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A Single Histidine Residue Is Essential for Zinc Inhibition of GABA ρ1 Receptors

Tian-Li Wang,[1,3] Abigail Hackam,[3] William B. Guggino,[1] and Garry R. Cutting[2,3]

Departments of [1]Physiology and [2]Pediatrics and [3]Center for Medical Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The GABA ρ1 subunit, cloned from a human retina library, can form homooligomeric receptors with properties similar to $GABA_C$ receptors characterized in retinal cells. The divalent cation Zn^{2+}, abundant in the CNS and retina, was found to inhibit GABA ρ1 receptors in a voltage-independent manner. Varying the extracellular pH from 7.4 to 5.6 significantly reduced this inhibitory effect. This pH profile suggested that one or more histidine residues might play a role in the interaction between Zn^{2+} and the GABA ρ1 receptor. Site-directed mutagenesis revealed that a single histidine residue (His 156) in the putative extracellular domain of ρ1 was critical for Zn^{2+} sensitivity. Substitution of this amino acid with tyrosine (H156Y) created a functional GABA receptor with agonist and channel properties indistinguishable from wildtype. However, the H156Y mutant was insensitive to Zn^{2+}, even at concentrations as high as 1 mM. Mutation to aspartic acid, an amino acid that can interact with Zn^{2+} in other proteins, preserved sensitivity to Zn^{2+} but abolished the pH-dependent effect. This histidine residue is also involved in Ni^{2+} and Cd^{2+} interaction since the H156Y mutation completely suppressed the inhibition effects of these two cations. These data demonstrate that an extracellular histidine residue is critical for transition metal cation sensitivity of GABA ρ1 receptors.

[Key words: retina, transition metal cations, pH dependence, voltage independence, site-directed mutagenesis, Xenopus oocyte expression]

The transition metal Zn^{2+} is abundant in many regions of the brain, most notably the mossy fiber system of the hippocampus (Haug, 1967) and in several ocular tissues, particularly the outer retina (Hirayama, 1990; Wu et al., 1993). One of the proposed functions of Zn^{2+} in the CNS is to modulate pre- and/or postsynaptic ion channels including $GABA_A$ and glutamate receptors, voltage-gated Na^+ channels and K^+ channels (Legendre and Westbrook, 1990; Harrison and Gibbons, 1994). The mechanism of Zn^{2+} antagonism of $GABA_A$ receptors has been suggested by electrophysiological studies of native and recombinant receptors. Whole-cell patch-clamp recordings of isolated neuronal cells revealed that the inhibition of Zn^{2+} is reversible and the blocking effect is noncompetitive and voltage independent (Westbrook and Mayer, 1987; Legendre and Westbrook, 1990). Furthermore, $GABA_A$ receptors in spinal neurons can be inhibited by extracellular Zn^{2+} but not by Zn^{2+} that is injected intracellularly (Celentano et al., 1991). These results indicate that Zn^{2+} does not physically access the channel pore but interacts with residues in the extracellular domain of the $GABA_A$ receptors. These residues appear to be distinct from those that form the recognition sites for GABA, benzodiazepines, barbiturates, picrotoxin, and steroids (Legendre and Westbrook, 1990; Celentano et al., 1991; Smart, 1992). Studies of recombinant vertebrate $GABA_A$ receptors demonstrate that sensitivity to Zn^{2+} is dependent upon subunit composition. Receptors composed of α and β subunits are sensitive to Zn^{2+} whereas inclusion of γ subunits creates GABA receptors that are insensitive to Zn^{2+} (Draguhn et al., 1990; Smart et al., 1991). This situation appears to be more complex, since coexpression with δ subunits or variation in the α subunit type (α1 vs α2 or α3) moderate the Zn^{2+} insensitivity of receptors containing γ subunits (Saxena and Macdonald, 1994; White and Gurley, 1995).

A novel class of GABA receptor called type C has been identified in retina (Feigenspan et al., 1993; Qian and Dowling, 1993). These receptors exhibit a number of characteristics that distinguish them from $GABA_A$ receptors including a lack of sensitivity to bicuculline, barbiturates, and benzodiazepines. Many of these distinct properties are shared by GABA receptors formed of ρ subunits (Shimada et al., 1992; Wang et al., 1994). These subunits were identified in retina raising the possibility that retinal $GABA_C$ receptors are formed partially or wholly of ρ subunits. $GABA_C$ receptors are present in cell types in regions of the retina with high Zn^{2+} concentrations, and it appears that Zn^{2+} acts as a mixed antagonist for this class of GABA receptor (Dong and Werblin, 1995). Likewise, GABA-gated chloride currents generated by homooligomeric ρ1 and ρ2 receptors expressed in *Xenopus* oocytes are inhibited by Zn^{2+}, and Zn^{2+} inhibition of GABA ρ1 receptors displays competitive and noncompetitive components (Calvo et al., 1994; Wang et al., 1994; Chang et al., 1995). We report that a single residue in the putative extracellular domain is critical for sensitivity of ρ1 receptors to Zn^{2+} and two other transition metal cations, Ni^2 and Cd^{2+}. This study establishes a molecular basis for Zn^{2+} modulation of $GABA_C$ receptors in the outer retina.

Materials and Methods

Chimera construction. A ρ2ρ1-B chimeric cDNA encoding amino acids 1 to 62 of ρ2 and amino acids 72 to 473 of ρ1 was generated by two-step polymerase chain reaction as previously described (Yon and Fried, 1989). The primers used were: 5′-ρ2 primer: 5′-CGGCGGATCCA-

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Correspondence should be addressed to Garry R. Cutting, M.D., Center for Medical Genetics, Johns Hopkins University, School of Medicine, CMSC 1004, 600 North Wolfe Street, Baltimore, MD 21287.

CAGGCTACTGGAAAGCAGC-3′ ρ2ρ1-B hybrid primer: 5′-TCCGGA-AGGGAAAGCCTCAGCAGCCTTCTGAGGATAGATGAC-3′ 3′-ρ1 primer: 5′-CTGCAGCTTTCCAAAGCT-3′. The final PCR amplification product was subcloned into the PCR II vector (TA cloning kit, Invitrogen). Authenticity of the chimeric construct was confirmed by dideoxynucleotide sequencing of the entire cDNA.

