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A Novel γ-Aminobutyric Acid Receptor Subunit (ρ_2) Cloned from Human Retina Forms Bicuculline-Insensitive Homooligomeric Receptors in *Xenopus* Oocytes

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The ρ_2 subunit, a novel GABA receptor subunit, has been cloned from a human retinal complementary DNA library. This subunit shares 74% amino acid sequence identity with the ρ_1 subunit that forms homooligomeric bicuculline-, barbiturate-, and benzodiazepine-insensitive GABA receptors. The ρ_2 subunit also forms homooligomeric GABA-activated chloride channels when expressed in *Xenopus* oocytes. The amplitudes of the whole-cell currents of ρ_2 receptors are always smaller than those of ρ_1 receptors, but the affinity and cooperativity of GABA are very similar. Like the ρ_1 subunit, the currents generated by ρ_2 are insensitive to $GABA_A$ receptor modulators including bicuculline, hexobarbital, and diazepam and can be reversibly inhibited by $ZnCl_2$. Homooligomeric ρ_2 and ρ_1 receptors are less sensitive to muscimol and picrotoxin, and desensitize slower than $GABA_A$ receptors. These data demonstrate that homooligomeric receptors formed by ρ_2 and ρ_1 subunits have a number of electrophysiologic and pharmacologic characteristics that differ from receptors formed by $GABA_A$ receptor subunits. The distinctive properties of ρ receptors are very similar to those of bicuculline-insensitive GABA-gated chloride channels identified in retina, suggesting a molecular basis for this form of GABA receptor in visual pathways.

[Key words: GABA, GABA receptor, chloride channel, retina,* Xenopus *oocyte, bicuculline insensitivity]

GABA is the major inhibitory neurotransmitter in the CNS. At least three types of GABA receptors have been identified. $GABA_A$ receptors are multiunit ligand-gated Cl^- channels that can be antagonized by bicuculline and picrotoxin and potentiated by barbiturates and benzodiazepine (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Delorey and Olsen, 1992). $GABA_B$ receptors are activated by baclofen and appear to control K^+ and Ca^{2+} channels through GTP-binding proteins but are insensitive to bicuculline, picrotoxin, and any known $GABA_A$ modulators (Nicoll, 1988). In visual pathways, a group of GABA receptors have been described with distinct pharmacologic profiles and have been classified as $GABA_C$ receptors (Johnston, 1986). These receptors conduct Cl^- currents but are not responsive to typical $GABA_A$ receptor modulators (e.g., bicuculline, barbiturates). GABA receptors with these properties have recently been identified in bovine, rat, and perch retina (Woodward et al., 1992; Feigenspan et al., 1993; Qian and Dowling, 1993); however, the composition of these bicuculline-insensitive GABA receptors at the molecular level is unknown.

Based on the homology of the complementary DNA (cDNA) sequences encoding the individual subunits, $GABA_A$ receptor subunits can be divided into four subunit families: α, β, γ, and δ (Schofield et al., 1987; Shivers et al., 1989; Ymer et al., 1990). Receptor subunits within a particular family share a high degree of amino acid sequence identity (70–80%). There is approximately 30–40% amino acid sequence identity among the different subunit families. $GABA_A$ receptor subunits expressed singly in *Xenopus* oocytes and mammalian cells can form functional homooligomeric receptors although very inefficiently (Blair et al., 1988; Pritchett, 1988). Coexpression of $GABA_A$ subunits from different classes has been shown to be necessary to obtain GABA-gated Cl^- channels with properties similar to those observed *in situ* (Schofield et al., 1987; Levitan et al., 1988; Shivers et al., 1989; Polenzani et al., 1991). Different combinations of $GABA_A$ receptor subunits display distinctive properties. For example, different combinations of various α subunit (α_1–α_5) with β and γ subunits display different affinities for GABA and varying levels of cooperativity (Sigel et al., 1990). Coexpression of the γ_2 subunit with α and β subunits leads to full acquisition of benzodiazepine sensitivity (Pritchett et al., 1989a,b).

A new family of GABA receptor subunits, the ρ family, has been cloned from a human retina cDNA library (Cutting et al., 1991, 1992). Two members of this family, ρ_1 and ρ_2, share 74% amino acid sequence identity. When the ρ subunit family is compared to $GABA_A$ receptor subunits, amino acid sequence identity ranges from 30% to 38%. Northern blot analysis has shown that ρ_1 messenger RNA is highly expressed in retina (Cutting et al., 1991). Previous studies using the *Xenopus* oocyte expression system demonstrated that ρ_1 efficiently forms homooligomeric GABA-gated Cl^- channels that were insensitive to bicuculline, pentobarbital, and benzodiazepine (Shimada et al., 1992). In this study, we demonstrate that the ρ_2 subunit can form homooligomeric receptors in *Xenopus* oocytes with pharmacologic features similar to ρ_1 receptors and have characterized additional distinctive properties of this novel class of GABA receptor subunits.

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Materials and Methods

RNA preparation. RNA transcripts were synthesized *in vitro* from linearized pR5-2.0 plasmid containing the ρ_1 cDNA and pR24-3.0 containing the ρ_2 cDNA using T3 and T7 RNA polymerase (Promega, Madison, WI), respectively. The reactions were carried out in 100 μl of buffer containing 40 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 10 mM NaCl, 100 U of RNasin (Promega), 500 μM each ATP, CTP, UTP, 100 μM GTP (Pharmacia, Piscataway, NJ), 800 μM G(5')ppp(5')G (New England Biolabs, Beverly, MA), 5 μg of plasmid DNA, and 100 U of RNA polymerase (Promega) for 2 hr at 37°C (Maniatis et al., 1982). The RNA transcripts were precipitated twice with NH_4OAc and ice-cold ethanol then resuspended in DEPC-treated H_2O. RNA concentration was determined by a UV spectrophotometer (Beckman DU-40, Columbia, MD). The purity of RNA was confirmed by electrophoresis in a 1.5 M formaldehyde agarose gel.

