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Allosteric interactions between γ-aminobutyric acid, benzodiazepine and picrotoxinin binding sites in primary cultures of cerebellar granule cells. Differential effects induced by γ- and δ-hexachlorocyclohexane

Carmen Vale [a], Anna Pomés [a], Eduard Rodríguez-Farré [b], Cristina Suñol [a,*]

[a] *Department of Neurochemistry, Institut d'Investigacions Biomèdiques de Barcelona, CSIC, Jordi Girona 18–26, E-08034 Barcelona, Spain*
[b] *Department of Pharmacology and Toxicology, Institut d'Investigacions Biomèdiques de Barcelona, CSIC, Jordi Girona 18–26, E-08034 Barcelona, Spain*

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Abstract

Allosterism between γ-aminobutyric acid (GABA), benzodiazepine and picrotoxinin recognition sites on the $GABA_A$ receptor was studied in primary cultures of cerebellar granule cells. The increase in [^{3}H]flunitrazepam binding induced by GABA was inhibited by bicuculline and picrotoxinin and the decrease in [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding mediated by GABA was reverted by bicuculline. The effects of hexachlorocyclohexanes (the convulsant γ- and the depressant δ-isomers, both acting at the picrotoxinin recognition site) on GABA and benzodiazepine sites were studied. δ-Hexachlorocyclohexane, but not the γ-isomer (lindane), increased [^{3}H]flunitrazepam binding in a concentration-dependent manner (EC_{50}: 8.3 μM). This increase in [^{3}H]flunitrazepam binding was reduced by bicuculline and picrotoxinin. The γ-isomer reduced the increase in [^{3}H]flunitrazepam binding induced by GABA or δ-hexachlorocyclohexane. Neither δ- nor γ-hexachlorocyclohexane inhibited [^{3}H]GABA binding. Moreover, the inhibition of [^{35}S]TBPS binding induced by δ-hexachlorocyclohexane was not reverted by bicuculline. The results obtained in this study in vitro agree with the pharmacological properties and the effects of γ- and δ-hexachlorocyclohexane in vivo. It is concluded that δ-hexachlorocyclohexane acts as a positive allosteric modulator and γ-hexachlorocyclohexane acts as a non-competitive antagonist of the $GABA_A$ receptor.

Keywords: Hexachlorocyclohexane isomer; Lindane; $GABA_A$ receptor; Flunitrazepam binding; GABA (γ-aminobutyric acid) binding; TBPS (*t*-butylbicyclophosphorothionate) binding; Cerebellar granule cell, cultured

1. Introduction

The γ-aminobutyric acid (GABA) receptor/Cl^- ionophore complex ($GABA_A$ receptor) is an oligomeric protein that has separate but allosterically interacting binding sites for the endogenous neurotransmitter GABA, for benzodiazepines and for picrotoxinin-like convulsants. A wide spectrum of drugs, toxic agents and metals modify the $GABA_A$ receptor function by directly interacting with these binding sites or with other not yet well described sites present in this receptor complex (Sieghart, 1992; Macdonald and Olsen, 1994; Narahashi et al., 1994). Known allosteric modulations include the enhanced binding of benzodiazepine agonists by GABA, the enhanced GABA-induced Cl^- flux by benzodiazepines and barbiturates and the different modifications of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS, ligand of choice for the picrotoxinin recognition site) binding induced by GABA, benzodiazepines and barbiturates.

A series of compounds acting at the picrotoxinin recognition site by competitively inhibiting [^{35}S]TBPS binding have convulsant activity: among them, picrotoxinin, pentylenetetrazol and the organochlorine pesticides γ-hexachlorocyclohexane (lindane) and cyclodienes (Cole and Casida, 1986; Fishman and Gianutsos, 1988; Suñol et al., 1989; Pomés et al., 1993). In addition, these compounds inhibit the GABA-induced Cl^- flux (Wafford et al., 1989; Narahashi et al., 1992; Woodward et al., 1992; Pomés et al., 1994b; Tokutomi et al., 1994). Modulation of the other well characterized binding sites at the $GABA_A$ receptor by these picrotoxinin-like compounds has been poorly studied. Early studies using brain membrane preparations showed that the pesticide γ-hexachlorocyclohexane

* Corresponding author. Tel.: (34-3) 400-6152; Fax: (34-3) 204-5904; e-mail: csenqi@cid.csic.es

does not affect [^{3}H]flunitrazepam or [^{3}H]GABA or [^{3}H]muscimol binding (Cattabeni et al., 1983; Abalis et al., 1985). However, rats treated with γ-hexachlorocyclohexane show an increase in [^{3}H]flunitrazepam and a decrease in [^{3}H]muscimol binding in almost all the cerebral regions studied, which may be secondary to the inhibition of TBPS binding by lindane acting as an allosteric antagonist of the $GABA_A$ receptor (Solà et al., 1993). Lindane, picrotoxinin and picrotoxinin-like cage convulsants potentiate the protective effect of NaCl against heat inactivation of [^{3}H]flunitrazepam binding sites (Squires and Saederup, 1989). In addition, the cytotoxic action of lindane on cultured cortical neurons is reduced by GABA and flunitrazepam (Pomés et al., 1994a).

The δ-isomer of hexachlorocyclohexane is a central nervous system depressant that antagonizes convulsions induced by lindane and pentylenetetrazol (McNamara and Krop, 1948; Fishman and Gianutsos, 1988; Tusell et al., 1993). It also inhibits [^{35}S]TBPS binding although with lower potency than the convulsant γ-hexachlorocyclohexane (Pomés et al., 1993). However, in contrast to the γ-isomer, it increases the GABA-induced Cl^- flux (Woodward et al., 1992; Pomés et al., 1994b; Nagata and Narahashi, 1995).

The different neuropharmacological behaviour of γ- and δ-isomers can be accounted for by an inhibitory or facilitatory effect at the $GABA_A$ receptor (Pomés et al., 1994b) or by a different mechanism of action of both isomers (Pessah et al., 1992; Cristòfol and Rodríguez-Farré, 1994). One argument in favour of a common target on the picrotoxinin recognition site that elicits opposite responses is the similarity of effects with butyrolactones. These compounds differentially modify GABA-activated currents depending on their convulsant properties but both inhibit [^{35}S]TBPS binding and the convulsant compounds are more potent (Holland et al., 1990). However, in support of the hypothesis of a different target site for γ- and δ-isomers, it has been reported that δ-hexachlorocyclohexane inhibits, in a stereoselective manner, the [^{3}H]ryanodine binding to the Ca^{2+} channel of cardiac sarcoplasmic reticulum, skeletal muscle and the brain cortex (Pessah et al., 1992). It has also been reported that γ- and δ-hexachlorocyclohexane modify the K^+-evoked release of [^{3}H]noradrenaline by interacting with presynaptic molecular processes involving changes in Cl^- permeability and intracellular Ca^{2+} homeostasis, respectively (Cristòfol and Rodríguez-Farré, 1994).

