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Characterization of Bicuculline/Baclofen-Insensitive γ-Aminobutyric Acid Receptors Expressed in *Xenopus* Oocytes I. Effects of Cl^- Channel Inhibitors

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SUMMARY

Poly(A)$^+$ RNA from bovine retina expressed γ-aminobutyric acid (GABA)-activated membrane current responses in *Xenopus* oocytes, consisting of two pharmacologically distinct components. One component ($I_{G\text{-}Aret}$) was mediated by $GABA_A$ receptors, and the other component ($K_{G\text{-}BR}$) by atypical GABA receptors that were resistant to inhibition by bicuculline and insensitive to activation by baclofen. To further characterize the bicuculline/baclofen-insensitive GABA receptors, electrical recordings were made measuring the sensitivity of $I_{G\text{-}BR}$ to two Cl^- channel inhibitors, *t*-butylbicyclophosphorothionate (TBPS) and picrotoxin. For purposes of comparison, effects of TBPS and picrotoxin were also assayed on currents mediated by $GABA_A$ receptors expressed in oocytes by rat cerebral cortex RNA ($I_{G\text{-}Actx}$). The main finding of this study was that TBPS was a surprisingly weak inhibitor of $I_{G\text{-}BR}$, whereas $I_{G\text{-}Actx}$ was potently suppressed. Assays on maximum responses indicated that $I_{G\text{-}Actx}$ was at least 500 times more sensitive to TBPS than was $I_{G\text{-}BR}$ (IC_{50} values of approximately 0.2 μM and > 50 μM, respectively). Moreover, inhibition of $I_{G\text{-}Actx}$ by micromolar concentrations of TBPS was largely insurmountable, whereas the weak inhibitory effects on $I_{G\text{-}BR}$ showed strong dependence on agonist concentration. For example, 10 μM TBPS reduced maximum $I_{G\text{-}Actx}$ by >90%, an effect that was not significantly reversed by 10-fold increases in the concentration of agonist. In contrast, the same concentration of TBPS caused a 2-fold increase in the EC_{50} for $I_{G\text{-}BR}$ but had only marginal (<5%) inhibitory effects on maximum responses. Picrotoxin inhibited both types of current, but assays on maximum responses indicated that $I_{G\text{-}Actx}$ was approximately 30 times more sensitive than $I_{G\text{-}BR}$ (IC_{50} values of approximately 1 and 30 μM, respectively). Inhibitory effects of picrotoxin on $I_{G\text{-}BR}$ again showed strong dependence on agonist concentration, but in this case there was also a clear insurmountable component. Comparisons between $I_{G\text{-}Actx}$ and $I_{G\text{-}Aret}$ suggested that $GABA_A$ receptors expressed by either brain or retina RNA showed approximately the same sensitivity to TBPS and picrotoxin. Our experiments indicate that the bicuculline/baclofen-insensitive GABA receptors expressed by retina RNA differ markedly from $GABA_A$ receptors in their sensitivity to TBPS and picrotoxin. Defining the structural features responsible for these differences at the molecular level will provide a further means of investigating the complex mechanisms underlying interactions between inhibitors and GABA-activated Cl^- channels.

Poly(A)$^+$ RNA from mammalian brain expresses a diversity of neurotransmitter receptors in *Xenopus* oocytes (see Refs. 1 and 2 for reviews). Fidelity of receptor expression is generally very good and, once expressed in oocytes, receptors are accessible to detailed electrical and pharmacological characterization (e.g., Refs. 3–5). For example, poly(A)+ RNA from mammalian cerebral cortex or chick optic lobe expresses subunits of $GABA_A$ receptors that are assembled to form functional receptor-channel complexes with properties similar to those studied *in situ* (6–11). $GABA_A$ receptors expressed in oocytes are ligand-gated Cl^- channels that show desensitization, are competitively antagonized by Bic, positively modulated by barbiturates, benzodiazepines, and 3α-OH-pregnanolones, negatively modulated by β-carbolines, and inhibited by picrotoxin and the neurosteroid pregnenolone sulfate (8–11). Expression of functional recombinant $GABA_A$ receptors in oocytes has further extended the utility of the system, allowing detailed studies into the effects of subunit composition on $GABA_A$ receptor pharmacology and electrical properties (12, 13).

Unlike cerebral cortex RNA, which predominantly expresses $GABA_A$ receptors, poly(A)$^+$ RNA from mammalian retina con-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; Bac, baclofen; Bic, bicuculline; DMSO, dimethylsulfoxide; γ-HCH, γ-hexachlorocyclohexane (lindane); 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 bicuculline/baclofen-insensitive GABA receptors expressed by retina RNA; TBPS, *t*-butylbicyclophosphorothionate.

sistently expresses GABA responses that appear to be mediated by two pharmacologically distinct types of receptor (14). One component of retina GABA responses is clearly mediated by $GABA_A$ receptors, consisting of a Cl^- current that shows desensitization, sensitivity to Bic, and modulation by barbiturates, benzodiazepines, and steroids. The other component is also a Cl^- current, but this response shows little desensitization, is resistant to Bic, and is largely or wholly insensitive to $GABA_A$ receptor modulators (11, 14). The Bic-resistant GABA response is neither activated by Bac enantiomers nor blocked by 2-hydroxysaclofen, indicating that the current is not due to activation of $GABA_B$ receptors (14–16). Taken together, these results suggest that bovine retina RNA expresses novel mammalian GABA receptors with qualitatively distinct pharmacology. Separate experiments have shown that poly(A)$^+$ RNA from cow, pig, and rabbit retina all express GABA receptors with these properties, whereas RNA from chick and frog retina predominantly, or exclusively, express receptors with $GABA_A$-type pharmacology (17).[2]

In terms of electrical properties, the Bic/Bac-insensitive GABA receptors expressed by mammalian retina RNA appear to be related to the $GABA_A$ family of receptors. Both types of receptor gate membrane currents that are carried substantially by Cl^- and show sensitivity to picrotoxin and γ-HCH (14, 18). Interestingly, the cDNA encoding a human GABA receptor that is highly enriched in retina has recently been cloned (19). Sequence data show that this receptor, denoted $GABA_{\rho 1}$, is related to $GABA_A$ receptors, and expression studies in oocytes show that homooligomeric receptors form ligand-gated Cl^- channels that are strongly blocked by picrotoxin. Furthermore, initial pharmacological characterization indicates that $GABA_{\rho 1}$ receptors are resistant to Bic and are insensitive to $GABA_A$ receptor modulators (20). Detailed pharmacology, combined with further studies at the molecular level, will be required to determine precisely what roles $GABA_{\rho 1}$ subunits play in the Bic-resistant GABA responses expressed by native retina poly(A)$^+$ RNA.

GABA receptors that are insensitive to both Bic and Bac have not, thus far, been clearly identified in retinal tissues (see Ref. 21 for a review). Developing a broad pharmacological profile for the Bic/Bac-insensitive GABA receptors expressed by native poly(A)$^+$ RNA is therefore important, (i) to develop drugs that can selectively probe receptor function *in situ*, (ii) for comparative studies with cloned receptors, to begin to investigate the subunit composition of Bic/Bac-insensitive receptors expressed by poly(A)$^+$ RNA, and (iii) to provide the pharmacological background for investigating which structural features, at the molecular level, are responsible for the functional disparities with more "conventional" $GABA_A$ receptors.