Oocyte preparation and RNA injection. Adult female *Xenopus laevis* frogs (*Xenopus* I, MI) were anesthetized with ice and several ovarian lobes removed. Oocytes were defolliculated by digestion with 1 mg/ml collagenase A (Boehringer–Mannheim Biochemicals, Indianapolis, IN) in Ca^{2+}-free OR 2 saline (in mM: NaCl, 100; KCl, 2; $MgCl_2$, 1; HEPES-Tris, 5; pH 7.5) at room temperature for 3 hr with gentle agitation. After digestion, oocytes were washed five times with OR 2 solution and five times with modified Barth's solution (MBS; in mM: NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $Ca(NO_3)_2$, 0.3; $CaCl_2$, 0.4; $MgSO_4$, 0.8; Tris-HCl, 15; and penicillin, 50 μg/ml, or streptomycin, 50 μg/ml) as described previously (Lu et al., 1990). Stage V and VI oocytes were selected and stored at 19°C in MBS. Oocytes were injected after 24 hr with 50 nl of RNA by a positive-displacement micropipette (Drummond, PA). In all experiments either 20 ng of ρ_2 RNA or 1 ng of ρ_1 RNA was injected into the oocytes. To minimize variations in expression efficiency between different batch of oocytes, comparisons of specific parameters between ρ_2 and ρ_1 were always performed in *Xenopus* oocytes from the same batches.

Nuclear injection of plasmid DNA expression vectors. The picrotoxin inhibition assays were performed using a nuclear injection strategy to enhance expression efficiency as reported by Swick et al. (1992). The ρ_1 cDNA was subcloned into the EcoRI site and the ρ_2 cDNA was subcloned into ScaI site of the nuclear expression vector pMT3. Injection of either pMT3-ρ_1 (15 ng in 1 μl) or pMT3-ρ_2 (15 ng in 1 μl) plasmid into *Xenopus* oocyte nuclei produced efficient expression after 72 hr. The plasmid was coinjected with the pMT2-SEAP vector (5 ng in 1 μl), which encodes the secreted form of alkaline phosphatase (Swick et al., 1992). An automatic pressure generator (IM-200, Narishige USA) was used to perform nuclear injection. Oocytes secreting alkaline phosphatase were selected for electrophysiology experiments.

Electrophysiology. Two-microelectrode voltage-clamp experiments were performed in normal frog saline (mM: NaCl, 96; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 1; HEPES-NaOH, 5; pH 7.4) at room temperature (22°C) using a 0.8 ml chamber 3–4 d after RNA injection. Microelectrodes filled with 3 M KCl, having resistance between 0.5 and 2 MΩ, were used for voltage clamping and current measurements. Drugs were applied by continuous superfusion to oocytes by gravity as described by Lu et al. (1990). Flow rate was 0.3 ml/sec and the dead volume of the perfusion system was 0.5 ml. Complete replacement of the bath solution took approximately 3 sec. The perfusion system and the time periods of solution exchanges were optimal for the study of slow-phase desensitization of ρ receptors (which occurred in seconds to minutes). Voltage pulse protocols and data acquisition were performed by means of a PCLAMP software package, version 5.51 (Axon Instruments, Burlingame, CA). Holding potential was −70 mV in all cases.

Chemicals. GABA, picrotoxin, muscimol, $ZnCl_2$, diazepam and bicuculline were obtained from Sigma, St. Louis, MO. Hexobarbital and bicuculline (Research Biochemicals International, Natick, MA) were gifts from Dr. George Uhl. Dilution of reagents with normal frog saline were prepared immediately before use from fresh stock solutions.

Data analysis and statistics. Statistical significance is tested by paired one-tail and two-tail Student's t test. Significant difference between two data sets is defined as $p < 0.01$. Data are reported as mean ± standard error (SE). The dose–response and the drug–inhibition curves were fitted and drawn by a N-FIT program provided by The University of Texas, Galveston. The equation used for dose–response relation was $I_{(x)} = I_{max}/1 + (K_d/x)^n$ (Hill equation). The equation for drug inhibition curve was $I_{(x)} = I_{max}/1 + (x/K_d)^n$ (inverse Hill equation). In both equations, x represents the drug concentration, K_d is the concentration for eliciting half-maximal response ($\frac{1}{2}I_{max}$), and n is the Hill coefficient.

