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Characterization of Bicuculline/Baclofen-Insensitive (ρ-like) γ-Aminobutyric Acid Receptors Expressed in *Xenopus* Oocytes. II. Pharmacology of γ-Aminobutyric Acid$_A$ and γ-Aminobutyric Acid$_B$ Receptor Agonists and Antagonists

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SUMMARY

Poly(A)$^+$ RNA from mammalian retina expresses bicuculline/baclofen-insensitive γ-aminobutyric acid (GABA) receptors in *Xenopus* oocytes with properties similar to those of homooligomeric GABA$_{\rho 1}$ receptors. The pharmacological profile of these ρ-like receptors was extended by measuring sensitivities to various GABA$_A$ and GABA$_B$ receptor ligands. For direct comparison the same compounds were also assayed with GABA$_A$ receptors expressed by rat brain RNA. The potency sequence for heterocyclic GABA analogues at the GABA$_\rho$-like receptors was GABA (1.3) > muscimol (2.3) > isoguvacine (100) (approximate EC_{50} in parentheses; all EC_{50} and K_b values given in μM). Both muscimol and isoguvacine were partial agonists at the ρ-like receptors. 4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol ($K_b \simeq 32$), piperidine-4-sulfonic acid ($K_b \simeq 85$), and isonipecotic acid ($K_b \simeq 1000$) acted primarily as competitive antagonists, showing little or no activity as agonists. The sulfonic acid GABA analogue 3-aminopropanesulfonic acid was also a competitive antagonist ($K_b \simeq 20$). Conformationally restricted GABA analogues *trans*- and *cis*-4-aminocrotonic acid (TACA and CACA) were agonists at the ρ-like receptors. TACA ($EC_{50} \simeq 0.6$) had twice the potency of GABA and was 125 times more potent than CACA ($EC_{50} \simeq 75$). Z-3-(Amidinothio)propenoic acid, an isothiouronium analogue of GABA, had little activity as an agonist but instead acted as a competitive antagonist ($K_b \simeq 20$). At concentrations of >100 μM, bicuculline did have some weak competitive inhibitory effects on the GABA$_\rho$-like receptors ($K_b \simeq 6000$), but it was at least 5000 times more potent at GABA$_A$ receptors. Strychnine ($K_b \simeq 70$) and SR-95531 ($K_b \simeq 35$) also were competitive inhibitors of the ρ-like receptors but were, respectively, 20 and 240 times more potent at GABA$_A$ receptors. The GABA$_B$ receptor ligands baclofen, phaclofen, and saclofen (1-100 μM) had no appreciable effects on the ρ-like receptors. In contrast, 3-aminopropylphosphonic acid, the phosphonic acid analogue of GABA, acted as a competitive antagonist ($K_b \simeq 10$), and 3-aminopropylphosphinic acid and 3-aminopropyl(methyl)-phosphinic acid were moderately potent antagonists ($K_b \simeq 1.7$ and 0.8, respectively). δ-Aminovaleric acid was also an antagonist ($K_b \simeq 20$), whereas 4-aminobutylphosphonic acid, the phosphonic acid analogue of δ-aminovaleric acid, was only a weak inhibitor ($K_b \simeq 600$). In terms of structure-activity relationships, our experiments suggest that incorporation of the carboxyl or amino groups of GABA into 3-isoxazolol or piperidine rings either reduces agonist potency at the GABA$_\rho$-like receptors or results in analogues that act as competitive antagonists. Similarly, substitution of the carboxyl group for sulfonic acid or of the amino group for isothiouronium generates antagonists. The relative activities of TACA and CACA clearly suggest that GABA interacts with the ρ-like receptors in extended conformations and appears to distinguish these receptors from previously postulated GABA$_C$ receptor sites. The 4-chlorophenyl substituent of baclofen and related GABA$_B$ receptor antagonists almost wholly prohibits functionally relevant interactions with the ρ-like receptors. However, some phosphonic and phosphinic analogues of GABA, normally catagorized as selective GABA$_B$ receptor agonists, show a clear potential for acting as antagonists. These results should be useful for designing drugs that interact selectively with mammalian bicuculline/baclofen-insensitive GABA receptors and for investigating the mechanisms by which ligands interact with GABA-gated Cl^- channels.

Xenopus oocytes injected with poly(A)$^+$ RNA from chick or rat brain express functional GABA receptors that have electrical and pharmacological profiles similar to those of GABA$_A$ receptors studied *in situ* (e.g., Refs. 1-9). Expression of functional GABA$_B$ receptors in oocytes has recently been reported using RNA isolated from rat cerebellum (10). Oocytes injected with poly(A)$^+$ RNA from mammalian retina express GABA-activated membrane current responses made up of two pharmacologically distinct components. One component is mediated by GABA$_A$ receptors and consists of a Cl^- current that desensitizes, is competitively antagonized by bicuculline, is blocked by picrotoxin, TBPS, and γ-hexachlorocyclohexane, and is positively modulated by barbiturates, benzodiazepines, and 3α-

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hydroxypregnanolones (6–9). The other component is also a Cl^- current that shows sensitivity to picrotoxin and γ-hexachlorohexane (8, 9), but this current is resistant to bicuculline (6), is weakly and surmountably blocked by TBPS (9), and is not significantly modulated by barbiturates, benzodiazepines, or steroids (6, 7). Furthermore, the bicuculline-resistant current is neither activated by the $GABA_B$ receptor agonist baclofen nor appreciably suppressed by the antagonist 2-hydroxysaclofen (6). These results indicate that poly(A)$^+$ RNA from mammalian retina expresses GABA receptors with a distinct pharmacological profile.

cDNAs encoding novel GABA receptor subunits that are highly enriched in human and bovine retina have recently been cloned (11, 12). These receptors, denoted $GABA_{\rho 1}$ and $GABA_{\rho 2}$, show clear sequence similarities (30–40%) to $GABA_A$ subunits. However, expression studies in oocytes indicate that homooligomeric receptors assembled from $GABA_{\rho 1}$ subunits form GABA-gated Cl^- channels that are insensitive to bicuculline, baclofen, and $GABA_A$ receptor modulators, raising the issue of whether such receptors should be classified as a separate group, i.e., $GABA_C$ (13). At present it remains unclear whether the bicuculline/baclofen-insensitive GABA responses expressed by retina poly(A)$^+$ RNA are wholly mediated by homooligomeric $GABA_{\rho 1}$ receptors or whether more complex subunit combinations are involved. In this report we refer to these receptors as $GABA_\rho$-like, or simply as ρ-like, receptors. Whatever the situation with respect to subunit composition, experiments using poly(A)$^+$ RNA and cloned genes both raise the possibility that subclasses of GABA receptors in mammalian retina, and perhaps other regions of the brain, have novel electrical and pharmacological properties largely uncharacterized *in situ* (e.g., Ref. 14). This contention has recently been confirmed by studies reporting bicuculline/baclofen-insensitive GABA responses in neurons dissociated from fish and rat retinae (15, 72).

In the present study we have extended the pharmacological profile of the $GABA_\rho$-like receptors by comparing actions of various commonly used $GABA_A$ and $GABA_B$ receptor ligands on GABA-activated Cl^- currents expressed in oocytes by poly(A)$^+$ RNA from bovine retina. In terms of electrical properties (6) and structural similarities at the molecular level (11), the ρ-like receptors appear to be related to $GABA_A$ receptors. Hence, to serve as a control, the potency of each agonist and antagonist was also assayed at $GABA_A$ receptors expressed by rat cerebral cortex RNA.

Materials and Methods

RNA extraction, size fractionation, and receptor expression in oocytes. Preparation of poly(A)$^+$ RNA and size fractionation of retina RNA were as described previously (e.g., Refs. 6 and 7). In this study, 10 separate RNA preparations were made from bovine retina and three from rat cerebral cortex. All retina preparations were size-fractionated to amplify expression of GABA responses; this was usually necessary for detailed characterization of drug effects. Follicle-enclosed *Xenopus* oocytes, at stages V and VI of development (16), were microinjected with 75–100 ng of total poly(A)$^+$ RNA from cerebral cortex or retina or 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 were defolliculated approximately 48 hr after injection by treatment with collagenase (17).

Electrophysiology and data analysis. 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), using a two-electrode voltage clamp. All drugs were applied to oocytes in Ringer solution by bath perfusion.

Actions of various GABAergic agonists and antagonists were assayed on $I_{G\text{-}Actx}$, $I_{G\text{-}Aret}$, and $I_{G\text{-}BR}$. Detailed quantitative analyses were restricted to comparisons between $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$, with effects on $I_{G\text{-}Aret}$ being assayed qualitatively. The two components of GABA responses expressed by retina RNA were distinguished using bicuculline methobromide (Fig. 1). For responses elicited by 100 μM GABA, 100 μM bicuculline was more than sufficient to abolish $I_{G\text{-}Aret}$. For responses elicited by 0.1–1 mM GABA, equimolar concentrations of bicuculline were used to ensure abolition of $I_{G\text{-}Aret}$ (6–9). A similar approach was used to distinguish effects of other agonists that activated both components of the GABA responses expressed by retina RNA.

Potencies of the different antagonists used in this study were initially assayed on $I_{G\text{-}Actx}$ elicited by 10 μM GABA and $I_{G\text{-}BR}$ elicited by 1 μM or 10 μM GABA. IC_{50} values were calculated by regression, measuring effects on $I_{G\text{-}Actx}$ elicited by 50 μM GABA (35–45% of maximum response) and $I_{G\text{-}BR}$ elicited by 1 μM GABA (20–40% of maximum response) (9). Relative potencies of agonists were determined in each case by direct comparison with currents elicited by GABA in the same oocyte. Concentration-response curves for $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$ were constructed as described previously (6–9). Actions of antagonists were characterized by measuring effects on full concentration-response curves. 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 (18). Dissociation constants for antagonists (K_b) were either determined by Schild regression or, assuming simple competitive inhibition, estimated from shifts in EC_{50} induced by a single concentration of antagonist (usually 100 μM), where dose ratio = 1 + [antagonist]/K_b (e.g., Ref. 19).

Drugs. 3-APMPA and ZAPA (some experiments) were from Cookson Chemicals Ltd. (Southampton, UK). (*R*)-(+)- and (*S*)-(−)-Baclofen (hydrochloride salts), DAVA, 2-hydroxysaclofen, isoguvacine, isonipecotic acid, phaclofen, P-4-S, saclofen, SR-95531 [2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)] pyridazinium bromide, and THIP were from Research Biochemicals Inc. (Natick, MA). THIP was also a generous gift from Dr. Arne Schousboe (Royal Danish School of Pharmacy, Copenhagen, Denmark). 4-ABPA, 2-AEPA, 3-APA, 3-APS, bicuculline methobromide, GABA, DAVA (some experiments), muscimol, and strychnine were from Sigma. CACA, TACA, and ZAPA (some experiments) were from Tocris Neuramin (Bristol, UK). CGP 35348 [3-aminopropyl(diethoxymethyl)phosphinic acid] was generously pro-

ABBREVIATIONS: GABA, γ-aminobutyric acid; 4-ABPA, 4-aminobutylphosphonic acid; 2-AEPA, 2-aminoethylphosphonic acid; 3-APMPA, 3-aminopropyl(methyl)phosphinic acid (SK&F 97541); 3-APA, 3-aminopropylphosphonic acid; 3-APPA, 3-aminopropylphosphinic acid (alternatively termed 3-aminopropylphosphonous acid) (CGP 27492); 3-APS, 3-aminopropanesulfonic acid; CACA, *cis*-4-aminocrotonic acid; DAVA, δ-aminovaleric acid (alternatively termed 5-aminovaleric acid); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $I_{G\text{-}Actx}$, membrane current elicited through activation of $GABA_A$ receptors expressed by cerebral cortex RNA; $I_{G\text{-}Aret}$, membrane current elicited through activation of $GABA_A$ receptors expressed by retina RNA; $I_{G\text{-}BR}$, membrane current elicited through activation of $GABA_\rho$-like receptors expressed by retina RNA (BR denotes bicuculline-resistant); P-4-S, piperidine-4-sulfonic acid; TACA, *trans*-4-aminocrotonic acid; TBPS, *t*-butylbicyclophosphorothionate; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride; ZAPA, *Z*-3-(amidinothio)propenonic acid.

