Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11756–11760, December 1995
Neurobiology

Cloning of a γ-aminobutyric acid type C receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block

(ρ subunits/inhibitory neurotransmission/ligand-gated chloride channels, GABA receptor)

DONGXIAN ZHANG, ZHUO-HUA PAN, XISHAN ZHANG, AMY D. BRIDEAU, AND STUART A. LIPTON*

Laboratory of Cellular and Molecular Neuroscience, Children's Hospital and Program in Neuroscience, Harvard Medical School, Boston, MA 02115

Communicated by George Wald, Harvard University, Cambridge, MA, August 11, 1995

ABSTRACT **Ionotropic receptors for γ-aminobutyric acid (GABA) are important to inhibitory neurotransmission in the mammalian retina, mediating $GABA_A$ and $GABA_C$ responses. In many species, these responses are blocked by the convulsant picrotoxinin (PTX), although the mechanism of block is not fully understood. In contrast, $GABA_C$ responses in the rat retina are extremely resistant to PTX. We hypothesized that this difference could be explained by molecular characterization of the receptors underlying the $GABA_C$ response. Here we report the cloning of two rat GABA receptor subunits, designated rρ1 and rρ2 after their previously identified human homologues. When coexpressed in *Xenopus* oocytes, rρ1/rρ2 heteromeric receptors mimicked PTX-resistant $GABA_C$ responses of the rat retina. PTX resistance is apparently conferred in native heteromeric receptors by rρ2 subunits since homomeric rρ1 receptors were sensitive to PTX; rρ2 subunits alone were unable to form functional homomeric receptors. Site-directed mutagenesis confirmed that a single amino acid residue in the second membrane-spanning region (a methionine in rρ2 in place of a threonine in rρ1) is the predominant determinant of PTX resistance in the rat receptor. This study reveals not only the molecular mechanism underlying PTX blockade of GABA receptors but also the heteromeric nature of native receptors in the rat retina that underlie the PTX-resistant $GABA_C$ response.**

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (1). In the GABA receptor family, the $GABA_A$ receptor gates a Cl^- channel, while the $GABA_B$ receptor is coupled to a guanine nucleotide binding protein (2). These receptors have been characterized by their differential responses to selective agonists and antagonists. $GABA_A$-mediated responses can be blocked by a selective antagonist, bicuculline, and $GABA_B$-mediated effects can be activated by a specific GABA analog, baclofen (3). Recently, a third type of GABA response has been described that, unlike the others, is bicuculline and baclofen insensitive and has been designated $GABA_C$ (2, 4). Like $GABA_A$, the $GABA_C$ response is mediated by a Cl^- current, but the pharmacology is clearly different. $GABA_C$ responses were first reported in the retina (5–12) but recently have also been found in the hippocampus (13).

GABA ρ subunits, cloned from the human retina by Cutting and colleagues (14–17), have been postulated to underlie $GABA_C$ responses. The pharmacology of human ρ receptors is similar to that reported for $GABA_C$ responses of rat and fish retinal neurons or *Xenopus* oocytes injected with bovine retinal mRNA (5–7, 14, 16, 17). Strikingly, however, $GABA_C$ responses are quite sensitive to picrotoxinin (PTX) in each of these preparations except rat retinal neurons (6, 8). Although PTX is thought to block vertebrate GABA receptors by acting directly or allosterically in the channel (4, 18), the molecular mechanism of its action is still not completely understood. Human ρ subunits are capable of forming functional homomeric receptors, but heretofore the native subunit composition of receptors formed from ρ subunits was not clear. One hypothesis for the difference in PTX sensitivity of rat $GABA_C$ responses involves formation of heteromeric receptors (6). Thus, we searched for possible variants of GABA ρ subunits in the rat retina.

Here we report the cloning of two GABA subunits from the rat retina that are homologous to the previously reported human ρ subunits (14, 15). Most importantly, one of the rat subunits, unlike its human homologue, contains a single amino acid difference of fundamental importance to the pharmacology of the native responses. Functional expression in *Xenopus* oocytes demonstrated that this subunit (ρ2) is responsible for PTX resistance of the rat receptor. Site-directed mutagenesis confirmed that PTX resistance in the native receptor is determined by a single amino acid, a methionine instead of a threonine in the second membrane region (M2) of the ρ2 subunit compared to the ρ1 subunit. Preliminary reports of our findings have been presented (19, 20).†

MATERIALS AND METHODS

cDNA Cloning. Reverse transcription PCR experiments were conducted using postnatal day 12 rat retinal total RNA with forward (5′-TGCNACATGGACTTCNGCC-3′) and reverse (5′-CGAACACAAAGCTGACCCAG-3′) primers based on the sequence of the human GABA ρ1 subunit (N = mixed nucleotides of G, C, A, and T). cDNA fragments thus obtained were subcloned, sequenced, and used as probes to screen a λ ZAP II (Stratagene) rat retinal cDNA library. Bluescript plasmids were excised from positive clones and analyzed by automated sequencing to yield the rat ρ1 subunit. Point mutations were introduced into cDNA clones by double-stranded, site-directed mutagenesis (Stratagene) and confirmed by sequencing.