Site-directed mutagenesis and in vitro *RNA synthesis.* Oligonucleotide-mediated mutagenesis was performed on ρ1 uracil-containing single-strand DNA using the Muta-gene Phagemid *in vitro* mutagenesis kit (Bio-Rad) or by double-stranded mutagenesis with the Transformer Site-Directed Mutagenesis Kit (Clontech). The sequences of the mutagenic oligos were as follows: H78Y, 5′-CTGAAATCATAGTCATCTATC-3′; H120Q, 5′-CTTCCAGTACTGCCTCAGG-3′; H156C, 5′-GCGTTTG-GAGCACACGAAAAACA-3′; H156D, 5′-GCGTTTGGAGTCCACG-AAAAACA-3′; H156N, 5′-GCGTTTGGAGTTCACGAAAAACA-3′; H156Y, 5′-GCGTTTGGAGTACACGAAAAACA-31; H163N, 5′-GG-TGGTGTCGTTGATGAAGGAGC-3′; H163Y, 5′-CTGTGGTGGTGT-CGTAGATGAAGGAGCG-3′; H248Q, 5′-GGTGGTGGTCTGAAATT-CCTG-3′; H274Y, 5′-GAAGATGTAGCGCCGCAACG-3′; H447N, 5′-CAATGGCGTTGGTATCGATTC-3′.

Mutations were confirmed by dideoxynucleotide sequencing the mutated nucleotide and approximately 100 nucleotides flanking each side of the mutation. Capped cRNA was synthesized *in vitro* using a Megascript Kit (Ambicon). Both ρ1 and mutant plasmid DNAs were linearized with Bam HI, and cRNA was synthesized from each DNA template using T3 RNA polymerase.

Oocyte preparation and electrophysiology. Oocyte preparation and RNA injection were performed as described previously (Wang et al., 1994). Stage V or VI oocytes isolated from adult female *Xenopus* (Nasco, Michigan) were injected with either 5 or 10 ng of ρ1 RNA or 5 to 50 ng of mutant RNA in a 50 nl volume by a positive-displacement micropipette (Drummond, PA). In experiments comparing responses of mutant receptors to those of ρ1 receptors, the amount of mutant cRNA injected was adjusted to obtain whole-cell currents that were within 20% of those generated by 5 ng of ρ1 cRNA.

Voltage clamp. GABA-induced currents were recorded from individual oocytes using the two-electrode voltage-clamp technique with an Axonclamp-2A amplifier (Axon Instruments, CA) as described previously (Wang et al., 1994). Voltage pulse protocols and data acquisition were performed by a pCLAMP computer software (Version 5.51, Axon Instruments, CA). Two days after RNA injection, oocytes were placed in a 800 μl recording chamber with bath solution (ND96) continuously perfused by gravity at a flow rate of 300 μl/sec. ND96 contains 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES (pH 7.4). GABA, with or without other drugs, was applied to the oocyte by bath perfusion. The rate of agonist application was suitable for ρ1 receptors since the GABA-induced currents show minimal desensitization as observed by others (Amin and Weiss, 1994; Calvo et al., 1994). The current reached a peak within 2 sec, and there was little or no decline in current during 2–3 min of GABA application. Since Zn^{2+} inhibition of GABA ρ1 receptors is agonist dependent (Calvo et al., 1994; Chang et al., 1995), the same concentration of GABA (5 μM) was used in all experiments examining metal cation inhibition. The pH values of ND96 was adjusted to 5.6, 6.5, and 7.4 using 0.5 *N* NaOH. GABA and $ZnCl_2$ were obtained from Sigma, St. Louis, MO, and $NiCl_2$ and $CdCl_2$ were from Aldrich Chemicals, Milwaukee, WI. These chemicals were dissolved in ddH_2O as 1 M stocks then diluted in ND96 to obtain test concentrations. Recording electrodes, filled with 3 M KCl, with resistances of 0.50–3 MΩ were used for recordings. The membrane potential was held at −50 mV continuously unless a current–voltage clamp protocol was performed (Figs. 1, 5). The trial number (n) represents the number of oocytes studied. Each oocyte served as its own pretreatment control. A minimum of three oocytes from at least two frogs were studied per experiment.

Data analysis. In all experiments except Figure 5, the current observed in the presence of drug(s) was subtracted from the current in the absence of any drug (corresponding to background current of the oocyte) by pCLAMP software. Data shown in Figure 5 were the original tracings before and after drug application without any subtraction. The N-fit program (The University of Texas, Galveston) was used to perform curve fitting of dose-dependent relations. The GABA dose–response relation was constructed at a wide range of GABA concentrations. The Hill equation $I(x) = I_{max}/1+(EC_{50}/[GABA])^n$ was used to fit the normalized data points; I_{max} is the maximal current response eliciting by GABA; EC_{50} is the concentration of GABA that elicits half-maximal response (1/2 I_{max}); n represents the Hill coefficient. To quantify the inhibitory effect of antagonist upon GABA-induced currents, the inverse Hill Equation $I(x) = I_{max}/1+([Antagonist]/IC_{50})^n$ was used to fit the normalized current response versus antagonist concentration. IC_{50} represents the concentration of Zn^{2+} that blocks half-maximal current response; I_{max} is the maximal current eliciting by GABA in the absence of Zn^{2+}; n represents the inverse Hill coefficient. The top of dose–

