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Effects of Hexachlorocyclohexanes on γ-Aminobutyric Acid Receptors Expressed in *Xenopus* Oocytes by RNA from Mammalian Brain and Retina

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SUMMARY

Poly(A)$^+$ RNA from rat cerebral cortex expresses γ-aminobutyric acid (GABA)-activated membrane current responses in *Xenopus* oocytes, mediated by $GABA_A$ receptors ($I_{G\text{-}Actx}$). In contrast, RNA from bovine retina expresses GABA responses composed of two pharmacologically distinct Cl^- currents, one mediated by $GABA_A$ receptors ($I_{G\text{-}Aret}$) and the other by atypical GABA receptors that are resistant to bicuculline and are not activated by baclofen ($I_{G\text{-}BR}$). The pharmacology of the bicuculline/baclofen-insensitive GABA receptors was further investigated by comparing actions of hexachlorocyclohexane (HCH) enantiomers on GABA-activated membrane currents expressed in oocytes by brain and retina RNA. γ-HCH (lindane) was a potent inhibitor of $I_{G\text{-}Actx}$, with suppression of currents detectable at concentrations as low as 50 nM. The IC_{50} for γ-HCH, calculated from inhibitory effects on maximum $I_{G\text{-}Actx}$ (current elicited by 3 mM GABA), was 7.3 ± 3 μM. Inhibitory effects of γ-HCH on $I_{G\text{-}Aret}$ were qualitatively similar to those described for $I_{G\text{-}Actx}$. In contrast, α-HCH and δ-HCH induced clear positive modulation of $I_{G\text{-}Actx}$ elicited by low (e.g., 10 μM) concentrations of GABA. Thresholds for the modulatory effects of α-HCH and δ-HCH were between 100 and 300 nM, with maximum levels of potentiation (5–7-fold) between 20–50 μM. Potentiation of $I_{G\text{-}Actx}$ by α- and δ-HCH was reversible and largely insensitive to the benzodiazepine antagonist flumazenil (1 μM). Assays on maximum $I_{G\text{-}Actx}$ indicated that α-HCH (10–100 μM) caused only marginal reductions in response (≤15%), whereas δ-HCH had stronger inhibitory effects (IC_{50}, 20–30 μM). At concentrations between 0.1 and 50 μM, β-HCH induced only 10–25% facilitation of $I_{G\text{-}Actx}$ elicited by 10 μM GABA and had no clear effects on maximum responses. $I_{G\text{-}BR}$ was also potently inhibited by γ-HCH. Thresholds for detecting reductions in current were ~20 nM, and the IC_{50} calculated from effects on maximum responses was 5.8 ± 2 μM. However, neither α-HCH nor δ-HCH (1–100 μM) induced any potentiation of $I_{G\text{-}BR}$. α-HCH had some weak inhibitory effects that were largely surmountable, whereas δ-HCH and β-HCH were essentially inactive. These experiments raise the possibility that α- and δ-HCH constitute a novel class of $GABA_A$ receptor modulators, which might prove to be useful for investigating the mechanisms underlying regulation of $GABA_A$ receptors. In addition, our results indicate that bicuculline/baclofen-insensitive GABA receptors expressed by retina RNA resemble $GABA_A$ receptors in their sensitivity to γ-HCH but are largely insensitive to α-HCH and δ-HCH.

Poly(A)$^+$ RNA extracted from mammalian brain or chick optic lobe expresses functional $GABA_A$ receptors in *Xenopus* oocytes, that have electrical and pharmacological properties similar to those of receptors studied *in situ*. (e.g., Refs. 1–7). Poly(A)$^+$ RNA from mammalian retina likewise expresses $GABA_A$ receptors, but retina RNA also expresses GABA receptors with atypical pharmacology, which show resistance to Bic, are not modulated by benzodiazepines, barbiturates, or steroids, and are neither activated by Bac nor blocked by 2-hydroxysaclofen (6, 7). Like $GABA_A$ receptors, the Bic/Bac-insensitive GABA receptors expressed by retina RNA gate Cl^- channels that are sensitive to picrotoxin (6). However, separate experiments indicated that TBPS had only weak, surmountable, inhibitory effects on the Bic/Bac-insensitive GABA receptors, whereas inhibitory effects on $GABA_A$ receptors were potent and substantially insurmountable (38). The apparent differences in sensitivity to TBPS suggested that it would be worthwhile to characterize effects of other drugs thought to interact with $GABA_A$ receptors at the picrotoxin binding site.

γ-HCH (generic name, lindane) is still widely used as an

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ABBREVIATIONS: GABA, γ-aminobutyric acid; Bac, baclofen; Bic, bicuculline; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; α-, β-, γ-, and δ-HCH, α-, β-, γ-, and δ-stereoisomers of hexachlorocyclohexane; $I_{G\text{-}Actx}$, membrane current elicited through activation of γ-aminobutyric $acid_A$ receptors expressed by cerebral cortex RNA; $I_{G\text{-}Aret}$, membrane current elicited through activation of γ-aminobutyric $acid_A$ receptors expressed by retina RNA; $I_{G\text{-}BR}$, membrane current elicited through activation of bicuculline-resistant γ-aminobutyric acid receptors expressed by retina RNA; TBPS, *t*-butylbicyclophosphorothionate.

insecticide and as an ectoparasiticide in the treatment of scabies and pediculosis. Early studies showed that γ-HCH was a strong convulsant in mammals, whereas other enantiomers had only weak effects or acted as CNS depressants (e.g., Ref. 8). The toxicology of γ-HCH in mammals has yet to be fully resolved (e.g., Refs. 9–11), but there is now considerable evidence to suggest that $GABA_A$ receptors are an important target (12). For example, (i) binding of [^{3}H]dihydropicrotoxinin and [^{35}S] TBPS to rat brain membranes was potently inhibited by γ-HCH (13–15); (ii) γ-HCH antagonized GABA-stimulated $^{36}Cl^-$ influx into rat or mouse brain vesicles (16, 17); (iii) electrical recordings in rat dorsal root ganglion cells showed that GABA-activated membrane currents were attenuated by γ-HCH (18); (iv) picrotoxin and γ-HCH were shown to inhibit GABA-gated Cl^- channels in crayfish muscle by similar mechanisms, which did not involve reductions in single-channel conductance or mean channel open time but appeared to be due to stabilization of closed states (19); and (iv) γ-HCH-induced seizures in rats were shown to be modulated by GABAergic drugs in ways consistent with an *in vivo* interaction with $GABA_A$ receptors (20).

In the present study, we used electrical assays to compare effects of α-HCH, β-HCH, γ-HCH, and δ-HCH on $GABA_A$ receptors expressed in oocytes by poly(A)$^+$ RNA from rat cerebral cortex with effects on Bic/Bac-insensitive GABA receptors expressed by bovine retina RNA.

Materials and Methods

RNA extraction, size fractionation, and expression in oocytes. RNA was extracted from rat cerebral cortex and bovine retina using the phenol-chloroform procedure (7). Retina poly(A)$^+$ RNA was size fractionated by centrifugation on 10–30% sucrose density columns (21), and fractions showing enrichment for RNA encoding GABA receptors were assayed by expression in oocytes (6, 22). Two separate RNA preparations were made from bovine retina and one from rat cerebral cortex. Follicle-enclosed *Xenopus* oocytes, at stages V and VI of development (23), were microinjected with 75–100 ng of total poly(A)$^+$ RNA from cerebral cortex or retina and 20–30 ng of size-fractionated RNA from retina (injection volume, 50–100 nl). Oocytes were stored in Barth's medium [in mM: NaCl, 88; KCl, 1; $CaCl_2$, 0.41; $Ca(NO_3)_2$, 0.33; $MgSO_4$, 0.82; $NaHCO_3$, 2.4; HEPES, 5; pH adjusted to 7.4 with NaOH; usually with 0.1 mg/ml gentamycin] and defolliculated by treatment with collagenase (24).