***In Vitro* Transcription, Expression, and Oocyte Recording.** Rat ρ subunits were studied by using a two-microelectrode voltage clamp in the *Xenopus laevis* oocyte expression system 2–9 days after injection of 20–60 ng of complementary RNA (cRNA) (21). Drugs were applied via a rapid superfusion system. Data were sampled at 2.2–11.1 Hz at room temperature.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GABA, γ-aminobutyric acid; PTX, picrotoxinin; hρ1, human GABA ρ1 subunit; rρ1, rat GABA ρ1 subunit; cRNA, complementary RNA.

*To whom reprint requests should be addressed at: Children's Hospital, 300 Longwood Avenue, Enders Building, Suite 361, Boston, MA 02115.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U21070).

RESULTS

Cloning Rat GABA ρ Subunits. Screening a rat retinal cDNA library with probes obtained by reverse transcription PCR, we isolated clones of high sequence identity with the previously cloned human GABA ρ subunits (14, 15). One of these clones contained an open reading frame of 1422 bp encoding a predicted protein of 474 amino acids. This protein shared 95% (449/473) identical amino acids with the human ρ1 receptor subunit (hρ1) and was therefore designated rat ρ1 (rρ1; Fig. 1). The major differences between hρ1 and rρ1 occurred near the N terminus and between M3 and M4; the conserved regions among all GABA receptors, such as the Cys–Cys loop and the membrane regions, were preserved. The nonidentical amino acid residues did not alter the predicted functional motifs, including glycosylation and phosphorylation sites (14). A second clone, designated rρ2, had a deduced amino acid sequence that shared 91% (423/465) amino acid identity and the same length as hρ2 (Fig. 1) (15). Comparison of hρ2 and rρ2 revealed one additional phosphorylation site for cAMP-dependent kinase in rρ2 between M3 and M4. Like the human ρ subunits, rρ1 and rρ2 were quite similar to each other, especially in the membrane-spanning regions. Thus, we were surprised to find a methionine in rρ2 in place of a threonine in rρ1 at a site that is highly conserved among all other ligand-gated Cl^- channels in the M2 region.

```
Rat ρ1    ....MKFGIFLLWWGWVLAAESTVHWPGREVHEPSKKGSRPQRQRRGAHD  46
Human ρ1  ....MRFGI__LWWGWVLAT__RMHWPGREVHEMSKKG.R_Q_Q_REVHE  45
Rat ρ2    MPYFMRLAL__..FCLMALV__R.................K_R_K_WTGHL  32
Human ρ2  MPYFTRLIL__..FCLMVLV__R.................K_K_K_WTGQV  32

Rat ρ1    DAHKQGSPILKRSSDITKSPLTKSEQLLRIDDHDFSMRPGFGGPAIPVGV  96
Human ρ1  DAH_QV_PILRRSP_I__SPLT_SEQ___IDD___S___G__________  95
Rat ρ2    ETS_P._HLYKKNL_V__IRTG_PRP___VED___T___A__________  81
Human ρ2  EMP_P._HLYKKNL_V__IRKG_PQQ___VDE___S___A__________  81

Rat ρ1    DVQVESLDSISEVDMDFTMTLYLRHYWKDERLSFPSTNNLSMTFDGRLVK 146
Human ρ1  _____________________________K____S_P_TN_L_______ 145
Rat ρ2    _____________________________R____A_P_SS_R_______ 131
Human ρ2  _____________________________K____A_S_AS_K_______ 131

                                                        |---
Rat ρ1    KIWVPDMFFVHSKRSFIHDTTTDNVMLRVQPDGKVLYSLRVTVTAMCNMD 196
Human ρ1  ______M_________I_______V____Q___K____L_V_______ 195
Rat ρ2    ______V_________T_______I____F___H____M_I_______ 181
Human ρ2  ______V_________T_______I____F___H____M_I_______ 181

          ----------|
Rat ρ1    FSRFPLDTQTCSLEIESYAYTEDDLMLYWKKGNDSLKTDERISLSQFLIQ 246
Human ρ1  __R____T_______I_____ED____Y__K_ND_____R_______ 245
Rat ρ2    __H____S_______L_____DE____Y__N_DE_____K_______ 231
Human ρ2  __H____S_______L_____DE____H__N_DE_____K_______ 231

                                        M1
Rat ρ1    EFHTTTKLAFYSSTGWYNRLYINFTLRRHIFFFLLQTYFPATLMVMLSWV 296
Human ρ1  E____TK_____________________________________ 295
Rat ρ2    K____SR_____________________________________ 281
Human ρ2  K____SR_____________________________________ 281

                 M2                                  M3
Rat ρ1    SFWIDRRAVPARVPLGITTVLTMSTIITGVNASMPRVSYIKAVDIYLWVS 346
Human ρ1  _____R_____P___T_____S_____________IK_______ 345
Rat ρ2    _____H_____S___M_____S_____________IR_______ 331
Human ρ2  _____R_____S___T_____T_____________VK_______ 331

          ------
Rat ρ1    FVFVFLSVLEYAAVNYLTTVQERKERKLREKISCTCGLPQPRGVMLDSSY 396
Human ρ1  _____________________R__Q___E_LP_TS_LPPPRTAM__GN_ 395
Rat ρ2    _____________________Q__R___D_FP_TC_MLHSRTMT__GS_ 381
Human ρ2  _____________________R__R___E_FP_MC_MLHSKTMM__GS_ 381

Rat ρ1    SDGEVNDLGGY....MPENGEKPDRMMVQLTLASERGSPQRKSQRGSYVS 442
Human ρ1  _DG_V_D_DN_....MPENG_KP_RMM_Q_T_AS_RSSPQR_SQRSSYVS 441
Rat ρ2    _ES_A_S_AG_PRSHILPEE_RQ_KIV_H_A_NS_LTSSRK_GLLKGQMG 431
Human ρ2  _ES_A_S_AG_PRSHILTEE_RQ_KIV_H_G_SG_ANAARK_GLLKGQTG 431

                                    M4
Rat ρ1    MRI..NTHAIDKYSRIIFPAAYILFNLIYWSIFS 474
Human ρ1  MR_..D_________I____A__L_______I__ 473
Rat ρ2    LY_FQN_________L____F__V_______V__ 465
Human ρ2  FR_FQN_________L____S__F_______V__ 465
```

