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An N-Terminal Histidine Is the Primary Determinant of α Subunit-Dependent Cu^{2+} Sensitivity of $\alpha\beta3\gamma2L$ $GABA_A$ Receptors

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ABSTRACT

Copper (Cu^{2+}) is a physiologically important cation and is released from nerve terminals. Cu^{2+} modulates $GABA_A$ receptor currents in an α subunit subtype-dependent manner; $\alpha1\beta3\gamma2L$ receptors are more sensitive to Cu^{2+} than $\alpha6\beta3\gamma2L$ receptors. We compared the effect of Cu^{2+} on $\alpha\beta3\gamma2L$ receptors containing each of the six α subtypes and generated $\alpha1/\alpha6$ chimeras and mutants to determine the functional domain(s) and specific residues responsible for α subtype-dependent differences in Cu^{2+} sensitivity. Whole-cell $GABA_A$ receptor currents were obtained from L929 fibroblasts coexpressing wild-type, chimeric and mutant α subunits with $\beta3$ and $\gamma2L$ subunits. Maximal Cu^{2+} inhibition of $\alpha1\beta3\gamma2L$ and $\alpha2\beta3\gamma2L$ receptor currents was larger (52.2 ± 3.0 and 59.0 ± 2.5%, respectively) than maximal inhibition of $\alpha3\beta3\gamma2L$, $\alpha4\beta3\gamma2L$, $\alpha5\beta3\gamma2L$, and $\alpha6\beta3\gamma2L$ receptor currents (22.6 ± 3.1, 19.2 ± 3.4, 20.2 ± 4.8, and 21.2 ± 3.6%, respectively). Receptors containing chimeric constructs with $\alpha1$ subtype N-terminal sequence between residues 127 and 232 were inhibited by Cu^{2+} to an extent similar to those with $\alpha1$ subtypes, suggesting that this N-terminal region (127–232) contains a major determinant for high Cu^{2+} sensitivity. $\alpha1$ subtype residues V134, R135, and H141 in a **VR**AECPM**H** motif (**VQ**AECPM**H** in the $\alpha2$ subtype) conferred higher Cu^{2+} sensitivity, and the H141 residue was the major determinant in the motif. The $\beta3$ subtype M2 domain residue H267, which is a major determinant of Zn^{2+} inhibition, and $\alpha6$ subtype M2-M3 loop residue H273, which is responsible for the increased Zn^{2+} sensitivity of the $\alpha6$ subtype, also seemed to contribute to Cu^{2+} inhibition. These data suggest that the N-terminal **VR(Q)**AECPM**H** motif in $\alpha1$ and $\alpha2$ subtypes is the major determinant of increased subtype-dependent inhibition by Cu^{2+}, that residue H141 is the major determinant in that motif, and that Cu^{2+} may also interact with $GABA_A$ receptors at sites similar to or overlapping Zn^{2+} sites.

Fast inhibitory synaptic transmission in the mammalian central nervous system is mediated primarily by $GABA_A$ receptors, which are members of a superfamily of ligand-gated ion channels including nicotinic acetylcholine, glycine, and 5-hydroxytryptamine type 3 receptors. $GABA_A$ receptors are composed of a pentameric combination of subunits that form an intrinsic chloride ion channel. Each $GABA_A$ receptor subunit has a putative membrane topology consisting of a large N-terminal extracellular domain, four membrane spanning domains (TM1–TM4) and an extracellular C terminus. A large number of $GABA_A$ receptor subunit subtypes have been identified; $\alpha(1–6)$, $\beta(1–4)$, $\gamma(1–3)$, δ, ϵ, π, and θ subunit subtypes (Macdonald and Olsen, 1994; Rabow et al., 1995; Davis et al., 1997; Hedblom and Kirkness, 1997; Bonnert et al., 1999). Most native $GABA_A$ receptors in brain are thought to be composed of $\alpha\beta\gamma$ or $\alpha\beta\delta$ subunit combinations (Macdonald and Olsen, 1994; McKernan and Whiting, 1996).

