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Location of a High Affinity Zn^{2+} Binding Site in the Channel of $\alpha1\beta1$ γ-Aminobutyric $Acid_A$ Receptors

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ABSTRACT

Zn^{2+} inhibits currents through γ-aminobutyric acid $(GABA)_A$ receptors. Its affinity depends on the subunit composition; $\alpha1\beta1$ receptors are inhibited with high affinity (IC_{50} = 0.54 μM). We sought to identify the residues that form this high affinity Zn^{2+} binding site. β1His267 aligns with α1Ser272, a residue near the extracellular end of the M2 membrane-spanning segment that we previously demonstrated to be exposed in the channel. The Zn^{2+} affinity of $\alpha1\beta1$ H267S was reduced by 300-fold (IC_{50} = 161 μM). Addition of a histidine at the aligned position in α1 creates a receptor, α1S272Hβ1, that should have five channel-lining histidines; the Zn^{2+} affinity was increased 20-fold (IC_{50} = 0.025 μM). Shifting the position of the histidine from the β1 subunit to the aligned position in α1 with the two mutants α1S272Hβ1H267S reduced the affinity (IC_{50} = 26 μM) compared with wild-type. We infer that the high affinity Zn^{2+} binding site involves β1His267 from at least two subunits. For two histidines to interact with a Zn^{2+} ion, the α carbons must be separated by <13 Å. This limits the separation of the subunits and provides a constraint on the possible quaternary structures of the channel. The ability of a divalent cation to penetrate from the extracellular end of the channel to β1His267 implies that the charge-selectivity filter, the structure that discriminates between anions and cations, is located at a more cytoplasmic position than β1His267; this is consistent with our previous work that showed that positively charged sulfhydryl-specific reagents reacted with an engineered cysteine residue as cytoplasmic as α1T261C.

The $GABA_A$ receptors are members of the ligand-gated ion channel gene superfamily and form anion-selective channels (Macdonald and Olsen, 1994; Karlin and Akabas, 1995; Sieghart, 1995). The functional receptor complex is formed as a pentamer of homologous subunits arranged pseudosymmetrically around the central channel axis (Unwin, 1993; Macdonald and Olsen, 1994; Nayeem *et al.*, 1994). Numerous $GABA_A$ receptor subunits have been cloned, including six α, four β, three γ, one δ, one ϵ, and three ρ subunits (Macdonald and Olsen, 1994; Sieghart, 1995). The subunits have a similar transmembrane topology with an ~200-amino acid extracellular amino terminus, three closely spaced membrane-spanning segments (M1, M2, M3), a cytoplasmic loop of variable length, a fourth membrane-spanning segment (M4), and an extracellular carboxyl terminus (Macdonald and Olsen, 1994). Using the substituted-cysteine-accessibility method, we have shown that the channel lining is formed, at least in part, by residues from the M2 segment (Xu and Akabas, 1996).

Functional receptors are formed by coexpression of α and β subunits, although the presence of the γ subunit is essential for benzodiazepine potentiation (Schofield *et al.*, 1987; Pritchett *et al.*, 1989; Gorrie *et al.*, 1997). In heterologous expression systems, the subunit stoichiometry for receptors formed by expression of the α and β subunits is uncertain; support has been provided for two α and three β subunits (Tretter *et al.*, 1997) and for three α and two β subunits (Im *et al.*, 1995; Kellenberger *et al.*, 1996), as well as other stoichiometries (Gorrie *et al.*, 1997). When the γ subunit is included, the stoichiometry seems to be two α, two β, and one γ subunit (Chang *et al.*, 1996; McKernan and Whiting, 1996; Tretter *et al.*, 1997). Some β subunits also form homomeric channels, but they tend to be constitutively open (Krishek *et al.*, 1996).

The divalent cation Zn^{2+} blocks GABA-induced currents

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A preliminary report of this work has appeared in abstract form [Horenstein J and Akabas MH (1997) *Soc Neurosci Abstr* **23**:112]. While preparing the manuscript, we were informed of a preliminary report describing similar results of mutation of the aligned residue in the β_3 subunit, β_3His292A [Wooltorton JRA, McDonald BJ, Moss SJ, and Smart TG (1997) *Br J Pharmacol* **122**(suppl):38P]. A full version of this work was subsequently published in *J Physiol (Lond)* **505:**633–640 (1997).

ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid type A receptor; ACh, acetylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CFFR, Ca^{2+}-free frog Ringer's solution; GABA, γ-aminobutyric acid; MTS, methanethiosulfonate; $MTSES^-$, methanethiosulfonate ethylsulfonate; $MTSET^+$, methanethiosulfonate ethyltrimethylammonium; pCMBS, *p*-chloromercuribenzenesulfonate.

with variable affinity depending on the subunit composition of the receptors (Westbrook and Mayer, 1987; Draguhn *et al*., 1990; Legendre and Westbrook, 1991; Smart *et al*., 1991; Harrison and Gibbons, 1994; Saxena and Macdonald, 1994; Smart *et al*., 1994; Chang *et al*., 1995; Wang *et al*., 1995). It is likely that the effects of Zn^{2+} are mediated by interactions with different sites in receptors with different subunit compositions (Harrison and Gibbons, 1994; Smart *et al*., 1994; Saxena *et al*., 1997). Receptors formed by coexpression of α and β subunits display the highest affinity for Zn^{2+} block with an IC_{50} of ~1 μM (Draguhn *et al*., 1990; Smart *et al*., 1991). In these receptors, Zn^{2+} block is slightly voltage dependent (Draguhn *et al*., 1990), implying that the binding site may be in the channel. The β homomeric channels also display high affinity Zn^{2+} block (Draguhn *et al*., 1990; Krishek *et al*., 1996). The addition of the γ2 subunit to the functional complex markedly reduced Zn^{2+} block so that 100 μM Zn^{2+} caused only 17% inhibition of the GABA-induced currents (Draguhn *et al*., 1990; Smart *et al*., 1991). Zn^{2+} has been proposed to inhibit GABA-induced currents by stabilizing the closed state of the receptor (Smart *et al*., 1994). We sought to identify the residues that form the high affinity Zn^{2+} binding site in the α1β1 $GABA_A$ receptor.