Electrophysiology and notation of membrane current responses. Using a conventional two-electrode voltage-clamp, electrical recordings were made in frog Ringer solution (in mM: NaCl, 115; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH adjusted to 7.0 with NaOH). All drugs were applied to oocytes by bath perfusion. Comparisons were made between the effects of Cl^- channel blockers on $I_{G\text{-}Actx}$, $I_{G\text{-}Aret}$, and $I_{G\text{-}BR}$. As before (6, 7), detailed quantitative analyses were generally restricted to comparisons between $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$, with effects on $I_{G\text{-}Aret}$ illustrated qualitatively. The two components of retina GABA responses were distinguished using 0.1–1 mM Bic methobromide to abolish $I_{G\text{-}Aret}$ (6, 7).

Pharmacological assays. Effects of HCH enantiomers were initially assayed on $I_{G\text{-}Actx}$ elicited by 10 μM GABA and on $I_{G\text{-}BR}$ elicited by 1 μM GABA. Suppression of $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$ by γ-HCH showed minor use-dependent components, but procedures for constructing concentration-responses curves were such that levels of inhibition were allowed to equilibrate fully. Concentration-response curves for $I_{G\text{-}Actx}$ were constructed in single oocytes, firstly under control conditions and then with HCH enantiomers, with incubation being maintained throughout intervals between GABA exposures. Different concentrations of GABA were applied for 1 min, with responses being separated by 1–20 min, depending upon GABA concentration; sufficient time to allow resensitization of currents. $I_{G\text{-}BR}$ showed little desensitization (6), and concentration-response curves were constructed either using responses separated by a 3–10-min wash or using a "stepped-ramp" procedure, where different concentrations of GABA were applied without intervening intervals of wash. Results using either procedure were indistinguishable.

Data analysis. Concentration-response curves were analyzed as described previously (7). EC_{50} values and slope factors (pseudo-Hill coefficients) were calculated using a nonlinear least squares curve-fitting program, based on a four-parameter logistic equation (25). IC_{50} values were determined from blocking effects on maximum GABA responses, i.e., $I_{G\text{-}Actx}$ elicited by 3 mM GABA and $I_{G\text{-}BR}$ elicited by 100 μM GABA with 100 μM Bic methobromide. Curves to measure specificallythe potency of inhibition were constructed over a range of γ-HCH concentrations (four to six points), and IC_{50} values were determined by regression.

Drugs. γ-HCH was from Sigma or Riedel-de Haen (United States Pestanal supplier, Cresent Chemical Co. Inc., Hauppauge NY); α-, β-, and δ-HCH were from Riedel-de Haen. Cross-contamination between enantiomers was ≤0.02%, except for β-HCH, which contained 0.17% δ-HCH, and γ-HCH, which contained 0.05% δ-HCH (supplier's specifications). All HCH stereoisomers were made up daily as 1, 10, or 100 mM stocks in DMSO. Solubility of the different HCH enantiomers in Ringer solution was checked before first use. At concentrations of ≤100 μM, α-HCH, γ-HCH, and δ-HCH appeared to remain fully dissolved in Ringer solutions (DMSO was used as a vehicle at levels up to 0.3%, v/v). On the other hand, β-HCH was appreciably less soluble and, at concentrations greater than about 50 μM, Ringer solutions progressively developed light suspensions of undissolved β-HCH (DMSO was used as a vehicle at levels up to 0.5%, v/v). Effects of DMSO applied alone were checked on the different GABA-activated currents, and in all cases there were no consistent changes in the amplitudes of responses or rates of response decay. Flumazenil (Ro 15–1788) was a generous gift from Hoffmann-La Roche (Nutley, NJ); other drugs were obtained from Sigma.

Results

Effects of γ-HCH on $I_{G\text{-}Actx}$

Effects of γ-HCH on $I_{G\text{-}Actx}$ were initially assayed on currents elicited by 10 μM GABA (responses that constituted 3–5% of maximum $I_{G\text{-}Actx}$). These experiments revealed that inhibition exhibited a degree of use dependence, such that, during incubations with γ-HCH, levels of inhibition could be increased (10–20%) by extended or repeated application of agonist. These effects were not characterized in any detail, and all records and data given in this study represent levels of inhibition where use-dependent effects had been allowed to equilibrate fully. Thresholds for detecting reductions in $I_{G\text{-}Actx}$ were at concentrations as low as 50 nM γ-HCH, with 0.1 and 1 μM γ-HCH reducing responses by 16 ± 7% (n = 3) and 50 ± 11% (n = 4), respectively (all values quoted as mean ± standard deviation) (Fig. 1A). Even when relatively low (0.1–1 μM) concentrations of γ-HCH were used, recovery from inhibition was slow, typically requiring 10–45 min before currents returned to control levels.

Further increases in γ-HCH concentrations were expected to cause correspondingly higher levels of inhibition. However, raising the concentration of γ-HCH to 10 μM did not cause much additional suppression of currents (Fig. 1B, *second record*). Furthermore, responses activated after a 2–5-min wash of 10 μM γ-HCH applications were consistently 30–55% smaller than currents elicited during incubation with the inhibitor (Fig. 1B, *third record*). The additional inhibitory effects apparent

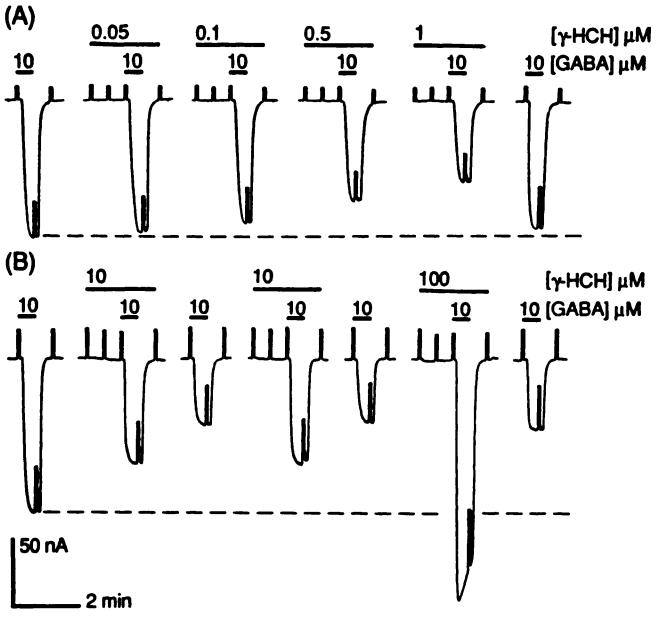

Fig. 1. Actions of γ-HCH on $I_{G\text{-}Actx}$ elicited by low concentrations of GABA. A and B are records taken from two separate oocytes. A, Concentrations of γ-HCH causing threshold levels of inhibition of $I_{G\text{-}Actx}$. In this case, inhibitory effects were marginal with 50 nM γ-HCH but were clear at 100 nM. For 0.1–1 μM γ-HCH, records are the second of two repeated GABA applications, where weak use-dependent effects had fully equilibrated. B, Effects of higher concentrations of γ-HCH. *First record*, control response; *second record*, response with 10 μM γ-HCH, reduced by only 35%; *third record*, response after 2-min wash of γ-HCH, now reduced by 60%; *fourth and fifth records*, response with 10 μM γ-HCH, potentiated with respect to residual inhibitory effects; *sixth and seventh records*, 100 μM γ-HCH increased current to 155% of control (see text for details). All records were separated by intervals of 4–6 min; *dashed lines*, levels of control responses. Oocytes were voltage-clamped at −70 mV, with the holding potential stepped at 30-sec intervals to −60 mV for 5–6 sec. Voltage steps were used to monitor membrane conductance and as markers for solution changes. Dead time of the perfusion system was 5–10 sec. Capacitative transients, picked up by the chart recorder upon potential steps, have been deleted during preparation of figures. Inward membrane currents are denoted by downward deflections. Unless otherwise stated, all following figures have the same recording conditions.