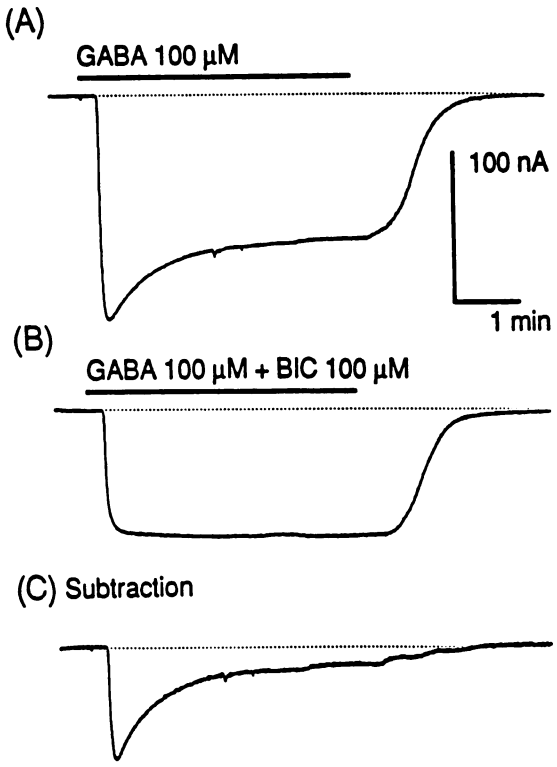

Fig. 1. Two pharmacologically distinct components of GABA-activated membrane current responses expressed by poly(A)$^+$ RNA from bovine retina. A, Full response elicited by application of 100 μM GABA. B, Bicuculline-resistant component ($I_{G\text{-}BR}$) elicited in the same oocyte by 100 μM GABA applied together with equimolar bicuculline methobromide. The bicuculline-resistant component appeared to show little desensitization. C, Subtracting B from A gave the bicuculline-sensitive component ($I_{G\text{-}Aret}$), which showed clear desensitization (85–90%) upon extended exposure to GABA. This oocyte was injected with size-fractionated retina RNA that showed relatively strong expression of $I_{G\text{-}Aret}$. The holding potential was −60 mV. Drugs were applied as indicated by *bars*, and the dead-time of the perfusion system was 5–15 sec. Unless otherwise stated, these recording conditions apply to all following records.

vided by CIBA-GEIGY Ltd. (Basel, Switzerland). Structures for most of these compounds are given in Fig. 2.

For the majority of experiments, drugs were made up freshly, each day of use, as concentrated (0.001–1 M) stocks in H_2O. 2-Hydroxysaclofen, phaclofen, saclofen, and ZAPA were made up in 0.1 M HCl. Occasionally, stock solutions were stored for up to 72 hr at 4° in the dark or were frozen at −20°; the activity of these stocks did not appear to be appreciably different from that of fresh solutions. The pH of all Ringer solutions was checked and readjusted to pH 7.0 when necessary. For example, at concentrations of >100 μM solutions of 4-ABPA, 2-AEPA, 3-APPA, baclofen enantiomers, DAVA, isoguvacine, THIP, and compounds made up in 0.1 M HCl became acidified and were readjusted to pH 7.0 with NaOH. In this context, it should be noted that in some oocytes mildly acidified Ringer solution itself elicits membrane currents consisting of two major components, (i) a smooth, maintained, apparently inward current associated with a reduction in membrane conductance primarily to K^+ and (ii) large oscillatory Cl^- currents due to increased activity of the phosphoinositide/Ca^{2+} messenger pathway (20).

Results

$GABA_A$ Receptor Agonists

Initial characterization indicated that the $GABA_\rho$-like receptors expressed by retina RNA had relatively high affinity for GABA (6). In the present study we examined the actions of nine $GABA_A$ receptor agonists on currents mediated by the ρ-like receptors, i.e., five heterocyclic analogues of GABA (muscimol, THIP, isoguvacine, isonipecotic acid, and P-4-S), the sulfonic acid analogue of GABA (3-APS), two enantiomers of 4-aminocrotonic acid (TACA and CACA), and an isothiouronium analogue (ZAPA) (Fig. 2). For purposes of comparison and control, the potency of each agonist was first assayed on $I_{G\text{-}Actx}$.

Muscimol. Muscimol was a strong agonist at rat brain $GABA_A$ receptors expressed in oocytes. Muscimol responses were associated with increases in membrane conductance, were sensitive to inhibition by bicuculline, and had the same voltage dependence as currents elicited by GABA. Both currents reversed between −25 and −30 mV, the equilibrium potential for Cl^- in oocytes (21), and showed clear outward rectification at holding potentials more negative than −60 mV (3, 6). Similar criteria were used to identify currents elicited by all following $GABA_A$ receptor agonists. Potency was compared by measuring concentration-response curves for GABA and muscimol in the same oocytes (Fig. 3A). The EC_{50} for GABA was 107 ± 37 μM (four experiments; all data are given as mean ± standard deviation), and the EC_{50} for muscimol was 27 ± 8 μM (four experiments). Slope values for the two curves were approximately the same (1.2 and 1.1, respectively), and maximum responses were of similar amplitude.

Muscimol also activated membrane current responses in oocytes expressing retina RNA. As described for GABA (6), these currents were associated with increases in membrane conductance and consisted of two superimposed components that could be distinguished by their sensitivities to bicuculline. Reversal potentials for both components were the same and corresponded to the equilibrium potential for Cl^- (between −25 and −30 mV). Muscimol seemed to be more potent than GABA in eliciting $I_{G\text{-}Aret}$ (data not shown) but was less active than GABA in eliciting $I_{G\text{-}BR}$ (Fig. 3B). Like responses generated by GABA (6), bicuculline-resistant currents activated by muscimol appeared to show very little desensitization upon extended exposure to agonist. It should be stressed, however, that the method of drug application used in this study (simple bath perfusion) is unable to resolve rapid desensitization, so it remains possible that there is an undetected component of $I_{G\text{-}BR}$ that decays with millisecond time constants.

Analysis of $I_{G\text{-}BR}$ concentration-response curves, using bicuculline to abolish $I_{G\text{-}Aret}$, indicated that the EC_{50} for GABA was 1.3 ± 0.1 μM (three experiments), whereas the EC_{50} for muscimol was 2.3 ± 0.3 μM (three experiments). In addition, maximum muscimol responses were only 73 ± 4% (three experiments) of the maximum currents elicited by GABA. Slope values for GABA ranged between 1.8 and 3.3, reconfirming the high levels of co-operativity that appeared to characterize gating of the $GABA_\rho$-like receptors (6, 7). Muscimol (100 μM) was tested for inhibitory effects on $I_{G\text{-}BR}$ and caused clear (20–25%) reductions in responses activated by 10 μM GABA (data not shown).

THIP. The bicyclo muscimol analogue THIP was a relatively weak agonist at rat cerebral cortex $GABA_A$ receptors expressed in oocytes. THIP elicited small bicuculline-sensitive Cl^- currents at concentrations as low as 5 μM, but more substantial responses (>1% of GABA maximum) required concentrations of >100 μM THIP. The EC_{50} for THIP was 831 ± 80 μM (three experiments), roughly 8 times higher than that for GABA and

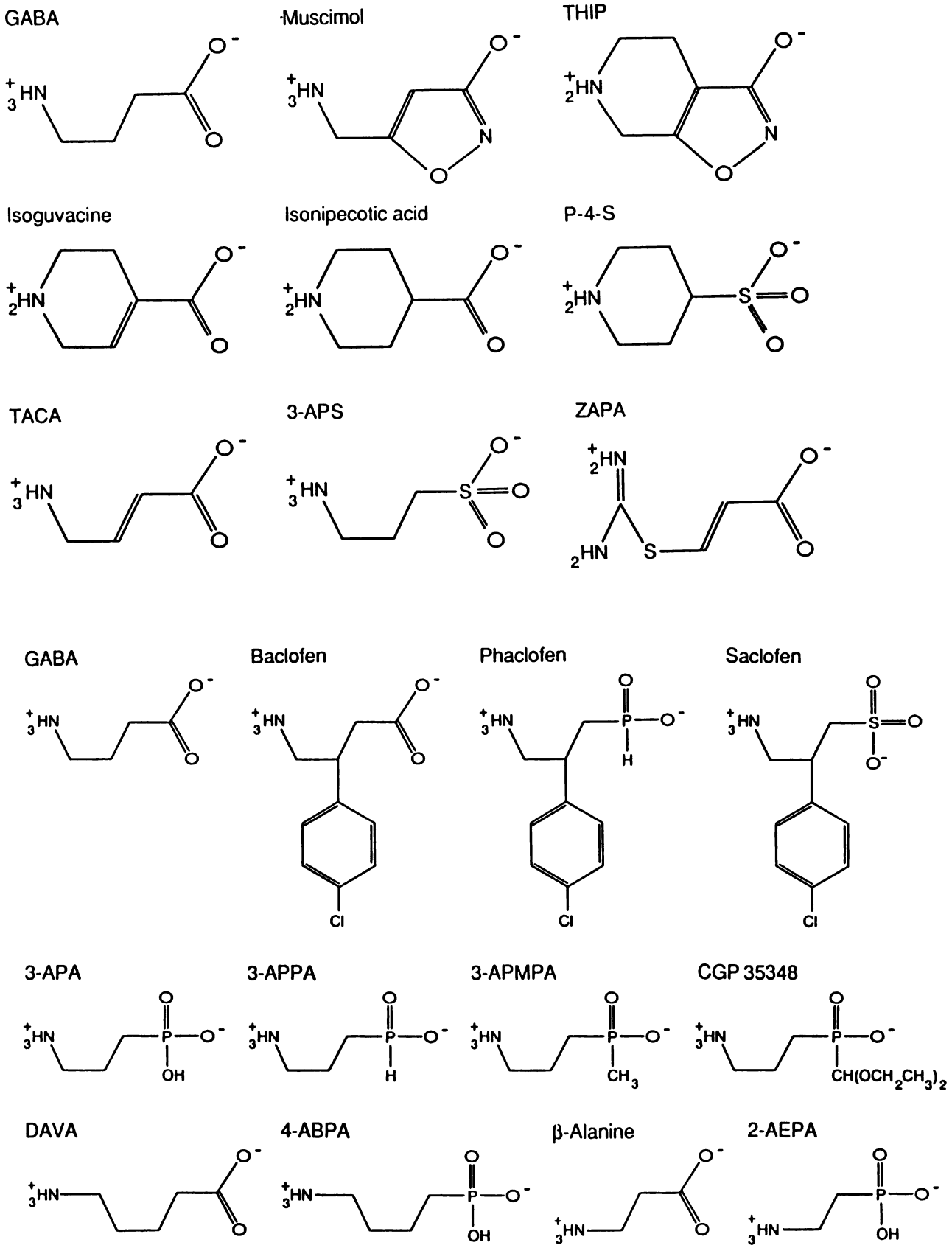

Fig. 2. Structures of $GABA_A$ receptor (*upper three rows*) and $GABA_B$ receptor (*lower three rows*) ligands assayed at $GABA_\rho$-like receptors expressed by bovine retina RNA.

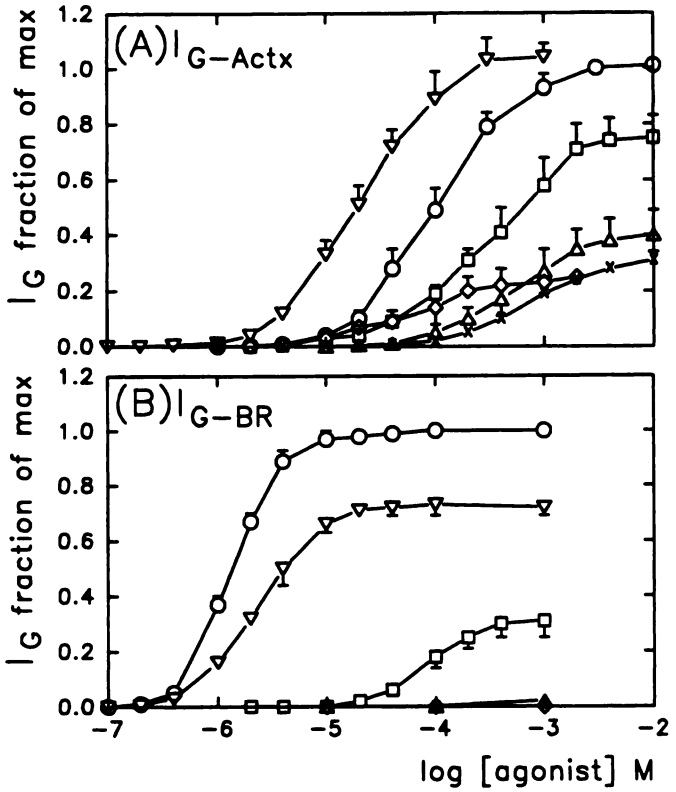

Fig. 3. Concentration-response curves comparing activities of heterocyclic $GABA_A$ receptor agonists at $GABA_A$ receptors expressed by brain RNA and at bicuculline/baclofen-insensitive GABA receptors expressed by retina RNA. A, Potency in eliciting I_{G-Actx}. ▽, Muscimol (four experiments); ○, GABA (four experiments); □, isoguvacine (three experiments); ◇, P-4-S (three experiments); △, isonipecotic acid (three experiments); ×, THIP (three experiments). B, Potency in eliciting I_{G-BR}. ○, GABA (three experiments); ▽, muscimol (three experiments); □, isoguvacine (three experiments). P-4-S, isonipecotic acid, and THIP showed marginal or no response. For these experiments, agonists were applied together with equimolar bicuculline to abolish any bicuculline-sensitive currents. In this and all following concentration-response curves, data points are the mean ± standard deviation expressed as a fraction of maximum response (I_{G-Actx} elicited by 3 mM GABA or I_{G-BR} elicited by 100 μM GABA with 100 μM bicuculline).