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Cations have been shown to modulate voltage- and ligand-gated ion channels (Ma and Narahashi, 1993; Smart et al., 1994; Fisher and Macdonald, 1998; Nagaya and Macdonald, 2001). Cu^{2+} plays important roles physiologically and pathologically as a cofactor for many enzymes or sources of free radicals (for review, see Pena et al., 1999). It has been reported that Cu^{2+} is released from nerve terminals in some brain regions during depolarization (Hartter and Barnea, 1988; Kardos et al., 1989), and Cu^{2+} levels are higher in brain than in other organs (Hui et al., 1977). The concentration of synaptic Cu^{2+} is estimated to be in the micromolar range (Kardos et al., 1989). Because it has been shown that Zn^{2+} is released from nerve terminals and can be a potential endogenous neuronal modulator (Assaf and Chung, 1984; Smart et al., 1994), most studies of cation modulation of ligand-gated ion channel have been on Zn^{2+}. However, several recent studies have shown that Cu^{2+} inhibited $GABA_A$ receptor currents in native and transfected cells (Ma and Narahashi, 1993; Narahashi et al., 1994; Fisher and Mac-

ABBREVIATIONS: ACh, acetylcholine.

donald, 1998; Sharonova et al., 1998). In addition, Cu^{2+} inhibited α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, *N*-methyl-D-aspartate, and glycine receptor currents (Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996) and blocked long-term potentiation in hippocampus (Doreulee et al., 1997). These findings imply that Cu^{2+} may play an important role in synaptic transmission as a modulator, possibly similar to the action of Zn^{2+}.

It has been demonstrated that Cu^{2+} modulates $GABA_A$ receptors in a subunit-subtype dependent manner: α1 subtype-containing receptors cotransfected with β3 and γ2L subunits are more sensitive to Cu^{2+} than α6 subtype-containing receptors (Fisher and Macdonald, 1998). In a previous study using α1/α6 chimera and α6/α1 chimeras made by replacing entire N-terminal sequence of α1 and α6 subunit with α6 and α1 sequences, respectively, from the middle of TM1 to the N terminus, we suggested that the functional domain regulating α subtype-dependent Cu^{2+} sensitivity of $GABA_A$ receptors was located in the N-terminal extracellular domain of α subunits (Fisher and Macdonald, 1998). In present study, we identify further the functional domains and specific residues responsible for α subtype-dependent Cu^{2+} sensitivity of αβ3γ2L $GABA_A$ receptors.

Materials and Methods

Construction of Chimeric and Mutant cDNAs. The chimeras were generated by interchanging restriction fragments between α1 and α6 cDNAs. Point mutations were generated using the QuikChange site-directed mutagenesis procedure and products (Stratagene, La Jolla, CA). Oligonucleotide primers were synthesized by the University of Michigan DNA synthesis core (Ann Arbor, MI). Sequences of chimeras and point mutants were verified by fluorescent DNA sequencing (University of Michigan DNA sequencing core).

Transient Transfection of L929 Cells. Full-length cDNAs for rat α1, α3, α5, α6, β3, γ2L, and human α2 $GABA_A$ receptor subtypes were subcloned into the pCMVneo vector and the rat α4 subtype cDNA was subcloned into the pRK5 expression vector. All of the cDNAs were transfected into the mouse fibroblast cell line L929 (American Type Culture Collection, Manassas, VA) using a modified calcium phosphate method (Chen and Okayama, 1987). Plasmids encoding α, β3, and γ2L $GABA_A$ receptor subtype cDNAs were added to the cells in 1:1:1 ratios of 4 μg each plus 4 to 8 μg of the plasmid-encoding sFv. After 4 to 6 h of incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in Bis-buffered saline for 30 s. L929 cells were maintained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For selection of transfected cells, the plasmid pHook-1 (Invitrogen) containing cDNA that encoded the surface antibody sFv was cotransfected into the cells. Twenty to 28 h later, the cells were passaged and mixed with 5 μl of magnetic beads coated with hapten. After a 30- to 60-min incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated using a magnetic stand. The selected cells were used for recording 18 to 28 h later.