The present study was aimed at determining whether γ- and δ-hexachlorocyclohexane interact with $GABA_A$ receptor binding sites other than the site of their primary action on [^{35}S]TBPS binding at the picrotoxinin recognition site and if so, whether this could explain the contrasting neuropharmacological profile of these isomers. The effect of these isomers on [^{3}H]flunitrazepam and [^{3}H]GABA binding was studied in neurons using primary cultures of cerebellar granule cells, which express full $GABA_A$ receptor characteristics (Belhage et al., 1986; Vaccarino et al., 1987; Pomés et al., 1993). Cerebellar granule cells were chosen to perform this study since this specific cerebellar layer has the richest density of [^{3}H]muscimol and [^{35}S]TBPS and a reasonably density of [^{3}H]flunitrazepam binding sites in the rat brain (Solà et al., 1993).

2. Materials and methods

2.1. Chemicals

Sources were as follows: δ- and γ-hexachlorocyclohexane were supplied by Promochem (Wesel, Germany). GABA, bicuculline methiodide, picrotoxinin, ryanodine, caffeine, cytosine arabinoside, 5-fluoro-2′-deoxyuridine, uridine, poly-D-lysine, kainate, trypsin, trypsin soybean inhibitor and DNAse were obtained from Sigma (St. Louis, MO, USA). Flunitrazepam was a gift from Productos Roche (Madrid, Spain). Foetal calf serum was from Sera-Lab (UK) and Dulbecco's modified Eagle's medium (MEM) was from Biochrom (Berlin, Germany).

2.2. Cell cultures

Primary cultures of cerebellar granule cells were obtained from cerebella of 7-day-old rats (Belhage et al., 1986). In brief, cells were dissociated by mild trypsinization (0.025% (w/v) trypsin at 37°C for 15 min) followed by trituration in a DNAse solution (0.004%, w/v) containing a soybean trypsin inhibitor (0.05%, w/v). The cells were suspended in Dulbecco's MEM (25 mM KCl, 31 mM glucose, 0.8 mM glutamine) supplemented with *p*-aminobenzoate, insulin, penicillin and 10% foetal calf serum. Kainate (50 μM) was added to eliminate GABAergic interneurons (Drejer and Schousboe, 1989). The cell suspension was seeded in 6- or 12-multiwell plates precoated with poly-D-lysine and incubated for 8–12 days in a humidified 5% CO_2/95% air atmosphere at 36.8°C. Cytosine arabinoside (20 μM) or a mixture of 5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine was added at 48 h to prevent glial proliferation.

2.3. [^{35}S]TBPS binding

[^{35}S]TBPS (> 60 Ci/mmol, New England Nuclear, Boston, MA, USA) binding in intact cultures was performed at 25°C in 50 mM Tris-citrate buffer (pH 7.4) containing 0.2 M NaCl (Pomés et al., 1993). Non-specific binding was determined in the presence of 100 μM picrotoxinin. Plates were washed three times in 1 ml/well of buffer and the binding reaction took place in the culture dish in a final volume of 1 ml consisting of incubation buffer, [^{35}S]TBPS (1–2 nM) and drug solutions. After 30 min incubation without shaking, cold buffer was added and

rapidly removed by suction and cells were rinsed three times in 1 ml cold buffer. Cells were collected in 1 ml 0.2 M NaOH (after 1 h at room temperature), neutralized with 2 N HCl and used to measure radioactivity and protein content. Experiments were usually performed simultaneously in four or more plates. In experiments involving organochlorine compounds control binding was determined in the presence of dimethyl sulfoxide (DMSO) ($\leq 0.1\%$ v/v).

2.4. [^{3}H]Flunitrazepam binding

Benzodiazepine binding to intact cultures was measured using 1.25–2.5 nM [^{3}H]flunitrazepam (> 80 Ci/mmol, Amersham Life Science, Amersham, UK) in 50 mM Tris-citrate buffer (pH 7.4) containing 0.2 M NaCl. Non-specific binding was determined in the presence of 20 μM flunitrazepam. Saturation curves were performed at 0°C with [^{3}H]flunitrazepam concentrations between 0.3 and 40 nM.

After checking that the increase in [^{3}H]flunitrazepam binding induced by GABA or δ-hexachlorocyclohexane at 0°C was the same as at 25°C we followed a similar procedure as mentioned above for [^{35}S]TBPS binding. This allowed us to perform [^{35}S]TBPS and [^{3}H]flunitrazepam binding in the same well when needed. In these cases, non-specific binding was determined in the presence of 100 μM picrotoxinin and 20 μM flunitrazepam.

2.5. [^{3}H]GABA binding

For [^{3}H]GABA binding, Triton X-100-treated freeze-thawed membranes were used (Horng and Wong, 1979; Maksay, 1988). Cells were harvested in 50 mM Tris-citrate buffer (pH 7.1), homogenized with a Polytron for 15 s and centrifuged at $48\,000 \times g$ for 20 min. The pellet was resuspended in distilled water, homogenized and centrifuged at $48\,000 \times g$ for 20 min. This second pellet was resuspended in 0.05% Triton X-100 for 10 min at 0–4°C, homogenized and centrifuged at $48\,000 \times g$ for 20 min, rinsed twice with buffer solution to eliminate Triton X-100 and frozen overnight. On the following day and before performing the binding assay, membranes were thawed, homogenized and centrifuged at $48\,000 \times g$ for 20 min. Membranes were suspended in 50 mM Tris-citrate. Binding assay aliquots (1 ml) in duplicate or triplicate were incubated by shaking in the presence of 4 nM [^{3}H]GABA (86 Ci/mmol, Amersham) for 20 min at 0°C. Non-specific binding was determined in the presence of GABA 1 mM. [^{3}H]GABA binding was determined using 25–70 μg protein per binding assay aliquot. Incubations were terminated by rapid filtration and rinses through glass-fiber filters (Whatman GF/B) using a Brandel M24 filtering manifold (Brandel Instruments, Gaithersburg, MD, USA). Saturation curves were performed with [^{3}H]GABA concentrations between 0.05 and 50 nM.

In all the experiments radioactivity was determined by liquid scintillation counting and proteins were determined by a micro-test using the Bradford method, with bovine serum albumin as a standard.

2.6. Data analysis

Values are means ± S.E.M. Data from saturation and inhibition curves were analyzed using the non-linear least-squares curve fitting program EBDA, LIGAND from Elsevier Biosoft and the GraphPad Inplot program. Concentration-response curves were fitted to sigmoid curves using the GraphPad Inplot program. Curves were compared using the *F*-test for paired experiments. Analysis of variance (one-way and two-way) followed by Duncan or Student's *t*-test were used.

