The modulation by chlormethiazole of the GABA_A-receptor complex in rat brain

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1 The interactions of chlormethiazole with γ -aminobutyric acid (GABA) synthesis and release, and with ligand binding to sites associated with the GABA_A-receptor complex and the GABA_B-receptor have been studied in the rat. The GABA_A-receptor was studied using [³H]-muscimol, [³H]-flunitrazepam was used to label the benzodiazepine modulatory site, and [³⁵S]-butylbicyclophosphorothionate ([³⁵S]-TBPS) to label the chloride channel.

2 Chlormethiazole had no effect on GABA synthesis in the cortex, hippocampus and striatum or on GABA release from cortical slices *in vitro*. Chlormethiazole did not displace [³H]-baclofen binding to the GABA_B-receptor.

3 Chlormethiazole (IC₅₀ = 140 μ M) and pentobarbitone (IC₅₀ = 95 μ M) both inhibited [³⁵S]-TBPS binding by increasing the rate of [³⁵S]-TBPS dissociation. In addition, chlormethiazole caused an apparent decrease in the affinity of [³⁵S]-TBPS binding.

4 Chlormethiazole enhanced the binding of [³H]-muscimol but had no effect on [³H]-flunitrazepam binding. In contrast, the sedative barbiturate pentobarbitone enhanced both [³H]-muscimol and [³H]-flunitrazepam binding.

5 It is concluded that the sedative and anticonvulsant effects of chlormethiazole are probably mediated through an action at the GABA_A-receptor. However, chlormethiazole does not interact with the GABA_A-receptor complex in an identical manner to the sedative barbiturate pentobarbitone.

Introduction

Chlormethiazole is used clinically as a sedative, hypnotic and anticonvulsant agent (Evans *et al.*, 1986). Many compounds with this profile of action have been shown to interact with the γ -aminobutyric acid_A (GABA_A)-receptor complex, and potentiate responses to GABA (see Simmonds & Turner, 1987 for review). The GABA_A-receptor is an oligomeric protein containing an ion channel permeable to Cl[−] ions. The activity of the ion channel is modulated not only by the binding of GABA to a specific binding site but also by the binding of several chemically distinct compounds (Schwartz, 1988 for review). Benzodiazepines bind to a specific site on the GABA_A-receptor and increase the frequency of the opening of the Cl[−] channel (Study & Barker, 1981), whereas barbiturates bind to a distinct and separate site and prolong the length of opening of the channel

(Barker & McBurney, 1979). Convulsant compounds such as picrotoxin and the cage convulsants such as *t*-butylbicyclophosphorothionate (TBPS) antagonise the effects of GABA by blocking channel opening. Barbiturates and some related compounds have been shown to interact with the TBPS/picrotoxin binding site labelled by [³⁵S]-TBPS in brain membranes (Squires *et al.*, 1983). In addition, sedative barbiturates will potentiate the binding of [³H]-muscimol and [³H]-flunitrazepam to the GABA_A-receptor (Leeb-Lundberg *et al.*, 1980; Whittle & Turner, 1982; Thyagarajan *et al.*, 1983; Lohse *et al.*, 1987).

Electrophysiological studies have shown that chlormethiazole can interact with GABA_A-receptors in a manner apparently analogous to that of barbiturates (Harrison & Simmonds, 1983; Hedlund *et al.*, 1987). In the present study we have examined the effects of chlormethiazole on ligand binding to sites

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associated with the GABA_A-receptor in rat brain cortex membranes. In addition, the effect of chlormethiazole on the synthesis of GABA in rat brain and on GABA release from rat cortical tissue prisms has also been examined.

A preliminary account of some of these findings has been presented to the British Pharmacological Society (Cross *et al.*, 1988).

Methods

Animals

Male Lister hooded rats (about 200 g) were used. They were housed in groups of 6 under a 12 h light/dark cycle with food and water available *ad libitum*.