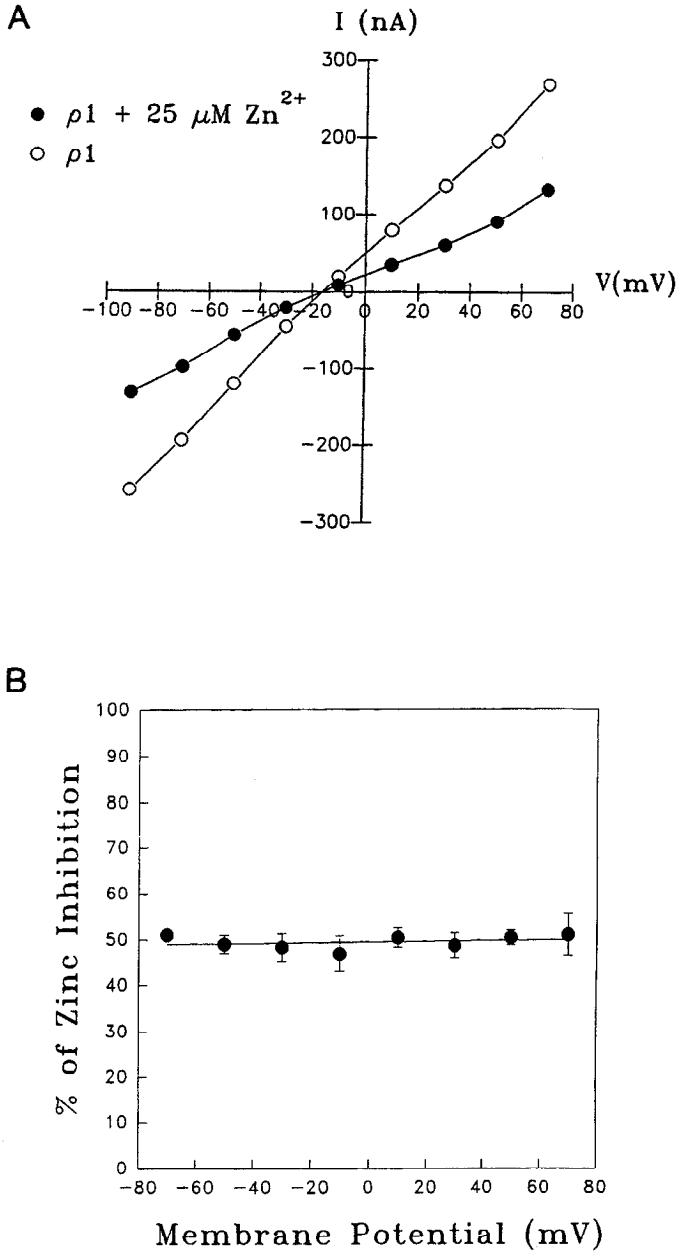

Figure 1. Voltage-independent inhibition of Zn^{2+} on GABA ρ1 currents. *A,* A representative current–voltage plot of ρ1 GABA-elicited currents before (○) and after (●) application of 25 μM Zn^{2+}. Similar results were observed in four different oocytes. At +50 mV, 50.4 ± 1.6% of current was inhibited (n = 4); at −50 mV, 49.0 ± 2.0% of current was inhibited (n = 4). *B,* Fractional blocking effect of Zn^{2+} at various voltage potentials. The inhibitory effect was measured as the ratio of GABA-induced current in the presence of Zn^{2+} to that in the absence of Zn^{2+} at a range of holding potentials (from −70 mV to 70 mV with 20 mV increment steps). GABA (5 μM) was used to elicit current response and 25 μM Zn^{2+} was applied to inhibit that current. Data are expressed as mean and SEM from four different oocytes. The data points fitted by linear regression produced a line that is parallel to the X-axis.

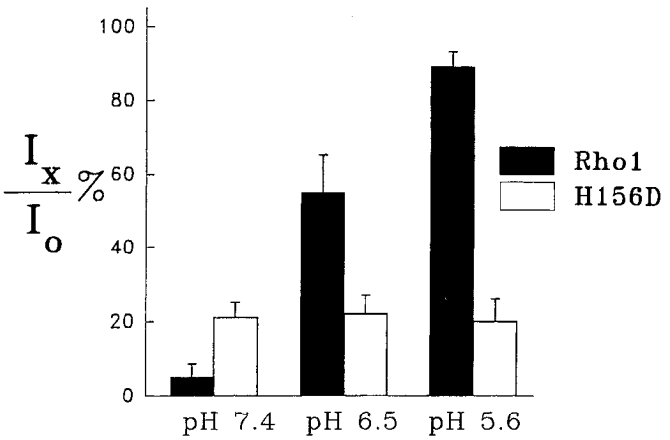

Figure 2. pH-Dependent Zn^{2+} inhibition effect on ρ1 and H156D receptors. GABA-gated currents were measured before or after exposure to Zn^{2+} at different pH values. Three different pH values of perfusion solution were tested: pH 5.6, pH 6.5, and pH 7.4. The data are normalized as I_X/I_O (percentage). I_O is the current response to 5 μM GABA; I_X is the current response to 5 μM GABA coapplied with 200 μM Zn^{2+} at each pH. Data: mean ± SEM. Four oocytes injected with ρ1 and four oocytes injected with H156D were studied at each pH.

response relations were determined when a 500-fold concentration increase did not change current amplitude by more than 1%. Student's *t* test was used to determine the statistical significant difference between two sample groups. In this study, independent sampling and comparison were used, and $p < 0.01$ was set as a significant level.

Results

Zn^{2+} inhibition of GABA ρ1 receptors is voltage independent and pH dependent

We and others have demonstrated that Zn^{2+} inhibits GABA ρ1 receptors in a dose-dependent fashion (Calvo et al., 1994; Wang et al., 1994; Chang et al., 1995). Further studies were performed to characterize the blocking effect of Zn^{2+} upon homooligomeric ρ1 receptors expressed in *Xenopus* oocytes. Zn^{2+} inhibited ρ1 receptors over a wide range of membrane potentials without significantly affecting the linearity of the current–voltage relationship (Fig. 1*A*). The fraction of GABA-gated currents blocked by 25 μM, Zn^{2+} was almost constant at each voltage potential (Fig. 1*B*). These observations indicate that Zn^{2+} inhibits GABA ρ1 receptors in a voltage-independent manner. The inhibitory effect of Zn^{2+} upon some native $GABA_A$ receptors can be influenced by changes in extracellular pH (Smart and Constanti, 1982). To evaluate this possibility for ρ1 receptors, the pH of the solution bathing the oocytes was varied from 7.4 to 5.6. Different external pH conditions did not significantly affect the magnitude of GABA-elicited currents in four oocytes studied (data not shown). However, Zn^{2+} inhibition of the currents activated by 5 μM GABA decreased as pH decreased (Fig. 2). At pH 7.4, 200 μM Zn^{2+} inhibited almost all currents (92.8 ± 3.6%); at pH 6.5, approximately half of the currents were inhibited (42.4 ± 8.3%), and at pH 5.6, about one-tenth of currents (12.2 ± 4.0%) were inhibited. These data demonstrate that decreasing extracellular pH affects Zn^{2+} interaction with ρ1 receptors.