In the present study, we have extended that pharmacological characterization of the Bic/Bac-insensitive GABA receptors expressed by bovine retina RNA through assaying effects of two Cl^- channel inhibitors, TBPS and picrotoxin. A previous study (22) had characterized the inhibitory effects of TBPS on chick $GABA_A$ receptors expressed in oocytes, but corresponding studies on mammalian $GABA_A$ receptors were not sufficiently detailed (7, 9). Sensitivity of the Bic/Bac-insensitive GABA receptors to TBPS and picrotoxin was, therefore, compared with the effects on $GABA_A$ receptors expressed by rat cerebral cortex RNA.

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

Materials and Methods

RNA extraction, size fractionation, and expression in oocytes. Preparation of poly(A)$^+$ RNAs and size fractionation of retina RNA were as described previously (e.g., Refs. 11, 14, 23, 24). Very briefly, RNA was extracted from rat cerebral cortex and bovine retina using the phenol-chloroform procedure (11). Retina poly(A)$^+$ RNA was size fractionated by centrifugation on 10–30% sucrose density columns, and fractions enriched in RNAs encoding GABA receptors were determined by assaying levels of expression in oocytes (14, 23, 24). In this study, four separate RNA preparations were made from bovine retina and two from rat cerebral cortex. All retina preparations were size fractionated to amplify expression of GABA responses, which was usually necessary for detailed pharmacological characterization. Preparatory experiments indicated that inhibitory effects of TBPS and picrotoxin on GABA responses expressed by fractionated and unfractionated RNA were similar. Follicle-enclosed *Xenopus* oocytes, at stages V and VI of development (25), 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, 40–100 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 (26).

Electrophysiology and notation of membrane current responses. Electrical recordings were made using a two electrode voltage-clamp 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. Using oocytes taken from the same frog, comparisons were made between the effects of TBPS and picrotoxin on $I_{G\text{-}Actx}$, $I_{G\text{-}Aret}$, and $I_{G\text{-}BR}$. As before (11, 14, 18), detailed 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 $I_{G\text{-}Aret}$ (14).

Measurement of concentration-response relationships for $I_{G\text{-}Actx}$. Concentration-response curves for $I_{G\text{-}Actx}$ were constructed in single oocytes, first under control conditions and then with one to three increasing concentrations of inhibitor. The possibility of underestimating potency through failing to account for use-dependent components of the inhibition (see text) was minimized (i) by using 1–1.5-min GABA exposures to allow use-dependent effects to equilibrate, and (ii) by maintaining continuous application of inhibitor throughout measurements, including intervals between GABA responses. Maintained exposure to the inhibitor minimized wash-out and allowed use-dependent effects to accumulate progressively and equilibrate throughout the course of experiments. For example, when concentration-response curves were measured with 0.1, 1.0, and 10 μM picrotoxin, responses elicited by repeated applications of 0.01, 0.1, or 3 mM GABA were approximately the same size, confirming that levels of inhibition had fully equilibrated.

Measurement of concentration-response relationships for $I_{G\text{-}BR}$. As described previously (14), $I_{G\text{-}BR}$ appeared to show little desensitization, even upon prolonged exposure to high concentrations of GABA. When measuring $I_{G\text{-}BR}$ in isolation from $I_{G\text{-}Aret}$, it was, therefore, unnecessary to separate repeatedly the individual GABA applications by time-consuming intervals of wash. Concentration-response curves were, thus, constructed using a "stepped-ramp" approach (Fig. 1). Curves measured using this procedure gave response thresholds, EC_{50} values, maximum currents, and slope factors that were essentially indistinguishable from those of curves constructed using responses separated by 5–15 min of wash. The comparative lack of desensitization in $I_{G\text{-}BR}$ also meant that minor use-dependent components were detect-

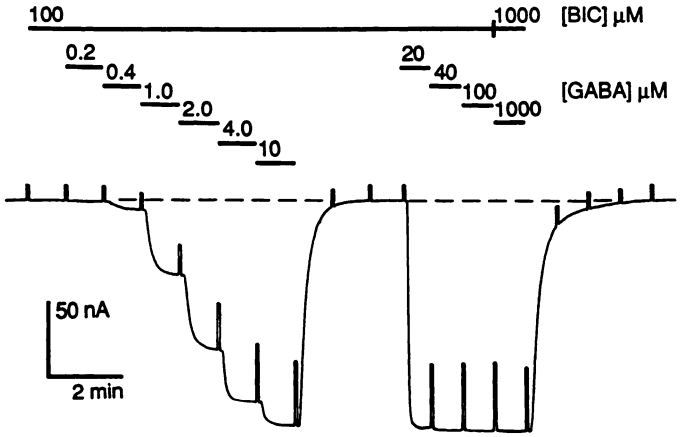

Fig. 1. Example of stepped-ramp GABA application used to determine concentration-response relationships for $I_{G\text{-}BR}$. An oocyte was sequentially exposed to 0.2–10 μM GABA, with no intervening periods of wash. Each concentration of GABA was applied for 1 min, sufficient time to allow equilibration of consecutive membrane current responses. In this case, the oocyte was then washed for 3 min, to re-establish the basal holding current, and subsequently exposed to 20–1000 μM GABA, concentrations that elicited currents only marginally larger than those elicited by 10 μM GABA. Solutions of 0.2–100 μM GABA all contained 100 μM Bic methobromide, to abolish the Bic-sensitive component (see text). Because Bic acts as a competitive inhibitor of $GABA_A$ receptors, 1 mM GABA required 1 mM Bic to ensure abolition of response. In this and following records, oocytes were voltage clamped at −70 mV, with the holding potential stepped at 1-min 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, partially picked up by the chart recorder upon steps in potential, have been deleted during preparation of figures. Inward membrane currents are denoted by downward deflection. Unless otherwise stated, similar recording conditions were used in all following figures.

able as slight reductions in current over the time course of a GABA application, superficially resembling slow desensitization. Membrane currents were, therefore, measured at the end of 1–1.5-min GABA applications, time enough to allow use-dependent components to equilibrate fully. The accuracy of these procedures in accounting for use-dependent effects was confirmed by checking the reproducibility of selected GABA responses after measurement of the full concentration-response curve, maintaining incubation with inhibitor throughout. The possibility that Bic (0.1–1 mM) significantly modified actions of the different Cl^- channel inhibitors on $I_{G\text{-}BR}$ was obviated by separate experiments assaying effects in the presence and absence of the antagonist; these showed no clear differences.

Data analysis. Concentration-response curves were analyzed as described previously (11, 18); 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 (27). IC_{50} values were determined from inhibitory effects on maximum GABA responses, i.e., for $I_{G\text{-}Actx}$, currents elicited by 3 mM GABA and, for $I_{G\text{-}BR}$, currents elicited by 100 μM GABA with 100 μM Bic methobromide. Each value was determined by regression from three or four independent curves constructed over a broad range of inhibitor concentrations (four to six points).