30 times higher than that for muscimol (Fig. 3A). Furthermore, maximum THIP responses were only 31 ± 3% of maximum currents elicited by GABA (three experiments). Simultaneous coapplication of 10 mM THIP reduced maximum (3 mM) GABA responses by <15%.

Recordings from oocytes expressing retina RNA showed that THIP was largely inactive in eliciting membrane currents. Responses were only appreciable when THIP was applied at concentrations of >100 μM and, moreover, these currents were sensitive to bicuculline, suggesting weak selective activation of I_{G-Aret}. Coapplication of THIP with equimolar bicuculline revealed no detectable activation of I_{G-BR}, even at concentrations as high as 1 mM. Because muscimol had shown some occlusion of GABA responses, THIP was likewise tested for antagonist activity at the ρ-like receptors. Assays of I_{G-BR} elicited by 1 μM GABA (30–40% of maximum response) showed that inhibition was indeed detectable, even at concentrations as low as 0.1–0.5 μM THIP. The IC_{50} for THIP measured under these conditions (standard for all following IC_{50} measurements) was 10 ± 0.4 μM (four experiments) (data not shown). Currents elicited by 10 μM GABA were reduced <10% by 100 μM THIP but were almost abolished by 1 mM THIP (Fig. 4A). Analysis of effects on I_{G-BR} concentration-response curves showed that THIP caused clear rightward shifts, without appreciably altering slope values (approximately 2) or decreasing maximum responses (Fig. 4B). Dose ratios were calculated from the shifts in EC_{50} induced by three concentrations of THIP (100, 500, and 1000 μM). The Schild regression of these data had a slope of 1.01 ± 0.04, consistent with models for competitive inhibition, and gave a K_b of 32 ± 5 μM (three experiments) (Fig. 4C). In separate experiments (i.e., different oocytes and RNA preparation), K_b was also determined by measuring the shift in EC_{50} induced by a single concentration of THIP (100 μM) and

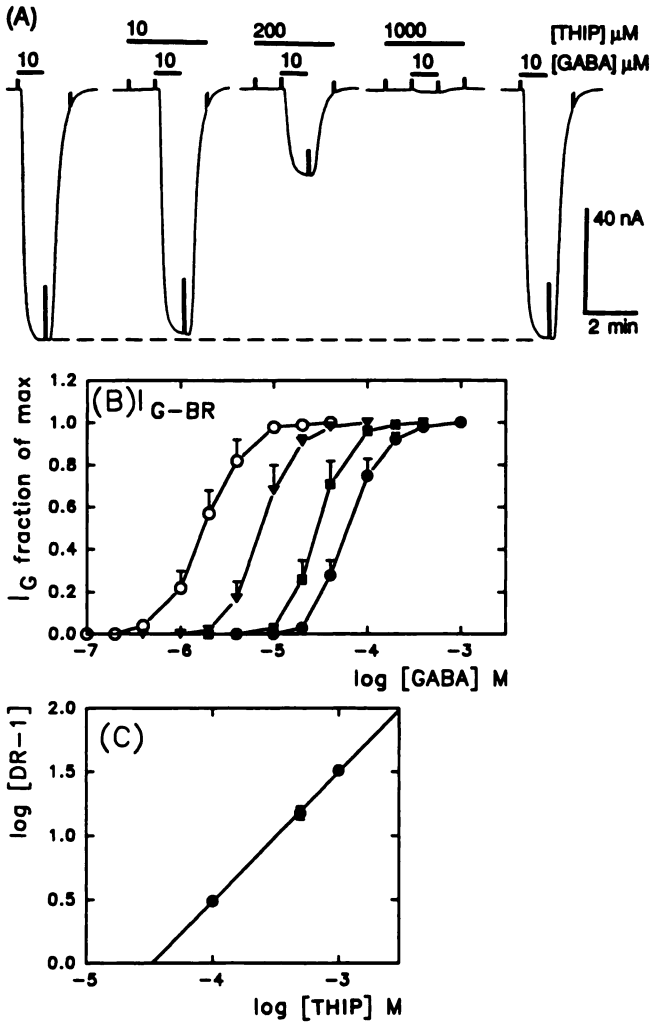

Fig. 4. A, Sample records illustrating inhibitory effects of 10–1000 μM THIP on I_{G-BR} elicited by 10 μM GABA; records are from the same oocyte. In all cases GABA was applied together with 100 μM bicuculline to ensure abolition of I_{G-Aret}. – – –, current amplitude under control conditions. The holding potential was −70 mV and was periodically pulsed to −60 mV (brief upward deflections). Pulses were used to monitor membrane conductance and also served as markers for solution changes. B, Inhibitory effects of THIP on I_{G-BR} concentration-response curves. ○, GABA control; ▼, GABA plus 100 μM THIP; ■, GABA plus 500 μM THIP; ●, GABA plus 1 mM THIP (three or four determinations for each data point). GABA and THIP were applied together with 100 μM bicuculline methobromide to abolish I_{G-Aret}. In this and all following figures *open symbols* denote currents directly activated by drugs (in this case GABA), whereas *closed symbols* show inhibitory effects of drugs on currents elicited by GABA. C, Schild regression of data presented in B, with dose ratios (*DR*) calculated from shifts in EC_{50} induced by different concentrations of THIP.

assuming simple competitive inhibition. In this case the K_b for THIP was 24 ± 7 μM (three experiments), within 1 SD of the value obtained from the Schild regression. K_b values for other moderately potent antagonists were estimated using this abbreviated procedure, making the same assumption of simple competitive inhibition.

Isoguvacine. Isoguvacine is an analogue of THIP in which the amino-group ring structure is maintained but the 3-isoxazolol ring is resubstituted with a carboxyl group, as in GABA. The potency of isoguvacine as an agonist at rat brain $GABA_A$ receptors expressed in oocytes was intermediate between that of GABA and that of THIP. The EC_{50} for eliciting $I_{G\text{-}Actx}$ was 305 ± 103 μM (three experiments), roughly 3 times higher than values for GABA but at least 2 times lower than values for THIP (Fig. 3A). Interestingly, maximum isoguvacine responses again appeared to be lower (25 ± 8%) than maximum currents elicited by GABA. Coapplication of 10 mM isoguvacine together with 3 mM GABA revealed only marginal (<10%) reductions in maximum GABA response.

Assays in oocytes expressing retina RNA showed that isoguvacine activated both $I_{G\text{-}Aret}$ and $I_{G\text{-}BR}$ but was a particularly weak agonist at the $GABA_\rho$-like receptors. The EC_{50} for eliciting $I_{G\text{-}BR}$ was 99 ± 7 μM (three experiments), approximately 80 times higher than that of GABA (Fig. 3B). Furthermore, maximum bicuculline-resistant currents activated by isoguvacine were only 31 ± 6% of currents elicited by GABA. Coapplication of 1000 μM isoguvacine together with 10 μM GABA resulted in 30–40% reductions in the current elicited by 10 μM GABA applied alone. The potency of isoguvacine in eliciting $I_{G\text{-}Aret}$ was not investigated in detail but appeared to be lower than that of GABA and higher than that of THIP.

Isonipecotic acid. The saturated isoguvacine analogue isonipecotic acid was appreciably less potent than isoguvacine as an agonist at rat brain $GABA_A$ receptors. The EC_{50} was 607 ± 189 μM (three experiments), roughly 6-fold higher than that of GABA (Fig. 3A). As seen with THIP and isoguvacine, maximum $I_{G\text{-}Actx}$ activated by isonipecotic acid appeared to be smaller than currents elicited by GABA or muscimol, in this case 40 ± 9%. Testing for inhibitory effects on currents elicited by GABA indicated that 10 mM isonipecotic acid reduced maximum GABA responses (3 mM GABA) by 10–15%.

Isonipecotic acid was almost wholly inactive as an agonist at the ρ-like receptors expressed by retina RNA (Fig. 3B). At 1 mM, isonipecotic acid activated small inward currents, but these were substantially blocked by equimolar bicuculline, indicating weak selective activation of $I_{G\text{-}Aret}$. Maximum bicuculline-resistant responses were <2% of maximum GABA responses. As described for THIP, isonipecotic acid did, however, have inhibitory effects on currents elicited by GABA. The IC_{50} measured under standard conditions was 195 ± 13 μM (three experiments), roughly 20 times higher than the value for THIP, indicating that the potency of inhibition was comparatively weak. Isonipecotic acid at 100 μM had little effect on $I_{G\text{-}BR}$ concentration-response curves but at 1 mM caused clear parallel rightward shifts that were not associated with decreases in maximum response (data not shown).

P-4-S. Strictly in terms of EC_{50} values, P-4-S, the sulfonic acid analogue of isonipecotic acid, appeared to have potency similar to that of GABA as an agonist at rat brain $GABA_A$ receptors expressed in oocytes. The EC_{50} for P-4-S was 102 ± 41 μM and that for GABA was 104 ± 33 (three experiments). However, the most conspicuous feature of P-4-S concentration-response curves was that maximum responses were consistently <30% of currents elicited by GABA (Fig. 3A). Furthermore, assays for inhibitory effects on $I_{G\text{-}Actx}$ elicited by GABA showed that coapplication of 10 mM P-4-S reduced 3 mM GABA responses by 60–75%.

As described for THIP, P-4-S had no detectable activity as an agonist at the $GABA_\rho$-like receptors (Fig. 3B). The only responses activated by 1 mM P-4-S were small bicuculline-sensitive currents, indicating low level activation of $I_{G\text{-}Aret}$. As with THIP and isonipecotic acid, assays of GABA-activated currents revealed clear inhibitory effects on $I_{G\text{-}BR}$. The IC_{50} measured under standard conditions was 24 ± 2 μM (three experiments), and 100 μM P-4-S more than doubled the EC_{50} for $I_{G\text{-}BR}$, from 2.1 ± 0.2 μM to 4.7 ± 0.4 μM (three experiments) (data not shown). Assuming simple competitive inhibition, this shift indicates a K_b of 81 ± 11 μM.

3-APS. The sulfonic acid GABA analogue 3-APS elicited $I_{G\text{-}Actx}$ at concentrations as low as 2 μM, but in this case maximum responses (currents elicited by 1–10 mM 3-APS) were <20% of currents elicited by GABA (data not shown). The EC_{50} for 3-APS was 131 ± 47 μM (three experiments). As described for P-4-S, maximum currents elicited by GABA (3 mM) were reduced 60–80% by simultaneous coapplication of 10 mM 3-APS.

3-APS had almost no activity as an agonist at the ρ-like receptors expressed by retina RNA. At concentrations up to 100 μM, 3-APS elicited $I_{G\text{-}BR}$ that was <3% of maximum GABA responses. As with THIP and P-4-S, 3-APS had clear inhibitory effects on $I_{G\text{-}BR}$, and the IC_{50} measured under standard conditions was 7.2 ± 1 μM (three experiments). At 100 μM, 3-APS increased the EC_{50} for $I_{G\text{-}BR}$ from 1.8 ± 0.3 μM to 11.4 ± 0.2 μM (three experiments), which translates into a K_b of 19 ± 3 μM.

TACA. The conformationally restricted analogue TACA had roughly the same agonist potency as GABA at the $GABA_A$ receptors expressed by rat brain RNA (Fig. 5A). Analysis of concentration-response curves gave an EC_{50} of 133 ± 50 μM (four experiments) and, unlike most of the heterocyclic GABA analogues and 3-APS, TACA activated maximum responses that were equal to those elicited by GABA.