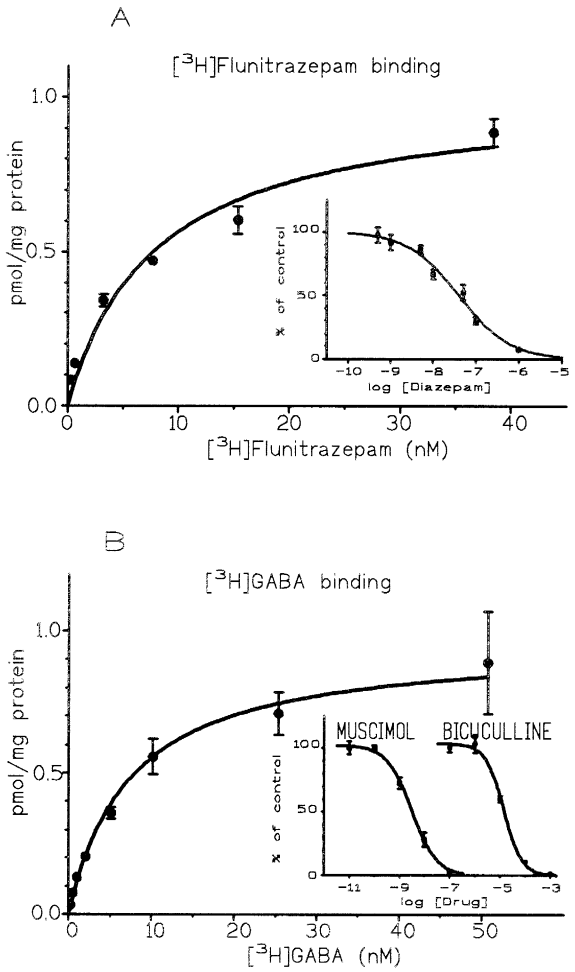

Fig. 1. Saturation curves for [^{3}H]flunitrazepam and [^{3}H]GABA binding to the $GABA_A$ receptor in cultured cerebellar granule cells. Results are means ± S.E.M. ($n = 2$–4) of a representative experiment. Non-specific binding accounted for < 15% of total. (A) [^{3}H]Flunitrazepam (0.3–40 nM) binding was performed on intact cultures of cerebellar granule cells. Inset: Inhibition by diazepam ($n = 6$–9). (B) Na^+-independent [^{3}H]GABA (0.05–50 nM) binding was performed in membranes obtained from cultured cerebellar granule cells. Inset: Inhibition by muscimol (■) and bicuculline (●) ($n = 3$–5).

3. Results

3.1. [^{3}H]Flunitrazepam and [^{3}H]GABA binding to cerebellar granule cells

Saturation curves for [^{3}H]flunitrazepam (0.3–40 nM) binding in intact cultures of cerebellar granule cells were obtained in 12-well plates. K_d values and Hill coefficients were determined from each individual plate. A Hill coefficient of 0.97 ± 0.08 ($n = 8$) accounted for a single class of binding sites with a K_d of 3.28 ± 0.22 nM ($n = 8$). B_{max} in four plates was 0.73 ± 0.06 pmol/mg protein (Fig. 1A). The inset of Fig. 1 shows inhibition of [^{3}H]flunitrazepam binding by diazepam (IC_{50}: 39 nM).

[^{3}H]GABA binding to the $GABA_A$ receptor in intact cultured cells can be masked by the binding of [^{3}H]GABA to its transport site, which is an energy- and Na^+,Cl^--dependent process (Enna and Snyder, 1975). Thus, the binding of GABA to $GABA_A$ receptors requires the use of a Na^+-free buffer. However, using intact cultures of cerebellar granule cells we did not find specific Na^+-independent [^{3}H]GABA binding in Tris-HCl buffer either at 0°C (4.7 nM [^{3}H]GABA binding: 59.2 ± 7.8, $n = 6$ and 48.8 ± 3.1, $n = 4$ fmol/mg protein in the absence or presence of 1 mM GABA, respectively) or at 25°C (4.3 nM [^{3}H]GABA binding: 77.2 ± 10.7, $n = 3$ and 59.3 ± 4.2, $n = 3$, fmol/mg protein in the absence or presence of 1 mM GABA, respectively). Therefore, to study the Na^+-independent GABA binding to the $GABA_A$ receptor we determined [^{3}H]GABA binding in membranes obtained from cultured cerebellar granule cells, which were treated with Triton X-100 and washed extensively. A unique class of sites with a K_d of 12.6 ± 2.4 nM ($n = 3$) and a B_{max} of 1.1 ± 0.3 pmol/mg protein ($n = 3$) was determined (Fig. 1B). This Na^+-independent [^{3}H]GABA binding was inhibited by muscimol (IC_{50}: 3 nM) and bicuculline (IC_{50}: 13 μM) (inset Fig. 1B).

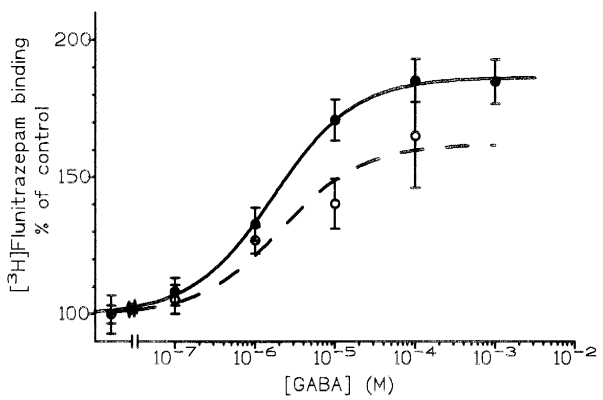

Fig. 2. Enhancement of [^{3}H]flunitrazepam binding by GABA. [^{3}H]Flunitrazepam binding was performed at 0°C (empty circles) or at 25°C (filled circles). Results (means ± S.E.M., $n = 3$–26) are expressed as percentage of control binding. Percentages were calculated for each individual experiment with respect to its control and values from different experiments were pooled. Control values for specific [^{3}H]flunitrazepam (1.4–2.7 nM) binding ranged from 104 to 230 fmol/mg protein.