Drugs. Picrotoxin was from Sigma and was made up daily as a 10 mM stock in H_2O or Ringer. TBPS was from Research Biochemicals Inc. (Natick, MA), was made up as 0.1–10 mM stocks in DMSO, and was stored at 4° for up to 1 week or at −20° for up to 2 weeks. In the vast majority of experiments, DMSO was diluted to ≤0.1% (v/v) in Ringer solutions, concentrations at which there were no appreciable effects on GABA responses. Occasionally, DMSO was diluted to concentrations ranging between 0.1 and 0.5% (v/v) and in these experiments might have caused up to 5% increases or decreases in currents, but, even these marginal effects were inconsistent.

Results

Use-Dependent Inhibitory Effects TBPS and Picrotoxin

Use-dependent effects of TBPS and picrotoxin on $I_{G\text{-}Actx}$. In oocytes expressing rat cerebral cortex RNA, 10 μM GABA elicited Bic-sensitive membrane current responses ($I_{G\text{-}Actx}$) that were reproducible after 1–2 min of wash, showed little desensitization, and had amplitudes ranging between 3 and 6% of maximum responses (14). During incubations with TBPS or picrotoxin (0.05–1 μM), repeated or extended applications of 10 μM GABA were associated with progressive increases in the levels of inhibition. For example, with 100 nM TBPS levels of inhibition were initially as low as 15–20% but steadily increased during the course of four or five 30-sec GABA applications to equilibrate at a maximum level of approximately

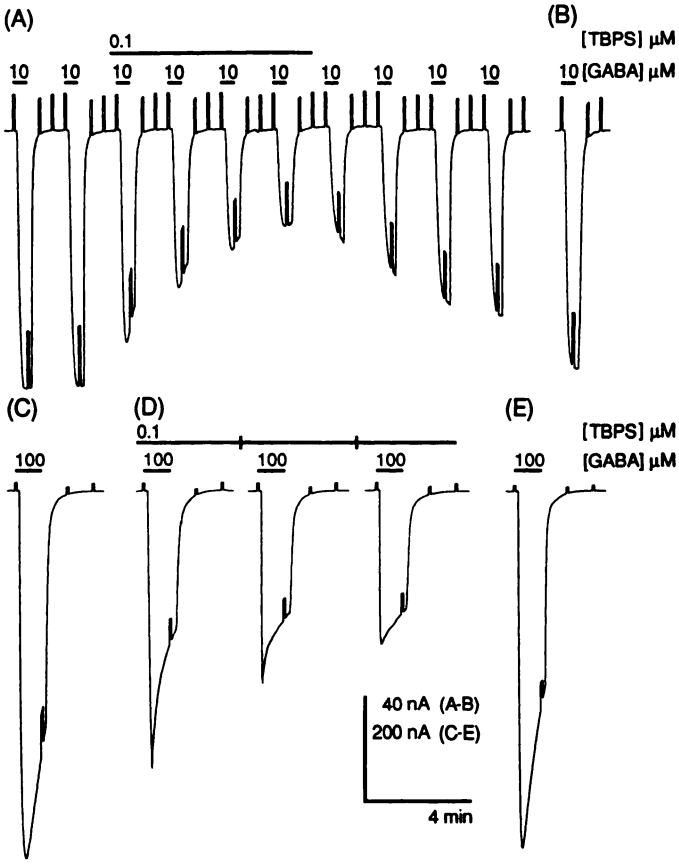

Fig. 2. Use-dependent effects characterizing inhibition of $I_{G\text{-}Actx}$ by TBPS; records were taken from the same oocyte, in the sequence indicated. A and B, Use dependence illustrated on currents elicited by 30-sec exposures to 10 μM GABA applied at 2-min intervals. A, Under control conditions, responses were reproducible and showed little desensitization; incubation with 100 nM TBPS was started 10 sec before the third application of GABA. Responses elicited during incubation with TBPS were progressively suppressed upon repeated exposure to GABA. B, Response after 23-min wash, showing >90% removal of inhibition. C–E, Use dependence illustrated on currents elicited by 1-min exposures to 100 μM GABA applied at 15-min intervals. C, Under control conditions, responses were reproducible but showed clear desensitization during 1-min exposures to GABA. D, Responses elicited during an extended incubation with 100 nM TBPS were also progressively suppressed upon repeated applications of GABA. E, Response after 45-min wash and two intervening applications of GABA; recovery was about 95%.

65% (Fig. 2A). These types of experiments strongly suggested that the inhibitory effects of both TBPS and picrotoxin were characterized by strong use-dependent components.

With concentrations of >10 μM GABA, $I_{G\text{-}Actx}$ showed increasingly pronounced levels of desensitization upon extended exposure to agonist and required 5–20-min intervals of wash to allow full resensitization of response (14). Use-dependent effects of TBPS and picrotoxin were also detectable on these larger responses and, during extended incubation with 100 nM TBPS, levels of inhibition measured on currents elicited by 100 μM GABA (approximately half-maximal responses) increased from 25–35% to levels as high as 65% after three 1-min applications of GABA (Fig. 1B). The strong use-dependent effects clearly suggested that suppression of $I_{G\text{-}Actx}$ by TBPS and picrotoxin was facilitated by channel activation. However, prolonged preincubations (40–60 min) with either inhibitor also resulted in increased levels of inhibition, implying that activation of the channel was not a prerequisite for inhibitory interactions. Washout of inhibition was also facilitated by applications of GABA, but this effect was not examined in any detail.

Use-dependent effects of TBPS and picrotoxin on GABA-activated currents expressed by bovine retina RNA. In oocytes expressing bovine retina RNA, 10 μM GABA elicited Bic/Bac-insensitive currents ($I_{G\text{-}BR}$) that were >90% of maximum responses and appeared to show little or no desensitization. The Bic-sensitive component of responses expressed by retina RNA ($I_{G\text{-}Aret}$) only became appreciable at concentrations of >10 μM GABA, with maximum currents being elicited by 1–3 mM GABA (14). As described for $I_{G\text{-}Actx}$, inhibitory effects of TBPS and picrotoxin on $I_{G\text{-}BR}$ were also characterized by use-dependent components; however, these effects were comparatively weak and were more difficult to illustrate unequivocally. For example, levels of inhibition induced by 1 μM picrotoxin on $I_{G\text{-}BR}$ elicited by 1 μM GABA (20–40% of maximum response) were already 70–75% upon the first application of GABA, even with no preincubation with inhibitor, and equilibrated to maximum levels of 80–85% by the second application of agonist (Fig. 3A). For $I_{G\text{-}BR}$, relatively brief (10–15-min) preincubations with either TBPS or picrotoxin were sufficient for full equilibration of inhibition, and any use-dependent effects were essentially undetectable under these conditions.