Results

Xenopus oocytes injected with ρ_2 RNA express GABA-induced currents (Fig. 1*A*) that are not found in either H_2O ($n = 5$) injected or antisense RNA injected oocytes ($n = 6$). Concentrations of glycine as high as 1 mM did not induce any currents in ρ_2 RNA-injected oocytes ($n = 6$, data not shown). In comparison with ρ_1, the average amplitude of current for ρ_2 RNA-injected oocytes was consistently smaller than that of ρ_1 RNA-injected oocytes. Comparing the currents induced by 5 μM GABA in Figure 1, *A* and *B*, it is clear that although 20 times more ρ_2 than ρ_1 RNA was injected, the currents generated by the ρ_2 receptor were significantly smaller ($p < 0.01$, $n = 10$). This was more evident from a close examination of the *I–V* relationship for both receptors depicted in Figure 1*D*. Although *I–V* relationships for both receptors were linear and the reversal potentials were not significantly different, the slope was much flatter for the ρ_2 receptor, suggestive of a lower whole-cell conductance. The reversal potential (the x-intercept of *I–V* relationship) for ρ_2 was −30 ± 4 mV ($n = 5$), and for ρ_1 was −28.5 ± 3 mV ($n = 5$; Fig. 1*D*). These values are close to the equilibrium potential of Cl^- in oocytes (Lu et al., 1990). When the Cl^- concentration in the bathing solution was reduced from 106 mM to 6 mM by replacing with gluconate, GABA-induced ρ_2 currents decreased (Fig. 1*C*). The reversal potentials of GABA-activated currents changed linearly with logarithmic Cl^- concentrations (Fig. 1*E*). The slope is 58 mV per 10-fold change of Cl^- concentration, which is very close to the theoretical value (59 mV per 10-fold change of concentration) estimated from Nernst equation at room temperature. These data confirm that ρ_2 forms GABA-activated Cl^--selective ion channels.

The amplitude of the induced currents was dependent on GABA concentration. Dose–response curves (Fig. 2) demonstrated that both ρ_2 and ρ_1 receptors have very similar sigmoid curves ($n = 5$). The threshold concentration of GABA required to elicit a detectable current for both receptors ranged from 0.01 μM to 0.1 μM. The magnitudes of both ρ_2 and ρ_1 receptor–generated currents showed a distinct concentration dependence saturating between 9 μM to 12 μM GABA. The GABA concentrations that elicited a half-maximal current responses (EC_{50}) were 1.8 ± 0.4 μM for ρ_2 and 1.7 ± 0.5 μM for ρ_1 receptors ($n = 5$). When the dose–response curves were plotted on double-logarithmic coordinates, the slopes of the initial phase (Hill coefficient; Sigel et al., 1990) are 2.3 ± 0.2 for ρ_2, and 2.5 ± 0.1 ($n = 5$) for ρ_1. There was no statistically significant difference either in the EC_{50} or the Hill coefficient between ρ_1 and ρ_2 receptors.

In this study, we selected several reagents including muscimol, bicuculline, hexobarbital, diazepam, picrotoxin, and $ZnCl_2$ to compare the pharmacologic profiles of ρ_2 and ρ_1 receptors. Data shown in Figure 3 are expressed as percentage of the currents elicited by 5 μM GABA. Muscimol, a $GABA_A$ receptor agonist, applied at 5 μM concentration, induces only 62.9 ± 8.3% ($n = 6$) and 72.3 ± 5.6% ($n = 5$) of the currents induced by 5 μM GABA for ρ_2 and ρ_1 receptors, respectively. Bicuculline, a $GABA_A$ receptor competitive antagonist, did not affect the magnitude of 5 μM GABA currents for either ρ_2 or ρ_1 receptors even when the concentration of bicuculline was increased to as high as 100 μM. Likewise, hexobarbital and diazepam, two common $GABA_A$ receptor potentiators used as antiepileptic and sedative medications, coapplied at concentrations ranging from 10 μM to 1 mM in the presence of 5 μM GABA, did not affect the magnitude of either ρ_2 or ρ_1 currents. Picrotoxin inhibited GABA-