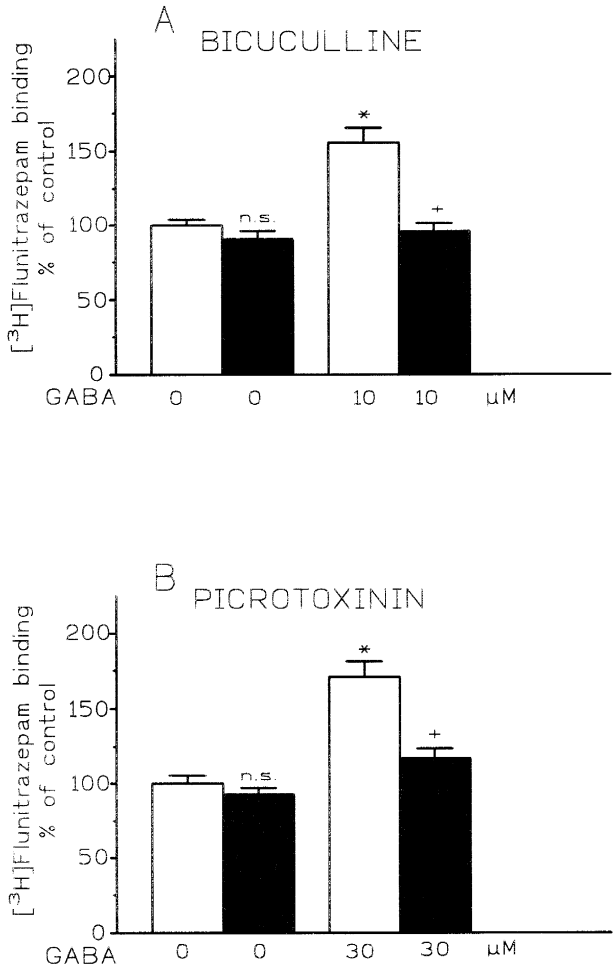

Fig. 3. Effect of $GABA_A$ receptor antagonists (black bars) on GABA-induced increase in [^{3}H]flunitrazepam binding. Bars are means ± S.E.M. of 6–14 determinations. (A) Inhibition by 20 μM bicuculline. Two-way analysis of variance showed significant differences ($P < 0.005$) for GABA ($F(1,46) = 22.5$); bicuculline ($F(1,46) = 30.7$) and a significant interaction between both factors ($F(1,46) = 13.0$). (B) Inhibition by 50 μM picrotoxinin. Two-way analysis of variance showed significant differences ($P < 0.005$) for GABA ($F(1,21) = 48.6$); picrotoxinin ($F(1,21) = 18.6$) and a significant interaction between both factors ($F(1,21) = 11.3$). * $P \leq 0.001$ vs. control; + $P \leq 0.001$ from GABA in the absence of antagonist; n.s.: not-significantly different from control (Student's *t*-test).

3.2. Coupling of GABA, benzodiazepine and picrotoxinin binding sites in cerebellar granule cells

[^{3}H]Flunitrazepam binding in intact cultured cerebellar granule cells was increased by GABA in a dose-dependent manner both at 0°C and at 25°C (Fig. 2). EC_{50} values for GABA-induced increase in [^{3}H]flunitrazepam binding were similar at both temperatures (1.7 μM and 2.1 μM, after fitting experimental data to sigmoid curves). The increase in [^{3}H]flunitrazepam binding induced by 10–30 μM GABA was inhibited by competitive and non-competitive (bicuculline and picrotoxinin, respectively) $GABA_A$ recep-

tor antagonists (Fig. 3). A more complex study at different concentrations of GABA was performed (Fig. 4). The concentration-response curve of the effect of GABA on [^{3}H]flunitrazepam binding was shifted to the right in the presence of bicuculline or picrotoxinin. Fitting the data of Fig. 4A to sigmoid curves revealed a rise in the EC_{50} value for GABA-inducing increase in [^{3}H]flunitrazepam binding from 1.6 μM to 56.3 μM (in the absence and presence of bicuculline, respectively) without modifying the maximum response (175% and 179% vs. control in the absence and presence of bicuculline, respectively). To determine whether the two sets of data (without/with bicuculline) differ significantly, we pooled them and fitted them to a common sigmoid curve (Motulsky and Ransnas, 1987). Statistical comparison of this fitting to those obtained using separate sets of data for each curve indicates that the two separate fits (without/with bicuculline) were much better than the fit by one common sigmoid curve ($F(3,3) = 91.5$; $P = 0.002$). Picrotoxinin increased the EC_{50} (from 1.8 μM to 9.2 μM in the absence and presence of picrotoxinin, respectively) and also reduced the maximum GABA response (from 171% to 141% vs. control in the absence and presence of picrotoxinin, respectively) (Fig. 4B). Again, separate fits for each set of data

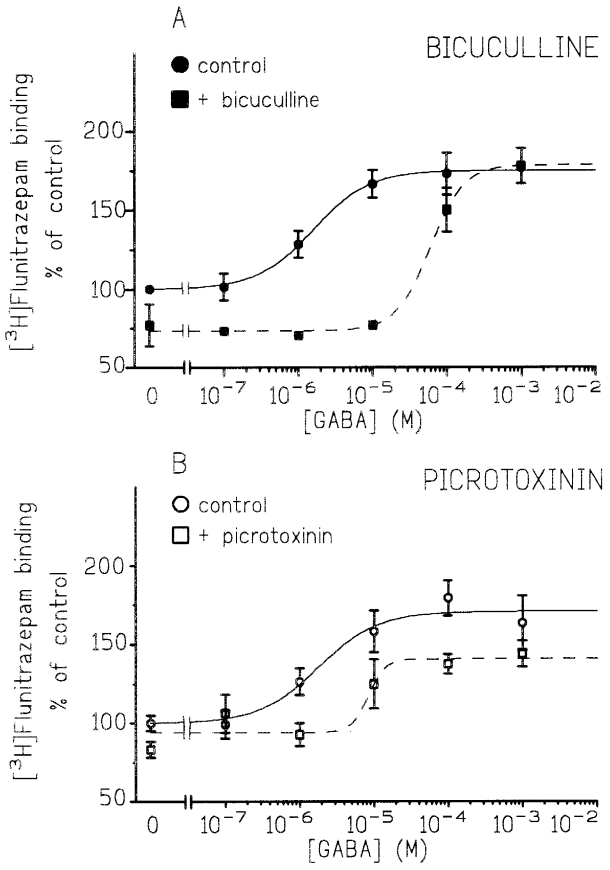

Fig. 4. Concentration-response curves for GABA-enhancing [^{3}H]flunitrazepam binding. Effect of 20 μM bicuculline (A) and of 50 μM picrotoxinin (B). Values are means ± S.E.M. of 3–6 determinations.