A histidine residue in the putative amino terminal domain of ρ1 confers sensitivity to Zn^{2+}

The pH dependency of the Zn^{2+} inhibition suggested that one or more histidine residues may play a critical role in the sensitivity of ρ1 receptors to this cation. Inspection of the predicted

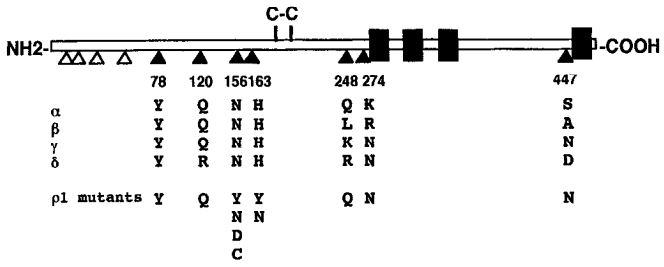

Figure 3. Schematic representation of the relative locations of the histidine residues of the GABA ρ1 subunit and the mutants used in this study. Amino acis are in *single letter code*. *C-C* indicates the location of a pair of highly conserved cysteine residues and *filled boxes* represent hydrophobic membrane-spanning segments M1 to M4. *Numbers* refer to the codons of the predicted amino acid sequence of the full-length cDNA (canonical methionine being codon 1) (Cutting et al., 1991). Histidines conserved between ρ1 and ρ2 subunits are indicated by *filled triangles* and four nonconserved histidines are shown by *open triangles*. Amino acids in human α1, human β1, human α2, and rat δ subunits in the same location as each filled triangle are shown in *single letter code* (Cutting et al., 1991).

amino acid sequence of ρ1 revealed eleven histidine residues. Ten are located in the putative extracellular N-terminal domain and one immediately precedes the fourth hydrophobic segment (Fig. 3). Seven of these histidine residues are conserved between ρ1 and the Zn^{2+}-sensitive ρ2 subunit (Fig. 3). To determine whether any of the four nonconserved histidines near the N-terminus of ρ1 influenced Zn^{2+} sensitivity, a chimera exchanging the N-terminal regions of ρ1 and ρ2 was created (ρ2ρ1-B) that retained the seven conserved histidines. This chimera generated robust currents in oocytes that, like ρ1, were sensitive to Zn^{2+} (Fig. 4*A*). The remaining seven histidines were then mutated individually and the resulting mutant was tested for sensitivity to 200 μM Zn^{2+}. This concentration was chosen since it almost completely inhibits ρ1 currents elicited by 5 μM GABA. The choice of the amino acid selected to substitute for histidine was based on the residues most frequently found at the same location in $GABA_A$ subunits (Fig. 3). Five histidine mutants (H78Y, H120Q, H248Q, H274N, and H447N) generated robust GABA-gated chloride currents that were sensitive to Zn^{2+} (Fig. 4*A*). The amino acid substituted for histidine in each case (asparagine, glutamine, or tyrosine) has not been shown to donate electrons to Zn^{2+} (Coleman, 1992). These results eliminated these five histidines as significantly contributing to the interaction between ρ1 receptors and Zn^{2+}.

Substitution of the histidine at codon 163 with either asparagine or tyrosine prevented the formation of functional GABA-gated receptors. GABA concentration was increased to 1 mM without response ($n = 8$). This result was not unexpected since this histidine is completely conserved among $GABA_A$ and ρ subunits, suggesting that it is critical for function. Mutation of the histidine residue at codon 156 to asparagine (H156N), the residue found in the corresponding location of all $GABA_A$ subunits, produced a mutant that did not respond to GABA up to 1 mM ($n = 4$). To eliminate the possibility that a second mutation had been inadvertently introduced elsewhere in the cDNA during mutagenesis, three independent H156N clones were studied. None of the mutants produced a functional GABA receptor. However, substitution with tyrosine (H156Y) resulted in functional channels that were insensitive to Zn^{2+}, even at concentrations as high as 1 mM (Fig. 4*B*). Two mutants were created in which the histidine at codon 156 was replaced with amino acids

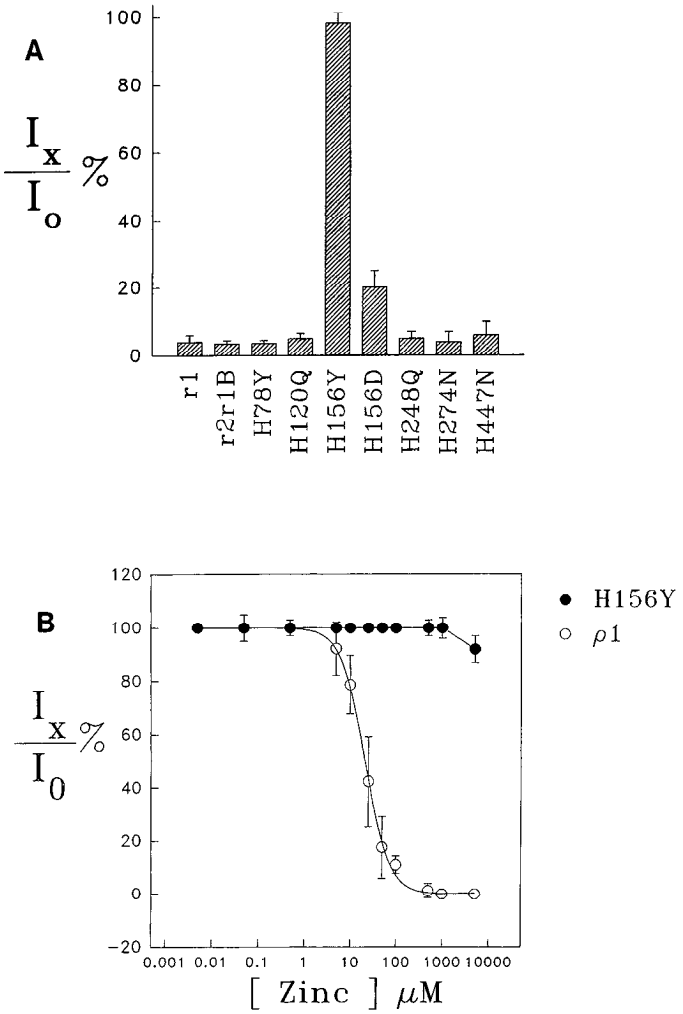

Figure 4. *A*, The effect of 200 μM Zn^{2+} on GABA-gated current of homooligomeric ρ1 (r1), ρ2ρ1-B (r2r1B) and ρ1 mutants. *B*, Inhibition effects of a wide range of Zn^{2+} concentration upon GABA-gated currents of ρ1 and the H156Y mutant. The Y-axis for both *A* and *B* are the normalized response, I_X/I_O%. I_O is the current response to 5 μM GABA and I_X is the current response to Zn^{2+} coapplied with 5 μM GABA. Data are presented as mean and SEM. $n = 5$ for each group in *A*; $n = 3$ for each group in *B*.