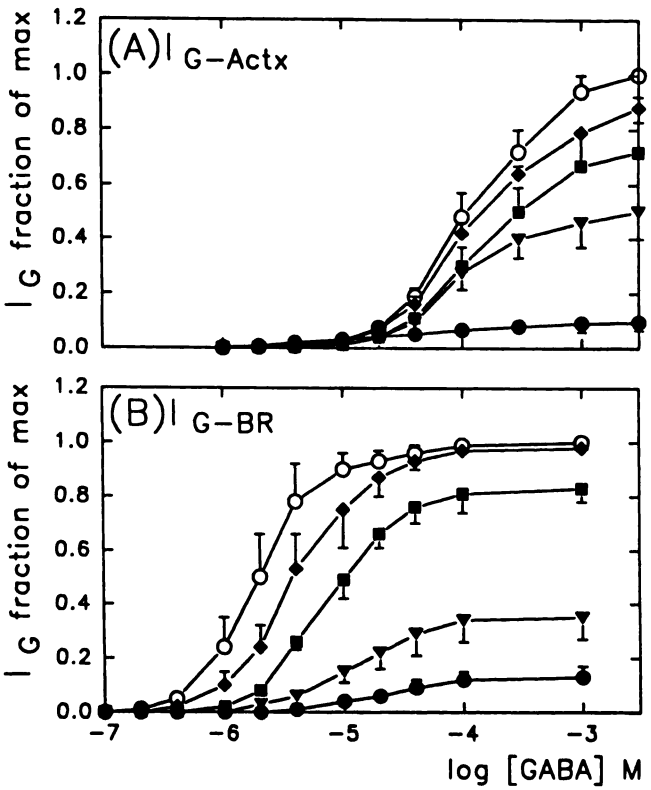

Fig. 2. Concentration-response curves comparing the effects of γ-HCH on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 6); ♦, GABA plus 0.1 μM γ-HCH (n = 3); ■, GABA plus 1 μM γ-HCH (n = 4); ▼, GABA plus 10 μM γ-HCH (n = 5); ●, GABA plus 100 μM γ-HCH (n = 6). B, $I_{G\text{-}BR}$. ○, GABA control (n = 6); ♦, GABA plus 0.1 μM γ-HCH (n = 4); ■, GABA plus 1.0 μM γ-HCH (n = 5); ▼, GABA plus 10 μM γ-HCH (n = 5); ●, GABA plus 100 μM γ-HCH (n = 4). For measurement of $I_{G\text{-}BR}$, 0.1–1 mM Bic methobromide was used to abolish $I_{G\text{-}Arel}$. Concentration-response curves were first constructed under control conditions and then remeasured, using the same oocyte, with different concentrations of γ-HCH. In this and all following graphs, data points are the mean ± standard deviation, expressed as a fraction of maximum control responses. Error bars have been omitted when smaller than the size of the symbols. I_G, membrane current response elicited by GABA.

upon wash were not rapidly removed, and subsequent reapplication of 10 μM γ-HCH actually appeared to increase responses. These experiments suggested that, in addition to strong inhibitory effects, micromolar concentrations of γ-HCH appeared to cause appreciable positive modulation of $I_{G\text{-}Actx}$ elicited by low concentrations of GABA. This was confirmed by measuring the actions of 100 μM γ-HCH, which paradoxically increased the GABA-activated currents by 50–150% (Fig. 1B, *sixth record*).

Effects of γ-HCH on $I_{G\text{-}Actx}$ were then examined over full concentration-response curves, revealing that inhibition was largely insurmountable (Fig. 2A). For example, maximum responses were reduced 52 ± 12% by 10 μM γ-HCH, whereas the EC_{50} appeared to be slightly reduced, from 119 ± 12 μM GABA under control conditions (n = 6) to 78 ± 14 μM with 10 μM γ-HCH (n = 4). Surmountable inhibition would have been associated with pronounced increases in EC_{50}. The IC_{50}, calculated from inhibition of maximum $I_{G\text{-}Actx}$ (current elicited by 3 mM GABA), was 7.3 ± 3 μM γ-HCH (n = 4). Estimation of IC_{50} values for currents elicited by lower concentrations of GABA was somewhat complicated by the weak facilitatory effects induced by high concentrations of γ-HCH. For example, currents elicited by 100 μM GABA (the approximate EC_{50}) were reduced approximately 50% by any concentration of γ-HCH between 1 and 20 μM.

Effects of γ-HCH on $I_{G\text{-}BR}$ and $I_{G\text{-}Arel}$

γ-HCH was initially tested for effects on $I_{G\text{-}BR}$ elicited by low concentrations of GABA. In contrast to the complex effects seen on $I_{G\text{-}Actx}$, 0.1–100 μM γ-HCH was clearly inhibitory at all concentrations tested and caused no potentiation of currents (Fig. 3, A and B). Thresholds for detecting reductions in $I_{G\text{-}BR}$ were as low as 20 nM γ-HCH. As described for $I_{G\text{-}Actx}$, during continuous applications of γ-HCH, extended or repeated exposures to GABA resulted in modest (10–15%) increases in levels of inhibition, again implying that inhibitory effects on $I_{G\text{-}BR}$ were characterized by a degree of use dependence (data not shown). Inhibition of $I_{G\text{-}BR}$ by γ-HCH was largely reversible, but full removal of effects required extended intervals of wash. For example, inhibition of $I_{G\text{-}BR}$ by low concentrations of γ-HCH (0.1–1 μM) was substantially washed out in 10–15 min, whereas effects of extended applications of 10–100 μM γ-HCH were not fully removed even after 45–60 min.

Examination of the effects of γ-HCH over full $I_{G\text{-}BR}$ concentration-response curves revealed that levels of inhibition