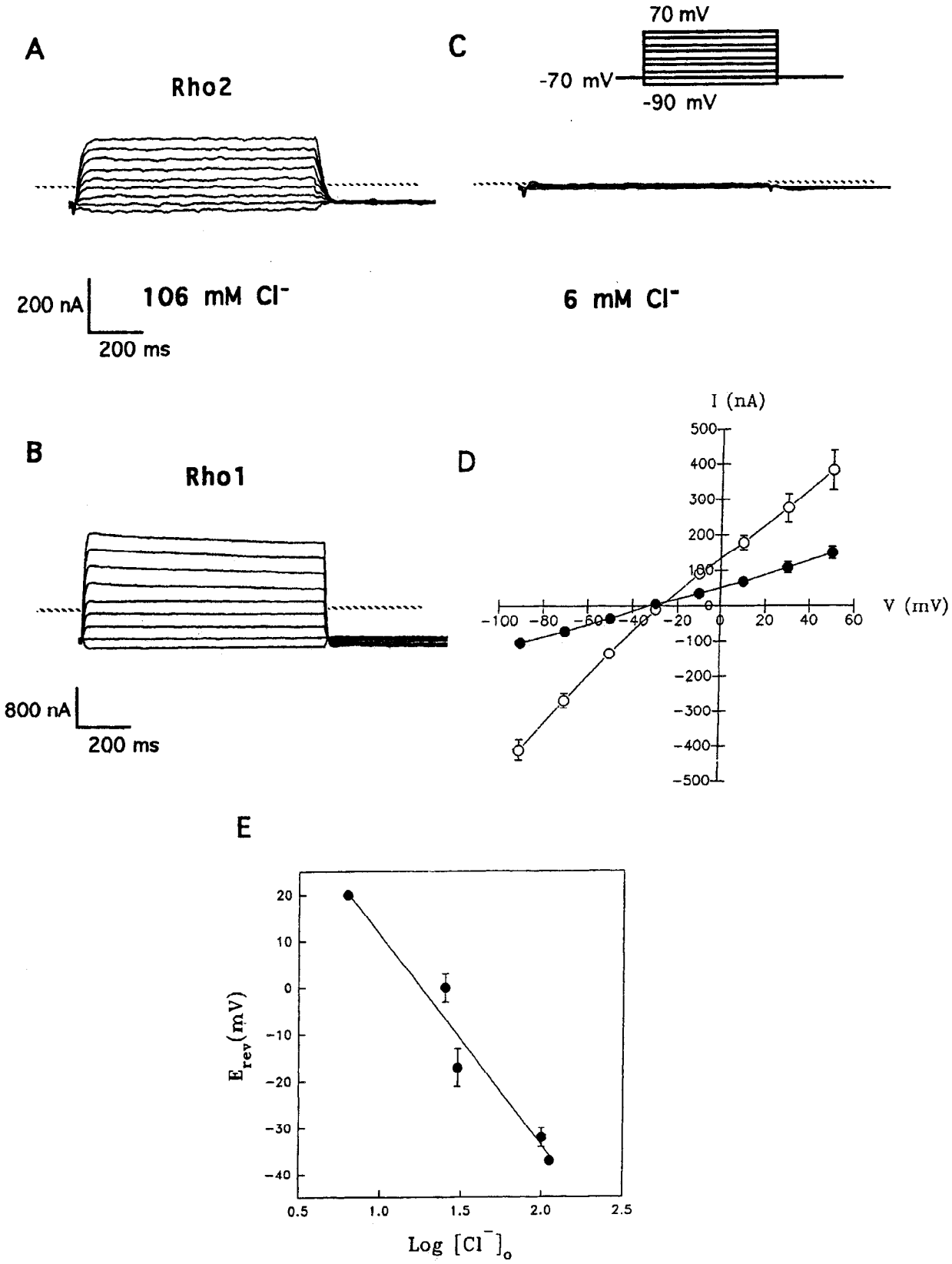

Figure 1. GABA-induced currents in ρ_2 and ρ_1 RNA-injected *Xenopus* oocytes. Currents were evoked by 5 μM GABA and were measured from oocytes injected with 20 ng of ρ_2 RNA (*A* and *C*) and 1 ng of ρ_1 RNA (*B*) at various command potentials (the voltage step protocol is indicated at right side). In *C*, the same oocyte as in *A* was perfused with 6 mM Cl^- for 20 min and then activated with 5 μM GABA. Data shown are net currents representing the GABA-induced currents subtracted from control currents (currents measured in the absence of GABA). *D*, Current–voltage (*I–V*) relationships of GABA ρ_2 and ρ_1 receptors. GABA (5 μM) was superfused to ρ_2-injected oocytes (●) and 1 μM GABA was superfused to ρ_1-injected oocytes (○). Negative currents correspond to inward currents. The GABA-activated currents reversed between −20 and −30 mV. Data were averages obtained from five different oocytes in each group, and expressed as mean ± SE (some of the ρ_2 error bars are small and within the data points). *E*, Effect of varying extracellular Cl^- concentration upon the reversal potential (●) of ρ_2 receptors. Data are expressed as mean ± SE (n = 3).

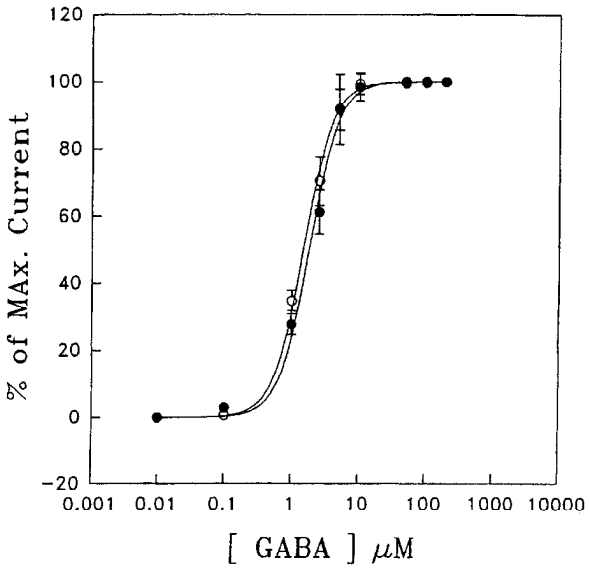

Figure 2. Dose–responses curve of GABA ρ_2 and ρ_1 receptors. Dose–response curves were constructed in a sequential manner with washes for 3 min between applications of different GABA concentrations. *Solid circles* depict the ρ_2 response, and *open circles*, the ρ_1 response. The data measured at each concentration were expressed as a percentage of the maximal current amplitude induced by 100 μM GABA. The holding potential was −70 mV. Data = mean ± SE (n = 5).