TACA was also a potent agonist at the $GABA_\rho$-like receptors. In this case, concentrations of TACA necessary to activate threshold responses were as low as 0.1 μM, and currents generated by 1 μM TACA were roughly double the currents elicited by the same concentration of GABA. Currents activated by 1–10 μM TACA had voltage dependence similar to that of GABA responses, were insensitive to bicuculline (10–100 μM), and showed little desensitization. As expected, higher concentrations of TACA (0.1–1 mM) elicited $I_{G\text{-}Aret}$ with approximately the same potency as GABA. Examination of concentration-response relationships confirmed that TACA was significantly more potent than GABA in activating $I_{G\text{-}BR}$ (Fig. 5B). The EC_{50} for TACA was 0.6 ± 0.2 μM (four experiments), whereas the EC_{50} for GABA in the same oocytes was 1.3 ± 0.2 μM (four experiments).

CACA. When compared with the *trans*-isomer, CACA was only a weak agonist at $GABA_A$ receptors expressed by rat cortex RNA. In oocytes where GABA elicited threshold $I_{G\text{-}Actx}$ at 1 μM, CACA activated threshold responses at approximately 100 μM, and the EC_{50} appeared to be >5 mM (Fig. 5A).

Oocytes expressing retina RNA also responded weakly to

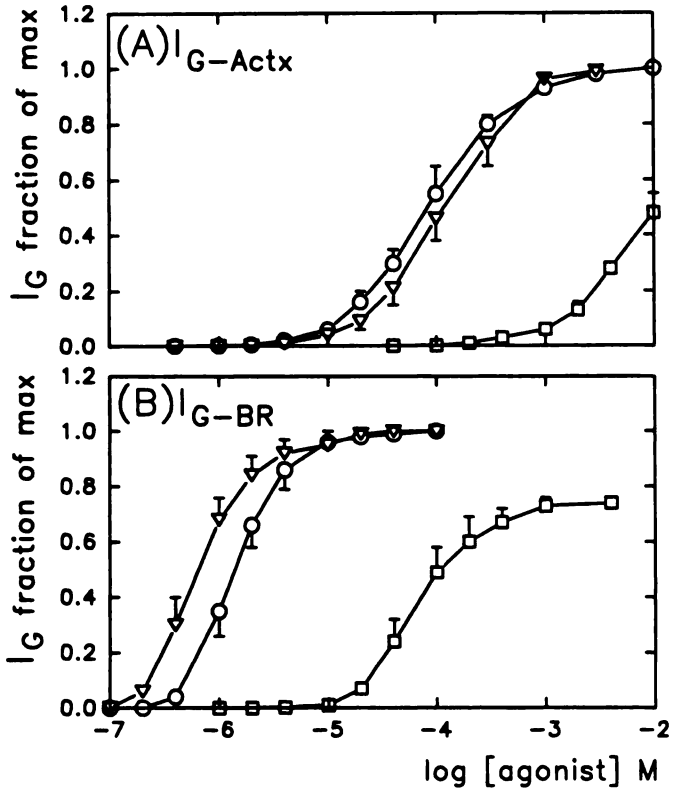

Fig. 5. Concentration-response curves comparing activities of TACA and CACA on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, Potency in eliciting $I_{G\text{-}Actx}$. ○, GABA; ▽, TACA; □, CACA (three to five determinations for all data points). B, Potency in eliciting $I_{G\text{-}BR}$. ○, GABA; ▽, TACA; □, CACA (three or four determinations for all data points). Agonists were applied together with equimolar bicuculline to abolish $I_{G\text{-}Aret}$.

CACA. Threshold responses were detectable at approximately 10 μM CACA, compared with 0.2 μM for GABA in the same oocytes (Fig. 5B). Currents activated by 0.1–1 mM CACA reversed at the equilibrium potential for Cl^-, appeared to show little desensitization, and were not appreciably blocked by 10–100 μM bicuculline, all confirming activation of $I_{G\text{-}BR}$. At millimolar concentrations, CACA was also a weak activator of $I_{G\text{-}Aret}$ (data not shown). Measurement of concentration-response relationships for the bicuculline-resistant component indicated that the EC_{50} for CACA was 74 ± 25 μM (six experiments). In addition, maximum $I_{G\text{-}BR}$ activated by CACA (1–2 mM) was only 70–80% of maximum responses elicited by GABA, suggesting that CACA was not a full agonist at the $GABA_\rho$-like receptors. As described for muscimol and isoguvacine, CACA was assayed for inhibitory effects on currents elicited by GABA, and in these experiments coapplication of 1 mM CACA reduced 10 μM GABA responses by 15–20%.

ZAPA. The isothiouronium analogue ZAPA was a potent agonist at $GABA_A$ receptors expressed by rat cerebral cortex RNA. Comparison of concentration-response curves showed that the EC_{50} for $I_{G\text{-}Actx}$ was 44 ± 15 μM (five experiments), approximately half the value for GABA, but also revealed that maximum ZAPA responses were only 69 ± 3% of the maximum responses elicited by GABA (data not shown). Simultaneous coapplication of 1 mM ZAPA did not cause any appreciable reduction in maximum GABA responses.

In contrast, ZAPA was relatively weak in eliciting membrane currents from oocytes expressing retina RNA. Furthermore, responses activated by 0.1–1 mM ZAPA showed clear desensitization and were substantially inhibited by equimolar bicuculline, indicating that currents were predominantly $I_{G\text{-}Aret}$. Recordings with 0.1–1 mM bicuculline showed that, like isonipecotic acid and 3-APS, ZAPA caused only marginal activation of $I_{G\text{-}BR}$. Maximum ZAPA responses were <5% of the maximum currents elicited by GABA and were too small for determination of meaningful EC_{50} values. As described for the heterocyclic GABA analogues, ZAPA was assayed for inhibitory effects on $I_{G\text{-}BR}$ elicited by GABA and likewise showed clear activity as an antagonist. The IC_{50} measured under standard conditions was 11 ± 2 μM (three experiments), with 100 μM ZAPA increasing the EC_{50} from 1.2 ± 0.1 to 7.6 ± 0.9 μM (four experiments) (data not shown). Assuming simple competitive inhibition, the K_b for ZAPA was 19 ± 3 μM.

$GABA_A$ Receptor Antagonists

Initial studies had shown that $I_{G\text{-}BR}$ could be pharmacologically distinguished from $I_{G\text{-}Aret}$ and $I_{G\text{-}Actx}$ by its relative insensitivity to bicuculline (6). In the present study we assayed the sensitivity of $I_{G\text{-}BR}$ to two additional antagonists, strychnine and SR-95531, but began by examining effects of bicuculline applied at high concentrations.

Bicuculline. As described previously (6), bicuculline was a potent competitive inhibitor of $I_{G\text{-}Actx}$ and $I_{G\text{-}Aret}$. The IC_{50} calculated from inhibitory effects on $I_{G\text{-}Actx}$ elicited by 50 μM GABA (35–45% of maximum response) was 1.7 ± 0.3 μM (four experiments). The K_b for bicuculline calculated from previously presented data (see Ref. 6) was 1.1 ± 0.4 μM (three experiments).

The $GABA_\rho$-like receptors were essentially insensitive to bicuculline applied at concentrations up to 100 μM. Inhibition of $I_{G\text{-}BR}$ did, however, become appreciable if bicuculline was applied at concentrations of >200 μM, and the IC_{50} measured under standard conditions was 1.7 ± 0.1 mM (three experiments). As with $I_{G\text{-}Actx}$, suppression of $I_{G\text{-}BR}$ by bicuculline was fully reversible and typically washed out in <1 min. At 1 mM, bicuculline appeared to cause a marginal rightward shift in the concentration-response curve for $I_{G\text{-}BR}$, increasing EC_{50} values from 1.3 ± 0.3 μM to 1.5 ± 0.3 μM (three experiments), without altering the slope of the curve (approximately 2) or the maximum response (Fig. 6). Assuming simple competitive inhibition, the K_b for bicuculline at the ρ-like receptors was 6.7 ± 2 mM.

Strychnine. The IC_{50} for strychnine measured on $I_{G\text{-}Actx}$ elicited by 50 μM GABA was 3.5 ± 1 μM (four experiments). Analysis of concentration-response curves indicated that 100 μM strychnine increased the EC_{50} for $I_{G\text{-}Actx}$ approximately 25-fold, from 97 ± 18 μM to 2.5 ± 0.8 mM (three experiments), indicating a K_b of 4.1 ± 1.5 μM (Fig. 7A).

$I_{G\text{-}BR}$ was also sensitive to strychnine, and the IC_{50} measured under standard conditions was 35 ± 15 μM (five experiments). Applied at 100 μM, strychnine caused a parallel rightward shift in the concentration-response curve for $I_{G\text{-}BR}$ but in this case only increased the EC_{50} from 1.1 ± 0.1 to 2.7 ± 0.2 μM (three experiments), corresponding to a K_b of 69 ± 8 μM (Fig. 7B).

SR-95531. SR-95531 was a potent inhibitor of $I_{G\text{-}Actx}$. The IC_{50} calculated from currents elicited by 50 μM GABA was 0.12 ± 0.02 μM (four experiments). At 10 μM, SR-95531 caused a pronounced parallel rightward shift in the concentration-response curve for $I_{G\text{-}Actx}$, increasing the EC_{50} from 78 ± 9 μM to 5.53 mM, giving a K_b of 0.14 ± 0.04 μM (Fig. 7A).

In contrast, the IC_{50} for SR-95531 on $I_{G\text{-}BR}$ was 17 ± 4 μM

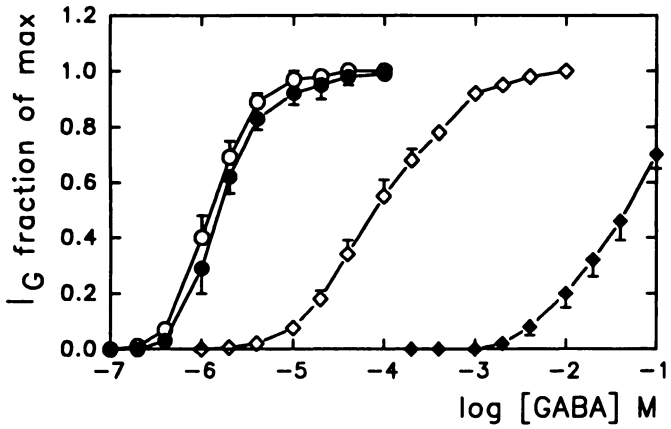

Fig. 6. Concentration-response curves comparing potency of bicuculline as an inhibitor of $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. ○, $I_{G\text{-}BR}$ elicited by GABA under control conditions (in these oocytes $I_{G\text{-}Aret}$ was insignificant at concentrations up to 100 μM); ●, $I_{G\text{-}BR}$ elicited by GABA with 1 mM bicuculline; ◇, $I_{G\text{-}Actx}$ elicited by GABA under control conditions; ◆, $I_{G\text{-}Actx}$ elicited by GABA with 1 mM bicuculline (three determinations for all data points).

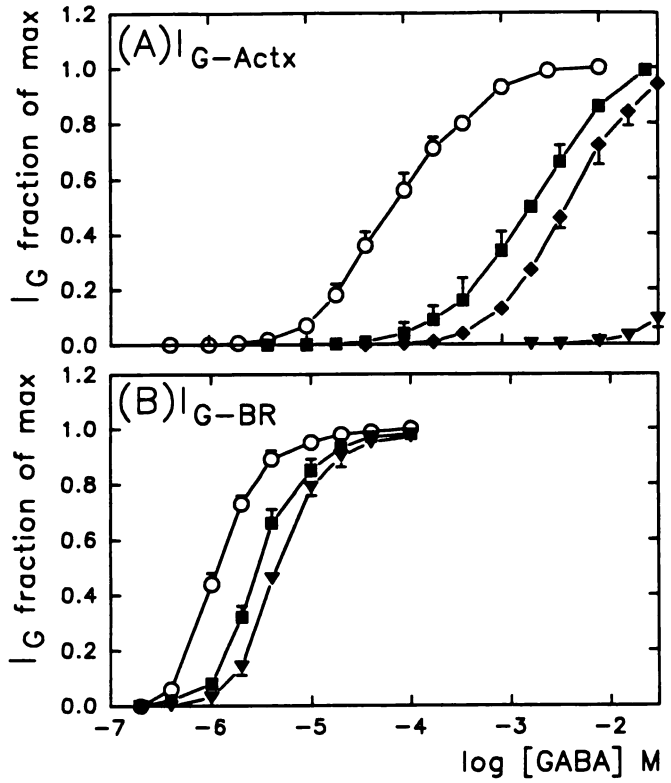

Fig. 7. Concentration-response curves comparing inhibitory effects of strychnine and SR-95531 on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, Inhibition of $I_{G\text{-}Actx}$. ○, GABA; ■, GABA plus 100 μM strychnine; ◆, GABA plus 10 μM SR-95531; ▼, GABA plus 100 μM SR-95531. B, Inhibition of $I_{G\text{-}BR}$. ○, GABA; ■, GABA plus 100 μM strychnine; ▼, GABA plus 100 μM SR-95531 (three determinations for all data points). GABA was applied together with equimolar bicuculline to abolish $I_{G\text{-}Aret}$.