FIG. 1. Comparison of deduced amino acid sequences of the GABA ρ1 and ρ2 subunits from human and rat. Conserved amino acids are listed only for rρ1. Differences between homologous subunits of different species are indicated (* for rρ1 vs. hρ1 and # for rρ2 vs. hρ2). The regions of the Cys–Cys loop (dashed line) and the four proposed membrane-spanning motifs (solid lines) are preserved across the human and rat genes.

Pharmacology of Recombinant Rat GABA ρ Subunits. In oocytes, rρ1 subunits, but not rρ2 subunits, formed functional homomeric GABA receptors. Many characteristics of homomeric rρ1 receptors were similar to those of human ρ receptors (16, 17), to $GABA_C$ responses encoded by bovine retinal mRNA (7), and to $GABA_C$ responses of primary retinal neurons (5, 6, 8–12). These properties included little if any desensitization to GABA and insensitivity to diazepam or pentobarbital (data not shown). The GABA dose–response curve for homomeric rρ1 receptors was well fit by the Hill equation with EC_{50} = 1.1 ± 0.1 μM (mean ± SEM; n = 17 oocytes) and a Hill coefficient of 2.3 ± 0.1 (Fig. 2*A*). Although this EC_{50} was slightly lower than those of their human ρ1 and ρ2 counterparts, reported to be 1.8 and 1.7 μM, respectively (17), the Hill coefficient was nearly identical (17). However, unlike the native $GABA_C$ responses of rat retinal neurons (6, 8), homomeric rρ1 receptors were relatively sensitive to PTX, similar to receptors formed from hρ1 or hρ2 subunits. PTX

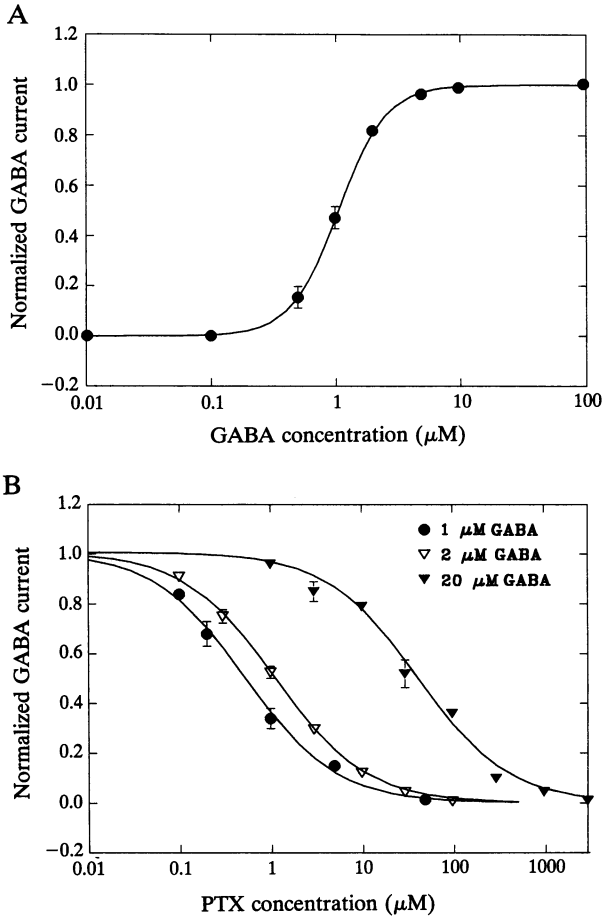

FIG. 2. GABA dose–response curve and PTX dose-inhibition curve for homomeric rρ1 receptors. (*A*) GABA activates rρ1 homomeric receptors with EC_{50} = 1.1 ± 0.1 μM (mean ± SEM; n = 17 oocytes) and a Hill coefficient of 2.3 ± 0.10. GABA responses were normalized to that of 100 μM GABA for each oocyte. (*B*) Dose-dependent inhibitory effects of PTX on GABA-evoked currents from homomeric rρ1 receptors. IC_{50} values of PTX for 1, 2, and 20 μM GABA were 0.52 ± 0.10 (n = 4), 1.1 ± 0.1 (n = 6), and 40.0 ± 5.5 (n = 8), respectively.