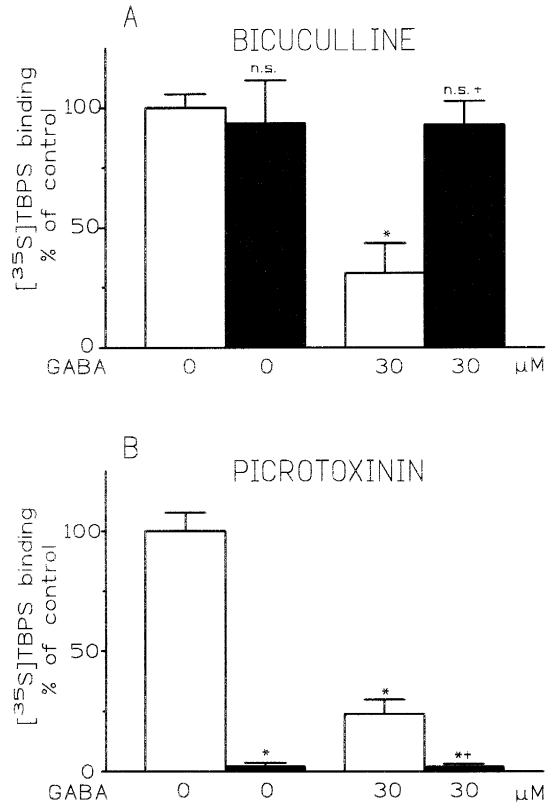

Fig. 5. Inhibition of [^{35}S]TBPS binding by GABA. Effect of $GABA_A$ receptor antagonists. Bars are means ± S.E.M. of 3–7 determinations. [^{35}S]TBPS binding in the absence (white bar) or presence (black bar) of $GABA_A$ receptor antagonists. Specific [^{35}S]TBPS (1.8–2.1 nM) binding of controls was 46.3 ± 1.9 (A) and 62.0 ± 2.3 (B) fmol/mg protein. (A) Effect of bicuculline (20 μM). Two-way analysis of variance showed significant differences ($P < 0.05$) for GABA ($F(1,14) = 9.14$) and a significant interaction between GABA and bicuculline ($F(1,14) = 6.9$). (B) Effect of picrotoxinin (50 μM). Two-way analysis of variance showed statistically significant differences ($P < 0.005$) for GABA ($F(1,21) = 54.7$), picrotoxinin ($F(1,21) = 130.6$) and a significant interaction between GABA and picrotoxinin ($F(1,21) = 50.1$). * $P < 0.001$ from control; + $P < 0.005$ vs. GABA in the absence of antagonist; n.s.: not-significantly different from control (Student's *t*-test).

(without/with picrotoxinin) were significantly better than a common fit ($F(3,4) = 9.48$; $P = 0.03$).

Allosteric interactions were also observed between GABA and TBPS binding sites since GABA significantly decreased the binding of [^{35}S]TBPS (Fig. 5). Bicuculline reverted the inhibition of [^{35}S]TBPS binding induced by GABA (Fig. 5A) whereas picrotoxinin by itself inhibited [^{35}S]TBPS binding and did not revert the effect of GABA (Fig. 5B).

3.3. Effect of the γ- and δ-hexachlorocyclohexane isomers on [^{3}H]flunitrazepam binding in primary cultures of cerebellar granule cells

Using the paradigm of the previous experiments we studied the effect of the convulsant and non-convulsant

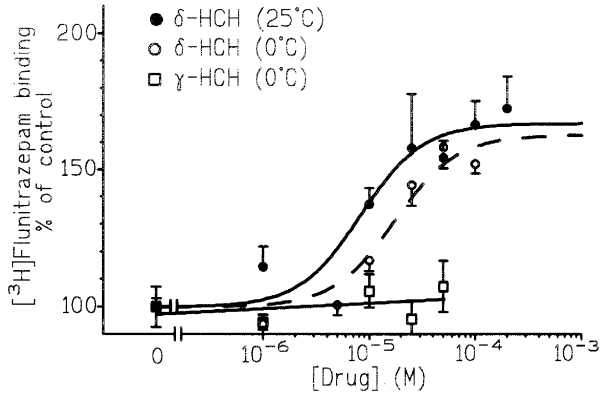

Fig. 6. Effect of δ- and γ-hexachlorocyclohexane on [3H]flunitrazepam binding in primary cultures of cerebellar granule cells. Results are expressed as means ± S.E.M. of 4–32 determinations. HCH: hexachlorocyclohexane.

hexachlorocyclohexane isomers (γ and δ, respectively) on [3H]flunitrazepam binding. Exposure of cultures to hexachlorocyclohexane isomers did not modify protein content (data not shown). Fig. 6 shows that the depressant compound (δ-hexachlorocyclohexane) increased [3H]flunitrazepam binding to intact cultures of cerebellar granular cells in a concentration-dependent manner. This effect was similar at 0°C and 25°C (EC_{50} values for δ-hexachlorocyclohexane-induced increase in [3H]flunitrazepam binding were 8.3 and 17.3 μM and the maximum effect was 167% and 162% at 25°C and 0°C, respectively). The convulsant compound γ-hexachlorocyclohexane (1–50 μM) did not significantly modify [3H]flunitrazepam binding. However, concentrations of γ-hexachlorocyclohexane that inhibit [35S]TBPS binding and reduce the GABA-induced Cl^- flux in neuronal cultures (Narahashi et al., 1992; Woodward et al., 1992; Pomés et al., 1993, 1994a,b; Tokutomi et al., 1994), also reduced the increase in [3H]flunitrazepam binding induced by GABA or δ-hexachlorocyclohexane. Fig. 7A shows that γ-hexachlorocyclohexane (50 μM) reduced the maximum effect of GABA (Fig. 7A) and the effect of δ-hexachlorocyclohexane at the range of concentrations used (Fig. 7B). Fitting the data of Fig. 7A or 7B to separate curves (without/with γ-hexachlorocyclohexane) is significantly better than the fitting to common curves ($F(3,4) = 69.2$;

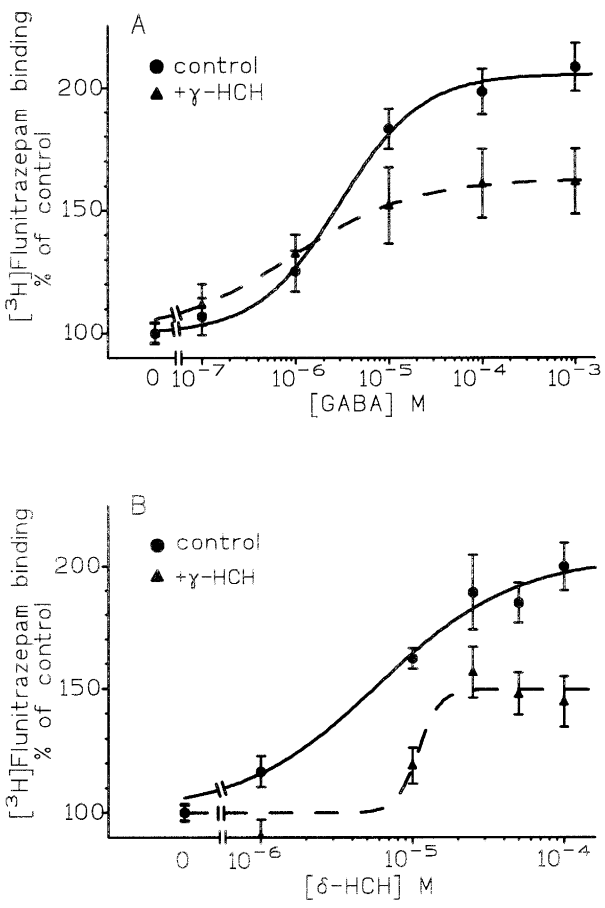

Fig. 7. Effect of γ-hexachlorocyclohexane (50 μM) on the concentration-response curves for GABA- (A) and δ-hexachlorocyclohexane- (B) induced increase in [3H]flunitrazepam binding. Values are means ± S.E.M. of 8–20 determinations. HCH: hexachlorocyclohexane.