(five experiments). Again, inhibitory effects of SR-95531 on $I_{G\text{-}BR}$ were fully reversible after 1–2-min intervals of wash. At 10 μM, SR-95531 had little effect on curves for $I_{G\text{-}BR}$ (data not shown), but 100 μM SR-95531 did cause a clear parallel rightward shift, increasing the EC_{50} from 1.1 ± 0.1 to 4.3 ± 0.4 μM, indicating a K_b of 35 ± 5 μM (Fig. 7B).

$GABA_B$ Receptor Agonists

Previous characterization of $I_{G\text{-}BR}$ showed that (±)-baclofen (1–100 μM) had no clear effects on the $GABA_\rho$-like receptors expressed by bovine retina RNA (6). In the present study we first checked whether individual enantiomers of baclofen showed any activity, to exclude the possibility of mutually antagonistic effects (22), and then assayed the phosphonic (3-APA), phosphinic (3-APPA), and methylphosphinic acid (3-APMPA) analogues of GABA (Fig. 2).

***(R)*-(+)- and *(S)*-(−)-Baclofen.** Electrical recordings from oocytes showing strong (0.5–3 μA) expression of rat cortex $GABA_A$ receptors indicated that neither (*R*)-(+)- nor (*S*)-(−)-baclofen (1–500 μM) caused any appreciable activation of $I_{G\text{-}Actx}$ or activation of membrane currents mediated by exogenous $GABA_B$ receptors (see Ref. 20). At concentrations up to 100 μM, both enantiomers had only marginal (<10%) inhibitory effects on $I_{G\text{-}Actx}$ elicited by 10 μM GABA. Assays with oocytes expressing retina RNA indicated that (*R*)-(+)- and (*S*)-(−)-baclofen were similarly inactive at $GABA_\rho$-like receptors (data not shown).

3-APA. The phosphonic acid analogue of GABA had no clear action as an agonist at $GABA_A$ receptors expressed by cortex RNA (Fig. 8A). At concentrations between 10 and 1000 μM, 3-APA did not elicit any appreciable membrane current responses, had little or no inhibitory effect on $I_{G\text{-}Actx}$ elicited by

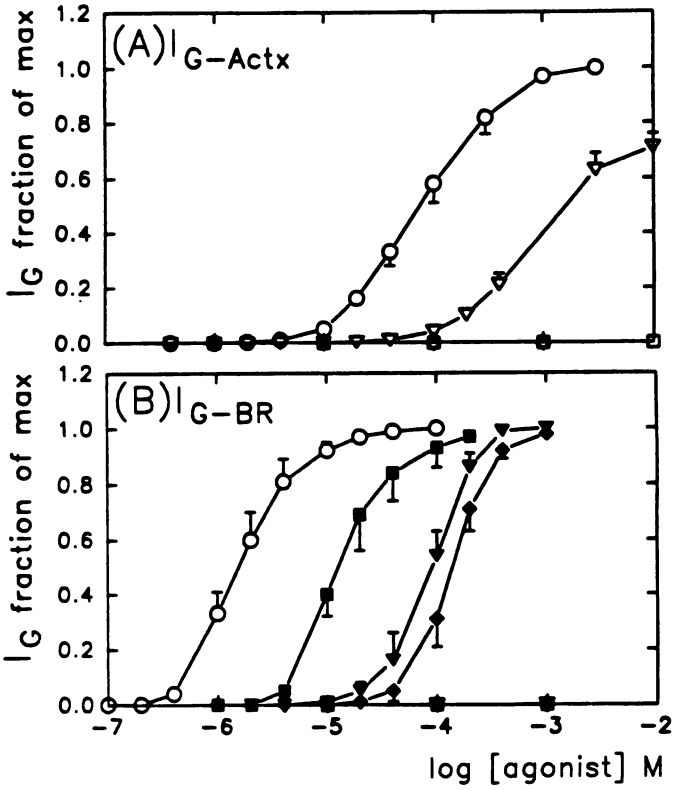

Fig. 8. Concentration-response curves comparing potencies of phosphonic and phosphinic acid analogues of GABA. A, Effects on $I_{G\text{-}Actx}$. ○, Composite control curve (five experiments); □, 3-APA (two experiments); ▽, 3-APPA (three experiments); ◇, 3-APMPA (two experiments). B, Effects on $I_{G\text{-}BR}$. ○, Composite control curve (13 experiments); ■, GABA plus 100 μM 3-APA (five experiments); ▼, GABA plus 100 μM 3-APPA (five experiments); ◆, GABA plus 100 μM 3-APMPA (three experiments). *Open symbols*, inactivity of all three drugs in eliciting $I_{G\text{-}BR}$. For measurements of $I_{G\text{-}BR}$ drugs were applied together with equimolar bicuculline methobromide.

50 μM GABA, and did not cause any significant shifts in concentration-response curves or reductions in maximum GABA responses (data not shown).

3-APA likewise showed little or no activity as an agonist at the $GABA_\rho$-like receptors (Fig. 8B). On the contrary, like the $GABA_A$ receptor agonists THIP, P-4-S, 3-APS, and ZAPA, 3-APA had clear inhibitory effects on $I_{G\text{-}BR}$. The IC_{50} determined under standard conditions was 7.1 ± 1.4 μM (five experiments). Inhibition was rapidly removed upon wash, even when millimolar concentrations of 3-APA were used, and control levels were re-established in <2 min. Analysis of effects on concentration-response curves showed that inhibition of $I_{G\text{-}BR}$ by 3-APA involved roughly parallel rightward shifts with no clear reduction in maximum response (Fig. 8B). At 100 μM, 3-APA increased the EC_{50} for $I_{G\text{-}BR}$ >7-fold, from 1.3 ± 0.4 μM to 9.8 ± 1 μM (four experiments). Dose ratios were calculated from increases in EC_{50} induced by 10, 100, and 1000 μM 3-APA (Fig. 9A). Schild regression of these values had a slope of 0.98 ± 0.11, consistent with simple competitive inhibition, and gave a K_b of 10.7 ± 2 μM (four experiments) (Fig. 9B).

3-APPA. In contrast to 3-APA, the phosphinic acid analogue of GABA acted as a weak agonist at $GABA_A$ receptors expressed by cortex RNA. The EC_{50} for 3-APPA was 938 ± 116 μM (three experiments), approximately 10-fold higher than that for GABA in the same oocytes, and maximum currents were 70–80% of maximum GABA responses (Fig. 8A). Coapplication of 100 μM 3-APPA with 10 or 50 μM GABA or of 10 mM 3-APPA with 1 mM GABA revealed no clear inhibitory effects on GABA-activated currents.

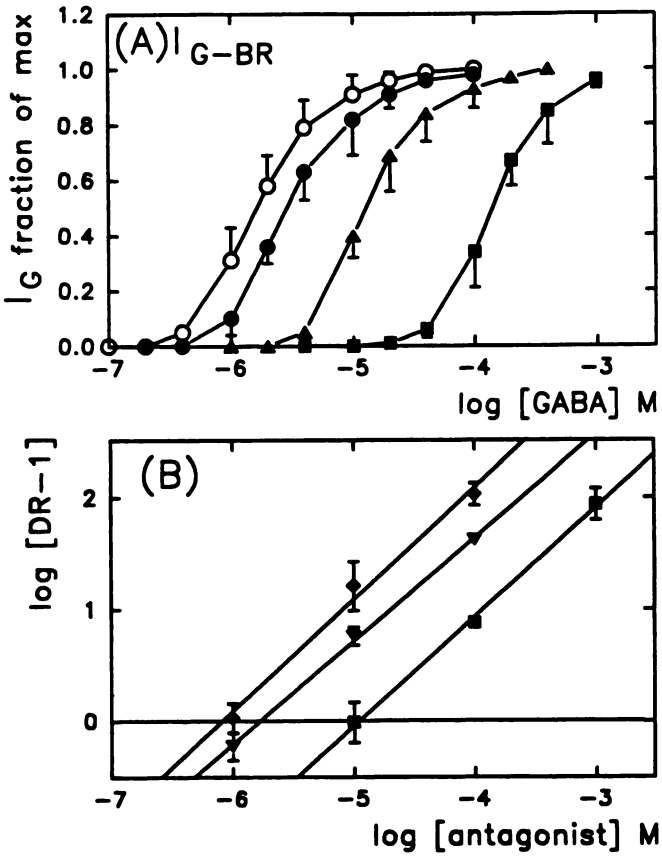

Fig. 9. A, Concentration-responses curves for $I_{G\text{-}BR}$, illustrating parallel rightward shifts induced by three concentrations of 3-APA. Dose ratios (*DR*) for Schild analysis were calculated from increases in EC_{50} induced by the different concentrations of antagonist. ○, GABA control; ●, GABA plus 10 μM 3-APA; ▲, GABA plus 100 μM 3-APA; ■, GABA plus 1 mM 3-APA (four separate curves for the control and each concentration of 3-APA). B, Schild regression for inhibition of $I_{G\text{-}BR}$ by phosphonic and phosphinic analogues of GABA. ■, 3-APA (four experiments); ▼, 3-APPA (four experiments); ♦, 3-APMPA (three experiments). Data points are mean ± standard deviation.

At concentrations of >200 μM, 3-APPA also activated small inward currents in oocytes expressing retina RNA. These responses were abolished by bicuculline, suggesting weak selective activation of $I_{G\text{-}Aret}$ (data not shown). Coapplication of 0.1–1 mM 3-APPA with equimolar bicuculline did not elicit $I_{G\text{-}BR}$ responses, indicating that 3-APPA had little or no activity as an agonist at the ρ-like receptors. 3-APPA did, however, have relatively potent inhibitory effects on currents elicited by GABA. In this case, the IC_{50} measured under standard conditions was 1 ± 0.1 μM (three experiments). As described for 3-APA, washout of inhibition was rapid, and 3-APPA did not suppress $I_{G\text{-}Aret}$. Inhibition of $I_{G\text{-}BR}$ by 3-APPA was also characterized by parallel rightward shifts in the concentration-response curves, with 100 μM 3-APPA increasing EC_{50} values >50-fold, from 1.6 ± 0.5 μM to 95 ± 21 μM (four experiments) (Fig. 8B). Dose ratios were calculated from shifts in EC_{50} induced by 1, 10, and 100 μM 3-APPA (four complete curves for each concentration of antagonist). The Schild regression had a slope of 0.97 ± 0.05 and gave a K_b of 1.7 ± 0.5 μM (four experiments) (Fig. 9B).

3-APMPA. Like 3-APA, the methyl analogue of 3-APPA had no clear activity at $GABA_A$ receptors expressed by rat cortex RNA (Fig. 8A). With oocytes that responded to 1 μM GABA, 0.01–1 mM 3-APMPA elicited only marginal (1–2 nA) inward currents, which were too small for further characterization. 3-APMPA also had no appreciable inhibitory effects on $I_{G\text{-}Actx}$ elicited by 10 or 50 μM GABA and did not cause reductions in maximum response.

3-APMPA had no detectable activity as an agonist at the $GABA_\rho$-like receptors but, like 3-APPA, was a comparatively potent inhibitor of GABA-activated currents. The IC_{50} for 3-APMPA was 0.53 ± 0.06 μM (three experiments), inhibition also washed out rapidly, and 3-APMPA had no clear effects on $I_{G\text{-}Aret}$. Concentration-response curves for $I_{G\text{-}BR}$ were shifted rightward by 3-APMPA, again without appreciable changes in slope or maximum response (Fig. 8B). The EC_{50} was increased >100-fold by 100 μM 3-APMPA, from 1.2 ± 0.2 μM to 137 ± 26 μM (three experiments). Dose ratios were calculated from increases in EC_{50} induced by 1, 10, and 100 μM 3-APMPA (three complete curves for each concentration of antagonist), and in this case the Schild regression had a slope of 1.0 ± 0.06 and gave a K_b of 0.8 ± 0.2 μM (three experiments) (Fig. 9B).

$GABA_B$ Receptor Antagonists

Previous characterization of $I_{G\text{-}BR}$ had indicated that the $GABA_\rho$-like receptors expressed by retina RNA were not appreciably inhibited by 10–100 μM 2-hydroxysaclofen (6). In the present study we assayed effects of another five $GABA_B$ receptors antagonists, i.e., phaclofen and saclofen (analogues of baclofen), CGP 35348 (an analogue of 3-APPA), DAVA (the five-carbon homologue of GABA), and 4-ABPA (the phosphonic acid analogue of DAVA) (Fig. 2).