inhibition of homomeric rρ1 responses was dependent on GABA concentration (Fig. 2*B*), similar to homomeric hρ1, hρ2, and other preparations (7, 9, 16, 17).

rρ2 Subunits Confer PTX Resistance. Although rρ2 subunits did not form functional homomeric receptors, coexpression of rρ1/rρ2 resulted in heteromeric GABA receptors that were resistant to PTX. We initially evaluated PTX sensitivity by using 100 μM PTX to block 2 μM GABA-evoked currents; responses in oocytes injected with rρ1 cRNA alone were almost completely blocked under these conditions (Fig. 3*A*). As we increased the percentage of rρ2 coinjected with rρ1 cRNA, the proportion of PTX-resistant GABA current increased (Fig. 3 *B* and *C*). The dependence of PTX resistance on rρ2 was evaluated quantitatively (Fig. 4*A*). The receptors underlying

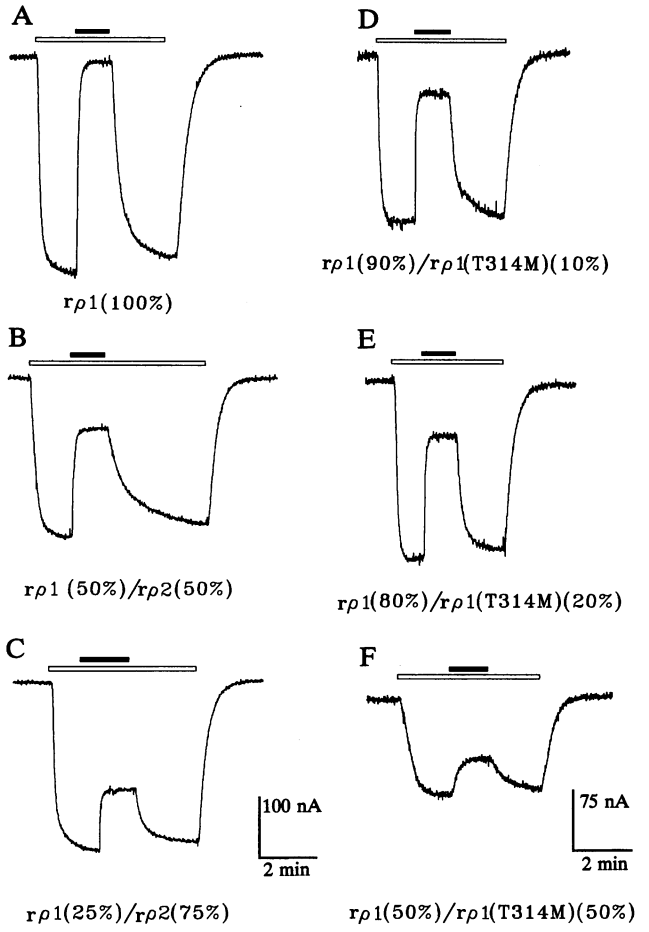

FIG. 3. Correlation of PTX resistance with the percentage of rρ2 or mutated rρ1(T314M) cRNA coinjected with rρ1 cRNA into *Xenopus* oocytes. A total of 30 ng of cRNA was injected into each oocyte, except in *C* and *F* where 60 ng was injected to increase the amplitude of the currents. PTX (100 μM; solid bar) was used to test the antagonist sensitivity of the responses to GABA (2 μM; open bar). (*A*) For homomeric rρ1 receptors, PTX at a concentration of 100 μM almost totally blocked current evoked by 2 μM GABA (degree of inhibition, 99.3% ± 0.3%, mean ± SEM; $n = 6$). (*B*) PTX blocked 74% ± 1.9% ($n = 6$) of the GABA-evoked current when 50% rρ1 cRNA was coinjected with 50% rρ2 cRNA. (*C*) PTX blocked 51% ± 4% ($n = 7$) of the GABA-evoked current when 25% rρ1 cRNA was coinjected with 75% rρ2 cRNA. (*D*) PTX blocked 78% ± 0.6% ($n = 4$) of the GABA-evoked current when 10% wild-type rρ1 cRNA was coinjected with 90% mutated rρ1(T314M) cRNA. (*E*) PTX blocked 64% ± 2.3% ($n = 4$) of the GABA-evoked current when 80% wild-type rρ1 cRNA was coinjected with 20% rρ1(T314M) cRNA. (*F*) PTX blocked 36% ± 4.0% ($n = 7$) of the GABA-evoked current when 50% wild-type rρ1 cRNA was coinjected with 50% rρ1(T314M) cRNA. GABA-evoked currents became smaller in magnitude as the percentage of mutated cRNA increased.