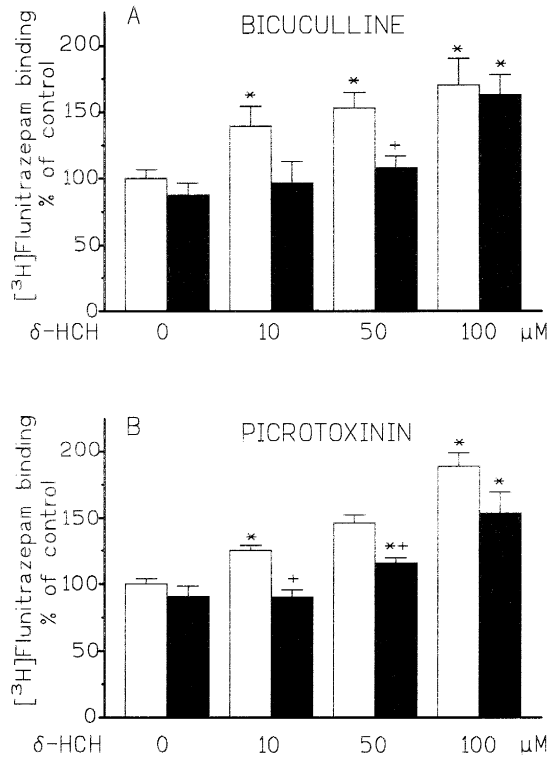

Fig. 8. Effect of $GABA_A$ receptor antagonists (black bars) on δ-hexachlorocyclohexane-induced increase in [3H]flunitrazepam binding. Bars are means ± S.E.M. of 3–14 determinations. HCH: hexachlorocyclohexane. (A) Effect of bicuculline (20 μM). Two-way analysis of variance showed significant differences ($P \leq 0.01$) for δ-hexachlorocyclohexane ($F(3,49) = 11.85$) and bicuculline ($F(1,49) = 11.0$). (B) Effect of picrotoxinin (50 μM). Two-way analysis of variance showed significant differences ($P < 0.01$) for δ-hexachlorocyclohexane ($F(3,63) = 42.69$) and picrotoxinin ($F(1,63) = 35.37$). * $P < 0.05$ vs. control (one-way ANOVA followed by Duncan test); + $P < 0.05$ vs. δ-hexachlorocyclohexane in the absence of antagonist (Student's *t*-test).

Table 1
[^{3}H]Flunitrazepam binding to intact cultured cerebellar granule cells – effect of ryanodine and caffeine

	[^{3}H]Flunitrazepam binding (% of control)
Control	100.00 ± 5.74 (5)
Ryanodine 0.1 μM	97.71 ± 3.24 (5)
Ryanodine 0.3 μM	85.46 ± 7.99 (5)
Ryanodine 3 μM	80.14 ± 10.28 (4)
Caffeine 10 mM	2.22 ± 1.02 (5) [a]

Results are means ± S.E.M. In parentheses, number of determinations. Control [^{3}H]flunitrazepam (2.3 nM) binding was 141 ± 8 (n = 5) fmol/mg protein. [a] $P < 0.05$ vs. control after one-way analysis of variance ($F(4,19) = 45.26$; $P < 0.001$).

$P < 0.005$ and $F(3,4) = 18.8$; $P < 0.01$, for GABA and δ-hexachlorocyclohexane, respectively).

Both competitive and non-competitive $GABA_A$ antagonists significantly reduced the increase in [^{3}H]flunitrazepam binding induced by δ-hexachlorocyclohexane at doses of up to 50 μM (Fig. 8). Two-way analysis of variance of the data shown in Fig. 8 reveals significant differences for the two main factors, antagonist (bicuculline, Fig. 8A, and picrotoxinin, Fig. 8B) and δ-hexachlorocyclohexane without interaction between factors.

We have observed that δ-hexachlorocyclohexane increased intracellular free Ca^{2+} levels in primary cultures of cerebellar granule cells by a mechanism involving mobilization of Ca^{2+} from intracellular stores (Rosa et al., 1996a). In relation with these results, it has also been reported that δ-hexachlorocyclohexane inhibits [^{3}H]ryanodine binding in microsomal brain preparations (Pessah et al., 1992). We studied the effect of ryanodine and caffeine (which affect intracellular Ca^{2+} mobilization, Miller, 1991) on [^{3}H]flunitrazepam binding, to examine the effect of a Ca^{2+} release from intracellular stores. Table 1 shows that ryanodine did not modify [^{3}H]flunitrazepam binding whereas caffeine strongly reduced it.

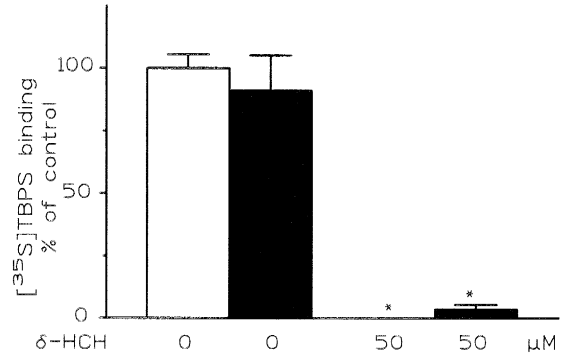

Fig. 9. Effect of bicuculline (20 μM; black bars) on [^{35}S]TBPS inhibition by δ-hexachlorocyclohexane. Bars are means ± S.E.M. of 5–6 determinations. Two-way analysis of variance showed significant differences ($P < 0.005$) for δ-hexachlorocyclohexane ($F(1,19) = 195.8$) without significant interaction between δ-hexachlorocyclohexane and bicuculline. * $P < 0.005$ vs. control (Student's t-test). HCH: hexachlorocyclohexane.

Table 2
Na^+-independent [^{3}H]GABA binding to membranes from cerebellar granule cells – effect of δ- and γ-hexachlorocyclohexane

Concentration (μM)	[^{3}H]GABA binding (% of control)	
	δ-HCH	γ-HCH
0	100.0 ± 1.5 (11)	100.0 + 3.0 (5)
0.01	99.8 ± 2.5 (9)	94.1 ± 4.5 (3)
0.1	100.6 ± 3.3 (10)	
1	94.9 ± 3.4 (9)	100.4 ± 4.8 (5)
10	94.2 ± 1.7 (5)	109.9 ± 1.3 (2)
50	93.4 ± 6.5 (8)	107.1 ± 1.3 (2)
100	87.6 ± 2.3 (5)	94.0 ± 4.4 (5)

Results are means ± S.E.M. In parentheses, number of determinations. [^{3}H]GABA (4 nM) binding in the absence of agents was 485 ± 68 fmol/mg protein (from 4 experiments, each performed in duplicate or triplicate). Non-specific binding accounted for less than 5% of total binding. HCH, hexachlorocyclohexane.