In proteins of known crystal structure, bound Zn^{2+} ions interact directly with two to four amino acids. The amino acids found at Zn^{2+} binding sites include histidine, cysteine, aspartate, or glutamate (Higaki *et al*., 1992; Regan, 1993; Berg and Shi, 1996). Because charged, water-soluble, sulfhydryl reactive reagents applied extracellularly have no effect on $GABA_A$ receptors (Xu and Akabas, 1993, 1996) it is unlikely that a cysteine residue is available to interact with extracellularly applied Zn^{2+}. In GABA receptors formed by the ρ1 subunit, a histidine residue in the extracellular domain was shown to mediate lower affinity Zn^{2+} block (IC_{50} = 16 μM) (Wang *et al*., 1995); histidine, however, is not conserved at the aligned position in other subunits. In the aligned sequences of $GABA_A$ receptor subunits, we noted that histidine was conserved at the position aligned with β1His267 in all β subunits but is not found at the aligned position in other subunit subtypes. This position aligns with α1Ser272 in the M2 membrane-spanning segment; we had previously shown that this residue was exposed in the channel lining (Fig. 1) (Xu and Akabas, 1996). We hypothesized that the high affinity Zn^{2+} binding site was formed by histidine residues from position β1 267 contributed by at least two β subunits.

Materials and Methods

Oligonucleotide-mediated mutagenesis. The cDNAs encoding the rat α1 and γ2 subunits in the pBluescript SK(−) plasmid (Stratagene, La Jolla, CA) were obtained from Dr. P. Seeburg (Max-Planck Institute for Medical Research, Heidelberg, Germany), and the β1 subunit in the pBluescript SK vector was from Dr. A. Tobin (University of California, Los Angeles). The subunits were excised from the pBluescript clones by restriction digestion with the enzymes α1 *Xho*I, β1 *Xba*I and *Hin*dIII, and γ2 *Eco*RI. The β1 and γ2 subunits were ligated into the pGEMHE vector (Liman *et al*., 1992) digested with the corresponding enzymes. For the α1 subunit, the *Xho*I cut fragment was blunt ended and ligated into pGEMHE, which had been digested with *Sma*I. Subcloning and orientation were confirmed by restriction digestion and DNA sequencing. The Altered-Sites Mutagenesis procedure (Promega, Madison, WI) was used to generate mutations as described previously (Xu *et al*., 1995). Mutations were confirmed by DNA sequencing.

Preparation of mRNA and oocytes. Plasmids were linearized with *Nhe*I for *in vitro* mRNA transcription with T7 RNA polymerase. *In vitro* mRNA transcription and the preparation and injection of *Xenopus laevis* oocytes were performed as described previously (Xu *et al*., 1995). Oocytes were injected with 10 ng of mRNA encoding the α1 and β1 subunits in a 1:1 ratio or with α1, β1, and γ2 subunits in a 1:1:1 ratio.

Electrophysiology. GABA-induced currents were recorded from individual oocytes under two-electrode voltage-clamp, at a holding potential of −50 mV. Electrodes were filled with 3 M KCl and had a resistance of <2 MΩ. The ground electrode was connected to the bath via a 3 M KCl/Agar bridge. Data were acquired and analyzed on a 486/33 MHz computer using a TEV-200 amplifier (Dagan Instruments, Minneapolis, MN), a Digidata 1200 data interface (Axon Instruments), and pCLAMP 6 software (Axon Instruments, Foster City, CA). The oocyte was perfused at 5 ml/min with CFFR (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM HEPES, pH 7.5, with NaOH) at room temperature. The recording chamber had a volume of ~0.25 ml.

Experimental protocol. In all experiments, GABA was applied at a concentration 10 times the GABA EC_{50} value of the mutant or wild-type unless otherwise indicated. Applications of GABA were separated by 3–5-min washes with CFFR. In all experiments used for analysis, the magnitude of the GABA-induced current changed by <10% between two consecutive applications of GABA.

To determine the Zn^{2+} IC_{50} value, an increasing series of Zn^{2+} concentrations were applied. Each concentration of $ZnCl_2$ was applied according to the following protocol: $ZnCl_2$ in CFFR, 1 min; $ZnCl_2$ plus GABA in CFFR, 20 sec; $ZnCl_2$ in CFFR, 30 sec; CFFR, 5 min.

The experiments to determine the effects of the sulfhydryl reagents on the β1H267C mutant were performed as described previously (Xu and Akabas, 1996). The sequence of reagents applied was GABA, 20 sec; GABA, 20 sec; sulfhydryl reagent, 1 min; GABA, 20 sec; GABA, 20 sec. For a given oocyte, the concentration of GABA used was either the GABA EC_{50} or 10 times the EC_{50} value of the receptor (see Table 1 for EC_{50} values). The fractional effect was taken as $[(I_{GABA,\ after}/I_{GABA,\ before}) - 1]$. The sulfhydryl reagents were dissolved in CFFR immediately before application.

Curve fitting. The concentration dependence of the inhibition of the GABA-induced currents by Zn^{2+} was fit with the Hill equation, $I/I_{max} = 1/[1 + (IC_{50}/Zn)^n]$, where IC_{50} is the concentration of Zn^{2+} that causes half-maximal inhibition, Zn is the concentration of Zn^{2+}, and n is the Hill coefficient, using either Prism 2.0 (GraphPAD, San Diego, CA) or custom software kindly provided by Dr. Juan Pascual (Columbia University, New York, NY).

Reagents. A 1 M stock solution of $ZnCl_2$ was prepared by adding sufficient HCl to eliminate all visible precipitates. All working solutions of $ZnCl_2$ were prepared daily by diluting the 1 M stock solution in CFFR. The addition of $ZnCl_2$ to CFFR did not change the pH of the solution.

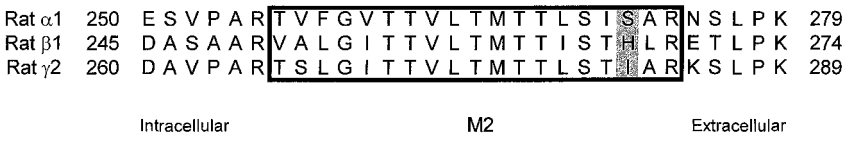

Fig. 1. Aligned sequences in and flanking the M2 membrane-spanning segments of the rat $GABA_A$ receptor α1, β1, and γ2 subunits. *Shaded*, residues aligned with β1His267; *, channel-lining residues identified in the α1 subunit by Xu and Akabas (1996).