Washing out of the inhibitory effects of TBPS and picrotoxin also showed some dependence on channel activity. One aspect of this dependence could be measured by assaying levels of recovery after a set interval of wash and comparing effects in the absence of agonist with levels after repeated or extended applications of GABA. For example, inhibitory effects of 1 μM picrotoxin on $I_{G\text{-}BR}$ elicited by 1 μM GABA were reversed by 60–70% after 7.5-min Ringer washes (data not shown), whereas recovery was almost 100% if the wash included three additional 40-sec applications of GABA (e.g., Fig. 3A). Another aspect was illustrated by measurement of the rate at which inhibition washed out, as a function of agonist concentration. This relationship could be assayed directly, because $I_{G\text{-}BR}$ showed only low levels of desensitization. For example, using extended application of 1 μM GABA, inhibitory effects of 1-min exposures to 50 μM picrotoxin washed out with a half-time of approximately 80 sec, with responses recovering to 80–90% of controls after 4 min (Fig. 3B). The same concentration of picrotoxin reduced 100 μM GABA responses by 50–60%, but under these

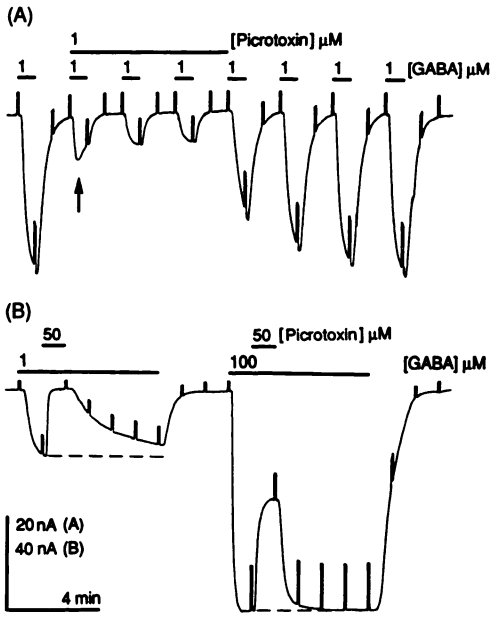

Fig. 3. A, Weak use-dependent effects characterizing inhibition of $I_{G\text{-}BR}$ by picrotoxin. Currents elicited by 40-sec applications of 1 μM GABA, repeated at 2-min intervals, were reproducible and equilibrated >95% before onset of wash. Incubation with picrotoxin was started by coapplication with the second exposure to GABA. This response was already strongly suppressed by the picrotoxin but did show a small initial peak of current (*arrow*), suggesting a weak use-dependent effect, whereas levels of inhibition appeared to have equilibrated fully in the two subsequent GABA responses. Recovery from inhibition was almost complete after 7.5 min of wash with three intervening exposures to GABA. B, Dependence of washout rate on agonist concentration. $I_{G\text{-}BR}$ elicited by an extended application of 1 μM GABA recovered only slowly after 1-min exposure to 50 μM picrotoxin. $I_{G\text{-}BR}$ elicited by 100 μM recovered approximately 10 times more rapidly after inhibition by the same concentration of inhibitor. Flow rate was 8 ml/min, and half-time for solution exchange was roughly 2.5 sec, not including dead-time of the perfusion system. Holding potential for these experiments was −80 mV.

conditions half-times for washing out of the inhibition were only 8–10 sec, a 10-fold increase in rate, with full recovery being complete within 2 min (Fig. 3B). Solution exchanges in the recording chamber had half-times between 2 and 3 sec (flow rates, 8–10 ml/min), which, to some extent, might have limited resolution of the faster dissociation rates. Similar experiments suggested that washing out inhibition induced by TBPS was characterized by a dependence on agonist concentration.

Inhibition of $I_{G\text{-}Aret}$ by TBPS and picrotoxin also appeared to be characterized by a degree of use dependence. Assay of effects on currents elicited by 100 μM GABA suggested that levels were similar to $I_{G\text{-}Actx}$, but accurate analysis was complicated by simultaneous effects on $I_{G\text{-}BR}$. Use-dependent components for inhibition of $I_{G\text{-}Aret}$ were not studied in any real detail.

Inhibitory Effects of TBPS and Picrotoxin Measured under Conditions where Use-Dependent Components Had Fully Equilibrated

Inhibition of $I_{G\text{-}Actx}$ by TBPS. $I_{G\text{-}Actx}$ was potently suppressed by TBPS. Assays on currents elicited by 10 μM GABA showed that threshold levels of inhibition were detectable upon application of 5–10 nM TBPS, and analysis of $I_{G\text{-}Actx}$ concentration-response relationships indicated that inhibitory effects of 0.1–10 μM TBPS were characterized by a strong insurmountable interaction (Fig. 4A). Currents elicited by 3 mM GABA