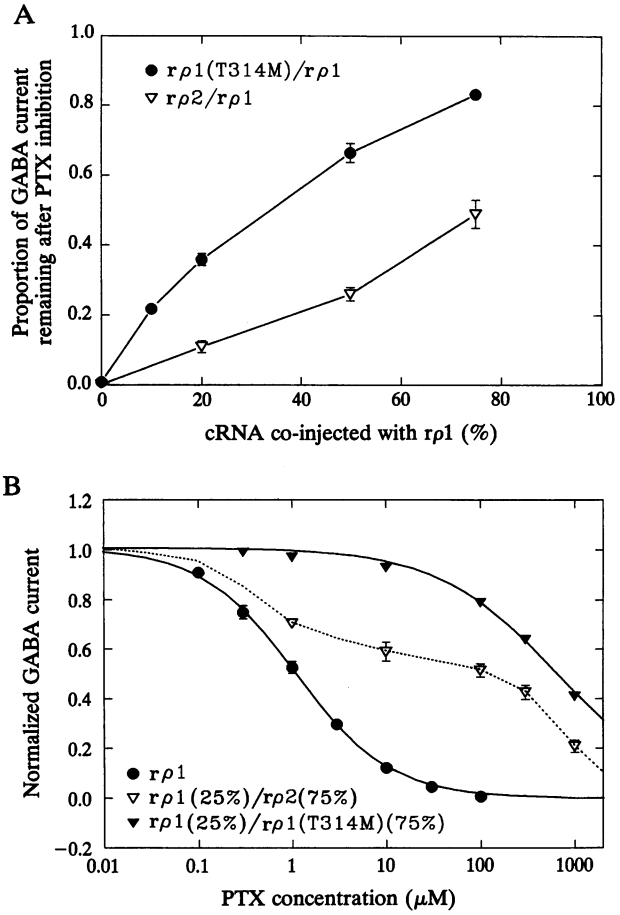

FIG. 4. Composition of ρ subunits influences the proportion of GABA current not blocked by a fixed concentration of PTX and the dose-inhibition curve of PTX. (*A*) Plot of the GABA current remaining in the presence of 100 μM PTX vs. percentage rρ2 cRNA (open triangles) or rρ1(T314M) cRNA (solid circles) coinjected with rρ1 cRNA. GABA responses were normalized to the maximal current evoked in each oocyte. (*B*) Dose-inhibition curves of PTX for 2 μM GABA responses from receptors expressed after coinjection of 25% rρ1 cRNA and 75% rρ2 cRNA (open triangles), coinjection of 25% rρ1 and 75% rρ1(T314M) (solid triangles), or injection of rρ1 subunits alone (solid circles). The dose-inhibition curve of PTX for rρ1/rρ2 subunits displays two components. IC_{50} of PTX for rρ1/rρ1(T314M) subunits was 634 ± 34 μM ($n = 8$).

the component of GABA current blocked by PTX may be composed predominantly of homomeric rρ1 subunits. This is suggested because the PTX dose-inhibition curve after coinjection of ρ subunits [rρ1(25%)/rρ2(75%); Fig. 4*B*] contained two components, one similar to that of homomeric rρ1 receptors (Fig. 4*B*) and a second shifted dramatically to the right.

Although the EC_{50} for heteromeric rρ1/rρ2 receptors was similar to that of homomeric rρ1 receptors, the absolute magnitude of GABA-evoked currents decreased as the proportion of injected rρ2 cRNA increased. This finding suggests that the presence of rρ2 results in a lower efficiency of receptor assembly, as recently proposed (17, 22). Alternatively, the presence of rρ2 may alter receptor–ionophore coupling or channel gating, reducing the overall input/output gain. In any event, the above results lead to two important conclusions. First, rρ1 and rρ2 subunits form functional heteromeric receptors that mimic PTX resistance of native $GABA_C$ responses in the rat retina. Second, PTX resistance appears to be based on the intrinsic structure of the rρ2 subunit.

Site-Directed Mutagenesis Elucidates the Basis for PTX Resistance. To investigate the molecular basis for PTX resistance, we analyzed the amino acid sequence predicted by rρ1

and rρ2. We looked at the M2 region because of reports that PTX acted at least in part by blocking ligand-gated Cl^- channels (refs. 23 and 24, but see refs. 18, 25, and 26). Strikingly, in this region the rρ2 clone differed from rρ1 by only 2 amino acids (Fig. 5). At the first of these sites, however, both rρ1 and hρ1 have a proline residue, while rρ2 and hρ2 have a serine. This difference cannot account for PTX resistance conferred by rρ2; data from Cutting and co-workers (27) suggest that, if anything, receptors composed of hρ2 subunits are more sensitive to PTX than hρ1 subunits, just the opposite of our findings with rat subunits. Moreover, this site is not conserved among GABA and glycine subunits (Fig. 5). In contrast, the amino acid residue at the second site of interest is conserved in all known GABA subunits as a threonine, except in rρ2 where a methionine resides. This residue was therefore likely to be critical for PTX resistance.