The MTS derivatives $MTSES^-$, $MTSET^+$, and MTS ethylammonium were synthesized as described previously (Stauffer and Karlin, 1994) or obtained commercially (Toronto Research Chemicals, North York, Ontario, Canada). These reagents react with cysteine and add $—SCH_2CH_2X$, the charged portion of the molecule onto the sulfhydryl; where X is SO_3^- for $MTSES^-$, $NH3^+$ for MTS ethylammonium, and $N(CH_3)_3^+$ for $MTSET^+$. The organic mercurial $pCMBS^-$ was obtained from Sigma Chemical (St. Louis, MO). It adds $HgC_6H_4SO_3^-$ onto the cysteine.

Results

Characterization of the mutants. We expressed all of the mutants in *X. laevis* oocytes as either $\alpha1\beta1$ or $\alpha1\beta1\gamma$ 2 combinations where the mutant subunit or subunits replaced the corresponding wild-type subunit or subunits. GABA-induced currents were observed in oocytes expressing each of the combinations. For all of the subunit combination, we determined the EC_{50} value for GABA and the Hill coefficient of the GABA dose-response relationship (Table 1). For wild-type $\alpha1\beta1$, the GABA EC_{50} value was 3.4 ± 0.7 μM and the Hill coefficient was 0.97 ± 0.12. The EC_{50} values of the mutants ranged from 5-fold smaller than wild-type for $\alpha1\beta1$H267S to 1.6-fold larger than wild-type for the $\alpha1$S272H$\beta1$ mutant. The EC_{50} value is a function of both the intrinsic affinity of the binding sites for GABA and the isomerization rate constants for transitions between the various closed, open, and desensitized states (Akabas *et al.*, 1992). Because channel gating involves conformational changes in the membrane-spanning segments to transduce ligand binding in the extracellular domain to opening of the gate near the cytoplasmic end of the channel (Xu and Akabas, 1996), mutations of residues in membrane-spanning segments can alter the isomerization rate constants. Thus, the observed changes in EC_{50} probably arise from effects of the mutations on the transduction process.

Residues forming the high affinity Zn^{2+} binding site. Zn^{2+} blocked GABA-induced currents arising from wild-type $\alpha1\beta1$ $GABA_A$ receptors with an IC_{50} value of 0.54 ± 0.02 μM (four experiments) (Fig. 2) similar to the IC_{50} values reported by other investigators for $\alpha_x\beta_y$ receptors (Draguhn *et al.*, 1990; Smart *et al.*, 1991). β1His267 aligns with α1Ser272, a residue that we had previously shown to be a channel-lining residue (Xu and Akabas, 1996). To determine whether β1His267 was involved in forming the high affinity Zn^{2+} binding site, we mutated the histidine to the amino acids found at the aligned positions in the α1 and γ2 subunits, serine and isoleucine, respectively (Fig. 1). The affinity for Zn^{2+} block of both mutants was reduced by >300-fold (Fig. 2); for $\alpha1\beta1$H267S, the IC_{50} value was 161 ± 40 μM (three experiments), and for $\alpha1\beta1$H267I, the IC_{50} value was 654 ± 113 μM (nine experiments) (Table 2). The residual inhibition likely arises from an interaction of Zn^{2+} with a different site or sites on the receptor; removal of the high affinity site has unmasked this previously unrecognized low affinity site or sites. The location of this low affinity binding site is unknown.

TABLE 1
GABA EC_{50} values

Receptor	EC_{50}	Hill	n
	μM		
αβ	3.4 ± 0.7	0.97 ± 0.12	3
αβH267S	0.7 ± 0.2	0.67 ± 0.05	3
αβH267I	2.2 ± 0.1	1.40 ± 0.24	3
αβH267C	4.6 ± 0.8	1.48 ± 0.24	7
αS272Hβ	5.6 ± 0.9	0.79 ± 0.12	2
αS272H βH267S	1.7 ± 0.6	0.83 ± 0.06	3
αS272H γI282H	No GABA-induced current		
βγI282H	No GABA-induced current		
αβγ	7.8 ± 0.7	0.96 ± 0.21	2
αS272H βH267Sγ	4.7 ± 1.5	0.64 ± 0.06	3
αβH267S γI282H	1.9 ± 0.3	1.03 ± 0.06	2
αS272H βH267S γI282H	2.9 ± 0.1	0.86 ± 0.21	2
αS272Hβγ	3.8 ± 0.7	1.07 ± 0.17	3
αβγI282H	3.4 ± 1.2	0.78 ± 0.14	2
αS272H βγI282H	2.4 ± 0.9	0.86 ± 0.05	3

Interactions with residues in other subunits. To investigate whether a histidine at the aligned position in the α1 subunit would interact with β1His267, we generated the mutant α1S272H and expressed it with the wild-type β1 subunit. With five histidines in the channel lining, the IC_{50} value of the $\alpha1$S272H$\beta1$ receptor was 0.025 ± 0.007 μM (three experiments) (Fig. 3); that the affinity was significantly higher than wild-type suggests that the aligned residues in different subunits are in close proximity in the channel lumen. To ensure that the higher affinity was not solely due to placement of the histidine in the α1 subunit, we