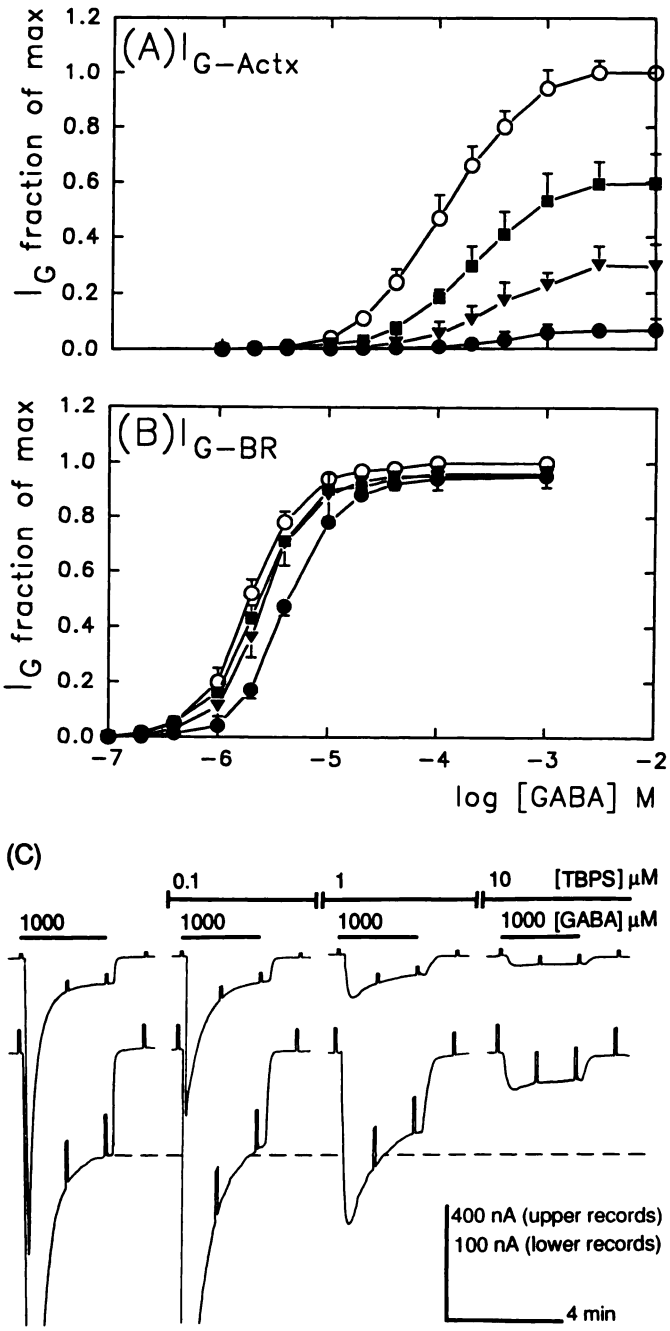

Fig. 4. A and B, Concentration-response curves comparing effects of TBPS on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, Inhibition of $I_{G\text{-}Actx}$. ○, GABA control (n = 5); ■, GABA plus 0.1 μM TBPS (n = 3); ▼, GABA plus 1.0 μM TBPS (n = 4); ●, GABA plus 10 μM TBPS (n = 3). B, Inhibition of $I_{G\text{-}BR}$. ○, GABA control (n = 4); ■, GABA plus 0.1 μM TBPS (n = 3); ▼, GABA plus 1.0 μM TBPS (n = 4); ●, GABA plus 10 μM TBPS (n = 4). In this and following concentration-response curves, data points are the mean ± standard deviation expressed as a fraction of the maximum control response, with error bars omitted when smaller than the size of symbols. For responses expressed by retina RNA, 0.1–1 mM Bic methobromide was used to abolish any $I_{G\text{-}Area}$. C, Comparison of relative sensitivities of peak and desensitized/plateau phases of $I_{G\text{-}Actx}$ to inhibition by TBPS. *Upper* and *lower records*, same responses, with lower records at increased gain to measure effects on the plateau phase (see text for details). – – –, Level of plateau under control conditions (high gain records). All responses were measured after use-dependent effects had been allowed to equilibrate fully.

(maximum responses) were reduced 41 ± 8% by 100 nM TBPS (all data given as mean ± standard deviation; n = 3), 71 ± 7% by 1 μM TBPS (n = 4), and >90% by 10 μM TBPS (n = 3). Levels of inhibition induced by 1–10 μM TBPS were only marginally reversed by increasing the concentration of GABA 10-fold, to 30 mM. Although effects of micromolar TBPS on $I_{G\text{-}Actx}$ were substantially insurmountable, levels of inhibition did appear to show some appreciable dependence on agonist concentration. For example, EC_{50} values for $I_{G\text{-}Actx}$ increased 3-fold with 1 μM TBPS, from 110 ± 3 μM under control conditions (n = 5) to 344 ± 44 μM (n = 4).

The IC_{50} for TBPS measured on maximum $I_{G\text{-}Actx}$ was 0.18 ± 0.01 μM (n = 3) but, due to the additional weak dependence on agonist concentration, values measured on currents elicited by low concentrations of GABA suggested somewhat higher potencies. For example, currents elicited by 10 μM GABA were suppressed 60–70% by 0.1 μM TBPS, implying that potency measured under these conditions would be at least twice that calculated from effects on maximum responses.

As described above, $I_{G\text{-}Actx}$ showed pronounced desensitization upon extended (2–3-min) exposure to high concentrations of agonist and, with 1–10 mM GABA, currents decayed to plateau levels that were roughly 10% of the peak response. Assay of effects of TBPS on peak and plateau responses suggested that the desensitized plateau phase was significantly more resistant to inhibition (Fig. 4C). Peak currents elicited by 1 mM GABA were reduced roughly 50% by 100 nM TBPS and 80% by 1 μM TBPS, whereas corresponding inhibition of the plateau current was only 10% and 25%, respectively. The IC_{50} for TBPS measured on the plateau phase of maximum $I_{G\text{-}Actx}$ was between 3 and 4 μM, approximately 20 times higher than values calculated on peak responses. Levels of inhibition on the plateau response also appeared to show some dependence on agonist concentration, but with high (5–10 μM) concentrations of TBPS inhibition was again predominantly insurmountable.

Inhibition of $I_{G\text{-}BR}$ by TBPS. In clear contrast to effects on $I_{G\text{-}Actx}$, TBPS was only a weak inhibitor of $I_{G\text{-}BR}$. Assays on responses elicited by 1 μM GABA showed that thresholds for suppression of currents ranged between 50 and 100 nM TBPS, 5–10 times higher than those for $I_{G\text{-}Actx}$. Moreover, analysis of effects on concentration-response curves indicated that inhibition of $I_{G\text{-}BR}$ by TBPS was almost wholly surmountable, with levels showing strong dependence on agonist concentration (Fig. 4B). At concentrations between 1 and 10 μM, TBPS caused modest lateral rightward shifts in concentration-response curves, without obviously decreasing slope values (approximately 2 for all curves) or maximum response. The EC_{50} for $I_{G\text{-}BR}$ was increased from 1.8 ± 0.1 μM under control conditions (n = 4) to 2.5 ± 0.1 μM with 1 μM TBPS (n = 4) and to 4.1 ± 0.1 μM with 10 μM TBPS (n = 4). This dependence on agonist concentration was obvious when effects on currents elicited by low and high concentrations of GABA were compared. For example, currents elicited by 2 μM GABA were reduced approximately 80% by 10 μM TBPS, whereas maximum responses (currents elicited by 100 μM GABA) were reduced by <5%.

Inhibitory effects of TBPS on maximum $I_{G\text{-}BR}$ were too weak for calculation of IC_{50} values, which complicated direct comparisons of potency with effects on $I_{G\text{-}Actx}$. Levels of inhibition induced by 50 μM TBPS on maximum $I_{G\text{-}BR}$ were clearly less than effects of 0.1 μM TBPS on $I_{G\text{-}Actx}$, suggesting that, measured under these conditions, $I_{G\text{-}BR}$ was at least 500 times more

resistant to TBPS than $I_{G\text{-}Actx}$. Comparison of effects on responses elicited by lower concentrations of GABA also indicated that $I_{G\text{-}BR}$ was relatively insensitive to TBPS. For example, $I_{G\text{-}BR}$ elicited by 1 μM GABA was inhibited 70–80% by 10 μM TBPS. This corresponded to levels of inhibition induced by 0.1 μM TBPS on $I_{G\text{-}Actx}$ elicited by 10 μM GABA, implying that, under conditions of low agonist concentration, $I_{G\text{-}BR}$ was still approximately 100 times less sensitive to TBPS than $I_{G\text{-}Actx}$.

These experiments suggested that there might be qualitative differences between the mechanisms by which TBPS inhibited $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. We therefore investigated whether even higher concentrations of TBPS gave any indication of an insurmountable component. Assay of effects on responses elicited by 100 μM GABA showed that 50 μM TBPS suppressed currents by approximately 10% (Fig. 5A). This effect was rapid in both onset and wash. To check whether the inhibition was evidence for an insurmountable interaction, experiments were repeated using 1 mM GABA to elicit maximum response, a 10-fold increase in agonist concentration (Fig. 5B). Under these conditions, TBPS reduced the response by <5%, suggesting that a substantial proportion of the inhibition still showed dependence on agonist concentration. Thus, even when high concentrations of TBPS were used, inhibitory effects on $I_{G\text{-}BR}$ did not show a clear insurmountable component. It should be noted, however, that use of TBPS at concentrations of >10 μM was somewhat compromised by limited solubility in Ringer and these solutions tended to contain light suspensions of what appeared to be undissolved TBPS. Concentrations quoted in the range of 20–50 μM TBPS are, therefore, probably slight overestimates of the real values.

