

Expression of mammalian γ -aminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes

(retina/membrane current/bicuculline sensitivity/barbiturates/picrotoxin)

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Contributed by R. Miledi, January 28, 1991

ABSTRACT γ -Aminobutyric acid (GABA), the major inhibitory neurotransmitter in mammalian brain, is known to interact with two classes of GABA receptors denoted GABA_A and GABA_B. Using *Xenopus* oocytes, we compared the electrical and pharmacological properties of GABA receptors expressed by poly(A)⁺ RNA isolated from mammalian brain and retina. RNA from cerebral cortex expressed GABA responses with features characteristic of currents mediated by GABA_A receptors. In contrast, RNA from retina expressed responses mediated by GABA_A receptors and, in addition, GABA responses that were insensitive to the GABA_A antagonist bicuculline and the GABA_B agonist baclofen and showed no modulation by barbiturates or benzodiazepines. The bicuculline/baclofen-insensitive GABA response was a Cl⁻ current that was blocked by picrotoxin but showed little desensitization or outward rectification. Our results suggest that mammalian retina contains RNAs encoding GABA receptors with distinct pharmacology.

In mammals, there are two well-characterized classes of receptors for the inhibitory neurotransmitter γ -aminobutyric acid (GABA). GABA_A receptors are ligand-gated Cl⁻ channels that are competitively antagonized by bicuculline, non-competitively blocked by picrotoxin, and allosterically modulated by barbiturates and benzodiazepines (1–3). Molecular cloning of cDNAs encoding GABA_A receptor subunits indicates that the receptors are heteromeric and comprised of up to four different subunits, found in a variety of closely related subtypes (e.g., refs. 4–7). In contrast, GABA_B receptors regulate K⁺ and Ca²⁺ channels through GTP-binding proteins and intracellular messenger pathways (8). These receptors have not been cloned but are presumed to belong to the superfamily of GTP-binding-protein-coupled receptors. GABA_B receptors are selectively activated by baclofen, are antagonized by phaclofen and 2-hydroxysaclofen, and are not affected by bicuculline, picrotoxin, or any of the GABA_A modulators (9, 10).

Xenopus oocytes are now widely used to study receptors and ion channels expressed after microinjection of either heterologous poly(A)⁺ RNA or RNAs transcribed from cloned cDNAs (for reviews, see refs. 11 and 12). GABA_A subunits are readily expressed in oocytes and assemble to form receptors that have electrical and pharmacological properties similar to those reported for cells *in situ* (e.g., refs. 4–7, 13–15).

Almost every neurotransmitter/neuromodulator identified in mammalian brain has also been found in retina (e.g., ref. 16). We used *Xenopus* oocytes to characterize neurotransmitter receptors expressed by retina RNAs, investigating whether there were any clear differences between the properties of brain and retina receptors. Initial studies, using

poly(A)⁺ RNA isolated from bovine retina, showed that retina RNA primarily expressed receptors to excitatory amino acids, glycine, and substance P (ref. 17 and unpublished results). Herein we report on the GABA receptors encoded by retina RNAs.

MATERIALS AND METHODS

Eleven poly(A)⁺ RNA preparations were made from bovine retina, 2 preparations were from rat cerebral cortex, and 1 preparation was from bovine cerebral cortex. Nine bovine retina RNA preparations, 1 bovine cortex preparation, and 1 rat cortex preparation were made using the phenol/chloroform procedure (18). Two bovine retina RNA preparations and 1 rat cortex preparation were made using the acid guanidinium thiocyanate method (19). *Xenopus* oocytes, at stages V and VI of development, were microinjected with approximately 100 ng of total poly(A)⁺ RNA in 50 nl. Five bovine retina RNA preparations were size-fractionated on 10–30% (wt/vol) sucrose density gradients (20). Oocytes were injected with approximately 25 ng of fractionated poly(A)⁺ RNA, in 50 nl. Two days after injection, enveloping ovarian tissues were removed by treatment with collagenase (21).