[³⁵S]-TBPS binding

The cerebral cortex was homogenised by an Ultra-Turrax in 50 volumes of 50 mM Tris-HCl pH 7.4 and centrifuged at 20,000 *g* for 20 min at 4°C. The resulting pellet was resuspended in 50 mM Tris-citrate buffer pH 7.4 containing NaCl (100 mM) and centrifuged at 20,000 *g* for 20 min at 4°C. The next resulting pellet was washed a further 4 times by resuspension in 50 mM Tris-citrate, NaCl (100 mM) buffer and centrifuged as previously. The final crude membrane pellet was resuspended in 40 volumes of the buffer and used immediately.

Binding assays contained 5 nM [³⁵S]-TBPS, 160 µl of membrane preparation and displacing agents in a total volume of 200 µl of 50 mM Tris-citrate buffer. Incubations were carried out in plastic microtitre plates, for 90 min at room temperature. Bound ligand was separated by rapid filtration and washing over glass fibre filters by use of a Skatron cell harvester (Hall & Thor, 1979). Non-specific binding was defined as that not displaced by 10 µM picrotoxin.

Saturation studies employed a range of [³⁵S]-TBPS concentrations from 2.5 to 200 nM, achieved by diluting [³⁵S]-TBPS with unlabelled TBPS. For studies of the dissociation of bound [³⁵S]-TBPS, membranes were incubated with 5 nM [³⁵S]-TBPS for 90 min at room temperature. Dissociation was initiated by the addition of unlabelled TBPS (final concentration 5 µM) with or without drugs under study and incubations were filtered at various times thereafter.

[³H]-flunitrazepam binding

Cerebral cortex was homogenised in 20 volumes of 0.32 M sucrose and centrifuged at 1000 *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 20,000 *g* for 20 min at 4°C. This pellet was washed

four times by resuspension in 10 mM sodium phosphate buffer containing 200 mM NaCl pH 7.4.

Binding assays contained membrane preparation, [³H]-flunitrazepam (1 nM final concentration) and drugs in 200 µl 10 mM sodium phosphate buffer, which contained 200 mM sodium chloride pH 7.4. Incubations were performed in plastic microtitre plates, for 30 min on ice and terminated by rapid filtration. Non-specific binding was defined as that not displaced by 10 µM diazepam.

[³H]-muscimol and [³H]-(-)-baclofen binding

A frozen osmotically-shocked membrane preparation from rat cortex was used for both [³H]-(-)-baclofen and [³H]-muscimol binding assays.

Frozen cortex was homogenized in 6 volumes ice-cold 10 µM Tris-HCl, pH 7.4, containing 0.32 M sucrose, by use of a Potter Elvehjem homogeniser. The homogenate was centrifuged at 500 *g* for 10 min. The resulting supernatant was stored on ice and the pellet re-homogenised and re-centrifuged twice so that the three pooled supernatants gave a total suspension of 18 volumes per g tissue. The supernatant was centrifuged at 30,000 *g* for 30 min at 4°C and the resulting pellet resuspended in 60 volumes of ice-cold distilled water by use of an Ultra Turrax homogeniser, this suspension was centrifuged and resuspended in distilled water as before. Membrane pellets were harvested by centrifugation at 30,000 *g* for 12 min at 4°C and stored at -20°C for [³H]-(-)-baclofen binding. For [³H]-muscimol binding, after freezing at -20°C overnight, membrane pellets were thawed at room temperature, resuspended in 90 volumes of distilled water and centrifuged at 30,000 *g* for 12 min at 4°C. All membrane pellets were stored at -20°C for up to 2 months before their use in binding assays.

For [³H]-muscimol binding (Williams & Risley, 1979), frozen membrane pellets were thawed, resuspended in ice-cold 50 mM Tris-HCl, pH 7.4 by use of an Ultra-Turrax homogeniser and centrifuged at 30,000 *g* for 10 min at 4°C. The resulting pellet was resuspended in buffer (1–1.5 mg protein ml⁻¹) and used immediately in the assay. The assay, which contained 10 nM [³H]-muscimol and approximately 0.2 mg membrane protein in 200 µl 50 mM Tris HCl (containing 50 mM KCl pH 7.4), was incubated on ice for 10 min and terminated by filtration.