Inhibition of $I_{G\text{-}Aret}$ by TBPS. Like $I_{G\text{-}Actx}$, $I_{G\text{-}Aret}$ was potently suppressed by TBPS. Inhibition of currents elicited by 100 μM GABA was detectable using 50 nM TBPS, and responses were reduced >60% by 1 μM TBPS. $I_{G\text{-}Aret}$ elicited by 100 μM GABA was effectively abolished by 10 μM TBPS, whereas $I_{G\text{-}BR}$ was largely unaffected (Fig. 6, A and B). In contrast to effects on $I_{G\text{-}BR}$, inhibition of $I_{G\text{-}Aret}$ by TBPS was clearly insurmountable.

Inhibition of $I_{G\text{-}Actx}$ by picrotoxin. As described for TBPS, $I_{G\text{-}Actx}$ was potently inhibited by picrotoxin. Assays on responses elicited by 10 μM GABA showed that suppression of currents was detectable as concentrations as low as 50 nM picrotoxin. Measurement of effects on full concentration-response curves

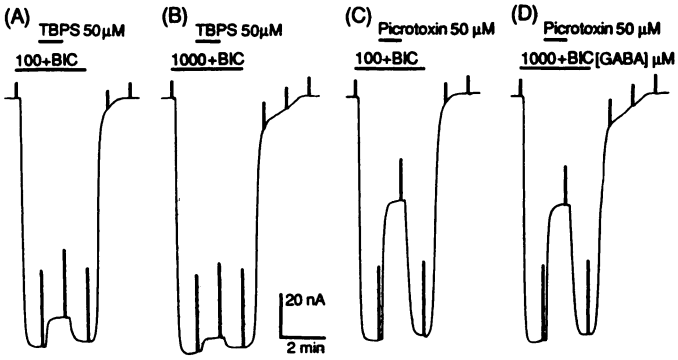

Fig. 5. Actions of TBPS and picrotoxin on pre-established maximum $I_{G\text{-}BR}$. A, Current activated by 100 μM GABA was suppressed only 11% by 50 μM TBPS. B, Increasing the agonist concentration 10-fold reversed this level of inhibition by >50%. C, Current activated by 100 μM GABA was suppressed 58% by 50 μM picrotoxin. D, Increasing the agonist concentration 10-fold reversed this level of inhibition by <5%.

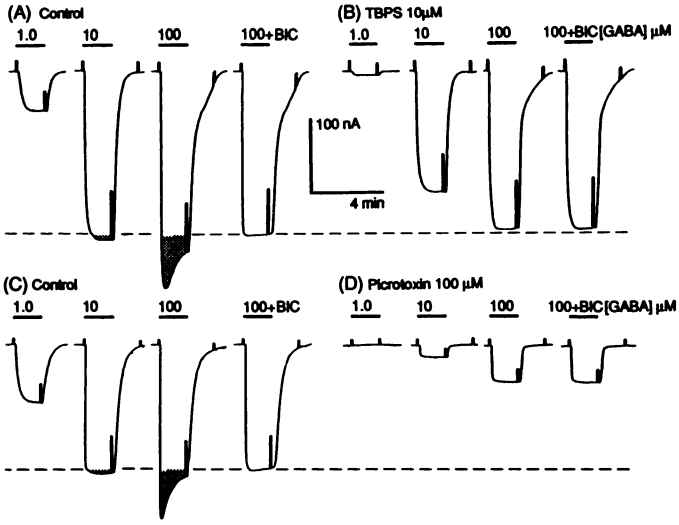

Fig. 6. Comparison of the effects of TBPS and picrotoxin on the two components of GABA responses expressed by retina RNA. A and B, Inhibition by TBPS; records from the same oocyte. A, Control responses before application of TBPS. Using concentrations of <10 μM GABA, responses consisted almost wholly of $I_{G\text{-}BR}$. Current elicited by 10 μM GABA was approximately maximal for $I_{G\text{-}BR}$ and threshold for $I_{G\text{-}Aret}$. Current elicited by 100 μM GABA was maximum $I_{G\text{-}BR}$, together with an appreciable $I_{G\text{-}Aret}$, which showed desensitization and was abolished by 100 μM Bic methobromide (*BIC*). B, GABA responses repeated during continuous incubation with 10 μM TBPS. $I_{G\text{-}BR}$ elicited by 1 μM GABA was reduced by >80%, but currents elicited by 10 and 100 μM GABA were reduced by only roughly 25% and 5%, respectively. In contrast, $I_{G\text{-}Aret}$ elicited by 100 μM GABA was effectively abolished by 10 μM TBPS. – – –, Maximum $I_{G\text{-}BR}$; *shading*, Bic-sensitive component of responses ($I_{G\text{-}Aret}$). C and D, Inhibition by picrotoxin; records from the same oocyte. C, Control responses before application of picrotoxin (as described in A). D, GABA responses repeated during continuous incubation with 100 μM picrotoxin. $I_{G\text{-}BR}$ elicited by 1 μM GABA was no longer detectable, and currents activated by 10 and 100 μM GABA were reduced by approximately 90% and 70%, respectively. $I_{G\text{-}Aret}$ elicited by 100 μM GABA was effectively abolished by 100 μM picrotoxin.

indicated that inhibition of $I_{G\text{-}Actx}$ was predominantly insurmountable (Fig. 7A). However, as with TBPS, inhibitory effects of picrotoxin did appear to show an appreciable dependence on agonist concentration. For example, with 100 μM picrotoxin the EC_{50} for $I_{G\text{-}Actx}$ increased from a control value of 122 ± 12 μM GABA (n = 5) to 337 ± 28 μM (n = 3). The IC_{50} for picrotoxin calculated from effects on maximum $I_{G\text{-}Actx}$ was 0.7 ± 0.1 μM (n = 3), indicating that potency was roughly 4 times lower than that of TBPS. Levels of inhibition measured on the fully desensitized plateau phase of $I_{G\text{-}Actx}$ again appeared to be weaker than effects measured on peak responses (data not shown).