Electrical recordings were made using a two-electrode voltage clamp, in a chamber (0.5 ml) continuously perfused (7–10 ml/min) with frog Ringer solution (115 mM NaCl/2 mM KCl/1.8 mM CaCl₂/5 mM Hepes, pH 7.0). All drugs were dissolved in the perfusing Ringer solution. Chloride substitutions were made by mixing normal Ringer with the appropriate volumes of a low Cl⁻ solution (115 mM sodium isethionate/2 mM KCl/1.8 mM CaCl₂/5 mM Hepes, pH 7.0). Zero-Na⁺ media were 115 mM Tris-HCl/2 mM KCl/1.8 mM CaCl₂, pH 7.0, or 115 mM choline chloride/2 mM KCl/1.8 mM CaCl₂/5 mM Tris-HCl, pH 7.0. Zero-Ca²⁺ Ringer was supplemented with 10 mM MgCl₂ and 1 mM EGTA. Membrane current responses were recorded from >200 oocytes, taken from >40 frogs, and in all cases uninjected oocytes gave no significant response to GABA. Intraoocyte injections of EGTA were made by pneumatic pressure ejection from micropipettes as described (22). Between 50 and 100 pmol of EGTA were injected, and chelation of intracellular Ca²⁺ was confirmed by monitoring abolition of Ca²⁺-gated Cl⁻ currents. (±)-Baclofen and 2-hydroxysaclofen were obtained from Research Biochemicals (Natick, MA). All other drugs and reagents were from Sigma.

Abbreviations: GABA, γ -aminobutyric acid; I_{G-A} , membrane current elicited through activation of GABA_A receptors; I_{G-ret} , membrane current response elicited by GABA in oocytes injected with retina RNA; I_{G-BR} , bicuculline-resistant GABA response.

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RESULTS

Size-Fractionation of Bovine Retina RNA. At a holding potential of -60 mV, oocytes injected with total poly(A)⁺ RNA from bovine retina responded to GABA with small (10–30 nA) inward membrane currents, which we will call I_{G-ret} . To increase the size of I_{G-ret} , total poly(A)⁺ RNA was size-fractionated by sucrose density gradient centrifugation. Fractions were injected into oocytes and assayed electrically to determine enrichment for mRNAs encoding GABA receptors. The fractionation profile for responses elicited by GABA appeared to be a single peak spread over 3 or 4 fractions of the 30 fractions collected, corresponding to RNAs of 3000–5000 nucleotides. Fractions showing the highest activity expressed responses approximately 10-fold larger than total poly(A)⁺ RNA (i.e., 50–300 nA). I_{G-ret} induced by both fractionated and unfractionated RNAs was then pharmacologically and electrically characterized. Using oocytes from the same frogs, I_{G-ret} was compared with the well-characterized GABA responses expressed by total poly(A)⁺ RNA isolated from bovine or rat cerebral cortex, previously shown to be mediated by GABA_A receptors (I_{G-A}) (e.g., refs. 13–15, 23–25). Most of the following experiments show data obtained from oocytes injected with fractionated bovine retina RNA. However, it was clear that size fractionation did not obviously alter any of the basic properties of I_{G-ret} (e.g., desensitization, dose–response characteristics, or sensitivity to antagonists and modulators) but simply increased the response amplitude.

Desensitization of GABA Responses. In agreement with the studies mentioned above, I_{G-A} showed marked desensitization with GABA concentrations >10 μ M. For example, in the case of I_{G-A} expressed by bovine cortex poly(A)⁺ RNA, responses elicited by 1 mM GABA desensitized by 50% within 27 ± 3 s of the maximum current and by $92 \pm 1.5\%$ after a 4-min exposure (all values are quoted as mean \pm SD; $n = 5$) (Fig. 1A). In contrast, I_{G-ret} showed comparatively little desensitization (Fig. 1B). By using oocytes from the same frog, extended exposure to 1 mM GABA caused I_{G-ret} to desensitize by only $12 \pm 3\%$ over 4 min ($n = 6$), and even 10 mM GABA applied for up to 15 min resulted in similar low levels of desensitization. More importantly, in most cases there appeared to be two quite distinct components to I_{G-ret} , with only the minor component desensitizing.