Electrophysiological Recording Techniques and Analysis of Whole-Cell Currents. For whole-cell recording, the external solution consisted of 142 mM NaCl, 8.1 mM KCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4, and osmolarity was adjusted to 295 to 305 mOsM. Recording electrodes were filled with an internal solution of 153 mM KCl, 1 mM $MgCl_2$, 5m EGTA, 10 mM HEPES, and 2 mM MgATP, pH 7.3, and osmolarity was adjusted to 295 to 305 mOsM. These solutions provided a chloride equilibrium potential near 0 mV. Patch pipettes were pulled from microhematocrit tubes made of soda lime glass (World Precision Instruments, New Haven, CT) on a P 87 Flaming Brown puller (Sutter Instrument Co., San Rafael, CA). The drugs were applied to cells using a modified U-tube delivery system with a 10-to-90% rise time of 70 to 150 ms (Greenfield and Macdonald, 1996). Currents were recorded with a List EPC-7 (List Electronics, Darmstadt, Germany) patch clamp amplifier. All experiments were performed at room temperature. Whole cell currents were analyzed using the programs Axoscope (Axon Instruments) and Prism (GraphPad, San Diego, CA). All whole-cell current amplitudes were obtained by measuring the peak current evoked during the application of GABA or GABA plus Cu^{2+}. All data for Cu^{2+} modulation of $GABA_A$ receptor currents were normalized to the response to GABA alone. Normalized concentration-response data for the different isoforms were fitted with a four-parameter logistic equation ($I = I_{max}/(1 + [10(\log EC_{50} - \log[drug])^{n_H}])$, where n_H is the Hill coefficient, and I represented currents expressed as a percentage of the current elicited by GABA alone (I_{max}). Data were presented as mean ± S.E.M. Statistical comparisons among $GABA_A$ receptor subunit combinations were performed with one-way analysis of variance, Newman-Keuls multiple comparison test.

Results

Cu^{2+} Inhibited α1β3γ2L to a Greater Extent Than α6β3γ2L Receptor Currents. Cu^{2+} inhibited α1-receptor currents (we use the short-hand notation of "α*n*-receptors" to signify "α*n*β3γ2L receptors") to a greater extent than α6-receptor currents. Cu^{2+} IC_{50} values for both receptors were similar, but the maximal Cu^{2+} inhibition of α1-receptor currents (52.2%) was greater than maximal inhibition of α6-receptors (21.2%) ($p < 0.001$) (Fig. 1 and Table 1). In a previous study, we reported that the Cu^{2+} concentration-response relationship curve for inhibition of $GABA_A$ receptor