Inhibition of $I_{G\text{-}BR}$ by picrotoxin. As suggested by our initial experiments (14), $I_{G\text{-}BR}$ was clearly inhibited by picrotoxin, and assays on currents elicited by 1 μM GABA showed that suppression of responses was detectable at concentrations as low as 50 nM. Analysis of concentration-response curves indicated that inhibition of $I_{G\text{-}BR}$ by picrotoxin showed strong dependence on agonist concentration but, in contrast to the effects of TBPS, was also characterized by a clearly insurmountable component (Fig. 7B). For example, 100 μM picrotoxin raised the EC_{50} of $I_{G\text{-}BR}$ from a control value of 1.8 ± 0.1 μM GABA (n = 7) to 12.9 ± 0.4 μM (n = 3), but this was associated with 60–70% reductions in maximum responses. Levels of suppression induced by 10–100 μM picrotoxin on

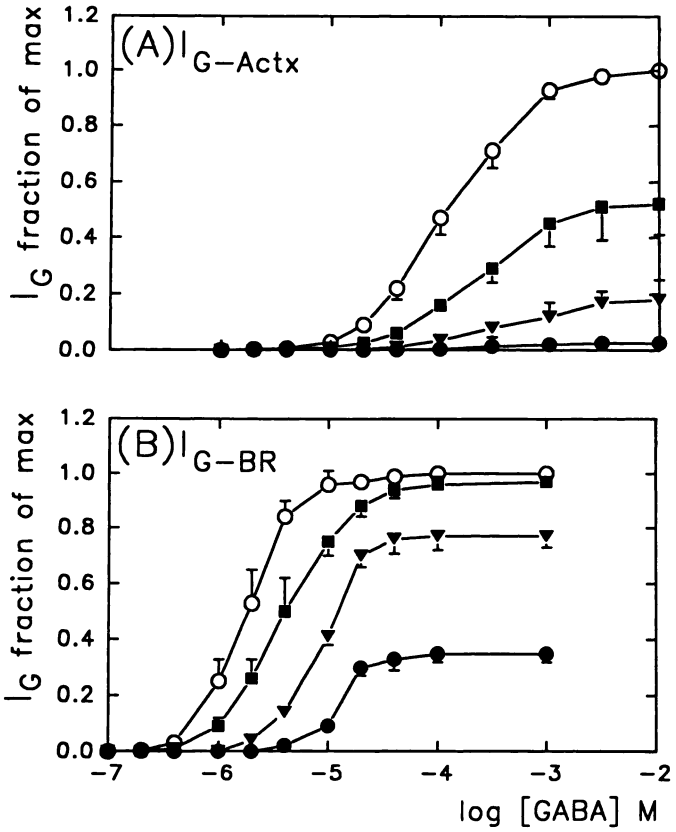

Fig. 7. Concentration-response curves comparing effects of picrotoxin on $I_{G\text{-}Actx}$ and $I_{G\text{-}BR}$. A, Inhibition of $I_{G\text{-}Actx}$. ○, GABA control (n = 5); ■, GABA plus 1.0 μM picrotoxin (n = 3); ▼, GABA plus 10 μM picrotoxin (n = 3); ●, GABA plus 100 μM picrotoxin (n = 3). B, Inhibition of $I_{G\text{-}BR}$. ○, GABA control (n = 7); ■, GABA plus 1.0 μM picrotoxin (n = 4); ▼, GABA plus 10 μM picrotoxin (n = 3); ●, GABA plus 100 μM picrotoxin (n = 3). Bic methobromide (0.1–1 mM) was used to abolish $I_{G\text{-}Aret}$.

maximum $I_{G\text{-}BR}$ were only slightly reversed by 10-fold increases in agonist concentration (Fig. 5, C and D).

The IC_{50} value measured on maximum responses was 26 ± 4 μM (n = 3), roughly 30 times higher than that determined for $I_{G\text{-}Actx}$. However, the strong dependence on agonist concentration translated into pronounced increases in potency if IC_{50} values were calculated on currents elicited by lower concentrations of GABA. For example, if inhibitory effects of picrotoxin were assayed on $I_{G\text{-}BR}$ elicited by 1 μM GABA, the IC_{50} was approximately 0.5 μM, implying a 50-fold increase in apparent potency compared with effects on maximum responses.

Inhibition of $I_{G\text{-}Aret}$ by picrotoxin. $I_{G\text{-}Aret}$ was strongly inhibited by picrotoxin. As described for $I_{G\text{-}Actx}$, inhibition of $I_{G\text{-}Aret}$ was detectable using 50–100 nM picrotoxin and was largely insurmountable. Currents elicited by 100 μM GABA were effectively abolished by 100 μM picrotoxin, whereas approximately 30% of $I_{G\text{-}BR}$ was still preserved (Fig. 6, C and D).

Discussion

Background. Inhibition of GABA-activated Cl^- channels by TBPS and picrotoxin has been the subject of detailed investigations in invertebrate muscle (28, 29), frog sensory neurons (30), and chick brain GABA receptors expressed in *Xenopus* oocytes (22). All these studies suggest that the mechanisms underlying inhibition are complex and could vary depending on the species or tissues examined. For vertebrate $GABA_A$ receptors, TBPS and picrotoxin have been reported to inhibit membrane current responses by either "mixed" or exclusively noncompetitive antagonism (22, 30, 31). In addition, blocking both drugs appeared to involve use-dependent components, such that levels of inhibition could be effects of enhanced by activating the channel with GABA (22, 30). The consensus from electrical recordings is that picrotoxin and TBPS suppress $GABA_A$ receptor activity by stabilizing closed forms of the Cl^- channel, and analysis of effects at the single-channel level indicate that inhibition is associated with pronounced decreases in the probability of channel opening, with no change in single-channel conductance and little or no reduction in mean channel open time (e.g., Refs. 32 and 33).

Binding studies suggest that TBPS and picrotoxinin interact with $GABA_A$ receptors at a common site, generally referred to as the picrotoxin or convulsant binding site (e.g., Refs. 34–36). Evidence from early studies on invertebrate muscle (37), taken together with the use-dependent component in the blocking mechanism, seemed to imply that picrotoxinin binding sites were located within the lumen of the Cl^- channel. On the other hand, more recent studies on crustacean muscle indicate that picrotoxin binding is largely unaffected by Cl^- substitutions, suggesting the interaction occurs at a relatively lipophilic site that is not within the channel itself (29).

Inhibitory effects of TBPS and picrotoxin on rat brain $GABA_A$ receptors. Our experiments showed that inhibitory effects of TBPS and picrotoxin on rat brain $GABA_A$ receptors expressed in oocytes were consistent with effects described using a variety of different techniques and assay systems (22, 28–38). (i) TBPS and picrotoxin both strongly suppressed $I_{G\text{-}Actx}$, but TBPS showed approximately 4-fold higher potency (compared on maximum responses). (ii) Inhibitory effects of TBPS and picrotoxin had clear use-dependent components, wherein onset, level, and washout of inhibition were facilitated by application of agonist. (iii) Under conditions where use-dependent effects were allowed to equilibrate fully, suppression of $I_{G\text{-}Actx}$ by both inhibitors appeared to be mixed, consisting of a predominantly insurmountable effect, indicating a noncompetitive interaction, but also showing some dependence on agonist concentration.