Comparison of Dose–Response Curves. Dose–response curves were constructed for I_{G-ret} and compared with I_{G-A}

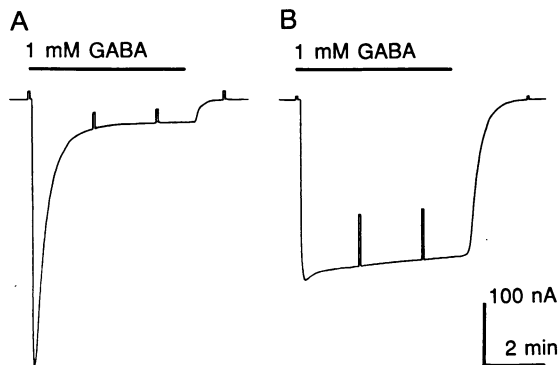


FIG. 1. Desensitization of membrane current responses evoked by GABA. (A) Current elicited by GABA in an oocyte injected with poly(A)⁺ RNA from bovine cerebral cortex. (B) Current elicited by GABA in an oocyte injected with poly(A)⁺ RNA isolated from bovine retina. For this and all following recordings, the holding potential was -60 mV, with periodic 5-s steps to -50 mV used to monitor membrane conductance. Inward currents correspond to downward deflections, with drugs applied as indicated by bars, and perfusion dead time between 5 and 10 s. Capacitative transients on steps in voltage have been touched out.

induced by bovine or rat cortex poly(A)⁺ RNA (Fig. 2). Maximum responses, elicited by 1–10 mM GABA, were 100–300 nA for I_{G-ret} and 300–3000 nA for I_{G-A} . Nevertheless, typical threshold concentrations for eliciting I_{G-ret} were between 0.2 and 0.4 μ M GABA, consistently lower than thresholds for I_{G-A} (1.0–2.0 μ M). I_{G-ret} showed a steep concentration dependence, with 10 μ M GABA already eliciting between 75 and 95% of the maximum response. The concentration of GABA required to elicit half-maximal responses (EC_{50}) was only 1.48 ± 0.32 μ M for I_{G-ret} ($n = 21$), as compared to 69.7 ± 14.3 μ M for I_{G-A} induced by rat cortex ($n = 21$) and 84.6 ± 15.3 μ M for I_{G-A} induced by bovine cortex ($n = 6$). When the dose–response curve for I_{G-ret} was plotted on double-logarithmic coordinates, the limiting slope value of the initial rising phase (calculated from the first five data points) was 2.69 ± 0.29 , indicating a high level of cooperativity. The corresponding slope values for I_{G-A} were 1.60 ± 0.04 (bovine cortex) and 1.63 ± 0.09 (rat cortex), similar to those reported in oocytes (13–15, 25).

Bicuculline/Baclofen-Insensitive GABA Responses. The clear differences between I_{G-ret} and I_{G-A} in desensitization and dose–response relationship prompted a more detailed characterization of the GABA response expressed by retina poly(A)⁺ RNA. I_{G-A} induced by bovine and rat cortex RNA was potently blocked by bicuculline, a specific GABA_A antagonist (see also refs. 13–15, 25). Inhibition was competitive, with 100 μ M bicuculline raising response thresholds from 2 μ M to about 200 μ M GABA (Fig. 3A). In striking contrast, I_{G-ret} was only partially blocked by 1–100 μ M bicuculline (Fig. 3B). A major component of the response, constituting 65–95% of the peak current, was essentially unaffected by the antagonist and even threshold responses, elicited by 0.2–0.4 μ M GABA, were not appreciably blocked by 1–100 μ M bicuculline. The only blocking effects on I_{G-ret} were on the small component that showed desensitization. This bicuculline-sensitive current had thresholds for activation between 5 and 10 μ M GABA and an EC_{50} between 50 and 100 μ M GABA and, with different RNA preparations and oocytes, usually constituted 5–35% of the total peak current. Inhibition of this minor component by bicuculline was competitive and similar in potency to that described for I_{G-A} (Fig. 3C and D).

These experiments showed that the major component of I_{G-ret} was pharmacologically distinct from the typical I_{G-A} . We therefore tested whether the current was due to expression of GABA_B receptors. (\pm)-Baclofen at 1–100 μ M, a GABA_B agonist (Fig. 4), and 2-hydroxysaclofen at 10–100 μ M, a GABA_B antagonist, were both essentially inactive on the bicuculline-resistant current. This clearly indicated that