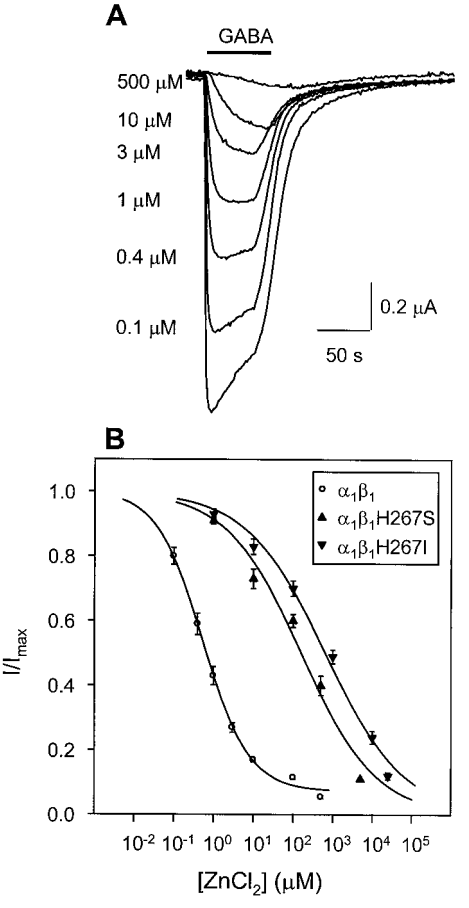

Fig. 2. High affinity Zn^{2+} inhibition of wild-type $\alpha1\beta1$ $GABA_A$ receptors is reduced in the mutants $\alpha1\beta1$H267S and $\alpha1\beta1$H267I. A, GABA-induced currents recorded by two-electrode voltage clamp from an oocyte expressing wild-type $\alpha1\beta1$ receptors in the presence of varying concentrations of $ZnCl_2$. B, Concentration dependence of the Zn^{2+} inhibition of GABA-induced currents for wild-type $\alpha1\beta1$ (○), $\alpha1\beta1$H267S (▲), and $\alpha1\beta1$H267I (▼). Mean ± standard error values are plotted; for some points, the *error bars* are smaller than the symbol. The data were fit by the Hill equation. *Solid lines*, calculated from the fitted equation.

examined the effect of Zn^{2+} on the receptor that at this position only contained histidine in the α1 subunit, α1S272Hβ1H267S; the IC_{50} value was 26 ± 1 μM (eight experiments) (Fig. 3, Table 2). The affinity of α1S272Hβ1H267S for Zn^{2+} was lower than that in wild-type but higher than that in the α1β1H267S receptor, a receptor with no histidines at this level of the channel.

Cysteine substitution for β1His267. It was hypothesized that Zn^{2+} inhibits GABA-induced currents by binding to and stabilizing the closed state of the $GABA_A$ receptor (Smart *et al.*, 1994). Therefore, we sought to determine whether β1His267 was accessible to interact with ions in the closed state of the channel. We used the substituted-cysteine-accessibility method (Akabas *et al.*, 1992; Xu and Akabas, 1993) to probe the accessibility of a cysteine residue substituted at position 267 in the closed state of the channel. In this approach, a cysteine residue is engineered into the protein. The cysteine-substitution mutant is expressed, and the ability of charged, sulfhydryl-specific reagents to react with the engineered cysteine residue is tested. The magnitude of the GABA-induced current is determined; then, the sulfhydryl reagent is applied for 1 min in the absence of GABA (i.e., in the closed state of the channel) and washed out, and the magnitude of the GABA-induced current is determined again. If the magnitude of the GABA-induced currents after application of the sulfhydryl reagent is significantly different than the magnitude of the GABA-induced currents before application of the sulfhydryl reagent, we infer that the sulfhydryl reagent has covalently modified the engineered cysteine. The sulfhydryl reagents we used are $pCMBS^-$, $MTSES^-$, and $MTSET^+$ (Xu and Akabas, 1996). Because these reagents react more rapidly with the thiolate anion (S^-) than with the neutral thiol (SH) (Hasinoff *et al.*, 1971; Roberts *et al.*, 1986) and because only cysteines on the water-accessible surface of the protein are likely to ionize to a significant extent, we assume that these reagents will only react with cysteine residues on the water-accessible surface of the protein at an appreciable rate. Covalent modification of a channel-lining cysteine may alter the single-channel conductance and/or the gating kinetics (Akabas *et al.*, 1994). To detect the effects of covalent modification, we used two different test concentrations of GABA: one at the GABA EC_{50} value and the other at 10 times the EC_{50} value. Zhang and Karlin (1997) have shown that applying the agonist at the EC_{50} value for the test responses before and after the reagent provides a more sensitive test for detecting covalent modification because it allows one to detect reaction when the effect of modification is a change in gating alone with little or no change in single-channel conductance. In contrast, application at 10 times the EC_{50} value will detect changes in conduction but should be insensitive to changes in gating.

A 1-min application of 0.5 mM $pCMBS^-$, 10 mM $MTSES^-$, or 1 mM $MTSET^+$ in the absence of GABA had no significant effect on wild-type α1β1 $GABA_A$ receptors whether the test concentration of GABA was at the EC_{50} or 10 times the EC_{50} value (Fig. 4C, *open bars*). For the α1β1H267C mutant, when the test concentration of GABA was 5 μM, the EC_{50} value for the mutant (Table 1), a 1-min application of 0.5 mM $pCMBS^-$, 10 mM $MTSES^-$, or 1 mM $MTSET^+$ in the absence of GABA potentiated the subsequent GABA-induced currents by ~50% (Fig. 4, A and C, *left*). For the α1β1H267C mutant, when the test concentration of GABA was 50 μM, 10 times the EC_{50} value for the mutant, a 1-min application of 1 mM $MTSET^+$ caused a 31% potentiation of the subsequent GABA-induced currents but a 1-min application of 0.5 mM $pCMBS^-$ or 10 mM $MTSES^-$ had no significant effect on the subsequent GABA-induced currents (Fig. 4, B and C, *right*). Thus, in the closed state of the receptor, the engineered cysteine was accessible to react with all three of the reagents. Therefore, we infer that the corresponding wild-type residue, β1His267, is on the water-accessible surface of the protein in the closed state of the channel and therefore available to interact with Zn^{2+}.