Phaclofen and saclofen. The phosphonic and sulfonic acid derivatives of baclofen were both essentially inactive as either antagonists or agonists at $GABA_A$ receptors expressed by rat cortex RNA. At concentrations ranging between 1 and 200 μM,

phaclofen and saclofen had only marginal (<10%) inhibitory effects on $I_{G\text{-}Actx}$ elicited by 10 μM GABA and they elicited no clear membrane current responses when applied alone.

Saclofen and phaclofen were similarly inactive as antagonists or agonists at the ρ-like receptors. At 100 μM, both drugs caused <10% suppression of $I_{G\text{-}BR}$ elicited by 1 μM GABA, and neither activated appreciable membrane currents when applied alone (data not shown).

CGP 35348. Like 3-APMPA, and in contrast to 3-APPA, CGP 35348 was essentially inactive on rat cortex $GABA_A$ receptors expressed in oocytes. In oocytes where threshold $I_{G\text{-}Actx}$ was activated by 0.4 μM GABA, 1–1000 μM CGP 35348 activated no detectable membrane current response and $I_{G\text{-}Actx}$ elicited by 10 μM GABA was inhibited <10% by 1 mM CGP 35348.

$GABA_\rho$-like receptors were also largely insensitive to CGP 35348. $I_{G\text{-}BR}$ elicited by 1 μM GABA was unaffected by 100 μM CGP 35348 and was reduced only 10–15% when the compound was applied at 1 mM (data not shown). At concentrations up to 1 mM, CGP 35348 did not itself activate membrane currents in oocytes injected with retina RNA.

DAVA. DAVA was a weak agonist at $GABA_A$ receptors expressed by rat cortex RNA. The EC_{50} was 1154 ± 337 μM (three experiments) (Fig. 10A), and maximum DAVA responses were only 35 ± 8% of currents elicited by GABA. At 100 μM DAVA did not inhibit $I_{G\text{-}Actx}$ elicited by 50 μM GABA, and 10 mM DAVA (simultaneous coapplication) did not appreciably reduce maximum GABA responses.

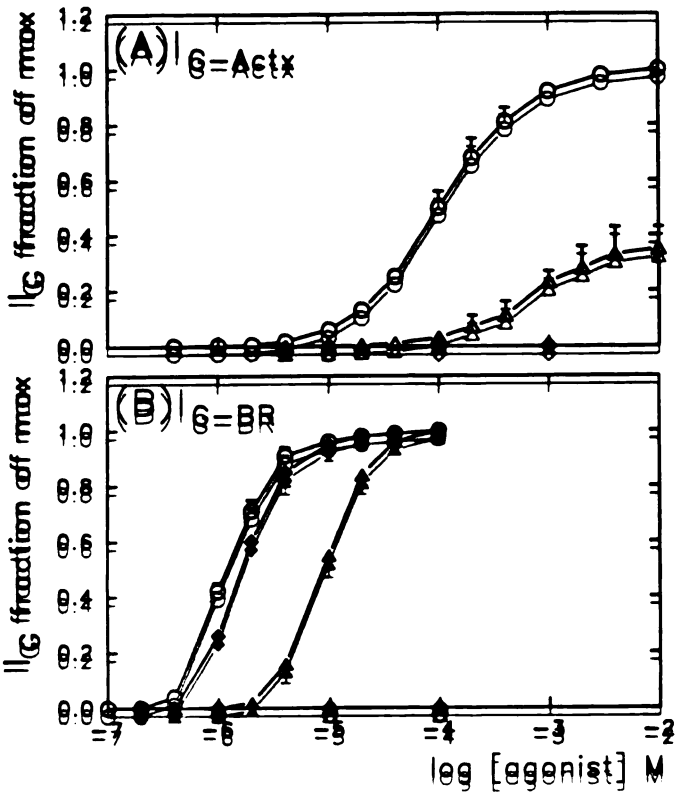

Fig. 10. Concentration-response curves comparing effects of DAVA and 4-ABPA. A, Effects on $I_{G\text{-}Actx}$. ○, GABA control (four experiments); △, DAVA (three experiments); ◇, 4-ABPA (two experiments). B, Effects on $I_{G\text{-}BR}$. ○, GABA control (five experiments); ▲, GABA plus 100 μM DAVA (three experiments); ◆, GABA plus 100 μM 4-ABPA (three experiments). Open symbols, inactivity of the two drugs in eliciting $I_{G\text{-}BR}$.

DAVA was essentially inactive as an agonist at the $GABA_\rho$-like receptors but, like 3-APA, did have clear inhibitory effects on currents elicited by GABA. The IC_{50} measured under standard conditions was 12 ± 2.8 μM (five experiments). The $I_{G\text{-}BR}$ concentration-response curve was again shifted rightward in a parallel manner, with 100 μM DAVA increasing EC_{50} values from 1.5 ± 0.2 to 9 ± 0.7 μM (Fig. 10B). Assuming simple competitive inhibition, this shift indicated a K_b for DAVA of 20.5 ± 3 μM (three experiments).

4-ABPA. The phosphonic acid analogue of DAVA, 4-ABPA (0.01–1 mM), had no detectable activity as an agonist at $GABA_A$ receptors expressed by rat cortex RNA and no clear inhibitory effects on $I_{G\text{-}Actx}$ elicited by 10 μM GABA.

At concentrations up to 1 mM, 4-ABPA also failed to activate the ρ-like receptors but did have appreciable, albeit weak, inhibitory effects on currents elicited by GABA. The IC_{50} appeared to be about 200 μM. Assay of effects on concentration-response curves showed that 100 μM 4-ABPA caused only marginal (≤20%) increases in EC_{50} (Fig. 10B), but by using 1 mM 4-ABPA it was clear that curves were shifted rightward in a parallel manner (data not shown). Again assuming simple competitive inhibition, the shifts indicated that 4-ABPA had a K_b of 625 ± 93 μM (three experiments).

Related Compounds that Are Not $GABA_B$ Receptor Ligands

In the context of the latter two compounds, we also tested activities of the three-carbon homologue of GABA (β-alanine), its phosphonic acid analogue (3-AEPA), and the neurotransmitter glycine; none of these can be considered ligands for $GABA_B$ receptors. β-Alanine was at least 1000 times less active than GABA as an agonist at the ρ-like receptors and at concentrations up to 1 mM had only marginal (≤15%) inhibitory effects on GABA-activated currents. 3-AEPA and glycine were similarly inactive.

Discussion

The overall purpose of this study was threefold, (i) to begin the process of identifying potential "lead structures" for drugs that interact selectively with the $GABA_\rho$-like receptors, (ii) to investigate how commonly used $GABA_A$ and $GABA_B$ receptor ligands might be expected to interact with these types of receptors *in situ*, and (iii) to provide some pharmacological background for future structure-function studies using mutated or chimeric receptors. Results are summarized in Table 1. Experiments on rat brain $GABA_A$ receptors were included simply as controls to allow direct comparison of drug potencies at receptors expressed in the same assay system; these experiments warrant only brief discussion. Effects of all of the various drugs on $I_{G\text{-}Aret}$ were at least qualitatively similar to effects on rat brain $GABA_A$ receptors and are not discussed further.

$GABA_A$ Receptor Ligands

Actions of heterocyclic GABA analogues at rat brain $GABA_A$ receptors. The discovery that muscimol was a potent agonist at mammalian $GABA_A$ receptors prompted development of a series of semi-rigid GABA analogues, wherein the conformational mobility of "GABA structure elements" were restricted by using heterocyclic rings. Binding studies and functional assays identified THIP, isoguvacine, isonipecotic acid, and P-4-S as $GABA_A$ receptor agonists (see Refs. 23–25 for reviews).

TABLE 1
Summary of results
All EC_{50} and K_b values are given in μM.

Drug	$GABA_A$ receptors	$GABA_\rho$-like receptors
GABA	Agonist (EC_{50} = 107)	Agonist (EC_{50} = 1.3)
$GABA_A$ agonists		
Muscimol	Agonist (EC_{50} = 27)	Partial agonist (EC_{50} = 2.3)
THIP	Agonist (EC_{50} = 831)	Antagonist (K_b = 32)
Isoguvacine	Agonist (EC_{50} = 305)	Partial agonist (EC_{50} = 99)
Isonipecotic acid	Agonist (EC_{50} = 607)	Antagonist (K_b = >100)
P-4-S	Partial agonist (EC_{50} = 104)	Antagonist (K_b = 81)
3-APS	Partial agonist (EC_{50} = 131)	Antagonist (K_b = 19)
TACA	Agonist (EC_{50} = 133)	Agonist (EC_{50} = 0.6)
CACA	Weak agonist (EC_{50} = >5000)	Agonist (EC_{50} = 74)
ZAPA	Agonist (EC_{50} = 44)	Antagonist (K_b = 19)
$GABA_A$ antagonists		
Bicuculline	Antagonist (K_b = 1.1)	Weak antagonist (K_b = 6700)
Strychnine	Antagonist (K_b = 4.1)	Antagonist (K_b = 69)
SR-95531	Antagonist (K_b = 0.14)	Antagonist (K_b = 35)
$GABA_B$ agonists		
Baclofen	Inactive (HTC, 500)*	Inactive (HTC, 500)
3-APA	Inactive (HTC, 1000)	Antagonist (K_b = 10)
3-APPA	Agonist (EC_{50} = 938)	Antagonist (K_b = 1.7)
3-APMPA	Inactive (HTC, 100)	Antagonist (K_b = 0.8)
$GABA_B$ antagonists		
Phaclofen	Inactive (HTC, 200)	Inactive (HTC, 200)
Saclofen	Inactive (HTC, 200)	Inactive (HTC, 200)
CGP-35348	Inactive (HTC, 1000)	Inactive (HTC, 1000)
DAVA	Agonist (EC_{50} = 1154)	Antagonist (K_b = 20)
4-ABPA	Inactive (HTC, 1000)	Antagonist (K_b = 625)

* HTC, highest tested concentration.

All of the heterocyclic GABA analogues assayed in this study clearly acted as agonists at rat cerebral cortex $GABA_A$ receptors expressed in oocytes, albeit with widely varying potencies. The rank order of potency was muscimol > GABA ≃ P-4-S > isoguvacine > isonipecotic acid > THIP (Table 1). This potency sequence is comparable, but not identical, to sequences measured by electrical recordings in rat hippocampal slices (26) and in assays of GABA-activated $^{36}Cl^-$ uptake using membrane vesicles prepared from mouse or rat brain (27, 28). The sequence also corresponds well to relative mean channel open times activated by the different agonists, measured on $GABA_A$ receptors in cultured mouse spinal cord neurons (29). However, for reasons that remain unclear, the absolute EC_{50} values determined in oocytes are 5–20 times higher than values reported in previous functional assays (26–28). This discrepancy is possibly the result of abnormal subunit composition of the receptors, expression in a foreign environment, or, at least partially, to differences in assay procedures. For example, in assays on population spikes (26) complete inhibition of activity is probably achieved at relatively low levels of $GABA_A$ receptor activation, which would tend to overestimate potency.

Aside from muscimol, heterocyclic GABA analogues activated maximum responses that were appreciably smaller than currents elicited by GABA. Ranking the agonists in terms of efficacy gave the sequence muscimol (1.0) > isoguvacine (0.75) > isonipecotic acid (0.40) > THIP (0.31) > P-4-S (0.25), where the approximate fraction of maximum GABA response is given in parentheses. Similar submaximal levels of response activation have been reported previously for P-4-S and THIP (26, 28). Of the heterocyclic analogues, only P-4-S appeared to have clear (60–75%) inhibitory effects on maximum responses elicited by GABA, implying that this compound is a true partial agonist/antagonist at the $GABA_A$ receptors (28). Inhibition of maximum GABA responses by high concentrations of isonipecotic acid and THIP was detectable but was not particularly convincing (<15%). For these agonists, failure to match the maximum currents elicited by GABA is most likely the result of a relatively low probability of channel opening even with high agonist concentrations. Alternatively, it remains possible that the agonists interact only with subpopulations of the GABA-sensitive receptor pool (30).

Actions of heterocyclic GABA analogues at $GABA_\rho$-like receptors. Of this group of compounds, only muscimol and isoguvacine had any appreciable activity as agonists at the ρ-like receptors expressed by retina RNA (Table 1). In terms of structure-activity relationships (Fig. 2), these results indicate that incorporation of the carboxyl group of GABA into a 3-isoxazolol ring (muscimol) or incorporation of the amino group into a piperidine ring (isoguvacine) serves only to reduce agonist potency and efficacy at ρ-like receptors. Recent pharmacological characterization indicates that homomeric human $GABA_{\rho 1}$ receptors are also less sensitive to muscimol than to GABA (13), further implicating these subunits in the bicuculline/baclofen-insensitive responses expressed by retinal poly(A)$^+$ RNA.