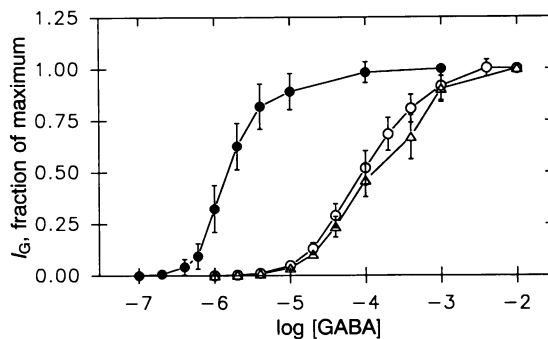


FIG. 2. Dose–response curves for I_G in oocytes injected with poly(A)⁺ RNA from various tissues. ●, Bovine retina ($n = 13$ –21; four RNA preparations); ○, rat cerebral cortex ($n = 11$ –21; two RNA preparations); Δ, bovine cerebral cortex ($n = 6$; one RNA preparation). In this and all following graphs, data points are the mean \pm SD, expressed as a fraction of maximum peak current, with error bars omitted when smaller than size of symbols.

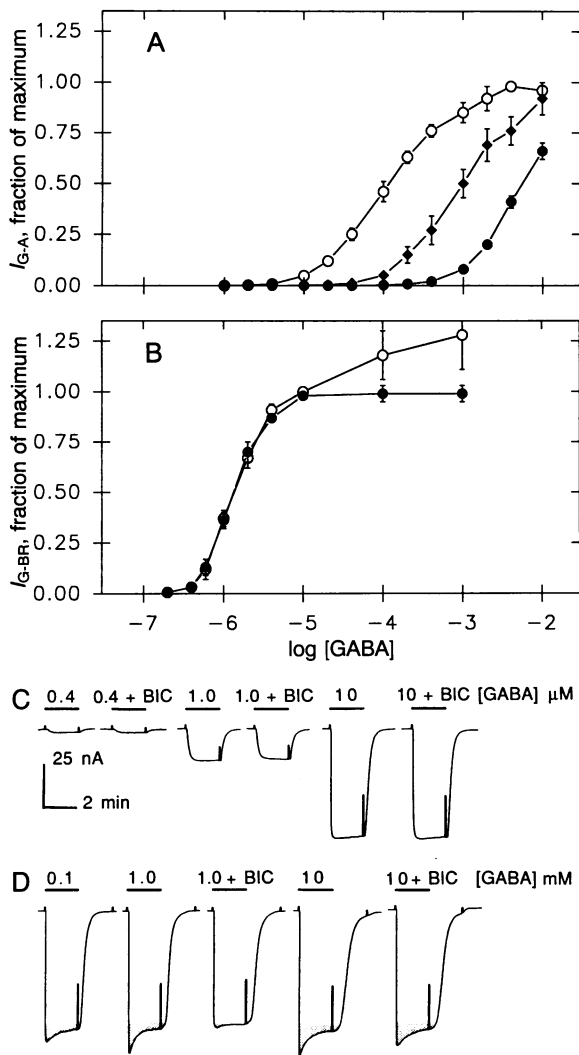


FIG. 3. (A) Bicuculline competitively blocked I_{G-A} induced by rat cortex RNA. \circ , GABA control; \blacklozenge , GABA plus 10 μM bicuculline (all methobromide); \bullet , GABA plus 100 μM bicuculline ($n = 3$). (B) Bicuculline was largely inactive on I_{G-ret} . \circ , GABA control; \bullet , GABA plus 100 μM bicuculline ($n = 5$). (C and D) Sample records of I_{G-ret} dose-response curve in a single oocyte. (C) At concentrations up to 10 μM GABA, responses showed little desensitization and were insensitive to 100 μM bicuculline methobromide (BIC). (D) At concentrations >10 μM GABA, the minor component became apparent, which showed desensitization and was inhibited by bicuculline. The bicuculline-sensitive component of I_{G-ret} is indicated by shading. When using 10 mM GABA, 100 μM bicuculline only partially blocked this component.

the response was not mediated by GABA_B receptors. We therefore termed the bicuculline-resistant component I_{G-BR} . Dose-response curves for I_{G-ret} (Fig. 2) were then reanalyzed taking currents elicited by 10 μM GABA as maximum