[³H]-baclofen binding was studied by using a modification of the method described by Bowery *et al.* (1983), in particular the binding assay was terminated by filtration rather than centrifugation. Frozen membrane pellets were thawed, resuspended in assay buffer (50 mM Tris-HCl, pH 7.4 containing CaCl₂, 2.5 mM) and incubated at room temperature for 45 min. The membranes were centrifuged at 30,000 *g*

for 12 min at 20°C and then washed in assay buffer a further three times, with 15 min incubations between each centrifugation. Final equilibrated membrane pellets were resuspended in assay buffer (1–2 mg protein ml⁻¹) and assayed immediately. The binding assay was performed in 96 well microtitre plates in a total incubation volume of 200 µl, and routinely utilized 30 nM [³H]-baclofen, and tissue homogenate (0.25 mg protein). After 10 min incubation at 25°C the binding was terminated by filtration through glass fibre filters by use of a Skatron semi-automatic cell harvester. Filters were rapidly washed with approximately 1 ml ice-cold assay buffer in a 2 s wash time. After drying, the radioactivity on the filters was measured by liquid scintillation counting. The non-specific binding of both [³H]-baclofen and [³H]-muscimol was defined with an excess of GABA.

GABA synthesis

The synthesis of GABA in the cortex, hippocampus and striatum was assessed by measuring the accumulation of GABA following an injection of amino-oxyacetic acid (AOAA), a method recently described and validated by Green *et al.* (1987). Briefly, animals were injected with chlormethiazole (35 mg kg⁻¹, i.p.). Animals were killed 60 min after administration of AOAA, by exposure of the head to a focussed high intensity microwave beam of power density 0.7 kW for 2.2 s. Cortex, hippocampus and striatum were removed from the brain and the GABA extracted in 2 mM HCl at 120°C for 15 min. Extracts were centrifuged at 5000 *g* for 5 min and the GABA concentration in the resultant supernatants was assayed by a slight modification of the enzymatic-fluorimetric assay of Baxter (1972), as described by Bowdler & Green (1982).

GABA release from rat cortical tissue prisms

Tissue prisms from whole cortex were prepared by cutting at 0.3 mm intervals in 2 planes (45° angle) with a McIlwain tissue chopper. The pooled slices were suspended in 30 ml Krebs bicarbonate buffer, pH 7.4 and bubbled with 95% O₂/5% CO₂ at 37°C. The medium composition was as follows (mM): NaCl 118.5, KH₂PO₄ 1.18, MgSO₄ 1.18, NaHCO₃ 25, glucose 10 and CaCl₂ 2.54. 'Basal' release was measured in the presence of 1 mM KCl and 'K⁺-evoked' release in 35 mM KCl. Tissue prisms were preincubated at 37°C for 1 h, the buffer being changed every 15 min. The buffer was discarded leaving a packed slice suspension. Aliquots of the slice suspension (60 µl, 1 mg protein) were distributed evenly into baskets made from plastic test tubes with nylon mesh bases. The baskets of slices were preincubated

in 1 ml of Krebs buffer for 10 min at 37°C. This, and all subsequent incubations contained 100 µM nipecotic acid. The baskets were blotted and immersed in a second set of vials which contained 1 ml fresh, pre-gassed buffer (containing drug where appropriate) and incubated for 5 min (basal release). The baskets were then blotted and transferred into another set of vials which contained 35 mM K⁺ buffer (containing drug where appropriate) for a further 5 min (K⁺-evoked release). After this incubation, the baskets were blotted and put into vials containing 1 ml of 0.1 M NaOH solution, SDS (0.7% w/v) and NaCO₃ (2% w/v), to solubilize the slices before analysis of the protein content by a phenol reagent technique (Dawson & Heatlie, 1984). The supernatants (800 µl) were removed and centrifuged at 4000 *g* for 5 min. The concentration of GABA in the supernatants was measured by h.p.l.c. as described by Procter *et al.* (1988).