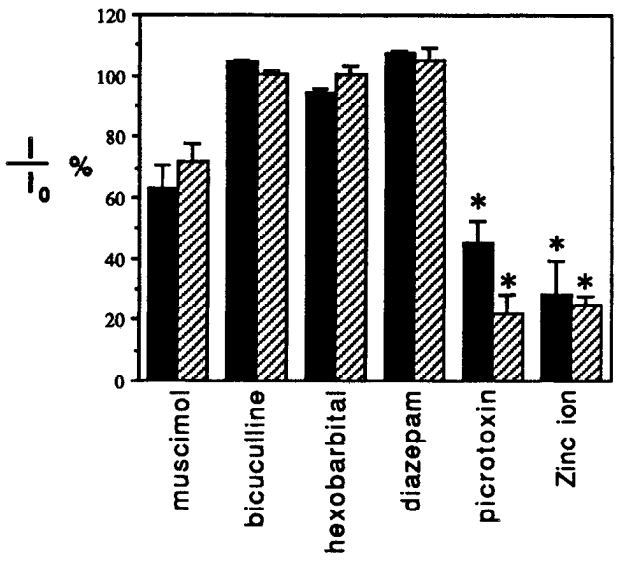

Figure 3. Pharmacological properties of GABA ρ_2 and ρ_1 receptors. *Solid columns* are the responses of ρ_2 RNA-injected oocytes, and *hatched columns* are the responses of ρ_1-injected oocytes to the following applications: 5 μM muscimol, 100 μM bicuculline + 5 μM GABA, 100 μM hexobarbital + 5 μM GABA, 100 μM diazepam + 5 μM GABA; 50 μM picrotoxin + 5 μM GABA, and 50 μM $ZnCl_2$ + 5 μM GABA. The peak current amplitudes were measured from oocytes exposed to 5 μM GABA (I_o) and to drugs coapplied with 5 μM GABA (I). Data were expressed as I/I_o percentages and plotted as mean ± SE from five determinations. *Asterisks* indicate drugs that have a statistically significant effect upon the GABA response.

induced currents in both ρ_2 and ρ_1 RNA injected oocytes. When 50 μM picrotoxin was coapplied with 5 μM GABA, the currents diminished by 55.1 ± 7.4% (n = 6) for ρ_2 receptors and 78 ± 6.2% (n = 7) of ρ_1 receptors. In the absence of GABA, application of picrotoxin to oocytes injected with either ρ_2 or ρ_1 mRNA did not cause any change in resting membrane potentials or currents (n = 3, data not shown).

The bicuculline-, barbiturate-, and benzodiazepine-insensitive properties of ρ_2 receptors are similar to those previously reported for ρ_1 receptors (Shimada et al., 1992). More extensive studies of the responses of ρ_2 and ρ_1 receptors to muscimol, picrotoxin, and Zn^{2+} were performed. The EC_{50} derived from the muscimol dose–response curves was 2 ± 0.2 μM for ρ_2 (n = 3) and 2.1 ± 0.3 μM for ρ_1 (n = 3). After normalization to their own maximal-response currents, the dose–response curves for muscimol and GABA for both ρ_2 and ρ_1 receptors were similar. Typically, currents were first detected at 0.1 μM of muscimol, with a steep rise that saturated at 10 μM.

Picrotoxin inhibition curves for ρ_1 receptors at three GABA concentrations are shown in Figure 4. The picrotoxin concentration that inhibited 50% of the maximal current response (IC_{50}) measured at 1 μM GABA was 0.88 μM (n = 5). The IC_{50} values increased dramatically with increased agonist concentration. At 5 μM GABA the IC_{50} was 27 ± 0.2 μM (n = 5) and at 20 μM GABA increased to 50 ± 0.3 μM (n = 5). Due to extremely low currents generated by ρ_2 receptors at 1 μM GABA, picrotoxin inhibition curves could be established only at GABA concentrations of 5 μM and 20 μM. Receptors formed from ρ_2 subunits displayed similar IC_{50} values to ρ_1 receptors. At 5 μM GABA the IC_{50} was 29 ± 0.6 μM (n = 5) and increasing the GABA concentration above saturation (to 20 μM) produced a modest increase in the IC_{50} value to 52 ± 0.8 (n = 5).

In the presence of 5 μM GABA, Zn^{2+} ions showed a potent inhibitory effect on ρ receptors with IC_{50} of 21 ± 5 μM (n = 3) for ρ_2 and 16.2 ± 4.5 μM (n = 3) for ρ_1 (Fig. 5). The GABA-induced currents generated by these two receptors was totally inhibited by ≥ 250 μM $ZnCl_2$. The EC_{50} values for picrotoxin and Zn^{2+} were not significantly different between these two ρ receptors. The inhibitory effect of $ZnCl_2$ for both ρ_2 and ρ_1 receptors was reversed by adding EDTA (1 mM; Fig. 5*B*). In the absence of $ZnCl_2$, EDTA did not influence GABA-induced currents (n = 4). This finding indicates that EDTA does not have a direct effect on the GABA-activated currents but can reverse Zn^{2+} inhibition by chelating Zn^{2+}.

Currents gradually decreased in oocytes expressing ρ_2 and ρ_1 receptors with longer recording times with GABA suggestive of receptor desensitization (Moss et al., 1992). Both receptors displayed slow-phase desensitization but at distinctly different rates (Fig. 6). After 70 sec of exposure to 5 μM GABA, ρ_1-generated currents decreased by 30 ± 7.2% of the maximum peak current (n = 4). In contrast, ρ_2-generated currents decreased only by 5 ± 0.7% (n = 6) after exposure for 70 sec to 5 μM GABA. The finding that ρ_2 receptors are more refractory to desensitization than ρ_1 receptors is consistent in different batches of oocytes.

Discussion

The GABA ρ_2 subunit cloned from human retina complementary DNA library represents the second member of a novel GABA receptor subunit class (Cutting et al., 1991, 1992). Expression of this subunit in *Xenopus* oocytes resulted in efficient formation of homooligomeric GABA-gated chloride channels. In contrast to ρ subunit expression, homooligomeric expression for $GABA_A$ receptor subunits is relatively inefficient in either *Xenopus* oocyte or mammalian expression systems (Blair et al., 1988; Pritchett et al., 1988). GABA-elicited currents from hom-