that can interact with Zn^{2+}: cysteine (H156C) and aspartic acid (H156D). Although the H156C mutant failed to produce currents, H156D generated a GABA-gated conductance that was sensitive to Zn^{2+} (Fig. 4). Unlike ρ1, however, Zn^{2+} inhibition of the H156D mutant was not affected by changes in extracellular pH (Fig. 2). Therefore, the nature of the residue at codon 156 is critical for Zn^{2+} sensitivity of ρ1 receptors.

Histidine at codon 156 is also critical for Ni^{2+} and Cd^{2+} inhibition of ρ1 receptors

A number of transition metal cations including Cd^{2+} and Ni^{2+} can inhibit $GABA_A$ receptors (Akimichi and Tachibana, 1986; Smart and Constanti, 1990; Celentano et al., 1991). Both cations also inhibit ρ1 currents (Calvo et al., 1994); the potency of Ni^{2+} inhibition was similar to that of Zn^{2+}, whereas Cd^{2+} was considerably weaker. The linearity of the current–voltage relationship was not affected by Ni^{2+} or Cd^{2+}, indicating that the blocking mechanism of these cations, like Zn^{2+}, was voltage independent (Fig. 5). To determine if these divalent cations share with Zn^{2+} the same site of interaction, the blocking efficiency of Ni^{2+} and Cd^{2+} upon the H156Y mutant was examined. At 250 μM of Ni^{2+} (Fig. 5*A*) and 500 μM of Cd^{2+} (Fig. 5*B*), ρ1 currents were almost completely eliminated, whereas H156Y currents were unaffected by the two cations at these concentrations. Like Zn^{2+}, Ni^{2+} and Cd^{2+} could not inhibit the chloride currents of the H156Y mutant receptors even at concentrations as high as 1 mM.

Mutation of the histidine residue at position 156 of ρ1 appears specific for divalent cation sensitivity

To determine whether the H156Y mutation had more pervasive effects upon receptor function, the agonist binding affinity and channel properties were studied in detail and compared to receptors formed by wild-type ρ1. Similar to ρ1, the H156Y mutant expressed robustly GABA-gated whole-cell currents ranging from 200 to 800 nA, depending on the batch of oocytes injected. The H156Y receptors had a linear current–voltage relationship (Fig. 5) and a chloride-selective reversal potential (Table 1). Its affinity to GABA (EC_{50} = 2.1 μM ± 0.3; $n = 3$) did not differ significantly from that of ρ1 (Table 1). The H156Y mutant is antagonized by picrotoxin with an IC_{50} of 34.1 ± 6.0 μM ($n = 3$) that is not significantly different from the value of ρ1 receptors (Table 1). These data suggested that Zn^{2+} sensitivity is conferred by a different region of ρ1 subunits than those involved with agonist binding, anion conduction, or picrotoxin interaction.

Increasing the ratio of coinjected H156Y to ρ1 cRNA progressively decreases the Zn^{2+} sensitivity of GABA-gated currents

It has been suggested that presence of γ subunits confers Zn^{2+} insensitivity to heterooligomeric $GABA_A$ receptors (Draguhn et al., 1990; Smart et al., 1991). To determine if the Zn^{2+} insensitive H156Y mutant had the same effect upon ρ1 receptors, *Xenopus* oocytes were injected with a constant amount of ρ1 cRNA and increasing amounts of H156Y mutant cRNA. As shown in Figure 6, oocytes injected with both cRNAs generated GABA-gated currents with Zn^{2+} sensitivity profiles that were distinct from those generated by ρ1 or by H156Y alone. Increasing amounts of H156Y mutant cRNA generated currents that rapidly became insensitive to Zn^{2+} inhibition. For example, 100 μM and 250 μM Zn^{2+} inhibited current from oocytes injected with a 1:1 ratio of ρ1 to H156Y, but not as potently as oocytes injected with ρ1 alone. When a 1:2 ratio of ρ1 to H156Y cRNA was injected, currents elicited by GABA were much less sensitive to 100 μM Zn^2 and less sensitive to 250 μM Zn^{2+}. At a ratio of 1:4, even though 20% of the injected cRNA was ρ1, the GABA-gated currents were virtually insensitive to Zn^2. These data suggest that coexpression of the H156Y mutant does affect the Zn^{2+} sensitivity of ρ1 receptors.

Discussion

In this study, the mechanism of Zn^{2+} inhibition of homooligomeric GABA ρ1 receptors was investigated at the molecular level. The inhibitory effect of this cation was voltage independent and attenuated by decreasing the pH of the bath solution. These results suggested an extracellular site of interaction distinct from the ion channel of the receptor. The pH dependency of the Zn^{2+} inhibitory effect raised the possibility that H^+ was interfering with the binding of this cation to an inhibitory motif. Studies of Zn^{2+}-interacting proteins have shown that this cation can be coordinated to four Cys residues or three amino acids,