Chemicals

[³⁵S]-TBPS (70–80 Ci mmol⁻¹), [³H]-flunitrazepam (80–85 Ci mmol⁻¹), [³H]-muscimol (15–20 Ci mmol⁻¹) and unlabelled TBPS were obtained from New England Nuclear, U.K. Phenobarbitone, pentobarbitone, diazepam, picrotoxin and GABA were supplied by Sigma Chemical Co., Poole. Chlormethiazole was obtained from Dr Ulf Lindberg, Astra Alab, Sweden.

Results

Ligand binding to the GABA receptor

Chlormethiazole (100 µM) did not inhibit [³H]-muscimol binding to the GABA_A-receptor or [³H]-baclofen binding to the GABA_B-receptor. In addition, chlormethiazole (100 µM) did not affect [³H]-flunitrazepam binding (data not shown, but see next section).

[³⁵S]-TBPS binding was fully displaced by GABA, picrotoxin and chlormethiazole (Figure 1). Pentobarbitone was also an effective inhibitor of [³⁵S]-TBPS binding, whereas phenobarbitone was less active (Figure 1).

[³⁵S]-TBPS binding to rat cortical membranes was saturable, the K_D being 70 ± 11 nM. In the presence of GABA, binding affinity was unchanged whereas the number of binding sites was significantly reduced (Table 1), consistent with non-competitive inhibition. Both chlormethiazole and picrotoxin decreased the apparent affinity of binding without significantly affecting the number of binding sites, consistent with an apparent competitive inhibition (Table 1).

Figure 1 Inhibition of [^{35}S]-butylbicyclopophosphorothionate ([^{35}S]-TBPS) binding by γ -aminobutyric acid (GABA) and drugs active at the GABA receptor. (■) picrotoxin, (□) GABA, (▲) pentobarbitone, (△) chlormethiazole, (×) phenobarbitone. Each curve is the mean of 3–4 experiments, s.e.mean for all points was less than 8%. IC_{50} values calculated from Hill plots were: GABA, $4.1 \pm 0.4 \mu\text{M}$; picrotoxin, $105 \pm 20 \text{ nM}$; chlormethiazole, $140 \pm 30 \mu\text{M}$ and pentobarbitone, $95 \pm 24 \mu\text{M}$.

Under control conditions the dissociation of [^{35}S]-TBPS was biphasic (Figure 2). The addition of chlormethiazole ($100 \mu\text{M}$ and 1 mM) increased the rate of dissociation, in the presence of 1 mM chlormethiazole only a single rapid dissociation was apparent. A similar change in the dissociation kinetics was observed in the presence of $300 \mu\text{M}$ pentobarbitone (Figure 2).

Stimulation of [^3H]-muscimol and [^3H]-flunitrazepam binding

Chlormethiazole, like pentobarbitone, stimulated [^3H]-muscimol binding in a dose-dependent manner (Figure 3a). Both compounds were approximately

Figure 2 Dissociation of bound [^{35}S]-butylbicyclopophosphorothionate ([^{35}S]-TBPS) in the presence of chlormethiazole and pentobarbitone. Membranes were incubated with [^{35}S]-TBPS to equilibrium. Dissociation was initiated by the addition of an excess ($5 \mu\text{M}$) of unlabelled TBPS, either alone (□) or in the presence of $100 \mu\text{M}$ chlormethiazole (△), 1 mM chlormethiazole (▲) or 1 mM pentobarbitone (●). The data are from a representative experiment, which was repeated at least 3 times.

Figure 3 (a) Stimulation of [^3H]-muscimol binding by pentobarbitone (■) and chlormethiazole (□). (b) Effects of pentobarbitone (■) and chlormethiazole (□) on [^3H]-flunitrazepam binding. Each point is the mean of 3 experiments, vertical lines represent s.e.mean.