The present experiments do not address questions relating to the precise mechanisms by which TBPS and picrotoxin interact with mammalian $GABA_A$ receptors. Nevertheless, our results strongly suggest that inhibitory effects are similar to the interactions previously characterized for frog, chick and rat $GABA_A$ receptors (22, 30, 33), and are wholly consistent with models proposing that picrotoxin binding sites show increased accessibility in the presence of agonist and that inhibition of currents occurs through stabilization of agonist-bound closed conformations of the Cl^- channel (32, 33). The rat brain $GABA_A$ receptors expressed in oocytes, therefore, appeared to be an appropriate control for the purpose of comparing effects of Cl^- channel inhibitors on Bic/Bac-insensitive GABA receptors.

Inhibitory effects of TBPS and picrotoxin on Bic/Bac-insensitive GABA receptors. Compared with effects on $I_{G\text{-}Actx}$, inhibition of $I_{G\text{-}BR}$ by TBPS and picrotoxin was associated with only weak use-dependent components. This suggests that binding sites for picrotoxin/TBPS on the Bic/Bac-insensitive GABA receptors are readily accessible in the absence of channel activity, whereas accessibility to corre-

sponding sites on $GABA_A$ receptors shows a more pronounced dependence on receptor activation.

The most striking differences between the Bic/Bac-insensitive GABA receptors and rat brain $GABA_A$ receptors involves sensitivity to TBPS. Compared with effects on $I_{G\text{-}Actx}$ TBPS was a particularly weak inhibitor of $I_{G\text{-}BR}$. But perhaps more importantly, inhibition did not appear to involve distinct noncompetitive interactions with the Bic/Bac-insensitive GABA receptors. Indeed, standard analyses of effects on concentration-response relationships indicated the type of strong dependence on agonist concentration that is consistent with competitive antagonism.

At this stage, explanations for why the Bic/Bac-insensitive GABA receptors are comparatively resistant to TBPS remain unclear. One possibility is simply that the relevant binding sites on these receptors have distinctly low affinity for TBPS. Alternatively, gating characteristics of the Bic/Bac-insensitive GABA receptors might be such that the Cl^- channels rarely assume the necessary agonist-bound closed conformations that can be stabilized by TBPS. Interestingly, the neurosteroid pregnenolone sulfate, which similarly inhibits $GABA_A$ receptors by decreasing the frequency of channel opening (39), is likewise largely inactive as an inhibitor of $I_{G\text{-}BR}$ (11).

There are also a variety of possible explanations for why inhibition of $I_{G\text{-}BR}$ by TBPS resembles a competitive effect. Perhaps the most likely is that the Bic/Bac-insensitive GABA receptors allow strong allosteric interactions between agonist and TBPS binding sites, such that high concentrations of agonist strongly facilitate dissociation of TBPS from the receptor. Our experiments, showing that wash rate is dependent upon agonist concentration, give some support for this type of effect. Binding studies have provided direct evidence for these effects on $GABA_A$ receptors (40), which probably explains the relative resistance of desensitized plateau-phase $I_{G\text{-}Actx}$ to inhibition. In this context, it is perhaps pertinent to note that, for nicotinic acetylcholine receptors, desensitization involves transitions to high affinity conformations (41–43). If the same is true for $GABA_A$ receptors, then high affinity/desensitized conformations would appear to show increased resistance to TBPS. Bic/Bac-insensitive GABA receptors are characterized by a distinctly high affinity for GABA, and it is even possible that receptor desensitization is too rapid to be resolved by bath perfusion techniques (e.g., Ref. 44), which would mean that $I_{G\text{-}BR}$ activated by high concentrations of GABA actually corresponds to a desensitized plateau-phase response. Another possibility is that the degree to which the Cl^- channels associated with Bic/Bac-insensitive GABA receptors assume closed conformations, sensitive to stabilization by TBPS, could be dependent on agonist concentration. Taking the most extreme case, the Bic/Bac-insensitive GABA receptors appear to show little desensitization, and it is even possible that at high agonist concentrations channels simply remain in open conformations. Electrical studies at the single-channel level should begin to resolve this particular issue. Lastly, the TBPS and agonist binding sites on the Bic/Bac-insensitive GABA receptors might actually be arranged in such a way as to allow direct steric interactions between agonist and inhibitor; i.e., TBPS has true competitive effects.

Inhibitory effects of picrotoxin on the Bic/Bac-insensitive GABA receptors were also characterized by strong dependence on agonist concentration, but at higher concentrations of inhibitor suppression of $I_{G\text{-}BR}$ was insurmountable, indicating a clear noncompetitive interaction. Possible reasons for the strong dependence on agonist concentration are similar to those discussed for TBPS but, if picrotoxin and TBPS act through the same site(s) on Bic/Bac-insensitive GABA receptors (corresponding to picrotoxin binding sites on $GABA_A$ receptors), then our results would suggest that even though mechanisms of inhibition show common features they are not identical. In contrast, suppression of $I_{G\text{-}BR}$ by γ-HCH is characterized both by strong dependence on agonist concentration and by a clear noncompetitive interaction (18). In terms of these criteria, mechanisms underlying inhibition of Bic/Bac-insensitive GABA receptors by γ-HCH and picrotoxin would appear to be similar.

As described in the introduction, initial pharmacological characterization of homooligomeric human $GABA_{\rho 1}$ receptors expressed in oocytes suggested distinctively high sensitivity to picrotoxin (19). This appears to be contrary to effects on the Bic/Bac-insensitive receptors expressed by bovine retina RNA, which are generally less sensitive to picrotoxin than are brain $GABA_A$ receptors. However, inhibitory effects of picrotoxin on $GABA_{\rho 1}$ receptors were tested on responses elicited by a single concentration of GABA (1 μM) and not over a full range of agonist concentrations. It therefore remains unclear whether the sensitivity of the $GABA_{\rho 1}$ receptors to picrotoxin shows strong dependence on agonist concentration. Currents elicited by 1 μM GABA were reported to be reduced 80% by 0.2 μM picrotoxin and to be abolished by 0.5–10 μM picrotoxin (19; but also see Ref. 20). In contrast, $I_{G\text{-}BR}$ elicited by 1 μM GABA was reduced <40% by 0.2 μM picrotoxin and 70–80% by 1 μM picrotoxin. If expression of homooligomeric $GABA_{\rho 1}$ receptors is indeed sufficient to produce responses with properties similar to those of $I_{G\text{-}BR}$, then any apparent discrepancy in sensitivity to picrotoxin could simply reflect differences between species (the $GABA_{\rho 1}$ clone is human rather than bovine). Another possibility is that the full properties of $I_{G\text{-}BR}$ are dependent upon coexpression of additional, and potentially novel, types of GABA receptor subunit.

In conclusion, previous experiments showed that RNA from mammalian retina expresses GABA receptors with atypical pharmacology, which are resistant to Bic, insensitive to Bac, and not modulated by barbiturates, benzodiazepines, or steroids (11, 14). The present study indicates that the Bic/Bac-insensitive GABA receptors are only weakly inhibited by TBPS and that effects of picrotoxin show strong dependence on agonist concentration. These results should be useful for the characterization of cloned receptors, for identifying Bic/Bac-insensitive GABA receptors *in situ*, and, ultimately, for structure-functions studies at the molecular level, designed to investigate mechanisms by which convulsants interact with GABA-activated Cl^- channels.

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