Based on the differences between the effects of modification as probed with GABA test concentrations at the EC_{50}

TABLE 2
Zn^{2+} IC_{50} values

Receptor	IC_{50}	IC_{50} (mut)/ IC_{50} (wt)	Hill	n
	μM			
αβ	0.54 ± 0.02	1	0.75 ± 0.03	4
αβH267S	161 ± 40	298	0.46 ± 0.06	3
αβH267I	654 ± 113	1211	0.43 ± 0.03	9
αS272Hβ	0.025 ± 0.007	0.05	0.64 ± 0.10	3
αS272H βH267S	26 ± 1	48	0.87 ± 0.03	8
αβH267C	23 ± 3	42	0.65 ± 0.06	5
αβγ	>1 mM	>1800		4
αS272H βH267Sγ	>1 mM	>1800		2
αβH267S γI282H	>1 mM	>1800		3
αS272H βH267S γI282H	>1 mM	>1800		2
αS272Hβγ	>1 mM	>1800		3
αβγI282H	>1 mM	>1800		3
αS272H βγI282H	>1 mM	>1800		5

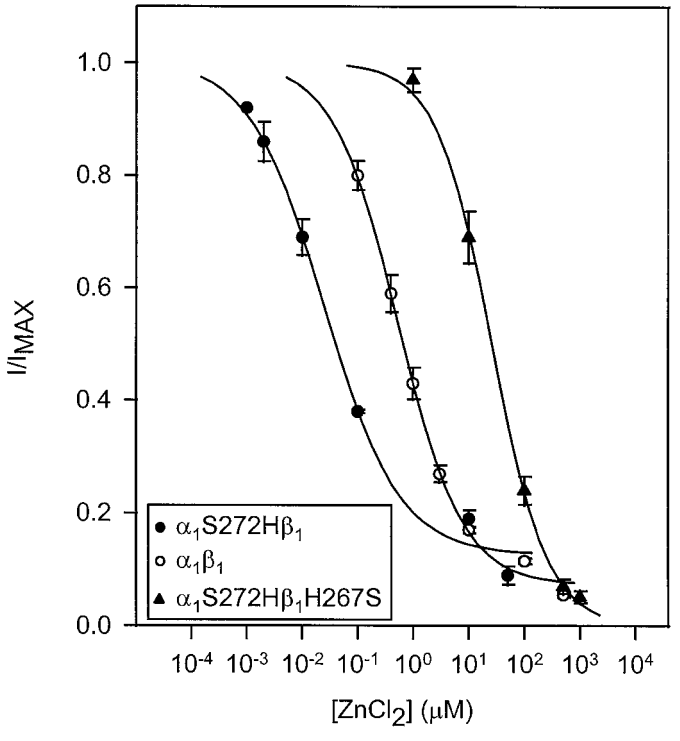

Fig. 3. The affinity for Zn^{2+} inhibition is increased in the α1S272Hβ1 receptor and decreased in the α1S272Hβ1H267S receptor relative to the wild-type α1β1 receptor. Concentration dependence of the Zn^{2+} inhibition of GABA-induced currents for wild-type α1β1 (○), α1S272Hβ1 (●), and α1S272Hβ1H267S (▲). Mean ± standard error values are plotted; for some points, the *error bars* are smaller than the symbol. The data were fit by the Hill equation. *Solid lines*, calculated from the fitted equation.

and 10 times EC_{50} value, we infer that the cationic reagent $MTSET^+$ alters conduction and probably also gating, but the major effect of modification by the anionic reagents $pCMBS^-$ and $MTSES^-$ is on gating. It was surprising that modification by all three reagents resulted in potentiation of the subsequent GABA-induced currents. Presumably, the presence of a charged residue, that is, the covalently modified cysteine, at this position stabilizes the open state; the mechanism, however, is unknown.

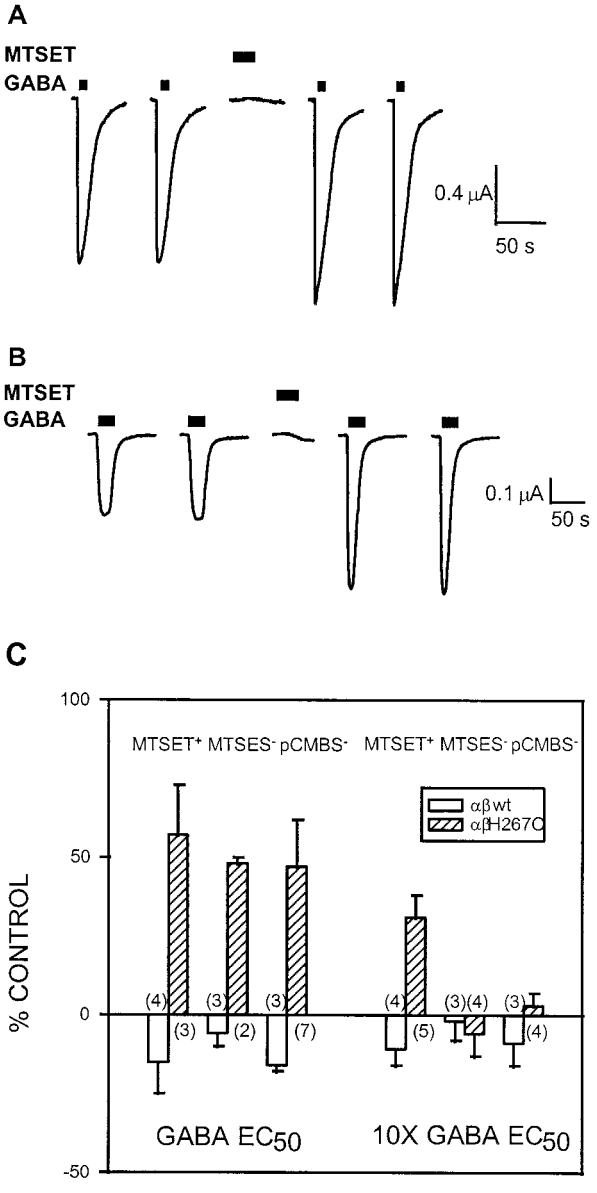

Fig. 4. The engineered cysteine in the mutant $\alpha1\beta1$H267C is accessible to covalent modification by charged, sulfhydryl-specific reagents applied extracellularly in the closed state of the receptor. A and B, Currents recorded from oocytes expressing the mutant $\alpha1\beta1$H267C by two-electrode voltage-clamp. *Bars over the traces*, periods during which reagents were applied to the oocytes. Holding potential was −50 mV. Note the increase in the magnitude of the two current responses after the application of $MTSET^+$ relative to the initial two responses. *Traces*, separated by a 5-min wash with CFFR. The GABA concentration was 5 μM in A and 50 μM in B. C, Effect of a 1-min application of 0.5 mM $pCMBS^-$, 10 mM $MTSES^-$, or 1 mM $MTSET^+$ applied extracellularly in the absence of GABA on wild-type (□) and $\alpha1\beta1$H267C (▨). The test concentrations of GABA were at the EC_{50} value (*left*) and at 10 times the EC_{50} value (*right*). Mean ± standard error values are plotted. *Parentheses*, number of oocytes tested. Positive effect, potentiation of the subsequent GABA-induced currents; negative effect, inhibition of the subsequent GABA-induced currents.