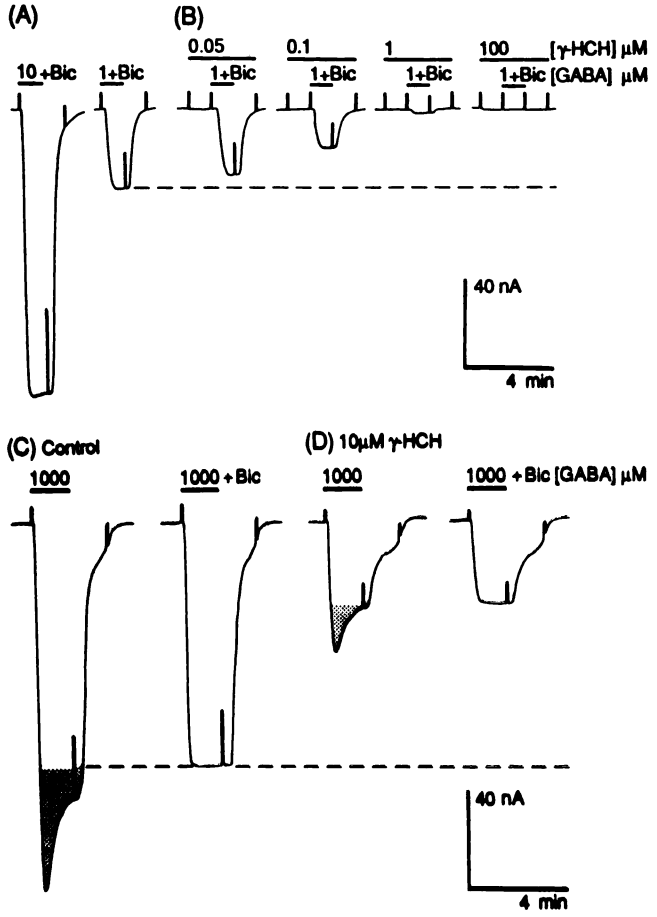

Fig. 3. A and B, Effects of γ-HCH on $I_{G\text{-}BR}$ elicited by 1 μM GABA; records are from the same oocyte. A, Control responses. B, Currents elicited by 1 μM GABA with increasing concentrations of γ-HCH. Inhibition was already clear with 50 nM γ-HCH, and high concentrations of γ-HCH caused no positive modulation but, instead, abolished responses. In each case, GABA was applied together with 100 μM Bic, to abolish $I_{G\text{-}Aret}$. *Dashed line*, level of control response elicited by 1 μM GABA. C and D, Effect of γ-HCH on $I_{G\text{-}BR}$ and $I_{G\text{-}Aret}$; records are from the same oocyte. C, Control responses. *Shading*, $I_{G\text{-}Aret}$, the Bic-sensitive component of GABA responses expressed by retina RNA. *Dashed line*, maximum $I_{G\text{-}BR}$. D, Responses elicited during extended and continuous application of 10 μM γ-HCH. Both components of the GABA responses expressed by retina RNA were inhibited by γ-HCH. *Bic*, 1 mM Bic methobromide.

showed appreciable dependence upon agonist concentration (Fig. 2B). Indeed, with low concentrations of γ-HCH, inhibition of $I_{G\text{-}BR}$ was almost completely surmountable. For example, 0.1 and 1 μM γ-HCH increased the EC_{50} from control values of 1.9 ± 0.1 μM (n = 6) to 3.8 ± 0.2 μM (n = 4) and 7.4 ± 0.3 μM (n = 5), respectively, but caused only minor reductions in maximum response. When concentrations of γ-HCH were raised above 1 μM, an insurmountable component became increasingly prominent (Fig. 2B). For example, with 10 μM γ-HCH the EC_{50} of $I_{G\text{-}BR}$ was further increased to 13 ± 0.6 μM (n = 5), but this rightward shift was accompanied by a 65 ± 8% reduction in maximum response. IC_{50} values calculated from effects on maximum responses (currents elicited by 100 μM GABA) were 5.8 ± 2 μM γ-HCH, not significantly different from values calculated for $I_{G\text{-}Actx}$. However, due to the strong dependence on agonist concentration, IC_{50} values measured from currents elicited by low concentrations of GABA suggested substantially higher potency.

γ-HCH also inhibited the Bic-sensitive component of responses expressed by retina RNA. When assayed on $I_{G\text{-}Aret}$ elicited by 100 μM GABA, threshold levels of inhibition were detectable using <1 μM γ-HCH. Inhibitory effects appeared to be largely insurmountable, with overall potency similar to that described for $I_{G\text{-}Actx}$ (Fig. 3, C and D).

Effects of HCH Enantiomers on $I_{G\text{-}Actx}$

As mentioned in the introduction, the various enantiomers of HCH have different effects on mammalian nervous systems (8). To investigate whether other enantiomers of HCH were able to interact with $GABA_A$ receptors, actions of α-HCH, β-HCH, and δ-HCH were assayed on receptors expressed in oocytes by rat cerebral cortex RNA.

Effects of α-HCH on $I_{G\text{-}Actx}$. At concentrations of ≤0.1 μM, α-HCH caused no detectable change in currents elicited by 10 μM GABA, but at concentrations between 0.1 and 1 μM this enantiomer induced 5–25% dose-dependent increases in response. At 10 μM, α-HCH caused surprisingly pronounced potentiation of $I_{G\text{-}Actx}$, increasing responses by 200–300% (Fig. 4A). Facilitatory effects induced by 2-min applications of 10 μM α-HCH were substantially (65–80%) washed out within 2 min, but complete removal of modulation typically required 10–15 min, suggesting there might be two components to the time course of recovery. Positive modulation of $I_{G\text{-}Actx}$ elicited by 10 μM GABA was maximal (500–700%) using 50 μM α-HCH, with little further increase or decrease in currents being detected with 100 μM α-HCH. Potentiation induced by high concentrations of α-HCH required correspondingly long intervals of wash for complete removal, and in some cases significant (15–20%) increases in response were still detectable after 60 min, even with numerous intervening applications of GABA.

Effects of α-HCH were then assayed over full $I_{G\text{-}Actx}$ concentration-response curves (Fig. 5A). At 10 μM, α-HCH caused clear leftward shifts, without significantly altering slope values or maximum responses. In these experiments, the EC_{50} for $I_{G\text{-}Actx}$ under control conditions was 82 ± 2 μM (n = 3) and was halved to 40 ± 1 μM by 10 μM α-HCH (n = 3). At 100 μM, α-HCH caused further potentiation, lowering EC_{50} values to 23 ± 1 μM (n = 3), but this effect appeared to be accompanied by modest (10–20%) reductions in maximum response. The transition between net facilitatory and inhibitory effects occurred around 100 μM GABA.

Effects of β-HCH on $I_{G\text{-}Actx}$. At concentrations of <1 μM, β-HCH had no clear effects on $I_{G\text{-}Actx}$ elicited by 10 μM GABA, and even at 10 μM, β-HCH induced only slight (10–20%) potentiation of responses (Fig. 4B). As described for α-HCH, complete removal of the facilitatory effects induced by 10 μM β-HCH required up to 15–20 min of wash. Raising concentrations of β-HCH to approximately 50 μM (saturation under assay conditions) resulted in only marginal increases in the levels of potentiation, and reversal of these effects required a >45-min wash. Over a range of concentrations between 1 and 50 μM, β-HCH caused no significant reduction or increase in currents elicited by 0.1–3 mM GABA and was not studied in further detail.

Effects of δ-HCH on $I_{G\text{-}Actx}$. Initial assays on currents elicited by 10 μM GABA showed that δ-HCH, like the α-enantiomer, acted as a positive modulator of $I_{G\text{-}Actx}$. Concentrations required to induce threshold increases in response were between 0.1 and 0.3 μM δ-HCH, usually slightly lower than thresholds for α-HCH assayed in the same oocyte. Levels of