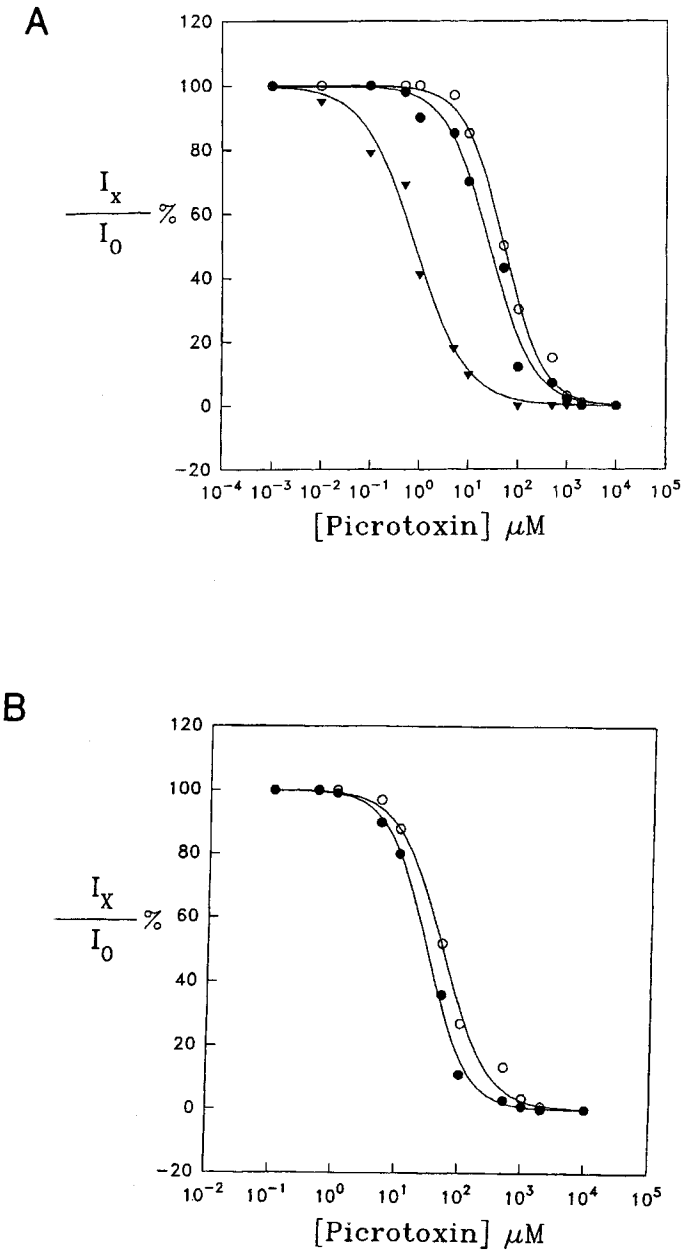

Figure 4. Dose-dependent inhibition curves for GABA ρ_1 (*A*) and ρ_2 (*B*) receptors by picrotoxin. Experiments were carried out for three concentrations of GABA for ρ_1 (*A*; 1 μM, ▼; 5 μM, ●; 20 μM, ○) and two concentrations for ρ_2 (*B*; 5 μM, ●; 20 μM, ○) over a wide range of picrotoxin concentrations. The data are expressed as I_x/I_o (%) versus x concentration of drugs. I_o is the current response of 1, 5, or 20 μM GABA; I_x is the current response of 1, 5, or 20 μM GABA coapplied with a certain concentration of picrotoxin. Data are mean ± SE (n = 5).

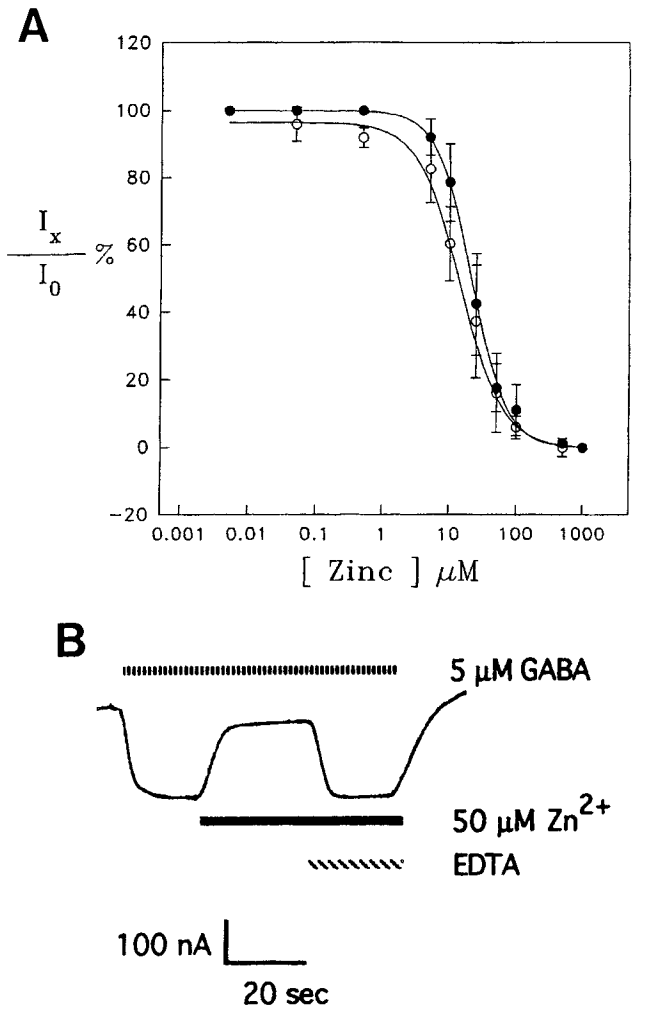

Figure 5. *A*, Dose-dependent inhibition curves for GABA ρ_2 and ρ_1 receptors over a wide range of Zn^{2+} concentrations. I_o and I_x are current responses of 5 μM GABA and 5 μM GABA coapplied with a certain concentration of Zn^{2+}. Data are mean ± SE (n = 5). *Solid circles* are ρ_2; *open circles* are ρ_1. *B*, Representative tracings of the effect of zinc ions on GABA ρ_1 currents from 5 μM GABA application and the complete reversal of zinc inhibition by EDTA. The *horizontal bars* correspond to the drug application periods. The membrane potential was continuously held at −70 mV.