3.4. Effect of δ-hexachlorocyclohexane on [^{35}S]TBPS binding in primary cultures of cerebellar granule cells

As we showed above, the increase in [^{3}H]flunitrazepam binding induced by δ-hexachlorocyclohexane was reduced by the competitive $GABA_A$ antagonist bicuculline. Therefore, δ-hexachlorocyclohexane acted in a similar way to GABA (Fig. 3). Since bicuculline reverted the effect of GABA on [^{35}S]TBPS binding, we decided to examine whether δ-hexachlorocyclohexane would act on TBPS binding in a similar way to GABA. Fig. 9 shows that bicuculline did not revert the inhibitory effect of δ-hexachlorocyclohexane on [^{35}S]TBPS binding. δ-Hexachlorocyclohexane (50 μM), picrotoxinin (50 μM) and the combination of both agents completely inhibited [^{35}S]TBPS binding (data not shown).

3.5. Effect of the γ- and δ-hexachlorocyclohexane isomers on [^{3}H]GABA binding in primary cultures of cerebellar granule cells

A second approach was undertaken to determine whether δ-hexachlorocyclohexane could directly interact with the GABA recognition site at the $GABA_A$ receptor. The effect of δ- and γ-hexachlorocyclohexane on the Na^+-independent [^{3}H]GABA binding was investigated. Table 2 shows the absence of effects of both hexachlorocyclohexane isomers on [^{3}H]GABA binding to membranes obtained from cerebellar granule cells.

4. Discussion

Allosteric modulation of flunitrazepam and TBPS/picrotoxinin binding by GABA, barbiturates, benzodiazepines, steroids, etc., has been shown to occur in

different tissue or neuronal preparations (Corda et al., 1993; Hu and Ticku, 1994; Klein et al., 1994; Slany et al., 1995; Unnerstall et al., 1981). Here we used primary cultures of cerebellar granule cells to study the $GABA_A$ receptor (Belhage et al., 1986; Vaccarino et al., 1987; Pomés et al., 1993). In addition, the primary cerebellar cultures were grown in the presence of kainic acid to eliminate GABAergic inhibitory neurons, thus providing a pure cerebellar granule cell preparation that does not release GABA (Drejer and Schousboe, 1989; Damgaard et al., 1996; C. Vale, unpublished results). This allows the study of the binding of [^{3}H]flunitrazepam and [^{35}S]TBPS minimizing the effects of GABA on the $GABA_A$ receptor complex during culture.

The results for [^{3}H]flunitrazepam and [^{3}H]GABA binding were in good agreement with those reported previously in cerebellar granule cells (Belhage et al., 1986; Vaccarino et al., 1987) and similar to those reported in cortical neuronal cultures (Bender and Hertz, 1984). We did not find specific Na^+-independent [^{3}H]GABA binding in intact cell cultures. However, we characterized Na^+-independent [^{3}H]GABA binding to the $GABA_A$ receptor (inhibition by bicuculline and muscimol, Fig. 1B) using extensively washed, Triton X-100-treated membranes. The use of a non-physiological buffer in the [^{3}H]GABA binding assay in intact cultured cells might have induced the release of endogenous substances from the cells that inhibited the binding of GABA and that otherwise were removed along the process of membrane preparation (Horng and Wong, 1979). It has also been long recognized that binding parameters of the GABA recognition site can be influenced by binding assay conditions (Madtes, 1984; Yang and Olsen, 1987). [^{3}H]Flunitrazepam binding to intact cells has been performed using cold Tris-citrate buffer (Bender and Hertz, 1984) or Locke's solution at 25°C (Vaccarino et al., 1987; Hu and Ticku, 1994). In this study [^{3}H]flunitrazepam binding was increased by GABA and δ-hexachlorocyclohexane at 0°C and at 25°C using Tris-citrate-NaCl buffer. This allowed us to perform binding assays simultaneously for [^{3}H]flunitrazepam and [^{35}S]TBPS, the latter requiring a Tris buffer at 22–25°C (Squires et al., 1983). However, care should be taken regarding buffer specificities for performing binding assays in intact cultured cells, since different cell types might require different buffer composition (unpublished results). The EC_{50} for GABA enhancing [^{3}H]flunitrazepam binding (around 2 μM) was of the same order of magnitude as that reported to induce $^{36}Cl^-$ influx in neuronal cultures (Hu and Ticku, 1994; Pomés et al., 1994b). Here it was clearly observed that the enhanced binding of [^{3}H]flunitrazepam induced by GABA is due to the action of GABA on its own recognition site, since the effect was competitively reverted by bicuculline (Fig. 3A and Fig. 4A). The fact that picrotoxinin also significantly reduced the GABA-induced increase in [^{3}H]flunitrazepam binding (Fig. 3B and Fig. 4B) indicates that blocking of the Cl^- channel may produce conformational modifications of the receptor protein complex that alter the GABA recognition site. Furthermore, the reduction of the maximum GABA response observed in the presence of picrotoxinin (Fig. 4B) agrees with the non-competitive action of this GABAergic antagonist. Other authors have also reported that the coupling between GABA and benzodiazepine receptors is allosteric since it was not modified by the benzodiazepine antagonist Ro 15-1788 (Klein et al., 1994). Moreover, bicuculline reverted the inhibitory effect of GABA on [^{35}S]TBPS binding in cultured cerebellar granule cells (Fig. 5) as it does in brain membranes (Squires et al., 1983), further indicating that this is an allosteric modification of TBPS/picrotoxinin binding sites induced by GABA action on its own recognition site.

Here we have shown that the depressant compound δ-hexachlorocyclohexane dose-dependently increased [^{3}H]flunitrazepam binding in a similar way to the inhibitory neurotransmitter GABA. Although the increase in [^{3}H]flunitrazepam binding induced by δ-hexachlorocyclohexane was inhibited by bicuculline, the effect of δ-hexachlorocyclohexane was not mediated by a direct interaction with the GABA recognition site, since [^{3}H]GABA binding was not modified in the presence of δ-hexachlorocyclohexane (Table 2). In addition, the inhibitory effect of δ-hexachlorocyclohexane on [^{35}S]TBPS binding was not reverted by bicuculline, thus precluding an action of δ-hexachlorocyclohexane on the GABA recognition site (Fig. 9). However, the requirement of a bicuculline-sensitive mechanism for δ-hexachlorocyclohexane mediating positive modulation of the $GABA_A$ receptor has been pointed out by Woodward et al. (1992). These authors observed that δ-hexachlorocyclohexane induces a positive modulation of GABA-activated currents in preparations containing the $GABA_A$ receptor but not in those containing the atypical bicuculline/baclofen-insensitive GABA receptor.