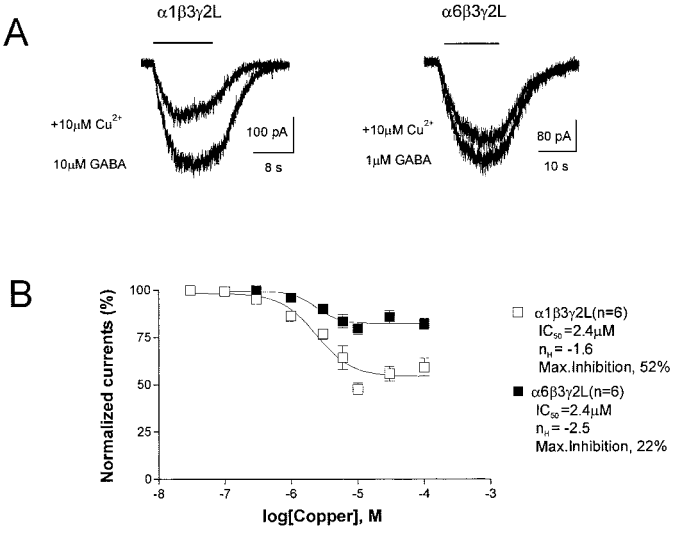

Fig. 1. Cu^{2+} sensitivity of wild-type α subunit subtype-containing receptors. A, representative whole-cell currents from L929 fibroblasts coexpressing α1 and α6 subtype-containing receptors with β3 and γ2L subunits. GABA or GABA plus Cu^{2+} was applied for 8 to 12 s (as indicated by horizontal bar) and voltage clamped at −50 mV. The GABA concentration used was near the EC_{50} value for each isoform, and the Cu^{2+} concentration used was 10 μM. B, Cu^{2+} concentration-response relationship for α1 and α6 subtype-containing receptors were obtained by normalizing peak response to GABA (near EC_{50} value for each isoform) plus each Cu^{2+} concentration as a percentage of maximum current response to GABA alone for each cells. Symbols and vertical bars represent means and S.E.M. Data were fitted with four-parameter logic equation. The average IC_{50} values for Cu^{2+} are presented in Table 1.

currents had a two population inhibition pattern for both α1- and α6-receptor currents with both "high-" and "low-affinity" block (Fisher and Macdonald, 1998). In the present study, we focused on the high affinity Cu^{2+} inhibition of GABA$_A$ receptor currents.

Cu^{2+} Modulated GABA$_A$ Receptor Currents in an α Subtype-Dependent Manner. We examined the effect of Cu^{2+} on currents from GABA$_A$ receptors containing the other α subtypes (α2, α3, α4, and α5). Each of the α subtype-containing receptors produced functional GABA$_A$ receptors when cotransfected with β3 and γ2L subunits in L292 fibroblasts. Cu^{2+} modulated GABA$_A$ receptor currents in an α subtype-dependent manner (Fig. 2A). Cu^{2+} inhibited α2-receptor currents (59.0%) to an extent that was similar to its effect on α1-receptor currents (52.2%) but to a greater extent than α3-, α4-, or α5-receptor currents (19.2 to 22.6%) (Fig. 2B and Table 1). The maximal Cu^{2+} inhibition of α3-, α4-, and α5-receptor currents was similar to the 21% inhibition of α6-receptor currents but was significantly less than that of α1-receptor currents (Fig. 2B and Table 1, $p < 0.001$). However, the Cu^{2+} IC_{50} values for all six wild-type α-subunit receptors (2.4 to 3.2 μM) were not different (Table 1). These results indicated that the maximal extent of Cu^{2+} inhibition varied with α subtypes rather than the IC_{50}.

The Proximal α Subtype N-Termini Contained the Domain That Determined the Maximal Extent of Cu^{2+} Inhibition. To determine which domain conferred the α subtype-dependent differences in Cu^{2+} sensitivity, we previously studied α1/α6 and α6/α1 chimeras, made by exchanging amino acid sequence from the N terminus to the middle of TM1 of the α subunits (α1 at residue 232 and α6 at residue 231) (Fig. 3A), coexpressed with β3 and γ2L subtypes [α1/α6(232/231)- and α6/α1(231/232)-receptors] (Fisher et al., 1997). α1/α6(232/231)-receptors were strongly inhibited by Cu^{2+} with maximal Cu^{2+} inhibition and IC_{50} values similar to those of α1-receptors, whereas α6/α1(231/232)-receptors were less inhibited by Cu^{2+} with maximal Cu^{2+} inhibition and IC_{50} similar to those of α6-receptors (Fig. 3B, Table 1). The results from these chimeric receptors suggested that the N termini were involved in the α subtype-dependent differences in maximal Cu^{2+}inhibition of GABA$_A$ receptor currents.