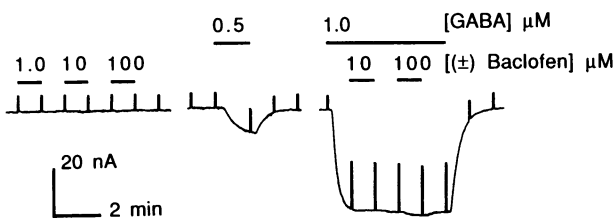


FIG. 4. (\pm)-Baclofen elicited no significant I_{G-BR} in an oocyte with response threshold at 0.2 μM GABA and showed no appreciable inhibition of the current elicited by 1 μM GABA.

responses, thereby excluding the superimposed bicuculline-sensitive component. The EC_{50} for I_{G-BR} alone was 1.38 ± 0.25 μM , and the slope value was 2.75 ± 0.26 . Electrical properties and pharmacology of I_{G-BR} were then further characterized by using 0.1–1 mM bicuculline and/or low concentrations of GABA to distinguish and exclude bicuculline-sensitive currents.

Electrical Properties of Bicuculline/Baclofen-Insensitive GABA Responses. At a holding potential of -60 mV, I_{G-BR} was an inward current associated with an increase in membrane conductance. The current had a reversal potential that ranged between -20 and -30 mV in oocytes taken from different frogs, corresponding to the equilibrium potential for Cl^- in oocytes, which shows similar variability (11). The bicuculline-sensitive component of I_{G-ret} was also associated with an increase in membrane conductance, and reversal potentials for the bicuculline-sensitive and -resistant components of I_{G-ret} and for I_{G-A} were indistinguishable. For example in one case, using oocytes from the same frog, reversal potentials were -28 ± 2 mV for I_{G-BR} ($n = 12$), -28 ± 1.5 mV for I_{G-A} expressed by bovine cortex RNA ($n = 6$), and -28 ± 2 mV for I_{G-A} expressed by rat cortex RNA ($n = 6$). As described (13–15), current-voltage relationships for I_{G-A} showed marked outward rectification in current at potentials more negative than about -60 mV. In contrast, the current-voltage relationship for I_{G-BR} showed comparatively little rectification even at potentials as negative as -140 mV (Fig. 5). Both currents showed Nernstian shifts in reversal potential when bathing Cl^- was substituted with the impermeant anion isethionate, giving approximately 55 mV shifts in reversal potentials for a 10-fold change in external Cl^- concentration. Complete substitution of bathing Na^+ with Tris or with choline or increasing bathing K^+ from 2 to 20 mM had no clear effect on the reversal potential of I_{G-BR} . These experiments confirmed that, like I_{G-A} , I_{G-BR} was associated with an increase in membrane conductance to Cl^- with no appreciable contribution by other ions. I_{G-BR} was largely unaffected by removal of extracellular Ca^{2+} or chelation of intracellular Ca^{2+} by intraoocyte injection of EGTA (23). This obviated the possibility that I_{G-BR} was generated indirectly, either through gating of extracellular Ca^{2+} or mobilization of intracellular Ca^{2+} , both of which would lead to activation of Ca^{2+} -gated Cl^- channels in oocytes (26–28). The electrical recordings also indicated that I_{G-BR} was not evoked through activation of an electrogenic GABA transporter, which would generally show no clear reversal poten-

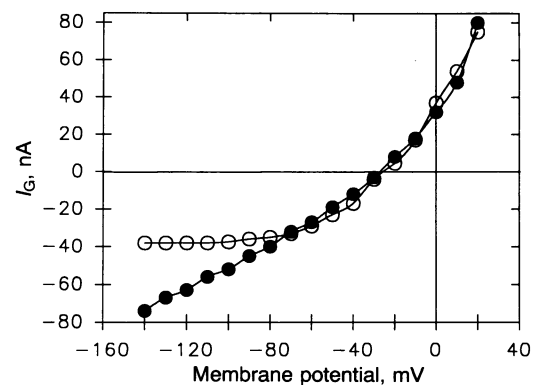


FIG. 5. Comparison of current-voltage relationships for I_{G-A} expressed by rat cortex RNA (\circ) and I_{G-BR} (\bullet). Concentrations of GABA were selected to elicit $\leq 10\%$ of maximum response and approximately the same current at -60 mV: 10 μM for I_{G-A} and 0.6 μM for I_{G-BR} . Curves were determined using repeated exposures to GABA at various holding potentials. A negative nA corresponds to inward current. Absence of outward rectification in I_{G-BR} was confirmed in six oocytes.

tial and would be dependent on external Na^+ (29). Moreover, the current was neither elicited nor significantly blocked by 10–100 μM nipecotic acid, an inhibitor and substrate of GABA transport (30).