ooligomeric ρ_2 (>100 nA) and ρ_1 (>1 μA) receptors in oocytes were at least 30-fold larger than those from $GABA_A$ homooligomeric receptors (<3 nA; Sigel et al., 1990). Spontaneous activation of picrotoxin blockable currents in the absence of agonist is believed to be due to inefficient assembly of homooligomeric $GABA_A$ receptors (Sigel et al., 1989; Zaman et al., 1992). This was not observed for homooligomeric receptors formed from ρ_2 or ρ_1 subunits. The ρ receptors display a high affinity toward GABA with an EC_{50} between 1.5 to 2 μM whereas EC_{50} values for $GABA_A$ receptors range from 1.5 to 100 μM (Schofield et al., 1987; Ymer et al., 1989a,b; Sigel et al., 1990). Significant positive cooperativity was also found in ρ receptors as indicated by a Hill coefficient ranging from 2.3 to 2.5, which is higher than those in $GABA_A$ receptors either in homooligomeric or heterooligomeric forms (Sigel et al., 1990; Polenzani et al., 1991). This result suggests that the ρ receptors either have a stronger allosteric interaction among subunits or have more GABA binding sites than receptors formed from $GABA_A$ subunits.

GABA receptors formed from ρ_2 subunits are insensitive to bicuculline (a competitive antagonist to $GABA_A$ receptors), diazepam, or barbiturates (typical potentiators for $GABA_A$ receptors). This pharmacologic profile is identical to that exhibited by ρ_1 receptors (Shimada et al., 1992) and indicates that these characteristics are conferred by sequences in common between the two subunits. Similar responses of ρ_2 and ρ_1 homooligomeric receptors to an extensive panel of agonists further supports this concept (Kusama et al., 1993a). Interestingly, a novel GABA

receptor subunit *Rdl* cloned from *Drosophila* forms bicuculline-insensitive homooligomeric GABA-gated chloride channels when expressed in *Xenopus* oocytes (ffrench-Constant, 1991, 1993). Similar to the ρ subunits, the *Rdl* receptor exhibits robust chloride currents (>1 μA). Comparison of the predicted amino acid sequences, however, reveals that homology of the *Rdl* subunit with the ρ subunits is no greater than with any of the $GABA_A$ subunits. This may suggest that robust homooligomeric expression and bicuculline insensitivity is conferred by groups of amino acids rather than single residues.

Additional properties of ρ_2 and ρ_1 receptors have been observed in this study that further distinguish this subunit class from conventional $GABA_A$ subunits. Muscimol, a potent GABA receptor agonist, induced a response that was only 60–70% of GABA-induced responses in ρ receptors. This finding is consistent with those reported for ρ_1 receptors expressed in either *Xenopus* oocytes or mammalian cells (Kusama et al., 1993b). In contrast, in $GABA_A$ receptors muscimol is typically more potent by almost 2–10-fold than GABA (Kusama et al., 1993b; Woodward et al., 1993). The inhibitory effect of picrotoxin upon ρ_1 receptors at 1 μM GABA was similar to that previously reported (Shimada et al., 1992). However, the picrotoxin sensitivity of ρ_1 receptors was very dependent upon agonist concentration. For example, 100 μM picrotoxin inhibited approximately 95% of currents elicited by 1 μM GABA, 80% of those elicited by 5 μM GABA but only 45% elicited by 20 μM GABA. The ρ_2 receptors displayed a similar profile of inhibition at the two higher concentrations of agonist (5 μM and 20 μM). The ρ receptors have higher IC_{50} values than reported for $GABA_A$ receptors at these agonist concentrations indicating that the ρ receptors are considerably less sensitive to the inhibitory effect of picrotoxin (Sigel et al., 1990; Woodward et al., 1992). This property was probably not appreciated in prior studies of the homooligomeric ρ_1 receptor since picrotoxin sensitivity was measured at only one concentration of agonist (1 μM GABA; Cutting et al., 1991; Shimada et al., 1992).

Zinc is thought to play a modulatory role for some ligand-gated channels because of its abundance in synaptic vesicles and nerve terminals and its release upon neuronal excitation (Howell et al., 1984; Xie and Smart, 1991). Studies of receptors formed from cloned $GABA_A$ subunits have shown that γ subunits are more resistant to Zn^{2+} inhibition than α and β subunits and coexpression of γ_1, γ_2, or γ_3 subunits with α and β subunits lead to the formation of Zn^{2+}-insensitive receptors (Draguhn et al., 1990). In this study, Zn^{2+} inhibited the Cl^- current of ρ receptors that was rescued by EDTA, indicating that ρ subunits have Zn^{2+} interacting regions. These results also suggest that Zn^{2+} could play a role in modulating bicuculline-insensitive GABA receptors in the retina.