To determine whether this amino acid residue contributes to PTX resistance, we performed site-directed mutagenesis to substitute a threonine for the methionine, yielding rρ2(M299T). Similar to wild-type rρ2, this mutant did not form functional homomeric GABA receptors. However, coexpression of rρ2(M299T) with rρ1 formed functional GABA receptors that were sensitive to PTX. These presumptive heteromeric receptors displayed virtually the same GABA dose–response and PTX-inhibition curves as homomeric rρ1 receptors (data not shown). This raises the possibility, however, that rρ2(M299T) cRNA was not functionally expressed and that only homomeric rρ1 subunits contributed to these responses. This possibility is highly unlikely because of evidence for the formation of heteromeric rρ1/rρ2(M299T) receptors; GABA-evoked currents after coinjection of rρ1/rρ2(M299T) cRNAs were substantially smaller than those recorded after injection of rρ1 cRNA alone but were very similar to their rρ1/rρ2 counterparts. Since rρ1/rρ2 receptors have a different pharmacology (PTX resistance) from homomeric rρ1 receptors, we infer that heteromeric receptors were indeed formed. Taken together, these results suggest that substitution of threonine for methionine at residue 299 of rρ2 eliminates PTX resistance of rρ1/rρ2 receptors. Since a methionine is normally present in this position, this site can account for the PTX resistance of native rat $GABA_C$ receptors.

A remaining question is whether PTX resistance can also be influenced by residue 295 of rρ2 (homologous to residue 310 in rρ1). To investigate this, we used the ability of rρ1 subunits to form homomeric receptors and made an additional mutant, rρ1(P310S). Compared to wild type, homomeric rρ1(P310S) receptors exhibited substantially smaller GABA-evoked currents. Consistent with the report of Cutting and co-workers (27), homomeric rρ1(P310S) receptors displayed a higher EC_{50} (9.1 ± 1.4 μM, mean ± SEM; $n = 5$) and a substantial reduction in the effect of GABA concentration on PTX inhibition. The IC_{50} value of PTX for 2 μM GABA (1.1 ± 0.1 μM; $n = 10$) was virtually the same as for 20 μM GABA (1.2 ± 0.1 μM; $n = 7$). With the alteration in PTX antagonism in this mutant, we could not quantitatively compare PTX sensitivity to wild-type rρ1 homomeric receptors. However, homomeric rρ1(P310S) receptors, like hρ2, appear to be more sensitive to PTX than their wild-type ρ1 counterparts. Thus, this residue cannot account for the PTX resistance conferred by the rρ2 subunit.

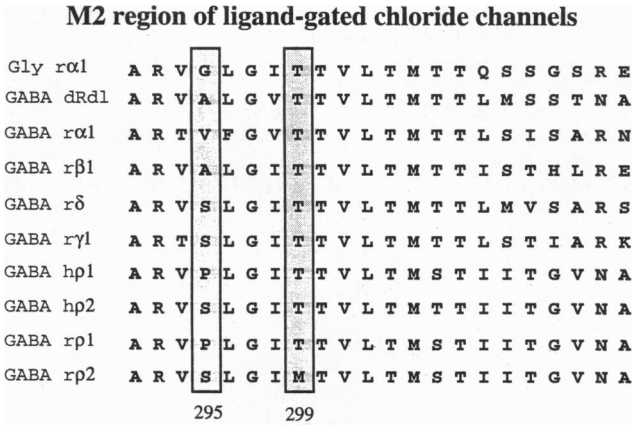

FIG. 5. Comparison of M2 regions of ligand-gated Cl^- channels, including the rat glycine α subunit; the *Drosophila* GABA *Rdl* subunit; the rat GABA α1, β1, γ1, δ, ρ1, and ρ2 subunits; and the human ρ1 and ρ2 subunits. The 2 amino acid residues different in rρ1 (residues 310 and 314) and rρ2 (residues 295 and 299) are indicated by shaded areas. Note that in the rρ2 subunit a methionine has replaced the threonine of the rρ1 subunit at a site that is highly conserved among ligand-gated Cl^- channels.

