General anaesthesia is generally accepted to be a consequence of a depression in central synaptic activity (Roth, 1979). If propanidid also causes a potentiation of the electrophysiological actions of GABA, as predicted by its effect on GABA-receptor binding, the interaction would provide an explanation for the general depressant effects of the drug at a molecular level. At the concentrations of propanidid found in serum during anaesthesia (10–50 μM) (Doenicke *et al.*, 1968), propanidid can enhance GABA-receptor binding substantially (Fig. 2*a*). While this paper was in preparation, a report appeared which described the ability of a steroid anaesthetic, alphaxalone, to potentiate the electrophysiological actions of GABA and enhance GABA-receptor binding (Harrison & Simmonds, 1984). An ability of several structurally diverse general anaesthetics to modulate the GABA-receptor complex would therefore appear to be a common and perhaps functional characteristic.

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