The inactivity of isonipecotic acid and P-4-S, compared with isoguvacine, implies that a reduction in the degree of planar structure of the piperidine ring, either alone or in conjunction with substitution of the carboxyl group for nonplanar sulfonic acid, is sufficient to effectively abolish agonist activity. Likewise, incorporating both amino and carboxyl groups into ring structures to generate THIP, a relatively rigid bicylic analogue of GABA, results in a complete loss of channel-gating activity.

Although THIP, isonipecotic acid, and P-4-S are essentially inactive as agonists at the $GABA_\rho$-like receptors, these compounds do act as competitive antagonists. In addition, experi-

ments showing that maximum GABA responses were occluded by coapplication of muscimol or isoguvacine indicate that both compounds are partial agonists/antagonists. The common way of explaining such effects is to assume that agonist binding sites on GABA receptors have different geometries depending on whether the Cl^- channel is in a closed or open configuration. In the case of the $GABA_\rho$-like receptors, heterocyclic GABA analogues such as THIP, isonipecotic acid, and P-4-S are able to interact with agonist binding sites in closed-channel conformations but are sterically hindered from interacting with open-channel conformations. These ligands occupy agonist binding sites but are unable to gate the Cl^- channel, and the result is competitive antagonism. Muscimol and isoguvacine, on the other hand, are able to bind sites in both closed-channel and open-channel conformations, promoting transitions between the two. However, open-channel binding site conformations of the ρ-like receptors appear to be only partially tolerant of the 3-isoxazolol and piperidine ring structures, so these analogues have significantly lower potency than GABA. Following this type of reasoning leads to the idea that the only critical differences between agonist binding sites on $GABA_\rho$-like receptors and $GABA_A$ receptors are restricted to open-channel conformations and that closed-channel sites have similar geometries. If this were actually the case, one would expect K_b values for the ρ-like receptors to correlate with EC_{50} values for the $GABA_A$ receptors. Cursory inspection of Table 1 shows that no such relationship exists. On the contrary, our results clearly suggest that both open-channel and closed-channel binding sites on the ρ-like receptors are quite distinct from corresponding sites on $GABA_A$ receptors.

Structure-activity relationships are notoriously unpredictable; nevertheless, these experiments begin to suggest that incorporating carboxyl or amino groups into heterocyclic ring structures is probably not an appropriate strategy to develop potent or selective agonists for $GABA_\rho$-like receptors. These modifications to the GABA structure element are associated with an increased potential for steric hinderance, a reduction in conformational mobility around amino and carboxyl groups, and dislocations of charge, any or all of which could reduce agonist potencies. It appears that steric constraints for open-channel conformations of the $GABA_\rho$-like binding sites are relatively severe, and it is even possible that ligands require a degree of rotational freedom around the amino and carboxyl groups to promote transitions between open-channel and closed-channel configurations. Heterocyclic analogues of GABA might, however, provide a means of generating competitive antagonists for the $GABA_\rho$-like receptors, and ideas for some potentially selective ligands are discussed below.

3-APS action at rat brain $GABA_A$ receptors. 3-APS, the sulfonic acid analogue of GABA (Fig. 2), has been characterized as a potent selective agonist at mammalian $GABA_A$ receptors (e.g., Ref. 31). In terms of EC_{50} value, 3-APS was a relatively strong agonist at rat brain $GABA_A$ receptors expressed in oocytes (Table 1) but, as with P-4-S, the dominant feature of 3-APS concentration-response curves was the low level of the maximum responses. As reported for $GABA_A$ receptors *in situ* (27, 28), pronounced inhibitory effects on $I_{G\text{-}Actx}$ strongly suggest that 3-APS acts as a partial agonist/antagonist at rat brain $GABA_A$ receptors expressed in oocytes.

3-APS action at $GABA_\rho$-like receptors. 3-APS had almost no activity as an agonist at $GABA_\rho$-like receptors but instead acted as a moderately potent competitive antagonist (Table 1). In particular, 3-APS was roughly 4 times more active than P-4-S, suggesting that the piperidine ring structure of P-4-S serves only to reduce the potency of antagonism. As described for THIP, isonipecotic acid, and P-4-S, these results suggest that 3-APS is able to bind to closed-channel conformations of the ρ-like receptors but is unable to promote channel gating. Open-channel conformations of the ρ-like receptor binding sites, therefore, appear to be sterically intolerant of substitution of the carboxyl group of GABA for a nonplanar sulfonic acid, whereas this substitution is at least partially tolerated by $GABA_A$ receptors. Again, it appears that steric constraints on open-channel binding site conformations of the $GABA_\rho$-like receptors are more stringent than those for $GABA_A$ receptors. Drawing parallels with effects of phosphonic and phosphinic acid substitutions (see below), it will be interesting to determine whether the less bulky sulfinic analogue of GABA (31) and related compounds either show markedly increased potency as antagonists or perhaps act as agonists.

TACA and CACA actions at rat brain $GABA_A$ receptors. The *trans*- and *cis*-enantiomers of 4-aminocrotonic acid are conformationally restricted analogues of GABA (Fig. 2) locked in extended and folded conformations, respectively. TACA was initially shown to act as an agonist at $GABA_A$ receptors in cat spinal cord neurons (32) but subsequent studies revealed interactions with $GABA_B$ receptors, implying that the ligand was relatively nonselective (33). CACA was substantially less active as an agonist at $GABA_A$ receptors (32, 34). However, additional binding studies using rat cerebellar membranes indicated that CACA was a relatively potent inhibitor of Ca^{2+}-independent, isoguvacine-independent, [^{3}H]GABA binding (34). Interestingly, this type of GABA binding was only partially inhibited by baclofen, in turn suggesting that CACA might be interacting with a population of GABA binding sites that were insensitive to both bicuculline and baclofen, putative $GABA_C$ receptors (34, 35) (reviewed in Ref. 36). A recent detailed study has shown that these sites might be responsible for up to 60% of specifically bound [^{3}H]GABA in rat cerebellar membranes (37). Assaying TACA and CACA with the $GABA_\rho$-like receptors expressed by retina RNA therefore addressed two interrelated questions, (i) whether GABA interacted with the receptors in extended or folded conformations and (ii) whether these ρ-like receptors corresponded to the putative $GABA_C$ sites described in rat brain.

TACA was almost equipotent with GABA in eliciting $I_{G\text{-}Actx}$ and was roughly 100 times more active than CACA (Table 1). This result serves to confirm that GABA preferentially interacts with rat brain $GABA_A$ receptors in extended, as opposed to folded, conformations.

TACA and CACA actions at $GABA_\rho$-like receptors. TACA was twice as potent as GABA at the ρ-like receptors (Table 1) and, as seen with $GABA_A$ receptors, roughly 100 times more active than CACA. Like muscimol and isoguvacine, CACA appeared to act as a partial agonist/antagonist. Firstly, these experiments indicate that the ρ-like receptors, particularly in open-channel configurations, preferentially interact with GABA in extended conformations. Secondly, the relative activities of TACA and CACA strongly suggest that the $GABA_\rho$-like receptors expressed by retina RNA are distinct from the putative $GABA_C$ receptor sites characterized in rat cerebellum, which were reported to be approximately 10 times more sensi-

tive to CACA than to TACA (35). At present, it is unclear whether the atypical GABA binding sites in cerebellum are associated with functional GABA receptors. Using poly(A)$^+$ RNA from rat cerebellum, expression studies in oocytes reveal strong expression of $GABA_A$ receptors and sporadic expression of $GABA_B$ receptors (10) but as yet no clear bicuculline/baclofen-insensitive GABA responses.[2] Nonetheless, it is perhaps worth mentioning that Northern blot analysis indicates that $GABA_{\rho 1}$ probes hybridize to a 3.9-kilobase mRNA from bovine cerebellum (11).

In terms of structure-activity relationships, the high potency of TACA shows that GABA analogues with partially restricted conformational mobility do not necessarily have reduced activity as agonists at the ρ-like receptors. Indeed, restricting mobility around the α-β carbons of GABA structure elements might be a simple way of increasing the activity of ligands, either as agonists or antagonists. For example, it will be interesting to determine whether the sulfonic, phosphonic, and phosphinic acid analogues of TACA have more potent inhibitory effects than 3-APS, 3-APA, and 3-APPA, respectively.

A word of caution is needed. Freshly made stocks were used for all experiments involving CACA and TACA, and levels of cross-contamination between enantiomers were assumed to be <0.1% (supplier's specification). Extended storage of solutions permits a degree of isomerization and results in levels of cross-contamination possibly as high as 1–2%. Without casting aspersions on the supplier's specification, it should be appreciated that CACA was roughly 100 times less active than TACA both at the $GABA_A$ receptors and at the ρ-like receptors. One percent contamination of CACA by TACA would therefore be sufficient to account for almost all of the activity assigned to CACA in this study.

ZAPA action at rat brain $GABA_A$ receptors. ZAPA is an isothiouronium analogue of GABA that has been shown to act at $GABA_A$ receptors in rat brain (35, 38). Like TACA and CACA, ZAPA is conformationally restricted between the α- and β-carbons, but in this case agonist activity resides in the *cis*-enantiomer.

Our experiments showed that ZAPA was a strong agonist at rat cortex $GABA_A$ receptors expressed in oocytes, having approximately twice the activity of GABA and half the activity of muscimol. Although ZAPA was a potent agonist, maximum responses appeared to be only 65–75% of maximum currents elicited by GABA. Explanations for this reduction in efficacy are similar to those discussed for THIP, isoguvacine, and isonipecotic acid.

ZAPA action at $GABA_\rho$-like receptors. In sharp contrast to actions at $GABA_A$ receptors, ZAPA was essentially inactive as an agonist at the ρ-like receptors, instead acting as a competitive antagonist. As discussed for 3-APS, it appears that the isothiouronium group of ZAPA is tolerated by closed-channel binding site conformations of the ρ-like receptors, resulting in the antagonist effect, but is not tolerated by open-channel binding site conformations. Our results suggest that ZAPA has some potential as a selective $GABA_A$ receptor agonist. Unfortunately, this is clearly compromised by the inhibitory interaction with $GABA_\rho$-like receptors, which would complicate interpretation of *in situ* experiments and probably renders the compound unsuitable for use in binding studies.

[2] R. M. Woodward and R. Miledi, unpublished observations.

Actions of $GABA_A$ receptor antagonists at $GABA_A$ and $GABA_\rho$-like receptors. Characterization of receptors assembled from $GABA_{\rho 1}$ subunits (11, 13) strongly suggests that the ρ-like receptors expressed by retina poly(A)$^+$ RNA are related to $GABA_A$ receptors. Consistent with this, the present study indicates that, at high concentrations, bicuculline has measurable inhibitory effects on $I_{G\text{-}BR}$. This inhibition appears to competitive, implying that suppression of currents is not due to nonspecific "plugging" of the channel. Nevertheless, ranking the three antagonists in terms of selectivity for $GABA_A$ receptors versus the ρ-like receptors gives the sequence bicuculline (6000) > SR-95531 (240) > strychnine (18), where the ratio of K_b values for $I_{G\text{-}BR}$ and $I_{G\text{-}Actr}$ is given in parentheses. Bicuculline clearly has the highest level of selectivity and should, therefore, remain the antagonist of choice for discriminating between the two types of receptors.

The convulsive alkaloid strychnine is most commonly used as a potent antagonist for glycine receptors (e.g., Ref. 39), although it has also been shown to have effects on mammalian and chick $GABA_A$ receptors (1, 40). Interactions with glycine and $GABA_A$ receptors, both ligand-gated Cl^- channels, in turn suggested that strychnine might have significant inhibitory effects on the ρ-like receptors. The present experiments indicate that strychnine is a moderately potent competitive antagonist at rat brain $GABA_A$ receptors expressed in oocytes but is approximately 20 times less active at the ρ-like receptors (Table 1). This result implies that any structural similarities between the ρ-like receptors and glycine receptors (11) clearly do not extend to the property of having high sensitivity to strychnine (41).

The aryl-aminopyridazine derivative of GABA, SR-95531, has been shown to act as a highly potent competitive antagonist of $GABA_A$ receptors in mammalian neurons (e.g., Ref. 42). In confirmation of these results, SR-95531 was the most potent antagonist assayed for rat brain $GABA_A$ receptors expressed in oocytes, being approximately 10 times more active than bicuculline and 30 times more active than strychnine (Table 1). Of the $GABA_A$ receptor antagonists assayed in this study, SR-95531 was also the most potent inhibitor at the ρ-like receptors. The clear inhibitory effects of SR-95531 at the ρ-like receptors are again consistent with apparent similarities to $GABA_A$ receptors at the molecular level (11) and suggest that antagonist binding sites on the two types of receptors are different but not wholly unrelated.