Cysteine residues are frequently involved in the formation of Zn^{2+} binding sites (Higaki *et al.*, 1992; Regan, 1993; Berg and Shi, 1996); therefore, we tested the effect of Zn^{2+} on $\alpha1\beta1$H267C. Zn^{2+} inhibited the GABA-induced currents; the IC_{50} value was 23 ± 3 μM (five experiments) (Table 2). Although the affinity of $\alpha1\beta1$H267C for Zn^{2+} was 40-fold lower than wild-type, it was 7- and 28-fold higher than the corresponding serine and isoleucine mutants at this position. Thus, we believe that Zn^{2+} is binding to the engineered cysteine or cysteines. Furthermore, covalent modification of the engineered cysteine by either 10 mM $MTSES^-$ or 5 mM $MTSET^+$ reduced the ability of Zn^{2+} to inhibit the $\alpha1\beta1$H267C. Before modification, Zn^{2+} inhibited $\alpha1\beta1$H267C with an IC_{50} of 23 ± 3 μM (five experiments) (Table 2); after modification of $\alpha1\beta1$H267C by either $MTSES^-$ or $MTSET^+$, the IC_{50} value for Zn^{2+} inhibition of GABA-induced currents was >1 mM (three experiments) (Fig. 5). Thus, covalent modification of the cysteine prevents it from interacting with Zn^{2+}.

Effect of the γ2 subunit on Zn^{2+} affinity. It has previously been shown that the IC_{50} value for Zn^{2+} inhibition of $GABA_A$ receptors containing the γ2 subunit is much higher than that for receptors formed from only the α and β subunits (Draguhn *et al.*, 1990; Smart *et al.*, 1991). Consistent with this, we found that the Zn^{2+} IC_{50} value for wild-type $\alpha1\beta1\gamma2$ was >1 mM (three experiments). In the γ2 subunit, the residue Ile282 aligns with β1His267 (Fig. 1). We mutated this residue to histidine, γ2I282H, and expressed the mutant with wild-type α1 and β1 subunits, but the Zn^{2+} IC_{50} value also was >1 mM (three experiments) (Table 2). Expression of the other combinations of histidines at this position α1S272Hβ1H267Sγ2I282H, and α1S272Hβ1γ2I282H, which should contain five histidines, also gave IC_{50} values of >1 mM (Table 2). Thus, inclusion of the γ2 subunit prevents the ability of Zn^{2+} to inhibit GABA-induced currents regardless of the number of histidines present at this level.

Discussion

Zn^{2+} is a high affinity inhibitor of $\alpha1\beta1$ $GABA_A$ receptors (Draguhn *et al.*, 1990; Smart *et al.*, 1991). We have shown that the Zn^{2+} affinity of $\alpha1\beta1$ $GABA_A$ receptors is reduced by >300-fold by mutation of β1His267 in the M2 membrane-spanning segment to either serine or isoleucine, the amino acids at the aligned positions in the α1 and γ2 subunits (Fig. 1). Furthermore, we have shown that β1His267 is exposed in the channel lining (Fig. 4) as we had previously shown for the aligned residue in the α1 subunit, α1Ser272 (Xu and Akabas, 1996). Histidine is conserved at the position aligned with β1His267 in all $GABA_A$ receptor β subunits but is not found at the aligned position in other subunit subtypes. Histidine residues frequently form part of metal ion binding sites (Higaki *et al.*, 1992; Regan, 1993; Berg and Shi, 1996). Thus, we infer that β1His267 participates in the formation of the high affinity Zn^{2+} site in $\alpha1\beta1$ $GABA_A$ receptors. The position of this residue near the extracellular end of the M2 channel-lining segment is consistent with the slight voltage dependence reported for Zn^{2+} block of $\alpha1\beta1$ $GABA_A$ receptors (Draguhn *et al.*, 1990). A different histidine in the extracellular amino-terminal domain of the GABA ρ1 subunit was impli-

cated in lower affinity Zn^{2+} inhibition of the $GABA_C$ receptor (Wang *et al.*, 1995).

Because the $GABA_A$ receptor is formed as a pentamer of subunits arranged pseudosymmetrically around the central channel axis (Unwin, 1993; Nayeem *et al.*, 1994), one would hypothesize that the aligned channel-lining residues from each subunit should be at approximately the same distance into the channel, thereby forming a ring of residues at a given level of the channel. Insertion of histidine into the aligned position in the α1 subunit and expression with wild-type β1, which should give five histidines at this level, increases the affinity for Zn^{2+} by 20-fold. This suggests that the aligned residues in different subunits are in close proximity in the channel lumen and that the presence of more histidines at this level allows for the formation of a higher affinity binding site.

To form a high affinity binding site, Zn^{2+} must interact with more than one residue. The affinity of a site will depend on the number of chelating residues, the relative position of the residues, and the local electrostatic environment (Higaki *et al.*, 1992; Regan, 1993; Berg and Shi, 1996). It is likely that at least two histidines form the high affinity Zn^{2+} binding site in the α1β1 $GABA_A$ receptor. The subunit stoichiometry of the α1β1 $GABA_A$ receptor is uncertain, and evidence has been reported for both two α and three β subunits (Tretter *et al.*, 1997) and three α and two β subunits (Im *et al.*, 1995; Kellenberger *et al.*, 1996; Gorrie *et al.*, 1997). We are uncertain why the affinity for Zn^{2+} is 50-fold higher when the histidine is in the β subunit in wild-type receptor compared with when the histidine is in the α subunit in the α1S272Hβ1H267S mutant (Table 2). There are several potential explanations for this difference in affinity. If the subunit stoichiometry is 2α:3β, then the high affinity site could be formed between histidines in the adjacent β subunits that would be present (Fig. 6A); alternatively, all three histidines might interact with the Zn^{2+} (Fig. 6A). The lower affinity observed when the histidine was in the α subunit would arise because there would be two histidines in nonadjacent subunits that might be less favorable for Zn^{2+} binding (Fig. 6B). Alternatively, neighboring channel-lining residues, such as β1Glu270, also might influence the interaction between the histidines and Zn^{2+}, thereby resulting in a higher affinity interaction when the histidine is in the β subunit compared with the α subunit, where the adjacent channel-lining residue is α1Asn275. Finally, the position of the α and β subunits relative to the channel may not be symmetrical, and the difference in the affinity is due to the asymmetry and not to the relative number of subunits.