Further Pharmacology of Bicuculline/Baclofen-Insensitive GABA Responses. The barbiturate pentobarbital potently modulates GABA_A receptors and in oocytes has been shown to potentiate I_{G-A} expressed by poly(A)⁺ RNAs from chicken optic lobe and rat brain (13–15, 23). Pentobarbital caused a similar potentiation of I_{G-A} expressed by bovine (data not shown) and rat cortex RNA (Fig. 6A). Potentiation of I_{G-A} was detectable using 0.5 μM pentobarbital, and 100 μM pentobarbital decreased the EC_{50} from 75 μM to approximately 1.5 μM GABA, with response thresholds as low as 30 nM GABA. In contrast, even at threshold concentrations of GABA, where potentiation should be pronounced, I_{G-BR} was largely or wholly unaffected by 1–100 μM pentobarbital (Fig. 6B). However, the bicuculline-sensitive component of I_{G-ret} , like I_{G-A} , was strongly potentiated by pentobarbital (Fig. 6B and C).

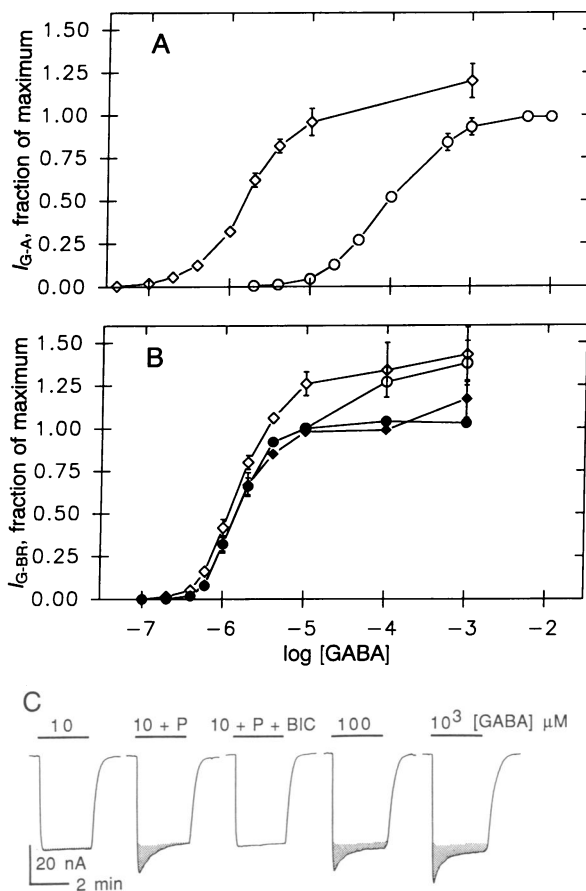


FIG. 6. (A) Pentobarbital potentiated I_{G-A} induced by rat cortex RNA. \circ , GABA control; \diamond , GABA plus 100 μM pentobarbital ($n = 3$). (B) Pentobarbital caused a marginal increase in I_{G-ret} with dose-response curves showing appreciable divergence around 10 μM GABA. \circ , GABA control; \diamond , GABA plus 100 μM pentobarbital ($n = 4$). Repeating curves in 100 μM bicuculline (methobromide) showed that I_{G-BR} was not potentiated by pentobarbital. \bullet , GABA plus 100 μM bicuculline; \blacklozenge , GABA plus 100 μM bicuculline plus 100 μM pentobarbital. (C) Pentobarbital potentiated the bicuculline-sensitive component of I_{G-ret} . For a control, 10 μM GABA elicited a maximum I_{G-BR} and threshold response for the bicuculline-sensitive component. In 100 μM pentobarbital (P), 10 μM GABA elicited additional current that showed desensitization and was blocked by 100 μM bicuculline (BIC). The bicuculline-sensitive component is indicated by shading.

I_{G-A} induced by cortex RNA was also modulated by benzodiazepines, though levels of potentiation were comparatively modest (see also refs. 15 and 31). For example, chlorazepate (10–100 μM) and diazepam (1–10 μM) caused 1.5- to 2-fold increases in responses elicited by 10 μM GABA. At the same concentrations, these benzodiazepines showed no significant effects on I_{G-BR} but did cause some potentiation of the bicuculline-sensitive component of I_{G-ret} .