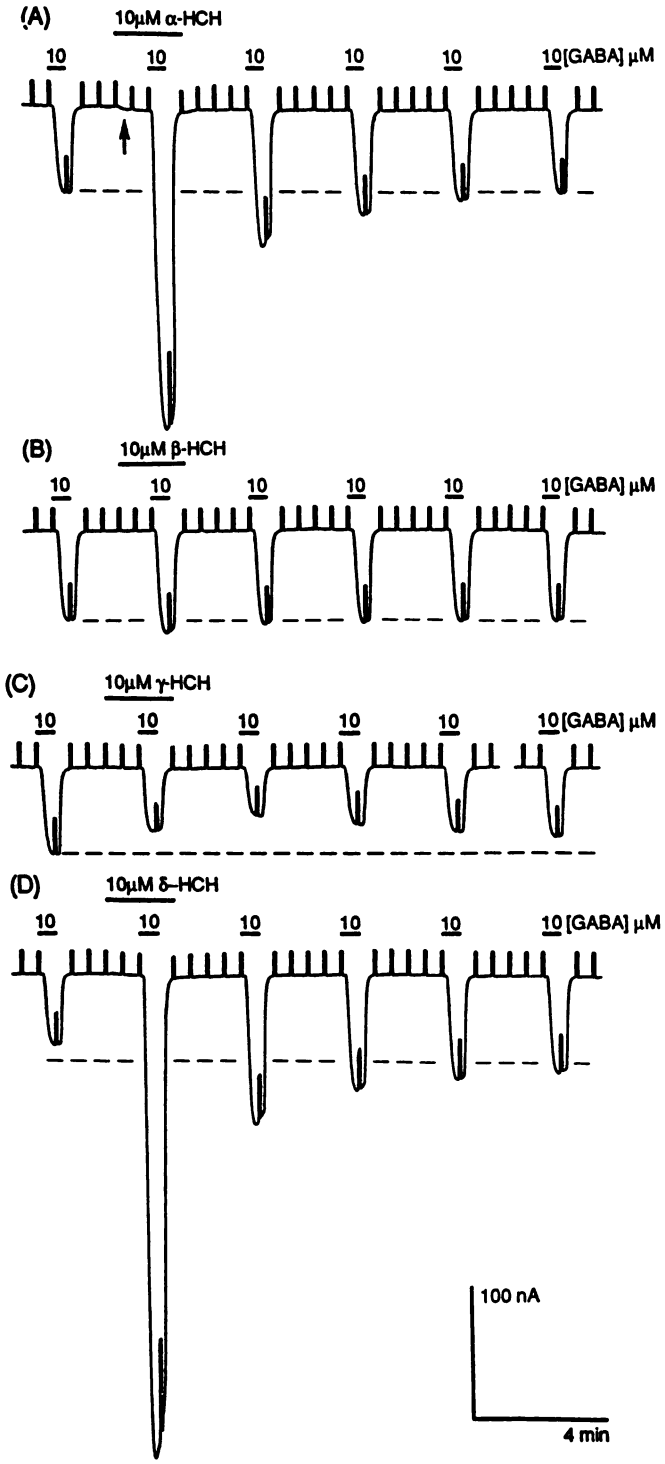

Fig. 4. Effects of HCH enantiomers on $I_{G\text{-}Actx}$ elicited by 10 μM GABA; all records (A–D) were taken from the same oocyte in the sequence indicated. A, Application of 10 μM α-HCH induced strong positive modulation of $I_{G\text{-}Actx}$ that washed out over an interval of approximately 10 min. In this oocyte, α-HCH itself elicited a small maintained inward current (*arrow*). (Further analysis showed that these responses did not appear to be mediated by interactions with $GABA_A$ receptors but were associated with decreases in membrane conductance, due to inhibition of K^+ currents present in the unstimulated oocyte membrane.) B, At 10 μM, β-HCH induced only weak positive modulation of $I_{G\text{-}Actx}$. C, At 10 μM, γ-HCH suppressed $I_{G\text{-}Actx}$ by 30%, and again the response upon a 2-min wash revealed 15% additional inhibition. Blocking effects of γ-HCH were refractory to wash. The final record taken after an additional 15-min interval shows that currents had not returned to control levels even after a 25-min wash and repeated applications of GABA. D, Although the control response showed some residual inhibition from the prior γ-HCH exposure, 10 μM δ-HCH induced strong positive modulation of $I_{G\text{-}Actx}$. Potentiation was washed out over a time course similar to that of effects induced by α-HCH but, in this case, a 10-min wash did not appear to remove the effect completely. *Dashed lines*, level of control response.

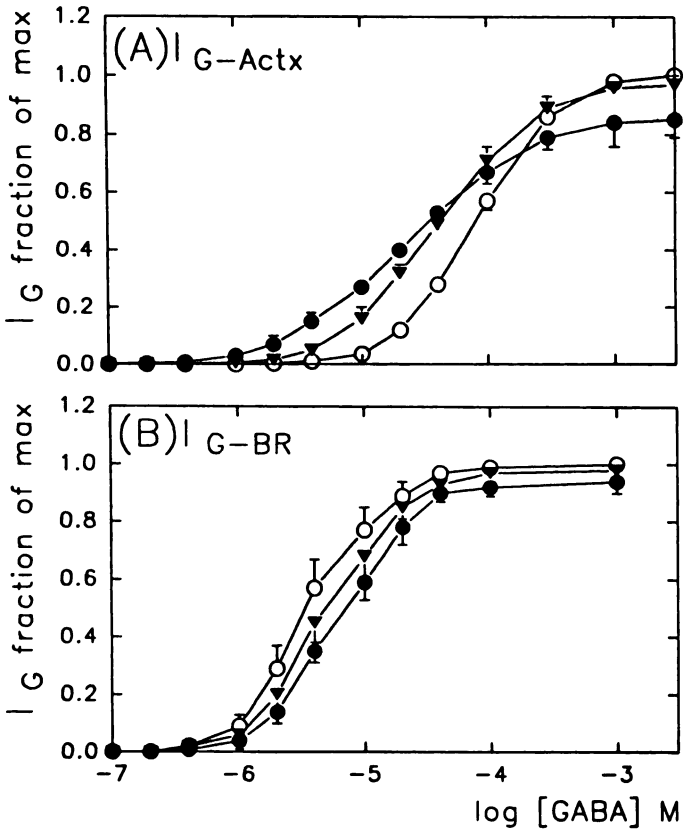

Fig. 5. Concentration-response curves comparing the effects of α-HCH on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 3); ▼, GABA plus 10 μM α-HCH (n = 3); ●, GABA plus 100 μM α-HCH (n = 4). B, $I_{G\text{-}BR}$. ○, GABA control (n = 3); ▼, GABA plus 10 μM α-HCH (n = 3); ●, GABA plus 100 μM α-HCH (n = 3).

potentiation induced by 10 μM δ-HCH typically ranged between 500 and 600%, again suggesting that δ-HCH was a little more potent than α-HCH (Fig. 4D). Washout of facilitatory effects followed similar time courses as those induced by α-HCH. Maximum levels of potentiation (650–750%) were induced by 20–50 μM δ-HCH, and complete washout of these effects was again protracted.

Effects of δ-HCH were also assayed over full $I_{G\text{-}Actx}$ concentration-response curves (Fig. 6A). As described for the α-enantiomer, δ-HCH applied at 10 μM acted principally as a positive modulator of $I_{G\text{-}Actx}$, causing leftward shifts in concentration-response curves, without appreciably changing slope values and causing only minor decreases in the amplitudes of maximum responses. The EC_{50} for $I_{G\text{-}Actx}$ was 79 ± 2 μM under control conditions and was lowered to 23 ± 2 μM by 10 μM δ-HCH, whereas maximum responses were reduced by only 13 ± 3% (n = 3) (Fig. 6A). The EC_{50} with 10 μM δ-HCH was approximately half that measured with the same concentration of α-HCH, confirming that the δ-enantiomer had higher potency. At 100 μM, δ-HCH induced further potentiation of currents elicited by low concentrations of GABA but, in con-