In X-ray crystal structures of proteins containing Zn^{2+} bound through histidine residues, the separation between the Zn^{2+} and the ε-amino group is ~2 Å (Higaki *et al.*, 1992). This distance and the size of histidine constrain the maximum separation of the α carbons of two histidine residues bound to a Zn^{2+} ion to <13 Å (Higaki *et al.*, 1992). Thus, in the Zn^{2+} bound state of the $GABA_A$ receptor, at the level of β1His267, the α carbons of the aligned residues in both adjacent and nonadjacent subunits must be <13 Å apart (Fig. 7B, *distances A–B and A–C*).

Our previous work demonstrated that picrotoxin, a rigid, roughly spherical molecule 9 Å in diameter, binds near the cytoplasmic end of the channel in the region of α1Val257 (Fig. 7, *distance D–E*) (Xu *et al.*, 1995). Because it reaches that site from the extracellular end of the channel, we inferred that the channel lumen must be ≥9 Å in diameter to the level of α1Val257 (Fig. 7) (Xu *et al.*, 1995). Our current results constrain the maximum separation of the α carbon atoms on nonadjacent subunits at the level of β1His267 to <13 Å. It should be noted, however, that these distances may be measured in different states of the receptor. Picrotoxin binds in the open state of the channel (Newland and Cull-Candy, 1992), and Zn^{2+} may bind in the closed state of the channel (Smart *et al.*, 1994). We do know, however, that charged sulfhydryl reactive reagents can react with engineered cysteine residues in the α1 M2 segment in the closed state of the channel (Xu and Akabas, 1996). These reagents would fit into a right cylinder 6 Å in diameter and 10 Å in length. Thus, in the closed state of the channel, the lumen must be ≥6 Å in diameter to allow these reagents to reach the engineered cysteine residues at positions more cytoplasmic than β1His267 (Xu and Akabas, 1996), but the α carbons of the residues aligned with β1His267 must be closer than 13 Å. The narrowest region in the channel was inferred, based on the size of the largest permeant anion, to be ~5.6 Å (Bormann *et al.*, 1987), but this must be at a position that is more cytoplasmic than the picrotoxin binding site (Fig. 7B, *distance F–G).*

If the channel were lined by five α helical M2 segments arranged perpendicular to the membrane, the separation between the aligned position on the surface of adjacent α helices would be ~4 Å and that between nonadjacent α-heli-

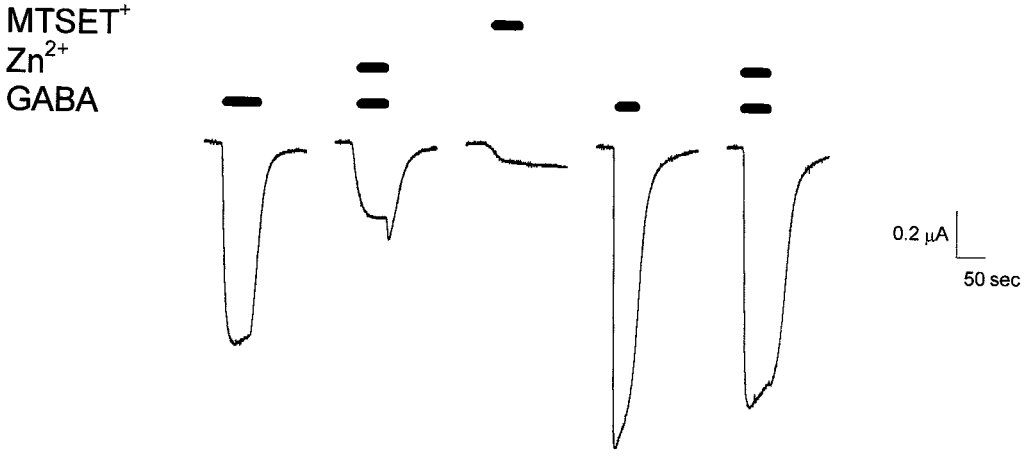

Fig. 5. Covalent modification of the engineered cysteine in the α1β1H267C mutant reduces the inhibition by Zn^{2+}. The reagents were applied during the periods indicated (*bars above the traces*) at 2 μM GABA, 5 mM $MTSET^+$, and 30 μM Zn^{2+}. Zn^{2+} inhibited the GABA-induced current by 63% before modification by $MTSET^+$ and by 14% after modification by $MTSET^+$. All other conditions were the same as for Fig. 4. Note that the rate of GABA-induced desensitization is increased after modification.

ces would be ~7 Å: The channel, however, is unlikely to be lined by five helices perpendicular to the membrane. In the 9 Å resolution structure of the ACh receptor reported by Unwin (1993), the putative M2 segments are not perpendicular to the membrane but rather angle out from the central channel axis toward the extracellular end of the channel (as illustrated in Fig. 7B). In the ACh receptor, we showed that residues near the extracellular end of the M1 membrane-spanning segment also were exposed in the channel lining, and we suggested that in the closed state, the M1 segments may intercalate between the M2 segments at the extracellular end of the channel (Akabas and Karlin, 1995). Thus, the 13 Å constraint on the separation of the α carbons seems to be reasonable given our current structural picture of the channel.

The $GABA_A$ receptor forms a nearly ideally anion-selective channel (Bormann *et al.*, 1987). The ability of a divalent cation, Zn^{2+}, to penetrate from the extracellular end of the channel to the level of β1His267 in the M2 membrane-spanning segment indicates that the charge-selectivity filter that discriminates between anions and cations must be located at a more cytoplasmic position than β1His267. This is consistent with our previous results (Xu and Akabas, 1996) that showed that cationic sulfhydryl reagents could react with cysteines substituted for residues as cytoplasmic as α1Thr261, which aligns with β1Thr256 (Fig. 7A). We inferred that the charge-selectivity filter is located at a more cytoplasmic position than these residues, perhaps near the cytoplasmic end of the channel where the channel seems to narrow and form a picrotoxin binding site (Xu *et al.*, 1995).