Picrotoxin is known to block currents mediated by both GABA_A and glycine receptors through interaction with the gating of Cl^- channels (2, 3). As reported in oocytes (23, 25, 32), I_{G-A} induced by brain poly(A)⁺ RNA was strongly inhibited by picrotoxin. Inhibition of I_{G-A} was largely non-competitive, with 10 μM picrotoxin reducing maximum currents by approximately 90% and 100 μM picrotoxin reducing maximum currents by >97%. I_{G-BR} , also a Cl^- current, was likewise reduced by picrotoxin but appeared to be significantly less sensitive to the blocker than I_{G-A} . At 1 mM GABA, 10 μM picrotoxin inhibited I_{G-BR} by approximately 25% and 100 μM picrotoxin inhibited I_{G-BR} by 65%.

DISCUSSION

These experiments reaffirm that poly(A)⁺ RNA from bovine or rat cerebral cortex expresses GABA responses that have the general pharmacological and electrical characteristics of currents mediated by GABA_A receptors. In contrast, poly(A)⁺ RNA from bovine retina expresses GABA responses with two superimposed, but distinct, components: (i) a minor component that desensitizes is blocked by bicuculline and is potentiated by pentobarbital and benzodiazepines and (ii) a major component that shows little desensitization is resistant to bicuculline, is not potentiated by pentobarbital or benzodiazepines, and is neither activated nor significantly blocked by baclofen, 2-hydroxysaclofen, or nipecotic acid. We therefore suggest that I_{G-ret} is elicited through activation of two distinct types of GABA receptors. The minor component appears to be mediated by GABA_A receptors, whereas the major component is mediated by GABA receptors with pharmacology which is distinct from that of either GABA_A or GABA_B receptors.

I_{G-A} and I_{G-BR} have one essential similarity in that both are Cl^- currents that show sensitivity to picrotoxin. This suggests that the receptors that mediate I_{G-BR} are possibly related to the GABA_A class of receptors. Expression studies using RNAs transcribed from cloned genes have shown that different subtype combinations of GABA_A receptor subunits form functional receptors with different affinities for GABA, voltage dependence, and rates of desensitization and, furthermore, that the benzodiazepine modulatory site requires γ subunits for full functional expression (e.g., refs. 4–7, 33–37). Nevertheless, thus far all heteromeric combinations of GABA_A subunits, and even possible homomeric receptors, have shown desensitization, potentiation by barbiturates, and, where reported, sensitivity to bicuculline. Therefore, in terms of pharmacology, the receptors that mediate I_{G-BR} appear to be quite distinct from other characterized GABA_A receptors.

The *Xenopus* oocyte expression system is normally used to study receptors and ion channels with properties that have already been characterized *in situ* by a variety of pharmacological and electrical techniques. In the experiments reported herein, oocytes expressed GABA receptors with qualitatively distinct pharmacology that have not, to our knowledge, been previously detected in the retina (e.g., ref. 38). Without corroborating reference to receptors studied *in situ*, using oocytes to study previously uncharacterized receptors raises a number of concerns requiring that some caution be exercised in the interpretation of results. For example, it remains possible that the oocyte incorrectly processes RNAs specif-

ically encoding retina GABA receptors or that properties of specifically retina GABA receptors could be modified by association with endogenous oocyte proteins. In this context, we should stress that incorrect processing of RNAs by oocytes would certainly be exceptional and that in general fidelity of receptor and channel expression is remarkably good (11, 12).

To conclude, our experiments show that poly(A)⁺ RNA isolated from mammalian retina expresses GABA receptors with distinct pharmacology in *Xenopus* oocytes. Further studies at the molecular level will establish the structure of these bicuculline/baclofen-insensitive GABA receptors and will determine if they are related to GABA_A subunits. Direct *in situ* studies will be required to determine whether functional bicuculline/baclofen-insensitive GABA receptors are expressed in retinal cells, and if so, their relevance to retina physiology.

We thank Ms. Laura Jaimes for valuable technical assistance and Dr. M. Panicker for helpful discussions. This work was supported by Grant R01-NS23284 from the U.S. Public Health Service. L.P. acknowledges support from FIDIA S.p.A. (Abano Terme, Italy) and Prof. A. Giotti.

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