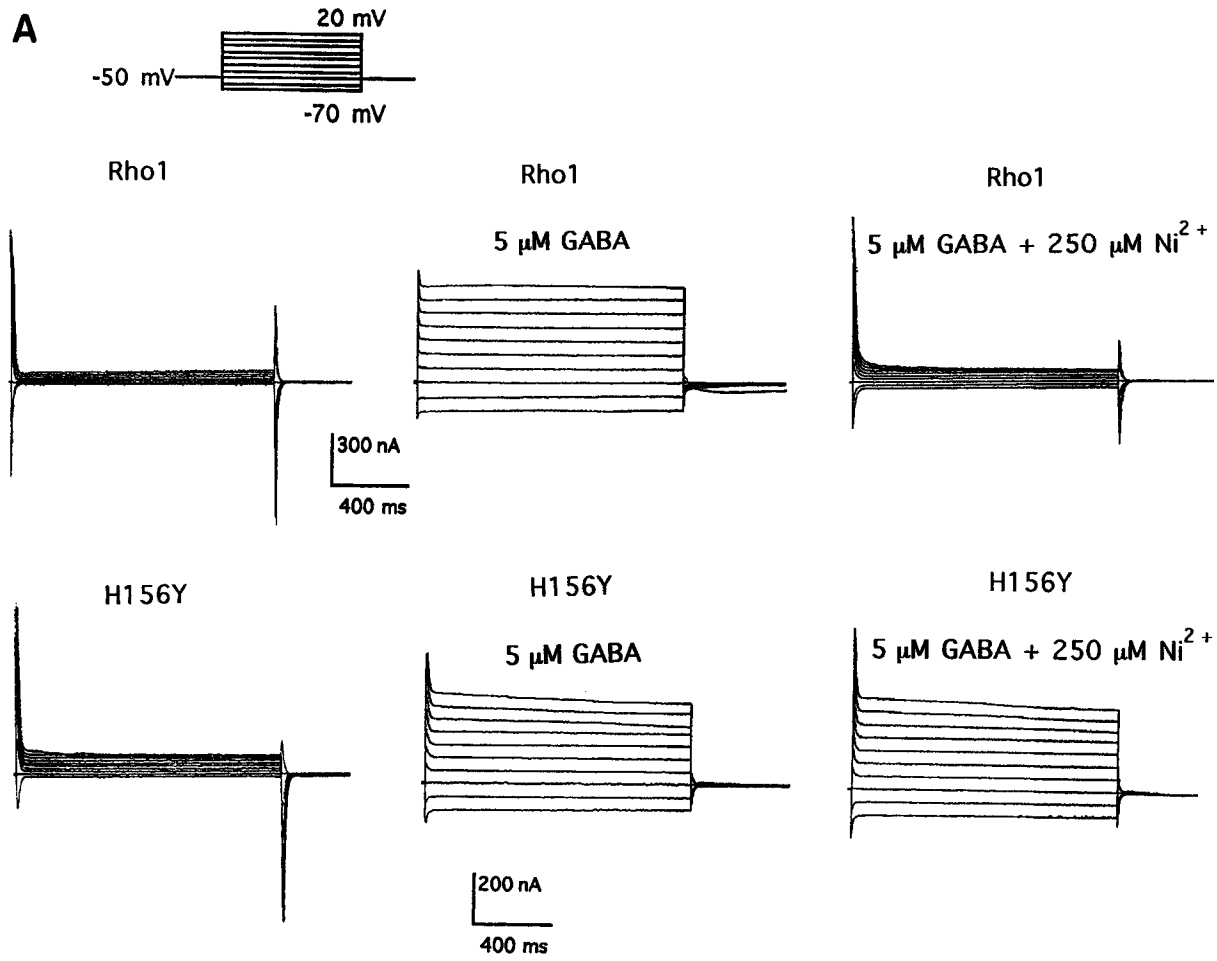

Figure 5. Inhibition effects of Ni^{2+} and Cd^{2+} on ρ1 and H156Y currents. In both *A* and *B*, the current tracings were recorded with a experimental protocol that held the membrane potential at −50 mV and stepped from −70 mV to 20 mV by 10 mV increments. *A*, Inhibition of ρ1 and H156Y currents by 250 μM Ni^{2+}. The *tracings* shown at the *left-hand side* are the intrinsic currents of the oocyte; the *tracings in the middle* are currents elicited by 5 μM GABA, and the *tracings shown on the right* are the responses of the same oocyte upon 250 μM Ni^{2+} and 5 μM GABA coapplication. *B*, Inhibition of GABA ρ1 and H156Y currents by 500 μM Cd^{2+}. The *tracings shown at the left-hand side* are the intrinsic currents of the oocyte; the *tracings at the middle* are the currents elicited by 5 μM GABA; and the *tracings shown at the right* are the responses of the same oocyte stimulated by 500 μM Cd^{2+} and 5 μM GABA coapplication. Similar recordings were obtained from five oocytes for each group.

usually a combination of Cys, His, Glu, and/or Asp residues, and an activated water molecule (Vallee and Auld, 1990; Coleman, 1992). Of the four amino acids, histidine has a titratable R group in solution in the pH range used in this study. We therefore speculated that Zn^{2+} may be interacting with a histidine residue that had a similar pK_R value in the protein as in solution. Site-directed mutagenesis of each histidine residue revealed that substitution of a single amino acid (His156) in the putative extracellular domain of ρ1 significantly altered Zn^{2+} sensitivity of ρ1 receptors. The almost complete elimination of the inhibitory effect of Zn^{2+} by this mutation indicates that Zn^{2+} inhibition of GABA-gated currents occurs by interaction with the GABA receptor as opposed to channel blockade or complexing with GABA or chloride ions.

X-ray crystallographic studies of Zn^{2+}-containing metalloenzymes indicate that the imidazole group of histidine can interact directly with Zn^{2+} (Vallee and Auld, 1990). Thus, His156 may form part of a Zn^{2+} binding pocket involving one or more other amino acids in the subunit. Alternatively, substitution of His156 with tyrosine may cause local changes in conformation that allosterically alter the interaction of Zn^{2+} with amino acids distinct from His156. The loss of function caused by the substitution of His156 with asparagine or cysteine supports this concept. However, the structural change would have to be quite discrete since responses to GABA and to picrotoxin and channel properties of H156Y receptors were indistinguishable from those of wild-type receptors (Table 1). Experiments involving the H156D mutant appear to provide the most convincing argument that His156 is directly involved in Zn^{2+} binding. Histidine to aspartic acid is a nonconservative substitution, yet robust currents were generated upon application of GABA, and Zn^{2+} sensitivity was retained. An important observation was the loss of the pH effect upon Zn^{2+} inhibition over the range used in the study (7.4 to 5.6). We propose that the aspartic acid residue coordinates with Zn^{2+}, but H^+ ions do not interfere with this interaction because of the low pK_R (3.86 in solution) of this amino acid. These data suggest that the nature of the amino acid at codon 156 plays a critical role in conferring Zn^{2+} sensitivity to the GABA ρ1 receptor.