To further localize the functional domain within the N-terminal domains, we created additional α1/α6 chimeras (Fig. 3A). When cotransfected with β3 and γ2L subunits in L292 fibroblasts, all α chimeric subtypes produced functional GABA$_A$ receptors. First, we exchanged the N-terminal amino acid sequences from the N terminus to before the cysteine loop (α1 at residue 126 and α6 at 125) to create α6/α1(125/126) and α1/α6(126/125) chimeric subunits. Maximal inhibition of α6/α1(125/126)-receptors by Cu^{2+} (44.4%) was similar to that of α1-receptors, and the magnitude of inhibition was significantly different from that of α6-receptors ($p < 0.001$) (Fig. 3B, Table 1). Maximal inhibition of α1/α6(126/125)-receptors (27.8%) was not significantly different from that of α6-receptors but was significantly smaller than that of α1-receptors ($p < 0.001$) (Fig. 3B and Table 1). These results suggested that the relevant functional domains were between α1 subtype residues 127 to 232 and α6 subtype residues 126 to 231.

Second, we exchanged the N-terminal amino acid sequences between α1 subtype residues 127 to 232 and α6 subtype residues 126 to 231 to create α6/α1/α6 (125/126;232/231) and α1/α6/α1(126/125;231/232) chimeric subunits. These chimeras exchanged the regions in the proximal N terminus that contains the functional domains responsible for α subtype-dependent Cu^{2+} inhibition. Maximal inhibition of α6/α1/α6 (125/126;232/231)-receptors by Cu^{2+} (51.4%) was similar to that of α1-receptors, and the magnitude of inhibition was significantly different from that of α6-receptors ($p < 0.001$) (Fig. 3B, Table 1). Maximal inhibition of α1/α6/α1(126/125;231/232)-receptors (28%) was not significantly different from that of α6-receptors (Fig. 3B and Table 1) but was significantly smaller than that of α1-receptors ($p < 0.001$). These results were consistent with the suggestion that the critical determinants for Cu^{2+} inhibition were located between the cysteine loop and the proximal N terminus, between α1 subtype residues 127 and 232 and α6 subtype residues 126 and 231.

Specific α Subtype N-Terminal Residues Determine the Extent of Cu^{2+} Inhibition. The chimera studies revealed that the regions between a site slightly N-terminal to the cysteine loop (127) and the middle of TM1 (232) of the α1 subtype contained the primary functional domain responsible for high Cu^{2+} sensitivity of GABA$_A$ receptor currents. Comparison of the sequences of α1-α6 subtypes between α1 positions 127 and 232 revealed a single set of residues close to the cysteine loop that were similar in α1 and α2 subtypes but different in α3–α6 subtypes. Residue 134 is a Val in the α1 subtype and equivalent residue 133 is also a Val in the α2

TABLE 1
Cu^{2+} sensitivity of wild-type and chimeric GABA$_A$ receptors

Receptor (αxβ3γ2L)	Max Inhibition	IC_{50}	n_H	n
	%	μM		
α1	52.2 ± 3.0	2.7 ± 0.3	−2.2 ± 0.5	6
α2	59.0 ± 2.5	3.1 ± 0.2	−2.4 ± 0.1	3
α3	22.6 ± 3.1	3.2 ± 0.5	−2.3 ± 0.2	5
α4	19.2 ± 3.4	3.1 (n = 1)	−3.0	5
α5	20.2 ± 4.8	2.8 ± 0.5	−2.8 ± 0.6	3
α6	21.2 ± 3.6	2.4 ± 0.3	−2.1 ± 0.3	6
α1/**α6**(126/125)	27.8 ± 0.1	4.1 ± 0.6	−3.0 ± 0.4	5
α6/**α1**(125/126)	44.4 ± 5.2	1.9 ± 0.5	−1.5 ± 0.2	3
α1/α6(232/231)	52.5 ± 2.9	3.7 ± 0.2	−3.4 ± 0.7	6
α6/α1(231/232)	16.7 ± 1.7	3.0 ± 0.8	−2.9 ± 0.2	3
α6/**α1**/α6(125/126;232/231)	51.4 ± 2.6	3.3 ± 0.3	−3.5 ± 0.4	5
α1/**α6**/α1(126/125;231/232)	28.0 ± 2.0	3.0 ± 0.3	−2.6 ± 0.2	3