Table 1 Interaction of chlormethiazole with the [^{35}S]-butylbicyclopophosphorothionate ([^{35}S]-TBPS) binding site

Compound	[^{35}S]-TBPS binding constants	
	K_D (nM)	B_{max} (fmol)
Control	70 ± 11	126 ± 17
GABA	78 ± 17	$78 \pm 9^*$
Picrotoxin	$203 \pm 32^*$	165 ± 30
Chlormethiazole	$233 \pm 72^*$	142 ± 30

Values are the mean $\pm$ s.e.mean of 3 experiments. Binding constants were derived from Scatchard plots using linear regression. $^*P < 0.01$ vs control (Student's t test, 2-tailed).

Figure 4 Effects of chlormethiazole (Cmz) in combination with pentobarbitone (Pent) on [^3H]-flunitrazepam binding (a) and [^3H]-muscimol binding (b). Both drugs were added at a final concentration of 1 mM. Results are the mean of 3 experiments, bars represent s.e.mean.

equipotent. The stimulation of [^3H]-muscimol binding by pentobarbitone (1 mM) was not significantly altered by 1 mM chlormethiazole (Figure 4). Pentobarbitone also enhanced [^3H]-flunitrazepam binding (Figure 3b). In contrast, chlormethiazole was without effect on [^3H]-flunitrazepam binding.

Table 3 The effect of chlormethiazole (Cmz) on γ -aminobutyric acid (GABA) release from rat cortical slices

	Release of endogenous GABA	
	Basal (1 mM K^+)	Stimulated (35 mM K^+)
Control	2.08 ± 0.27	5.26 ± 1.2
Cmz (100 μM)	2.17 ± 0.40	6.15 ± 0.78

Values are expressed as pmol GABA mg^{-1} protein 5 min^{-1} ; mean $\pm$ s.d. of 4 experiments. GABA release in the presence of Cmz was not significantly different from controls (Student's *t* test, 2-tailed).

However, chlormethiazole (1 mM) inhibited the stimulation of [^3H]-flunitrazepam binding produced by 1 mM pentobarbitone (Figure 4).

The effect of chlormethiazole on GABA synthesis

Acute administration of chlormethiazole (35 mg kg^{-1} , i.p.) did not alter the concentration of GABA in the cortex, striatum and hippocampus 40 min later (Table 2). The rate of synthesis of GABA, as indicated by the accumulation of GABA following an injection of aminooxyacetic acid, was also unaffected by chlormethiazole (Table 2).

The effect of chlormethiazole on GABA release

Chlormethiazole (100 μM) had no effect on the release of GABA from cortical slices under basal or depolarising (35 mM K^+) conditions (Table 3).

Discussion

In agreement with a previous study (Ogren, 1986), a single injection of chlormethiazole (35 mg kg^{-1} , i.p.) did not alter the concentration of GABA in the

Table 2 The effects of chlormethiazole (Cmz) on basal concentrations and aminooxyacetic acid (AOAA)-induced accumulation of γ -aminobutyric acid (GABA) in rat brain

	Hippocampus		Striatum		Cortex	
	Control	Cmz	Control	Cmz	Control	Cmz
Basal	2.13 ± 0.20 (10)	2.03 ± 0.03 (4)	2.74 ± 0.03 (10)	2.76 ± 0.21 (4)	1.46 ± 0.16 (10)	1.37 ± 0.11 (4)
AOAA	4.15 ± 0.35 (8)	3.83 ± 0.46 (8)	4.27 ± 0.70 (8)	4.14 ± 0.61 (9)	3.37 ± 0.56 (8)	3.32 ± 0.60 (8)
Synthesis rate	2.02	1.80	1.53	1.38	1.91	1.95

GABA concentrations are nmol g^{-1} tissue, mean $\pm$ s.d. The number of samples is given in parentheses. Synthesis rate is nmol g^{-1} tissue h^{-1} . There were no significant differences between controls and chlormethiazole-treated animals (one way analysis of variance).

brain. Moreover the rate of synthesis of GABA, as estimated by the aminooxyacetic acid method, was also unaltered. Treatment of cortical slices with chlormethiazole (100 μ M) was without effect on either the basal or potassium-evoked release of GABA. It would seem therefore that if the effects of chlormethiazole are mediated via the GABA system, this does not involve presynaptic mechanisms.