$GABA_B$ Receptor Ligands

Baclofen enantiomers and structurally related antagonists. $GABA_B$ receptors were initially identified and characterized in rat peripheral and central nervous tissues (43, 44). The two most striking features of their pharmacological profile were insensitivity to bicuculline and stereoselective activation by the muscle relaxant/antispasticity drug baclofen (β-4-chlorophenyl-GABA), with activity residing in the (−)-enantiomer (see Ref. 45 for review). [Although a potential source of confusion, we refer to the active enantiomer as (*R*)-(+)-baclofen, because optical rotation of the hydrochloride salt is opposite that of the free base.] The selectivity of baclofen for $GABA_B$ receptors was subsequently exploited in the search for antagonists. First to be developed was phaclofen, the phosphonic analogue, which was selective with respect to $GABA_A$ receptors but was only weakly active (46). This was followed by the sulfonic acid analogues 2-hydroxysaclofen and saclofen, which

showed similar selectivity but had 10–20 times higher potency than phaclofen (47, 48). Phaclofen and 2-hydroxysaclofen were successfully used to show that $GABA_B$ receptors mediated slow inhibitory postsynaptic potentials in many regions of rat brain (e.g., Refs. 49 and 50).

As expected, our experiments showed that baclofen enantiomers and related antagonists had no clear activity at rat cerebral cortex $GABA_A$ receptors expressed in oocytes. $GABA_\rho$-like receptors expressed by retina RNA were similarly insensitive to this group of compounds (Table 1). Firstly, this indicates that the use of racemic baclofen in the initial pharmacological characterization (6) had not masked effects of individual enantiomers (22). Secondly, the consistent inactivity of all of the compounds strongly suggests that the 4-chlorophenyl substituent on the β-carbon effectively prohibits functionally relevant interactions with the ρ-like receptors, in both closed-channel and open-channel configurations. In this context it is worth pointing out that phaclofen is the 4-chlorophenyl analogue of 3-APA and saclofen is the analogue of 3-APS, both of which act as moderately potent antagonists at the ρ-like receptors. Lastly, these results indicate that the selectivity of $GABA_B$ receptor agonists and antagonists with a 4-chlorophenyl substituent on the β-carbon is not compromised by their showing activity at $GABA_\rho$-like receptors. Indeed, preliminary assays show that the novel $GABA_B$ receptor ligand (±)-4-amino-3-(5-chloro-2-thienyl)butanoic acid (51) is also largely inactive at the ρ-like receptors,[2] which begins to suggest that ring structures or other large substituents on the β-carbon might generally result in loss of activity. It should also be noted, however, that β-phenyl-GABA has been shown to act as a displacer of GABA but not baclofen, which suggests that this analogue could be a ligand for some types of atypical GABA receptors (43).

Phosphonic and phosphinic acid analogues of GABA and related compounds. 3-APA, the phosphonic acid analogue of GABA, was first described as a weak central nervous depressant (31), an effect that was later shown to be bicuculline resistant (52). After the discovery of $GABA_B$ receptors, 3-APA was shown to act as a partial agonist/antagonist at peripheral $GABA_B$ receptors (53). The corresponding phosphinic acid analogue, 3-APPA, bears a closer structural resemblance to GABA and was found to be a potent selective agonist for $GABA_B$ receptors in central and peripheral nervous tissues (54, 55). Interestingly, effects of 3-APPA and baclofen do not always appear to correspond (56), adding to a growing body of evidence suggesting that mammalian $GABA_B$ receptors are heterogeneous. The relatively high activity of 3-APPA prompted development of various analogues, in particular 3-APMPA, which is a highly potent agonist at $GABA_B$ receptors in rat brain (57), and CGP 35348, which acts as an antagonist (57, 58).

Our experiments showed that 3-APA (≤1 mM) had no detectable interaction with rat cerebral cortex $GABA_A$ receptors expressed in oocytes. In terms of structure-activity relationships, substituting the carboxylate function of GABA for the bulkier nonplanar phosphonic acid group appears to result in complete loss of activity.

Corresponding assays of $GABA_\rho$-like receptors showed that 3-APA was likewise inactive as an agonist but did have clear activity as a competitive antagonist (Table 1). As described for 3-APS, the inhibitory effects suggest that 3-APA is able to interact with closed-channel binding site conformations of the ρ-like receptors, whereas the phosphonic acid group is not tolerated by open-channel conformations and 3-APA is thus unable to gate the Cl^- channel.

Somewhat to our surprise, 3-APPA showed appreciable activity as an agonist at the rat brain $GABA_A$ receptors expressed in oocytes (Table 1). It appears that the phosphinic acid group bears sufficient resemblance to the carboxyl group of GABA to permit interactions at agonist binding sites on $GABA_A$ receptors and, furthermore, gating of Cl^- channels. In contrast, the methyl and diethoxymethyl analogues (3-APMPA and CGP 35348, respectively) showed no clear activity at the $GABA_A$ receptors, either as agonists or antagonists, suggesting that attachment of bulkier substituents to the phosphinic acid function effectively prohibits such interactions.

Neither 3-APPA, 3-APMPA, nor CGP 35348 showed any activity as an agonist at the $GABA_\rho$-like receptors, but 3-APPA and 3-APMPA both acted as comparatively strong antagonists (Table 1). As described for phosphonic and sulfonic acid substituents, it appears that the phosphinic acid group is conducive to relatively strong interactions with closed-channel binding site conformations of the $GABA_\rho$-like receptors but fails to promote channel gating. The high activity of 3-APMPA implies that closed-channel binding sites on the ρ-like receptors might have a slightly greater tolerance for substitutions on the phosphorous atom than do corresponding sites on $GABA_A$ receptors, where the analogue was inactive. However, the inactivity of CGP 35348 at the ρ-like receptors clearly suggests that there are strict limitations to the size and type of such substituents, and it will be interesting to determine whether analogues incorporating short aliphatic chains or ring structures are antagonists (e.g., Ref. 57). These results also suggest that it will be appropriate to exercise caution when using phosphinic acid analogues of GABA to probe $GABA_B$ receptor function in retinal tissue.

The relatively high potency of 3-APMPA at the $GABA_\rho$-like receptors and the apparent inactivity at $GABA_A$ receptors suggest that this type of carboxyl substituent could be used to develop selective antagonists, that is, if appropriate structural modifications are made to abolish activity at $GABA_B$ receptors. Our studies with $GABA_A$ receptor ligands suggest that possible approaches would be either to incorporate the amino group into a heterocyclic ring or to make an appropriate amino group substitution. For example, the unsaturated or saturated piperidine rings of isoguvacine, isonipecotic acid, and P-4-S seem to prohibit interactions with $GABA_B$ receptors (44, 59). In contrast, closed-channel binding sites on the ρ-like receptors appear to tolerate this type of structural modification, albeit with some reduction in affinity with respect to GABA. Hence, a compound such as piperidine-4-(methyl)phosphinic acid, or probably better still unsaturated analogues of this molecule, might be expected to retain activity as competitive antagonists at the ρ-like receptors and also exhibit selectivity with respect to both $GABA_A$ and $GABA_B$ receptors. The same type of reasoning suggests that the methylphosphinic acid analogue of ZAPA might also be a selective antagonist. In this case, interactions at $GABA_B$ receptors should be prohibited by substitution of the amino group for isothiouronium.

Five-carbon and three-carbon homologues of GABA and their phosphonic acid analogues. DAVA, a weak agonist at $GABA_A$ receptors (59), was found to antagonize $GABA_B$ receptors in rat anococcygeus muscle (60). These effects sug-

gested that the corresponding phosphonic acid analogue (4-ABPA) might be a viable selective antagonist for $GABA_B$ receptors. Although 4-ABPA was largely devoid of actions at $GABA_A$ receptors, inhibitory effects on $GABA_B$ receptors were found to be weak (61). The 3-carbon GABA homologue, β-alanine, and its phosphonic acid analogue, 2-AEPA, were both found to be essentially inactive at $GABA_B$ receptors.

Our experiments confirmed that DAVA was a weak agonist at $GABA_A$ receptors, whereas 4-ABPA showed no activity (Table 1). DAVA had only inhibitory effects at the $GABA_\rho$-like receptors and, in this case, the phosphonic acid substitution resulted in a pronounced loss of activity as an antagonist. β-Alanine, 2-AEPA, and glycine were largely or wholly inactive, all of which serves to confirm that activity of ligands at the ρ-like receptors is critically dependent upon the degree of separation of the charged groups.

Comparisons between $GABA_\rho$-like receptors and other GABA receptors with atypical pharmacology. Extending the pharmacological characterization of the ρ-like receptors expressed by mammalian retina RNA permits comparisons with other vertebrate and invertebrate GABA receptors, which, from a pharmacological perspective, are not readily classified as belonging to either the $GABA_A$ or $GABA_B$ receptor families. These include a potentially novel GABA autoreceptor in rat spinal cord (62) and receptors in frog optic tectum (e.g., Ref. 63), frog sciatic nerve (64), and fish brain (65). In invertebrates, atypical GABA receptors have been characterized in muscles of *Ascaris suum*, a parasitic nematode (66), in the heart of the marine arachnid *Limulus polyphemus* (67), and in various insect tissues (e.g., Ref. 68). Putative $GABA_C$ receptor sites in rat cerebellum (34–37) have been discussed above (see TACA and CACA action at $GABA_\rho$-like receptors). Interestingly, although all of the atypical GABA receptors share the property of bicuculline resistance, none appear to have pharmacological profiles that correspond exactly to that of the $GABA_\rho$-like receptors. Our studies therefore give further support to the idea that atypical GABA receptors are heterogeneous (36).

Mutation studies. Recently, Sigel *et al.* (69) showed that point mutations at Phe-64 on α1 subunits can profoundly alter the affinity of $GABA_A$ receptors for agonists and antagonists. In particular, substitution of Phe-64 by leucine causes a 200-fold reduction in sensitivity to bicuculline. As pointed out by these authors, the homologous position in $GABA_{\rho 1}$ subunits is occupied by tyrosine (11). It will therefore be interesting to determine what effects substitution of Phe-64 for tyrosine have on $GABA_A$ receptor function and to what extent changes in a single amino acid can reproduce $GABA_\rho$-like pharmacology.

General considerations. Physiological functions have yet to be determined for $GABA_\rho$ receptors. Our experiments indicate that this type of receptor is distinctly expressed in conjunction with retinal $GABA_A$ receptors, and coexpression studies using cRNAs encoding $GABA_{\rho 1}$ subunits and conventional $GABA_A$ receptor subunits also tend to argue against coassembly (13). Preliminary *in situ* hybridization studies have located mRNA encoding $GABA_{\rho 1}$ subunits in rat retinal tissues (11). Furthermore, bicuculline/baclofen-insensitive GABA responses have recently been detected in fish and rat retinal neurons (15, 79). These experiments clearly indicate that GABA receptors with ρ-like pharmacology are actually expressed in the retina. If this is the case, it is tempting to speculate that intact properties such as high affinity for GABA, steep dependence upon agonist concentration (high levels of cooperativity), and low levels of desensitization would be useful in the fine control of graded potentials that characterize signaling between many retinal neurons. It is also intriguing that GABA release in mammalian retina does not appear to occur wholly via classical Ca^{2+}-dependent discharge of synaptic vesicles (70, 71). This raises the possibility that $GABA_\rho$ receptors are specifically designed in conjunction with a novel transmitter release mechanism.

Summary and Conclusion

Previous pharmacological characterization showed that $GABA_\rho$-like receptors expressed in *Xenopus* oocytes by retina RNA had high affinity for GABA and were inhibited by picrotoxin and γ-hexachlorocyclohexane but were insensitive to bicuculline and baclofen, were not functionally regulated by $GABA_A$ receptor modulators, and were only weakly sensitive to TBPS (6–9, 13). The present study indicates that the $GABA_\rho$-like receptors also have distinct sensitivities to a variety of other GABA receptor ligands. In particular, the $GABA_A$ receptor agonists THIP, isonipecotic acid, P-4-S, 3-APS, and ZAPA all act as competitive antagonists at the ρ-like receptors, showing essentially no activity as agonists. Furthermore, the $GABA_B$ receptor agonists 3-APPA and 3-APMPA are moderately potent antagonists. Our experiments begin to lay a foundation for the more detailed structure-activity studies that will be required to develop drugs that interact selectively with these novel mammalian GABA receptors.

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