Based on these results and on previous studies on the effect of δ-hexachlorocyclohexane showing both a depressant and an anticonvulsant profile (McNamara and Krop, 1948; Fishman and Gianutsos, 1988; Tusell et al., 1993), a facilitatory effect of GABA-induced Cl^- flux (Woodward et al., 1992; Pomés et al., 1994b; Nagata and Narahashi, 1995) and an inhibition of [^{35}S]TBPS binding (Pomés et al., 1993), we can putatively consider δ-hexachlorocyclohexane as a positive allosteric modulator at the $GABA_A$ receptor. Both picrotoxinin, γ-hexachlorocyclohexane and other convulsants that competitively inhibit [^{35}S]TBPS binding and inhibit the GABA-induced Cl^- flux are considered to be non-competitive $GABA_A$ receptor antagonists (Squires and Saederup, 1989; Wafford et al., 1989). The assumption that the δ-isomer acts as a positive allosteric modulator at the TBPS/picrotoxinin binding site would imply that its effect on [^{3}H]flunitrazepam binding should be reverted by picrotoxinin and γ-hexachlorocyclohexane. Fig. 7B and Fig. 8B show that γ-hexachloro-

cyclohexane and picrotoxinin indeed reduced the effect of δ-hexachlorocyclohexane on [^{3}H]flunitrazepam binding.

A facilitatory/inhibitory effect of δ-hexachlorocyclohexane on GABA-induced Cl^- flux depending on the concentrations has also been reported and suggests a complex allosteric interaction of δ-hexachlorocyclohexane with the $GABA_A$ receptor (Woodward et al., 1992; Pomés et al., 1994b). Woodward et al. (1992) have also reported that the facilitatory effect of δ-hexachlorocyclohexane on GABA-induced Cl^- flux was not reverted by the benzodiazepine antagonist Ro 15-1788, suggesting that this effect was not mediated by a direct interaction of δ-hexachlorocyclohexane at benzodiazepine binding sites. Although not directly acting at benzodiazepine sites, δ-hexachlorocyclohexane shares some pharmacological effects with diazepam, like for example, inhibition of the K^+-evoked release of noradrenaline in brain slices (Cristòfol and Rodríguez-Farré, 1993), increase in the GABA-induced Cl^- flux (Woodward et al., 1992; Pomés et al., 1994b) and reduction of c-fos expression induced by pentylenetetrazol and lindane (Vendrell et al., 1991). It is worth noting that the convulsant γ-isomer has opposite effects in all these systems.

It should be noted that the EC_{50} values for the increase in [^{3}H]flunitrazepam binding and for the inhibition of [^{35}S]TBPS binding induced by δ-hexachlorocyclohexane in primary cultures of cerebellar granule cells were similar (8 μM for [^{3}H]flunitrazepam binding, this work; 5–10 μM for [^{35}S]TBPS binding, Pomés et al., 1993). Other depressant compounds also induce both an increase in [^{3}H]flunitrazepam and an inhibition of [^{35}S]TBPS binding at concentrations that are of the same order of magnitude for both paradigms (Hawkinson et al., 1994).

A participation of intracellular Ca^{2+} in the effect of δ-hexachlorocyclohexane is not demonstrated here. δ-Hexachlorocyclohexane stereoselectively and dose-dependently releases Ca^{2+} from cardiac sarcoplasmic reticulum and inhibits [^{3}H]ryanodine binding in rat cardiac muscle and brain cortex (Pessah et al., 1992) and in cultured cerebellar granule cells (Rosa et al., 1996b). Ryanodine and caffeine, at concentrations reported to release Ca^{2+} from intracellular stores (Miller, 1991), did not mimic the effect of δ-hexachlorocyclohexane. Ryanodine did not modify [^{3}H]flunitrazepam binding and caffeine inhibited it (Table 1). Furthermore, it has been reported that caffeine reduces GABA responses at the $GABA_A$ receptor (Kardos and Blandl, 1994), an effect contrary to that produced by δ-hexachlorocyclohexane (Woodward et al., 1992; Pomés et al., 1993).

In contrast to the effect of δ-hexachlorocyclohexane on [^{3}H]flunitrazepam binding, the convulsant γ-isomer was ineffective. Nor did it produce any effect on the Na^+-independent [^{3}H]GABA binding. These results agree with those obtained using brain membrane preparations (Cattabeni et al., 1983; Abalis et al., 1985). A similar lack of effect on [^{3}H]flunitrazepam binding has been described for picrotoxinin in rat cerebellar membranes (Chweh et al., 1985). The concentrations of γ-hexachlorocyclohexane used in this work (up to 50 μM) were high enough to completely inhibit the [^{35}S]TBPS binding (Abalis et al., 1985; Pomés et al., 1993) and the GABA- or GABA + flunitrazepam-induced Cl^- flux in cultured neurons (Pomés et al., 1994a,b). Nevertheless, the effect of γ-hexachlorocyclohexane on GABA-induced increase in [^{3}H]flunitrazepam binding (Fig. 7A) agrees with its proposed mechanism of action as a non-competitive $GABA_A$ receptor antagonist (Wafford et al., 1989; Woodward et al., 1992; Pomés et al., 1993; Tokutomi et al., 1994). This further indicates the assembly of separate, although allosterically interacting, binding sites for GABA, benzodiazepines and picrotoxinin in the $GABA_A$ receptor.

These results agree with the pharmacological properties and the effects of γ- and δ-isomers in vivo (convulsant and depressant agents, respectively). We can conclude that δ-hexachlorocyclohexane acts as a positive allosteric modulator and γ-hexachlorocyclohexane acts as a non-competitive antagonist of the $GABA_A$ receptor. Whether the positive allosteric modulation of the $GABA_A$ receptor by δ-hexachlorocyclohexane is a consequence of its interaction with picrotoxinin/TBPS recognition sites at the Cl^- channel cannot yet be fully established. However, we suggest that the convulsant/depressant character of compounds that interact with the picrotoxinin recognition site can be predicted from their effect on [^{3}H]flunitrazepam binding.

These results further demonstrate the interrelation of the main neurotransmitter/drug recognition sites of the $GABA_A$ receptor: GABA influencing benzodiazepine and picrotoxinin recognition sites; convulsants, acting at the picrotoxinin binding site, that do not directly interact with benzodiazepine or GABA binding sites but reduce allosteric effects of GABA on benzodiazepine receptors; and depressants, primarily acting at the picrotoxinin recognition site, that influence benzodiazepine but not $GABA_A$ recognition sites. These results also extend the use of cultured cerebellar granule cells as a system to study the $GABA_A$ receptor functionality in vitro.

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