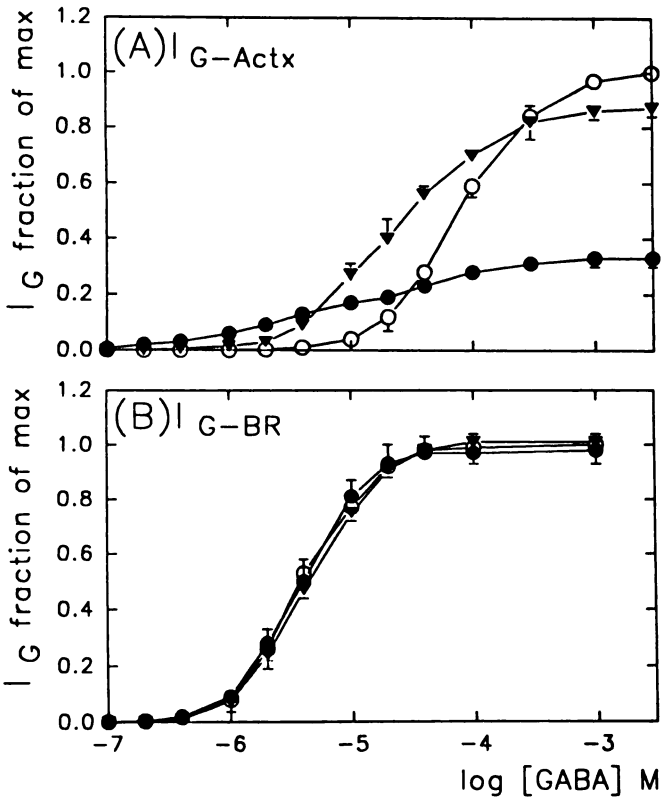

Fig. 6. Concentration-response curves comparing the effects of δ-HCH on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, $I_{G\text{-}Actx}$. ○, GABA control (n = 3); ▼, GABA plus 10 μM δ-HCH (n = 3); ●, GABA plus 100 μM δ-HCH (n = 3). B, $I_{G\text{-}BR}$. ○, GABA control (n = 4); ▼, GABA plus 10 μM δ-HCH (n = 4); ●, GABA plus 100 μM δ-HCH (n = 4).

trast to actions of α-HCH, this effect was accompanied by strong suppression of currents elicited by higher concentrations of GABA. Transitions from net potentiation to inhibition occurred at approximately 300 μM GABA with 10 μM δ-HCH and at 30 μM GABA with 100 μM δ-HCH.

Mechanism underlying potentiation of $I_{G\text{-}Actx}$ by α- and δ-HCH. As described previously (1–6), measurements of the voltage dependence of $I_{G\text{-}Actx}$ elicited by 10 μM GABA indicated that the current reversed between −25 and −30 mV, the equilibrium potential for Cl^- in oocytes (26), and showed strong outward rectification as holding potentials were made increasingly negative. Remeasurement of voltage dependence in 5–10 μM α-HCH or δ-HCH showed that potentiation was not due to any significant shifts in reversal potential and was not associated with decreases in levels of rectification (Fig. 7).

Levels of potentiation induced by α- and δ-HCH were similar to those induced by benzodiazepines such as diazepam and chlorazepate (5). Availability of the benzodiazepine antagonist flumazenil (Ro 15–1788) allowed us to investigate whether α- and δ-HCH induced positive modulation of $GABA_A$ receptors through interactions at benzodiazepine binding sites. $I_{G\text{-}Actx}$ elicited by 10 μM GABA was typically potentiated 150–200% by 2 μM diazepam, and this effect was almost abolished by coapplication of 1 μM flumazenil (Fig. 8A). In contrast, 2 μM α- or δ-HCH potentiated currents 50–150%, but this level of modulation was largely unaffected by 1 μM flumazenil (Fig. 8B). Similar experiments to investigate potential interactions of HCH enantiomers at barbiturate and steroid binding sites were not possible, due to lack of suitable antagonists.

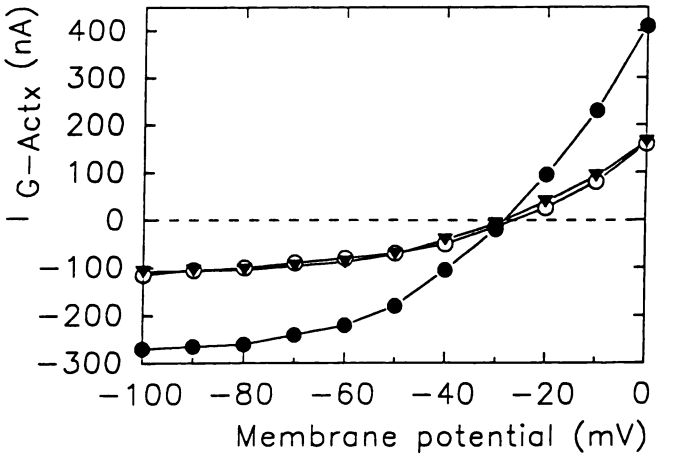

Fig. 7. Effect of δ-HCH on the voltage dependence of $I_{G\text{-}Actx}$; data are from the same oocyte. ○, Control current elicited by 10 μM GABA; ●, current elicited by 10 μM GABA with 5 μM δ-HCH; ▼, same curve scaled to the current elicited at −60 mV in the control curve. Voltage dependence of membrane currents was determined by clamping oocytes at a holding potential of 0 mV, eliciting GABA-activated currents, briefly stepping to different potentials during the maintained response (1-sec steps at increments of 10 mV), and then subtracting currents in the unstimulated membrane. The scaled response is plotted to illustrate that δ-HCH had no clear effect on levels of rectification found in control responses.

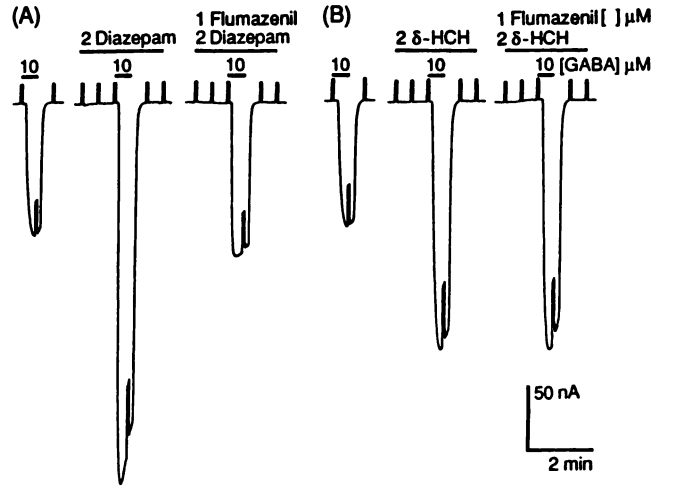

Fig. 8. Effects of flumazenil on positive modulation of $I_{G\text{-}Actx}$ induced by diazepam and δ-HCH; records are from two separate oocytes showing similar sensitivity to GABA. A, *First record*, control response elicited by 10 μM GABA; *second record*, current potentiated ~200% by 2 μM diazepam; *third record*, level of potentiation reduced to ~20% by 1 μM flumazenil. B, *First record*, control response; *second record*, current potentiated ~100% by 2 μM δ-HCH; *third record*, level of potentiation essentially unaffected by 1 μM flumazenil. Records were separated by a 2–4-min wash.

Interactions between Effects of Different HCH Enantiomers on $I_{G\text{-}Actx}$

Firstly, because β-HCH showed little activity as a modulator or inhibitor of $I_{G\text{-}Actx}$, this isomer was tested for antagonism of the inhibitory or facilitatory effects of other enantiomers. As described above, 50 μM β-HCH induced 20–25% potentiation of currents elicited by 10 μM GABA, and 1 μM γ-HCH reduced these responses by approximately 50%. Using the same concentrations, coapplication of the two enantiomers resulted in net reductions in current of approximately 45%, suggesting that β-

HCH had only marginal effects on the potency of γ-HCH. In addition, coapplication of 50 μM β-HCH caused no appreciable reductions in levels of potentiation induced by 5 μM δ-HCH, suggesting that the β-enantiomer did not act as an antagonist of modulatory effects.