As other investigators had shown (Draguhn *et al.*, 1990; Smart *et al.*, 1991), the presence of the γ2 subunit markedly reduces the affinity for Zn^{2+}. The mechanism of this inhibition is not solely due to the lack of a histidine at the aligned

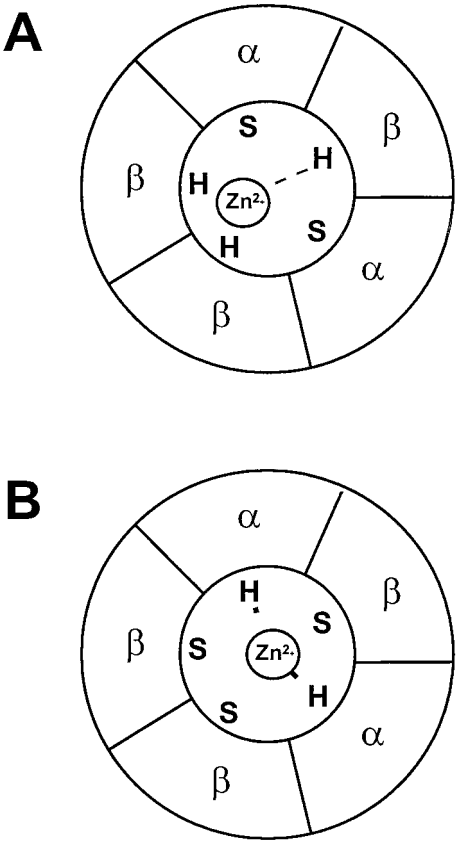

Fig. 6. Position of subunits and the histidine residues in the wild-type α1β1 receptor and in the α1S272Hβ1H267S double mutant relative to the channel and the bound Zn^{2+} ion. A, Top view of the channel formed by two α1 and three β1 subunits. B, Top view of the channel formed by two α1S272H and three β1H267S subunits.

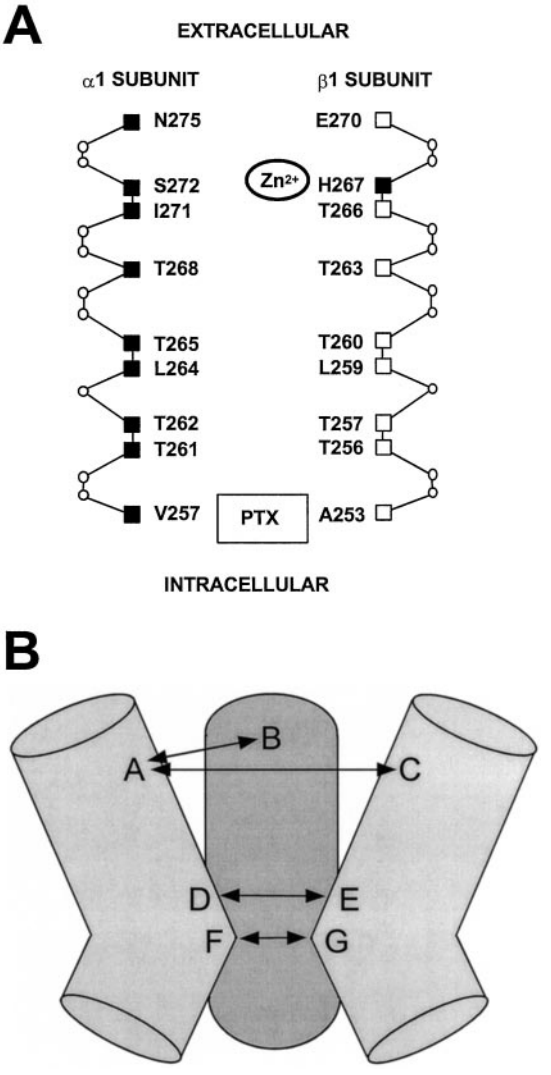

Fig. 7. Two views of the M2 membrane-spanning segments of the $GABA_A$ receptor, the positions of the Zn^{2+} and picrotoxin binding sites, and the inferred distances between subunits and within the channel. A, Channel-lining residues in the M2 segments of the α1 and β1 subunits. The channel-lining residues (■) in the α1 subunit were identified by the substituted-cysteine-accessibility method (Xu and Akabas, 1996). □, Aligned residues in the β1 subunit. *PTX*, inferred location of the picrotoxin binding site (Xu *et al.*, 1995). *Zn^{2+}*, location of the Zn^{2+} binding site determined in this report. B, Three-dimensional representation of the M2 segments lining the $GABA_A$ receptor channel. Three of the five M2 segments are shown; the front two have been removed. *Top*, extracellular end. The M2 segments are shown as kinked helices based on their inferred structure from the 9-Å resolution structure of the homologous ACh receptor (Unwin, 1993). *Points A–C*, level of β1His267, the Zn^{2+} binding site. *AB*, distance between adjacent subunits. *AC*, distance between nonadjacent subunits. The distances between the Cα carbons of the residues at these positions must be <13 Å. *Points D and E*, at the level of α1Val257, the picrotoxin binding site (Xu *et al.*, 1995). *DE*, must be ≥9 Å, the diameter of picrotoxin. *Points F and G*, at the narrowest region of the channel, the functional diameter of which was inferred to be ~5.6 Å based on the size of the largest permeant anion (Bormann *et al.*, 1987).

position in the channel because substitution of a histidine at that position did not increase the affinity for Zn^{2+} (Table 2). Thus, some other aspect of the γ2 subunit prevents Zn^{2+} inhibition of GABA-induced currents. Potential explanations include that (1) the γ2 subunit may sterically restrict the conformations of the other subunits, and particularly β1His267, from adopting the conformation to which Zn^{2+} binds; (2) the putative adjacent channel-lining residue γ2Lys285 may electrostatically interfere with Zn^{2+} binding; and (3) Zn^{2+} might still bind to the receptor complex containing the γ2 subunit, but the presence of the γ2 subunit prevents the conformational change induced by Zn^{2+} in the α1β1 receptor.

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