Chlormethiazole (100 μ M) did not inhibit the binding of [3 H]-muscimol to the GABA_A-receptor or [3 H]-baclofen binding to the GABA_B-receptor. It is unlikely therefore that chlormethiazole is a direct agonist at either the GABA_A- or the GABA_B-receptor. However, the results of the present study strengthen the previous suggestions that chlormethiazole interacts with the Cl⁻ channel of the GABA_A-receptor as it was an effective inhibitor of [35 S]-TBPS binding. Although the potency of chlormethiazole at the [3 H]-TBPS binding site appears weak in comparison with many other drug-receptor interactions, serum concentrations of chlormethiazole can reach 100–200 μ M at hypnotic doses (Kim & Khanna, 1983). The IC₅₀ of chlormethiazole in [35 S]-TBPS binding (140 μ M) was very similar to the potency of chlormethiazole in potentiating the electrophysiological response to GABA in rat cuneate nucleus (Harrison & Simmonds, 1983) and cultured rat spinal cord neurones (Hedlund *et al.*, 1987). The affinity of chlormethiazole in [32 S]-TBPS binding was similar to that of pentobarbitone (see legend to Figure 1).

Activity at [35 S]-TBPS binding clearly does not relate to anticonvulsant activity, as phenobarbitone is an effective anticonvulsant but is virtually inactive in [35 S]-TBPS binding, while pentobarbitone had a potency similar to chlormethiazole. It has been noted by others that the sedative actions of barbiturates may correlate with their activity at the site on the GABA_A-receptor labelled with [35 S]-TBPS (Whittle & Turner, 1982). By analogy with these barbiturates it would seem likely that the interaction

of chlormethiazole with the [35 S]-TBPS binding site may relate to its sedative effect. This conclusion is strengthened by observations that the sedative effects of chlormethiazole are potentiated by muscimol and aminooxyacetic acid (Ogren, 1986).

Saturation analysis suggested that both picrotoxin and chlormethiazole were competitive inhibitors of [35 S]-TBPS binding. This effect of chlormethiazole differs from that obtained for sedative barbiturates which lower the B_{max} of [35 S]-TBPS (Ticku & Ramanjaneyulu, 1984; Lohse *et al.*, 1987). The dissociation experiments demonstrate, however, that the interaction of chlormethiazole with the [35 S]-TBPS binding site is not truly competitive. Thus chlormethiazole increased the dissociation rate of bound [35 S]-TBPS suggesting an allosteric interaction. A similar effect has been noted previously for the sedative barbiturate, methohexitone (Lohse *et al.*, 1987), and in the present study with pentobarbitone. In this respect chlormethiazole seems to be acting in a similar manner to the sedative barbiturates. The studies on the enhancement of [3 H]-muscimol and [3 H]-flunitrazepam binding by chlormethiazole suggest that these interactions of chlormethiazole may well be distinct from that of the sedative barbiturates. Thus, whilst both chlormethiazole and pentobarbitone enhanced [3 H]-muscimol binding, in contrast to pentobarbitone, chlormethiazole had no effect on [3 H]-flunitrazepam binding (Figure 3b). It has been shown previously that chlormethiazole does not enhance [3 H]-diazepam binding (Leeb-Lundberg *et al.*, 1981). Moreover, when added in combination, 1 mM chlormethiazole effectively antagonised the stimulation of [3 H]-flunitrazepam binding induced by 1 mM pentobarbitone.

In conclusion, the present study provides *in vitro* evidence for the interaction of chlormethiazole with the GABA_A-receptor. This interaction may account for the sedative properties of chlormethiazole, and is similar, but not identical to the effects of barbiturates.

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