The proximity of a histidine at codon 163 and two cysteine residues to His156 raises the possibility of a Zn^{2+} binding motif similar to that observed in transcription factors containing Zn^{2+}-fingers (Fig. 3). However, the latter structure is formed by two Cys residues preceding two His residues with relatively specific distances between each amino acid (Jacobs, 1995). In addition,

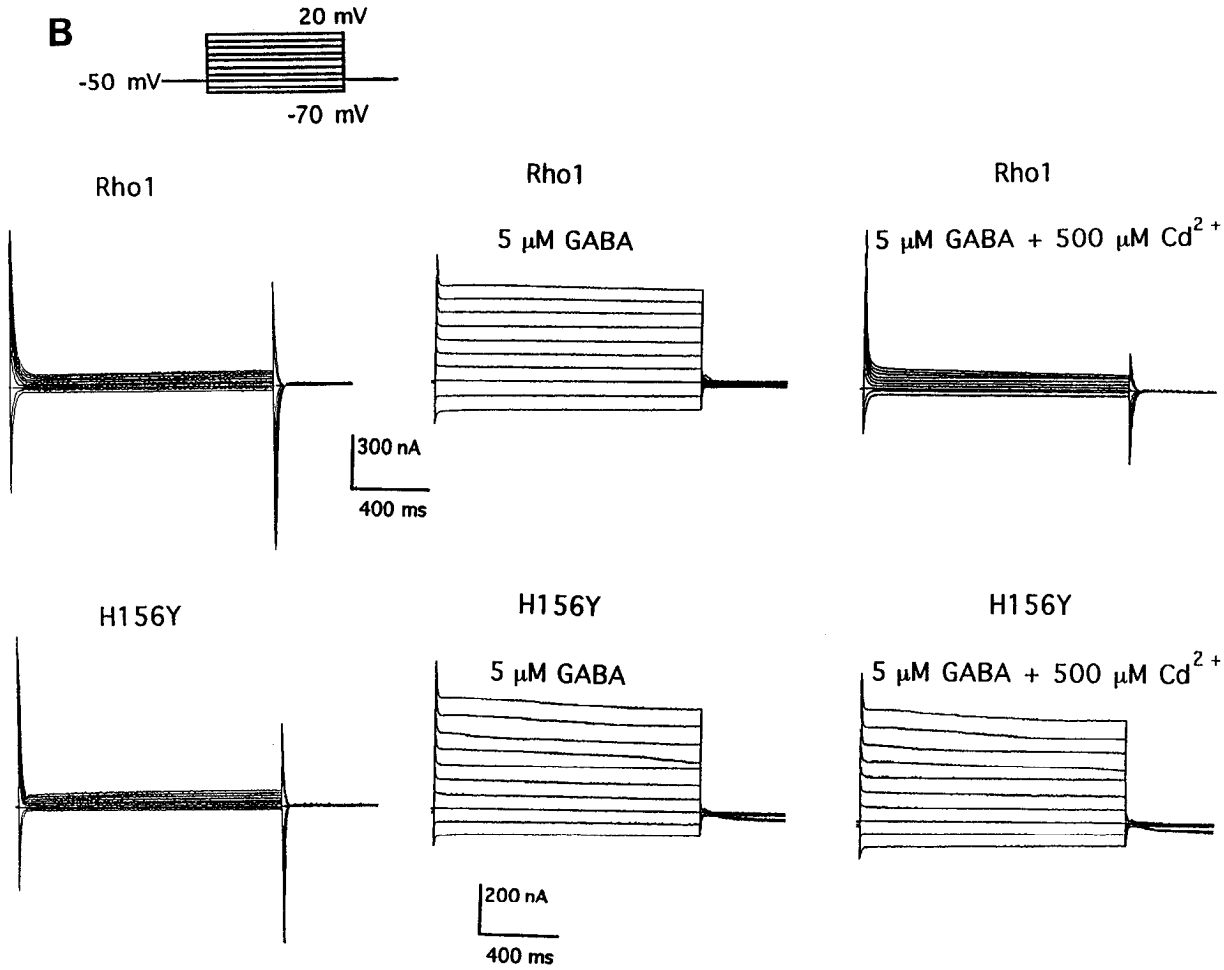

Figure 5. Continued.

the binding of Zn^{2+} to GABA ρ1 receptors is presumed to be dynamic so that Zn^{2+} can quickly bind or leave this pocket. This implies that the Zn^{2+}-binding moiety of GABA ρ1 receptors might be different from the motifs associated with structural Zn^{2+} atoms (Vallee and Auld, 1990; Jacobs, 1995). The multimeric structure of GABA receptors further complicates the conceptualization of the molecular components of the Zn^{2+} binding pocket. GABA ρ1 receptors, like other ligand-gated neurotransmitter receptors, are thought to be composed of five subunits. The deduced Hill coefficient of 1.5 from the Zn^{2+} inhibition curve (Fig. 4*B* and Table 1) is consistent with cooperative binding of two (or more) Zn^{2+} atoms. Therefore, Zn^{2+} could be binding to motifs in each subunit or pockets formed by multiple subunits. Expression of H156Y with ρ1 does appear to alter the Zn^{2+} sensitivity of ρ1 receptors. This could be due to the formation of H156Y:ρ1 heterooligomeric receptors with altered Zn^{2+} binding properties. Alternatively, H156Y may interfere with ρ1 translation efficiency, receptor assembly, or trafficking to the cell membrane. Finally, Zn^{2+} inhibition of ρ1 receptors is competitive at low concentrations (≤ 100 μM) and noncompetitive at higher doses (> 100 μM) suggesting more than one site of Zn^{2+} interaction or allosteric effects between separate Zn^{2+} and GABA binding sites (Chang et al., 1995). The complete loss of Zn^{2+} sensitivity of homooligomeric H156Y receptors across a wide range of Zn^{2+} concentrations (0.1 μM to 1 mM) is consistent with a single site of action. However, it remains possible that the H156Y mutation alters binding to multiple distinct sites by conformational changes of the multimeric receptor. Illustra-

Table 1. Comparison of several receptor properties of GABA ρ1 and H156Y receptors

				Zn^{2+} Inhibition	
	EC_{50} of GABA	IC_{50} of picrotoxin	Reversal potential	IC_{50}	Hill coefficient
ρ1	2.3 ± 0.4 μM	28.0 ± 5.0 μM	−20.5 ± 2.1 mV	16.2 ± 4.5 μM	1.5 ± 0.2
H156Y	2.1 ± 0.3 μM	34.1 ± 7.0 μM	−21.2 ± 2.0 mV	10 mM[a]	ND

ND, not determined. Data are expressed as mean ± SEM, $n = 3$ for each group.