Secondly, using $I_{G\text{-}Actx}$ elicited by 10 μM GABA, we characterized interactions between the positive modulation induced by α- or δ-HCH and the predominantly inhibitory effects of γ-HCH. For example, levels of potentiation induced by 1–5 μM α-HCH were first defined (Fig. 9, *first four records*), oocytes were then washed, to re-establish control responses, and effects of 1–5 μM α-HCH were reassayed when α-HCH was coapplied with 1 μM γ-HCH (Fig. 9, *last four records*). Control responses were reduced 40–50% by 1 μM γ-HCH, but application of 1–5 μM α-HCH still induced clear potentiation of these responses, with inhibitory effects of 1 μM γ-HCH being more than offset by coapplications of 2 μM α-HCH. Potentiated currents were reduced 35–45% by 1 μM γ-HCH, similar to reductions in control responses, suggesting that γ-HCH did not have specific inhibitory effects on the positive modulation induced by α-HCH. Repeating experiments with δ-HCH gave similar results (data not shown).

Effects of Other HCH Enantiomers on $I_{G\text{-}BR}$

For completeness, we lastly assayed effects of α-HCH, β-HCH, and δ-HCH on the Bic/Bac-insensitive receptors. In some cases, these experiments allowed us to cross-check weak effects on $I_{G\text{-}Actx}$ that might have been due simply to low level contamination between enantiomers.

Effects of α-HCH on $I_{G\text{-}BR}$. α-HCH was initially assayed for potentiation or inhibition of $I_{G\text{-}BR}$ elicited by 1 μM and 10 μM GABA. These experiments suggested that 1–100 μM α-HCH did not exert positive modulation of $I_{G\text{-}BR}$ but, on the contrary, had weak inhibitory effects when applied at concentrations >1 μM. As described for $I_{G\text{-}Actx}$, effects of α-HCH were then assayed over full $I_{G\text{-}BR}$ concentration-response curves (Fig. 5B). At concentrations >1 μM, α-HCH caused modest rightward shifts, associated with insignificant changes in slope values and minor reductions in maximum responses. For example, 100 μM α-HCH increased the EC_{50} of $I_{G\text{-}BR}$ from control values of 3.5 ± 0.3 μM (n = 3) to 4.9 ± 0.4 μM (n = 4), whereas maximum responses were reduced by only 6 ± 4%. Hence, in contrast to strong positive modulation apparent on $I_{G\text{-}Actx}$, α-HCH had only weak and predominantly surmountable inhibitory effects on $I_{G\text{-}BR}$.

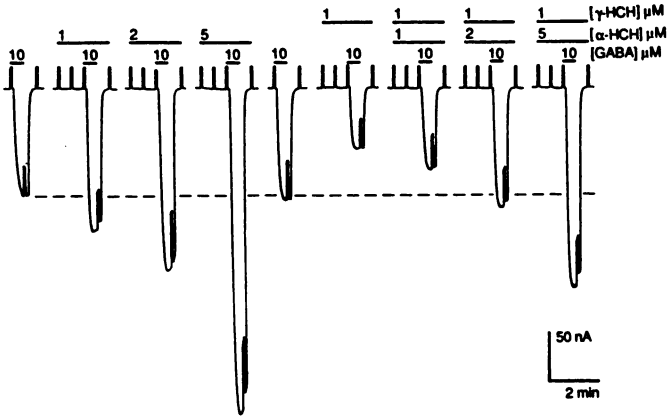

Fig. 9. Interactions between α-HCH and γ-HCH on $I_{G\text{-}Actx}$. *First record*, control response elicited by 10 μM GABA; *second* to *fourth records*, potentiation of currents induced by 1–5 μM α-HCH; *fifth record*, response after a 12-min wash returned to control levels; *sixth record*, control response, reduced ~45% by 1 μM γ-HCH (first application of γ-HCH not shown); *seventh* to *ninth records*, potentiation of currents by 1–5 μM α-HCH coapplied with 1 μM γ-HCH. Inhibitory effects of 1 μM γ-HCH appeared to be reversed by coapplication of 2 μM α-HCH. Records were taken from the same oocyte and, unless otherwise stated, intervals between records were restricted to 1 min, ensuring that any use-dependent inhibitory effects of γ-HCH remained fully equilibrated.

Effects of β-HCH on $I_{G\text{-}BR}$. Initial assays showed that 1–50 μM β-HCH had little or no effect on $I_{G\text{-}BR}$ elicited by either 1 μM or 10 μM GABA. Given the similar lack of activity on $I_{G\text{-}Actx}$, more detailed assays to characterize effects of β-HCH on $I_{G\text{-}BR}$ were omitted.

Effects of δ-HCH on $I_{G\text{-}BR}$. At 1–100 μM, δ-HCH showed little or no positive modulation of $I_{G\text{-}BR}$ elicited by 1 μM GABA but, unlike the α-enantiomer, δ-HCH also had no clear inhibitory effects. Interestingly, analysis of full concentration-response curves confirmed that, unlike the complex facilitatory and inhibitory interactions seen on $I_{G\text{-}Actx}$, 10–100 μM δ-HCH appeared to cause no appreciable positive modulation or inhibition of $I_{G\text{-}BR}$ (Fig. 6B).

Discussion

Effects of γ-HCH on GABA receptors expressed by brain and retina RNA. Previous electrophysiological studies have demonstrated inhibitory actions of γ-HCH on GABA-activated membrane currents in mouse dorsal root ganglion cells, crayfish stomach muscle, and insect neurons (18, 19, 27). Our experiments showed that membrane current responses mediated by rat cerebral cortex $GABA_A$ receptors, expressed in oocytes, were also suppressed by γ-HCH. The IC_{50} value was between 4 and 10 μM when measured on maximum responses but was closer to 1 μM when measured on currents elicited by 10 μM GABA. Analysis of full concentration-responses curves suggested that inhibition of $I_{G\text{-}Actx}$ was predominantly noncompetitive and resembled the effects of the Cl^- channel inhibitors picrotoxin and TBPS. Comparing potencies under the same assay conditions indicated that γ-HCH was approximately 7 times less active than picrotoxin and 40 times less active than TBPS as an inhibitor of $I_{G\text{-}Actx}$[2] (38).

Binding studies on mouse or rat brain membranes, measuring displacement of *t*-[^{3}H]butylbicycloorthobenzoate or [^{35}S]TBPS by γ-HCH, yielded IC_{50} values ranging between 0.5 and 5 μM (14, 28, 29). IC_{50} values calculated from inhibition of maximum $I_{G\text{-}Actx}$ were, therefore, only slightly higher than those predicted by displacement of TBPS and were also in rough agreement with the functional potency of γ-HCH determined by a $^{36}Cl^-$ uptake study using mouse brain vesicles, where the IC_{50} was reported to be 1 μM (17) (but see also Ref. 16). As reported for mouse dorsal root ganglion cells (18), inhibitory effects of γ-HCH on $I_{G\text{-}Actx}$ were relatively refractory to wash, particularly after extended applications of micromolar concentrations of inhibitor, but whether there is a truly irreversible component remains unclear. In general, our results indicated that $GABA_A$ receptors expressed in oocytes provided a suitable control for studies of the atypical GABA receptors expressed by retina RNA.

Currents mediated by Bic/Bac-insensitive GABA receptors were strongly inhibited by γ-HCH. Comparison of effects on maximum responses indicated that the potency of inhibition was similar to that described for $GABA_A$ receptors (IC_{50}, 4–8

μM). However, suppression of $I_{G\text{-}BR}$ by γ-HCH showed a relatively strong dependence on agonist concentration, such that IC_{50} values measured on currents elicited by 1 μM GABA were as low as 0.1 μM γ-HCH, suggesting potencies up to 60 times higher than values determined on maximum responses. Strong dependence on agonist concentration also characterized the inhibitory effects of picrotoxin on $I_{G\text{-}BR}$, and effects of TBPS were almost wholly surmountable (38). There are a variety of possible explanations for the distinctive dependence on agonist concentration, but perhaps the most likely is that the Bic/Bac-insensitive GABA receptors allow strong allosteric interactions between agonist and convulsant binding sites. Although questions relating to mechanism were not addressed directly, the effects of picrotoxin and γ-HCH showed sufficient similarities to suggest that the mechanisms of inhibition by these compounds share common features.