If the native "substitution" of a methionine for a threonine at residue 299 of rρ2 produced PTX resistance in heteromeric rat ρ receptors, then one might expect that site-directed mutagenesis of the homologous rρ1 residue 314 would produce PTX-resistant rρ1 mutant receptors. We therefore constructed the subunit rρ1(T314M). Unfortunately, homomeric channels formed from this subunit displayed only very tiny GABA-evoked currents. Instead, we studied PTX sensitivity of receptors formed by coexpressing rρ1/rρ1(T314M). GABA-evoked currents decreased in magnitude as the percentage of rρ1(T314M) cRNA increased (Fig. 3 *D–F*). The decrease in GABA-induced current was not due to a shift in the agonist dose–response curve since the EC_{50} was not significantly different for homomeric (rρ1/rρ1) vs. heteromeric rρ1/rρ1(T314M) receptors (data not shown). Qualitatively similar to the effect of rρ2 on wild-type heteromeric receptors, PTX resistance increased with the proportion of injected rρ1(T314M) cRNA (Figs. 3 *D–F* and 4*A*). However, the PTX dose-inhibition curve for rρ1(25%)/rρ1(T314M)(75%) was shifted to the right compared to rρ1(25%)/rρ2(75%) (Fig. 4*B*).

DISCUSSION

Our results suggest that the PTX resistance of $GABA_C$ responses in rat retina is due to rρ2 subunits in heteromeric rρ1/rρ2 receptors. Moreover, a single amino acid in the M2 region of rρ2 (methionine instead of threonine at residue 314 in rρ1) is responsible for PTX resistance in the native receptor. The rρ1 subunit differs from rρ2 by an additional amino acid in the M2 region, corresponding to proline residue 310 in rρ1 and serine residue 295 in rρ2. However, substitution of serine for proline at this site in rρ1 did not decrease PTX sensitivity. We therefore conclude that the proline/serine variation at this site is not the predominant factor conferring PTX resistance by the rρ2 subunit, although this site might affect PTX sensitivity under other conditions. In fact, in an invertebrate GABA receptor subunit the mutation of alanine to serine at a homologous site abolished PTX sensitivity (28). In an α-helical structure, the two amino acids corresponding to residues 310 and 314 of rρ1 could represent adjacent residues facing the pore (29), and thus each could affect PTX sensitivity. In any event, we find that a single amino acid residue located inside the predicted ion channel is critical for PTX action, lending further support to the notion that the channel itself is the major site of PTX blockade.

We also found that the amplitude of GABA-evoked currents of homomeric rρ1(T314M) or rρ1(P310S) receptors was significantly reduced compared to wild type. In addition, the GABA-induced currents of heteromeric rρ1/rρ1(T314M) receptors decreased in magnitude as we increased the amount of injected rρ1(T314M) cRNA (Fig. 3 *B* and *C*). These results suggest that substitution of amino acid residues at these two sites, presumably facing the channel pore (29), may also affect channel gating, as previously demonstrated for acetylcholine receptors (30). This may at least partially account for the fact that receptors formed from homomeric rρ2 subunits, containing serine and methionine residues at these two positions, displayed no detectable currents. Nonetheless, substitution of

the methionine with a threonine in rρ2 did not restore detectable GABA-evoked currents. In contrast to rρ2, hρ2 subunits can form functional homomeric receptors, but the currents are substantially smaller than their hρ1 counterparts (17). The N-terminal region is known to be critical for the assembly of functional ligand-gated receptors, including homomeric hρ2 receptors (22, 31, 32). It is therefore possible that defects in both assembly and channel gating in the rρ2 subunit prevent it from forming functional homomeric receptors.

It has been proposed that the two cloned human ρ subunits underlie the $GABA_C$ responses of native receptors (16). However, prior to the present study, the subunit composition of this type of GABA receptor had not been elucidated. We took advantage of the PTX-resistant property of the rat retinal $GABA_C$ receptor to discern the subunit composition of recombinant receptors. Since homomeric rρ1 receptors are sensitive to PTX, we are able to show that rρ1 and rρ2 subunits form functional heteromeric GABA receptors because of their PTX resistance. It is therefore very likely that the native PTX-resistant $GABA_C$ receptor in the rat retina is composed of both rρ1 and rρ2 subunits. However, the existence of additional ρ subunits cannot be excluded.

Our findings may provide an explanation not only for PTX resistance/sensitivity of $GABA_C$ receptors but also for other vertebrate GABA receptors that are coupled to Cl^- channels, including the $GABA_A$ subtype, which is inhibited by PTX (4, 33). PTX may therefore prove to be useful as a specific probe for examining motifs crucial to Cl^- permeability in a variety of receptor–channel complexes.

In summary, we demonstrate that a single amino acid residue in the rρ2 subunit renders heteromeric rρ1/rρ2 receptors resistant to PTX. Our results lend further support to the proposal that GABA ρ subunits underlie the previously reported $GABA_C$ responses in the retina of various species (6, 8). Recently, similar responses to GABA were reported in rat hippocampal neurons, suggesting that this form of ionotropic GABA receptor is more ubiquitous than previously thought (18). In a more general sense, the fact that a single amino acid residue can profoundly affect the PTX sensitivity of GABA receptors calls into question classification schemes based solely on pharmacological properties. Clearly, the ability to coassemble subunits to form functional heteromeric channels remains central to the definition of a receptor family. Our work, together with that of others (16), provides credence for the fulfillment of this concept by ρ subunits as the molecular basis for $GABA_C$ responses.