[a] Estimated IC_{50} value.

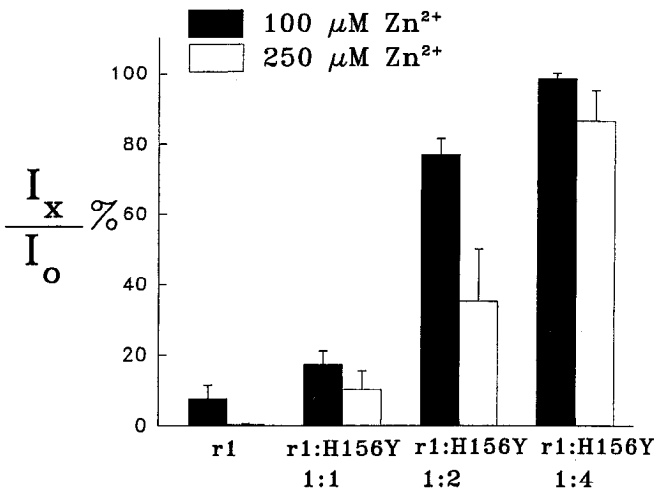

Figure 6. Coexpression of ρ1 and H156Y subunits. I_X/I_O% is the normalized response, I_O is the current response to 5 μM GABA, and I_X is the current response to 100 μM or 250 μM Zn^{2+} coapplied with 5 μM GABA. Data are expressed as mean and SEM (n = 3 for each group). Oocytes were injected with either ρ1 alone (10 ng) or coinjected with three different ratios of ρ1 (r1) and H156Y cRNAs. ρ1 (10 ng) was mixed with the appropriate amount of H156Y cRNA to achieve the desired RNA ratios.

tion of the importance of His156 should serve as a starting point to identify the precise molecular components of the divalent cation binding motif(s) in assembled GABA ρ1 receptors.

The GABA ρ subunits display 30 to 38% amino acid similarity to the $GABA_A$ subunits and share several functional similarities including sensitivity to Zn^{2+} (Cutting et al., 1991; Wang et al., 1994). A number of the characteristics of the interaction between Zn^{2+} and vertebrate $GABA_A$ receptors resemble those elucidated here for GABA ρ1 receptors. Both appear to have Zn^{2+} binding sites that are extracellular and distinct from the sites of action of GABA and picrotoxin (Celentano et al., 1991; Smart, 1992). Furthermore, each displays dose-dependent and voltage-independent inhibition (Westbrook and Mayer, 1987; Celentano et al., 1991; Calvo et al., 1994; Chang et al., 1995). However, there are two striking differences. Although Zn^{2+} inhibition of invertebrate $GABA_A$ receptors is pH dependent like ρ1 receptors, similar studies of rat sympathetic neurons failed to demonstrate the same effect upon vertebrate $GABA_A$ receptors (Smart, 1992). Secondly, the potency of other divalent cations differs between ρ1 and vertebrate $GABA_A$ receptors. The rank order of potency for ρ1 receptors was $Zn^{2+} \sim Ni^{2+} > Cd^{2+}$, as observed by others (Calvo et al., 1994). In studies of mature $GABA_A$ receptors in chick spinal cord neurons and rat superior cervical ganglion cells, Cd^{2+} has equal or greater potency than Zn^{2+}, and Ni^{2+} is considerably less inhibitory than Zn^{2+} (Smart and Constanti, 1990; Celentano et al., 1991). Recombinant $GABA_A$ receptors formed by α1 and β2 subunits expressed in mammalian cells display a potency rank order of $Zn^{2+} > Ni^{2+} > Cd^{2+}$ (Draguhn et al., 1990). Together, these observations suggest that the amino acid composition of the Zn^{2+}-interacting site differs between these two classes of receptors. Alignment of the ρ and $GABA_A$ subunit amino acid sequences and our mutagenesis studies support this contention. The $GABA_A$ subunits have a highly conserved asparagine in the location corresponding to the histidine conferring Zn^{2+} sensitivity to ρ1 receptors. Substitution of this residue with asparagine resulted in a nonfunctional receptor whereas similar substitutions in ρ1 (H274N and H447N) were tolerated, as were substitutions at codons 78 and 120 with amino acids that are highly conserved in $GABA_A$ subunits (Fig. 3). Therefore, despite sequence and functional similarities, $GABA_A$ and ρ1 receptors may have structurally diverse Zn^{2+} binding domains.

Numerous studies have documented that two pharmacologically distinct types of GABA receptors, $GABA_A$ and $GABA_C$, exist in the outer retina (Polenzani et al., 1991; Feigenspan et al., 1993; Qian and Dowling, 1993, 1994; Woodward et al., 1993; Lukasiewicz et al., 1994; Lukasiewicz and Werblin, 1994). Photoreceptor cells appear to have $GABA_A$ receptors, while horizontal cells have predominantly $GABA_C$ receptors and bipolar cells have both (Djamgoz, 1995). The functional significance for the presence of two types of receptor, particularly in the same cell, is unclear. However, a wider range of responses to GABA may be a particular requirement of the first retinal synapse. Consistent with the hypothesis is the recent observation that Zn^{2+} acts as a neuromodulator at this synapse but has different effects on the GABA-gated currents of horizontal versus bipolar cells (Wu et al., 1993; Chappell et al., 1995; Dong and Werblin, 1995). Since GABA ρ1 subunits appear to be an important constituent of $GABA_C$ receptors, the results of this study suggest a molecular basis for the diversity of Zn^{2+} interactions with retinal GABA receptors.

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