Effects of α-, β-, and δ-HCH on GABA receptors expressed by brain and retina RNA. Our experiments showed that α-HCH and δ-HCH induced relatively strong positive modulation of rat $GABA_A$ receptors expressed in oocytes, whereas β-HCH had only marginal effects. In addition, both α- and δ-HCH also appeared to have inhibitory effects on $I_{G\text{-}Actx}$, although these were weak compared with those of the γ-enantiomer. It should be stressed that the mechanism by which α- and δ-HCH potentiate GABA-activated currents remains wholly unclear. In particular, there is the fundamental question of whether positive modulation results from specific interactions of α- and δ-HCH with $GABA_A$ receptor complexes or from some type of "nonspecific" effect.

As mentioned earlier, binding studies indicate that γ-HCH inhibits GABA-activated Cl^- channels through interactions at, or close to, the picrotoxin binding site (13–15). α- and δ-HCH have also been shown to inhibit *t*-[^{3}H]butylbicycloorthobenzoate binding in mouse cerebellar membranes (28) and [^{35}S]TBPS binding in membrane homogenates from invertebrate tissues (30), albeit with lower potency than the γ-enantiomer. These studies suggest that α- and δ-HCH can act at the picrotoxin site, but the crucial issue remains whether this interaction is actually responsible for the potentiation of responses. In this context, it is worth noting that the idea of picrotoxin binding sites being able to mediate both inhibitory and facilitatory effects is by no means novel. For example, various γ-butyrolactones and γ-thiobutyrolactones that displace [^{35}S]TBPS from rat brain membranes have clearly been shown to potentiate or inhibit GABA-activated currents in rat hippocampal neurons, acting as convulsants or anticonvulsants depending on their alkyl substitutions (31, 32).

The likelihood of nonspecific effects arises because HCH enantiomers are lipophilic molecules that tend to partition preferentially into cell membranes. It is, therefore, possible that modulation of $GABA_A$ receptors is simply due to perturbations in the fluidity of lipid bilayers (33). This same possibility has been a recurrent issue concerning the modulatory effects of anesthetic steroids on $GABA_A$ receptors (e.g., Refs. 34 and 35). Interestingly, our previous studies indicated that 3-hydroxypregnanolones not only potentiated $I_{G\text{-}Actx}$ but also increased the rate of response decay, an effect that might be specific to $GABA_A$ receptors expressed in oocytes (7). Modulatory effects of α- and δ-HCH were not associated with appreciable increases in rates of desensitization, suggesting that any putative effects on membrane fluidity would appear to be different for these two classes of drug.

Comparison of the effects of α- and δ-HCH with those of other $GABA_A$ receptor modulators reveals only partial similarities, with no consistent correspondence to suggest a common underlying mechanism. For example, potentiation of $I_{G\text{-}Actx}$ by α-HCH and δ-HCH was not as pronounced as that induced by barbiturates or 3α-hydroxy-pregnanolones (3, 6, 7) but was similar to levels induced by diazepam or chlorazepate, at least in oocyte assays (4, 5). However, modulation of $I_{G\text{-}Actx}$ by α- and δ-HCH was largely insensitive to inhibition by the benzodiazepine antagonist flumazenil, clearly suggesting that the effect was not mediated by interactions at benzodiazepine binding sites. Facilitatory actions of α-HCH and δ-HCH showed thresholds between 0.1 and 0.3 μM, similar to the potency of pentobarbital in oocytes (3, 6) but weak compared with steroids and some benzodiazepines, which have effects in the nanomolar range (5, 7). Time courses for washing out the modulatory effects of 10–20 μM α- and δ-HCH appeared to be rapid compared with potentiation induced by diazepam or 3α-hydroxy-pregnanolones (7) but were slow compared with effects of pentobarbitol (3). The biphasic time course for washing out modulatory effects of α- and δ-HCH possibly reflects removal of the enantiomers from hydrophilic (fast) and hydrophobic (slow) compartments.

As described for barbiturates, benzodiazepines, and steroids (6, 7), the Bic/Bac-insensitive GABA receptors expressed by retina RNA showed no detectable positive modulation by either α-HCH or δ-HCH. The Bic/Bac-insensitive GABA receptors have relatively high affinity for GABA (EC_{50}, 1–2 μM) and high levels of co-operativity (slope values, approximately 2), and the inability to potentiate the current further with α- and δ-HCH appears to correspond to a general insensitivity to $GABA_A$ receptor modulators (6, 7). The inactivity of δ-HCH on the Bic/Bac-insensitive receptors deserves specific comment. Firstly, it indicates that inhibitory effects of δ-HCH on $I_{G\text{-}Actx}$ are not due to cross-contamination by γ-HCH. Secondly, it suggests either that γ-HCH binding sites on the Bic/Bac-insensitive receptors discriminate strongly between γ- and δ-enantiomers, whereas sites on $GABA_A$ receptors are less selective, or that γ-HCH and δ-HCH inhibit $I_{G\text{-}Actx}$ through functionally distinct mechanisms.

Although α- and δ-HCH have been characterized as anticonvulsants or depressants in mammalian brain (8, 36, 37), the targets and mechanisms underlying these effects remain unclear. Our experiments in oocytes appear to raise the possibility that $GABA_A$ receptors are a potential target for α- and δ-HCH and that anticonvulsant and depressant effects could be due to modulatory effects at GABAergic synapses. At present, this model has the attraction of simplicity but lacks any direct supporting evidence. More importantly, there seem to be clear discrepancies with previous studies assaying the anticonvulsive activities of different HCH enantiomers.

Positive modulation of brain $GABA_A$ receptors by HCH enantiomers has not, thus far, been demonstrated by electrophysiological studies on mammalian neurons. In particular, γ-HCH (10 μM) was reported to have substantial inhibitory effects on GABA-activated membrane currents in mouse dorsal root ganglion cells, whereas α-HCH (10 μM) appeared to have no effect (two experiments) (18). In this particular study, however, effects were assayed on currents elicited by a single concentration of GABA (10 μM), and it was unclear whether

the responses were close to threshold, and thus susceptible to potentiation, or already near maximum (18). It will, therefore, be necessary to clarify whether α- and δ-HCH cause positive modulation of $GABA_A$ receptors *in situ*. These effects could, for some reason, be specific to $GABA_A$ receptors expressed in oocytes, perhaps being dependent upon interactions between drugs and the foreign lipid environment into which receptors are inserted.

Finally, previous studies using a variety of assay systems have consistently reported that β-HCH has relatively strong depressant or anticonvulsant actions in mammals (8, 36, 37). Our experiments suggest that β-HCH has only marginal facilitatory effects on $I_{G\text{-}Actz}$, being approximately 200–500 times less active than α- or δ-HCH. Furthermore, the β-HCH used in this study was significantly contaminated by the δ-enantiomer (0.17%), raising the possibility that some of the effects induced by high concentrations of β-HCH were artifactual. These results tend to imply that β-HCH induces depressant or anticonvulsant actions through mechanisms that do not involve modulation of $GABA_A$ receptors, raising the possibility that anticonvulsant effects of α- and δ-enantiomers occur via similarly unrelated targets.

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