Although ρ_1 and ρ_2 receptors shared similar pharmacological features, they differ in several physiological aspects. For example, ρ_1 receptors are robustly expressed in *Xenopus* oocytes. In contrast, ρ_2 currents are significantly smaller even when we inject 20 times more RNA (20 ng) in the same batch of oocytes. This difference could be related to the intrinsic differences in the characteristics of ρ_2 and ρ_1 receptors. Alternatively, the reduced amplitude of ρ_2 receptors in oocyte expression system may be due to RNA instability, less efficient translation or subunit assembly, or defective processing and targeting to the plasma membrane. Both receptors desensitize during prolonged agonist application. The desensitization kinetics were independent of the level of protein expression in the oocytes. The rate of

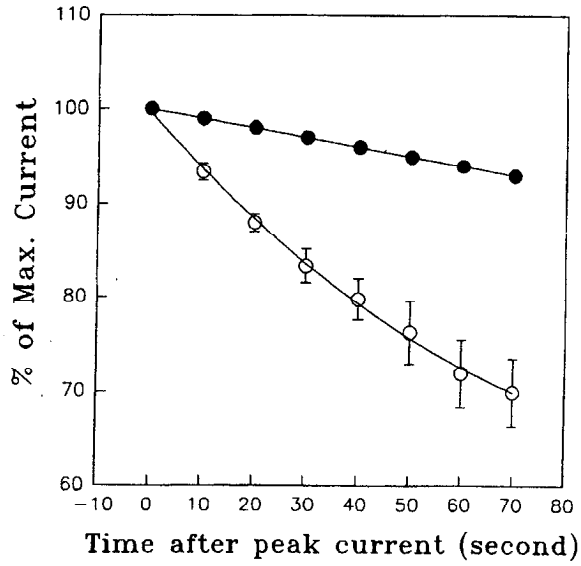

Figure 6. Desensitization of GABA ρ_1 and ρ_2 receptors with 5 μM GABA exposure. *Solid circles* are the tracing from ρ_2 RNA-injected oocytes (n = 6), and *open circles* are the tracing from ρ_1 RNA-injected oocytes (n = 6). The currents were measured at every 10 sec interval and only the currents at and after the maximal inward currents were plotted. The degrees of desensitization were represented as 1 − (decayed current/maximal current). Data are expressed as mean ± SE; the error bars of ρ_2 are too small and therefore are within the data points (●).

desensitization for ρ_1 receptors was consistently faster than that of ρ_2 receptors, suggesting an intrinsic difference between these two subunits. Interestingly, there are three potential protein kinase C (PKC) phosphorylation sites in the cytoplasmic loop of ρ_1 molecule, whereas ρ_2 sequence does not encode any consensus sequences for PKC phosphorylation. In view of recent reports that protein kinase A (PKA) or PKC may be involved in the mechanisms of desensitization (Browning et al., 1990; Porter et al., 1990; Revah et al., 1991), it is possible that modulation by PKC is responsible for the more rapid desensitization of the ρ_1 receptor.

ρ receptors have characteristics similar to a special group of GABA receptors in the nervous system from a variety of vertebrate species. Bicuculline- and barbiturate-insensitive GABA-gated Cl^- channels have been found in the visual pathways and certain specific areas in the CNS (Sivilotti and Nistri, 1989), and may be similar to the "$GABA_C$" receptors defined by Johnston (1986). When bovine retinal mRNA was expressed in *Xenopus* oocytes, the predominant GABA-activated Cl^- currents were unresponsive to bicuculline, barbiturates, and benzodiazepine (Polenzani et al., 1991; Woodward et al., 1992, 1993). These characteristics are strikingly similar to ρ receptor currents. Bicuculline-insensitive GABA-activated whole-cell currents with slow desensitization kinetics have also been characterized in white perch retinal horizontal cells and rat retinal bipolar cells (Feigenspan et al., 1993; Qian and Dowling, 1993). Muscimol was reported to be only a partial agonist for these receptors with EC_{50} values similar to those obtained for ρ receptors (Qian and Dowling, 1993; Woodward et al., 1993). Finally, bicuculline-insensitive GABA receptors characterized from bovine retina and rat retinal bipolar cells were considerably less sensitive to picrotoxin than $GABA_A$ receptors (Feigerspan et al., 1993; Woodward et al., 1993). The level of picrotoxin sensitivity dis-

played by these retinal GABA receptors was very similar to that reported here for homooligomeric ρ receptors.

Since homooligomeric receptors formed from ρ subunits display a number of features in common with bicuculline-insensitive GABA receptors characterized in retina, it is plausible that this type of GABA receptor may be composed entirely of ρ_1 or ρ_2 subunits. This hypothesis is supported by the identification of partial cDNA sequences from ρ-like subunits in rat and perch retina (R. Hazra and G. R. Cutting, unpublished observations). A second possibility is that bicuculline-insensitive GABA receptors *in vivo* are heteroologimers formed from more than one type of ρ subunit or from ρ subunits complexed with other subunits. Preliminary experiments suggest that ρ_1 and ρ_2 subunits can interact and form receptors with features distinct from homooligomeric ρ_1 and ρ_2 receptors (T.-L. Wang et al., unpublished observations). Regarding the latter possibility, coexpression studies suggest that ρ_1 subunits do not associate with $GABA_A$ subunits, although this has not been definitively proven (Shimada et al., 1992). In summary, ρ_2 and ρ_1 homooligomeric receptors expressed in *Xenopus* oocytes share a number of pharmacologic and electrophysiologic features that distinguish the ρ subunit class from the four classes of $GABA_A$ receptor subunits. The high degree of amino acid similarity between the ρ_1 and ρ_2 subunits provides an opportunity to determine the sequences responsible for these distinctive properties. Identification of the ρ receptor subunits increases the functional diversity of this GABA receptor family and appears to provide molecular evidence for the existence of bicuculline-insensitive GABA receptors in retina.

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