We thank Dr. S. Nakanishi for the kind gift of a rat retinal cDNA library. This work was supported in part by the American Foundation for AIDS Research and National Institutes of Health Grant R01 EY05477.

1. Roberts, E. (1986) in *Receptor Biochemistry and Methodology*, eds. Olsen, R. W. & Venter J. C. (Liss, New York), Vol. 5, pp. 1–39.
2. Johnston, G. R. A. (1986) in *Receptor Biochemistry and Methodology*, eds. Olsen, R. W. & Venter, J. C. (Liss, New York), Vol. 5, pp. 57–72.
3. Hill, D. R. & Bowey, N. G. (1981) *Nature (London)* **290,** 149–152.
4. Sivilotti, L. & Nistri, A. (1991) *Prog. Neurobiol.* **36,** 35–92.
5. Qian, H. & Dowling, J. E. (1993) *Nature (London)* **361,** 162–164.
6. Feigenspan, A., Wässle, H. & Bormann, J. (1993) *Nature (London)* **361,** 159–162.
7. Polenzani, L., Woodward, R. M. & Miledi, R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4318–4322.
8. Pan, Z.-H. & Lipton, S. A. (1995) *J. Neurosci.* **15,** 2668–2679.
9. Qian, H. & Dowling, J. E. (1994) *J. Neurosci.* **14,** 4299–4307.
10. Dong, C.-J., Picaud, S. A. & Werblin, F. S. (1994) *J. Neurosci.* **14,** 2648–2658.
11. Lukasiewicz, P. D., Maple, B. R. & Werblin, F. S. (1994) *J. Neurosci.* **14,** 1202–1212.
12. Matthews, G., Ayoub, G. S. & Heidelberger, R. (1994) *J. Neurosci.* **14,** 1079–1090.
13. Strata, F. & Cherubini, E. (1994) *J. Physiol. (London)* **480,** 493–503.
14. Cutting, G. R., Lu, L., O'Hara, B. F., Kasch, L. M., Montrose-Rafizadeh, C, Donovan, D. M., Shimade, S., Antonarakis, S. E., Guggino, W. B., Uhl, G. R. & Kazazian H. H., Jr. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2673–2677.
15. Cutting, G. R., Curristin, S., Zoghbi, H., O'Hara, B., Seldin, M. F. & Uhl, G. R. (1992) *Genomics* **12,** 801–806.
16. Shimade, S., Cutting, G. & Uhl, G. R. (1992) *Mol. Pharmacol.* **41,** 683–687.
17. Wang, T.-L., Guggino, W. B. & Cutting, G. R. (1994) *J. Neurosci.* **14,** 6524–6531.
18. Newland C. F. & Cull-Candy, S. G. (1992) *J. Physiol. (London)* **447,** 191–213.
19. Zhang, D., Pan, Z.-H., Brideau, A. D. & Lipton, S. A. (1995) *Invest. Ophthalmol. Visual Sci. Suppl.* **36,** 285 (abstr.).
20. Pan, Z.-H., Zhang, D., Brideau, A. D., Zhang, X. & Lipton, S. A. (1995) *Invest. Ophthalmol. Visual Sci. Suppl* **36,** 285 (abstr.).
21. Sullivan, J. M., Traynelis, S. F., Chen, H.-S. V., Escobar, W., Heinemann, S. F. & Lipton, S. A. (1994) *Neuron* **13,** 929–936.
22. Hackam, A. S., Wang, T.-L., Guggino, W. B. & Cutting, G. R. (1995) *Invest. Ophthalmol. Visual Sci. Suppl.* **36,** 285 (abstr.).
23. King, R. G., Nielsen, M., Stauber, G. B. & Olsen, R. W. (1987) *Eur. J. Biochem.* **169,** 555–562.
24. Inoue, M. & Akaike, N. (1988) *Neurosci. Res.* **5,** 380–394.
25. Smart, T. G. & Constanti, A. (1986) *Proc. R. Soc. London B* **227,** 191–216.
26. Yoon, K.-W., Covey, D. F. & Rothman S. M. (1993) *J. Physiol. (London)* **464,** 423–439.
27. Wang, T.-L., Hackam, A. S., Guggino, W. B. & Cutting, G. R. (1995) *Invest. Ophthalmol. Visual Sci. Suppl.* **36,** 285 (abstr.).
28. Ffrench-Constant, R. H., Rocheleau, T. A., Steichen, J. C. & Chalmers, A. E. (1993) *Nature (London)* **363,** 449–451.
29. Xu, M. & Akabas, M. H. (1993) *J. Biol. Chem.* **268,** 21505–21508.
30. Villaroel, A. & Sakmann, B. (1992) *Biophys. J.* **62,** 196–208.
31. Verrall, S. & Hall, Z. W. (1992) *Cell* **68,** 23–31.
32. Kuhse, J., Laube, B., Magalei, D. & Betz, H. (1993) *Neuron* **11,** 1049–1056.
33. Macdonald, R. L. & Olsen R. W. (1994) *Annu. Rev. Neurosci.* **17,** 569–602.