subtype. The equivalent residue is an Ile in the α3, α4, α5, and α6 subtypes (Fig. 4A). Residue 135 is an Arg in the α1 subtype, (Gln in residue 134 in the α2 subtype) and the equivalent residues in α3, α4, α5, and α6 subtypes are His (α3), Ser (α4 and α5), or Asn (α6) (Fig. 4A). There was another interesting residue within the cysteine loop. The α1 subtype has a His at residue 141 and a His residue also occurred in the equivalent position of the α2 and α3 subtypes. The equivalent residue was Arg for the α4 and α6 subtypes and Gln for the α5 subtype (Fig. 4A). Thus, the α1 subtype had an eight-amino acid **VR**AECPM**H** motif, whereas the α6 subtype had a modified eight-amino acid **IN**ADCPM**R** motif.

To determine whether these residues have a role in specifying the extent of Cu^{2+} inhibition of $GABA_A$ receptor channels, we made H141R, V134I/R135N, and V134I/R135N/H141R mutants in the α6/α1/α6(125/126;232/231) chimera and the "reverse" triple I133V/N134R/R140H mutation in the α1/α6/α1(126/127;231/232) chimera. All of these mutant chimeras produced functional $GABA_A$ receptors when coexpressed with β3γ2L subtypes. We chose the α6/α1/α6(125/126;232/231) and the α1/α6/α1(126/127;231/232) chimera constructs instead of wild-type subtype for mutation because each construct had only a small N-terminal part that conferred the Cu^{2+} response to the receptor. For example, the α6/α1/α6(125/126;232/231) chimera conferred α1 subtype Cu^{2+} sensitivity. This excluded the remainder of the α1 subtype residues as sites for determining the α1 subtype Cu^{2+} sensitivity and allowed us to identify the α1 subtype residues that switched the receptor to α6 subtype Cu^{2+} sensitivity.

Replacement of α1 subtype H141 with α6 subtype R140 [α6/α1/α6(125/126;232/231) H141R] resulted in a reduction in maximal Cu^{2+} inhibition of α6/α1/α6(125/126;232/231) receptors from 51.4 to 23.3% (Fig. 4B, Tables 1, 2), a level of inhibition similar to that of α6-receptors (21.2%) and α1/α6/α1(126/125;231/232)-receptors (28.0%). The IC_{50} for Cu^{2+} was not significantly different for the mutant receptor compared with that of the α6/α1/α6(125/126;232/231)-receptor (Table 2). In contrast, the reverse mutation, R140H, [α1/α6/α1(126/125;231/232) R140H] did not change maximal Cu^{2+} inhibition or IC_{50} of α1/α6/α1(126/125;231/232)-receptors (29.0%).

Combination of the V134I and R135N mutations [α6/α1/α6(125/126;232/231) V134I/ R135N] caused a small reduction in maximal Cu^{2+} inhibition of α6/α1/α6(125/126;232/231)-receptors from 51.4 to 42% (Table 1). Combination of all three mutations (V134I, R135N, and H141R) further decreased the maximal Cu^{2+} inhibition of α6/α1/α6(125/126;232/231)-receptors to 17.8%, a value similar to that of α6-receptors (21.2%) (Table 1). These data suggested that H141 is the major determinant and V134 and R135 are minor determinants of high Cu^{2+} sensitivity of α1-receptors.

We examined the Cu^{2+} sensitivity of the "reverse" triple mutation I133V/N134R/R140H in α1/α6/α1(126/125;231/

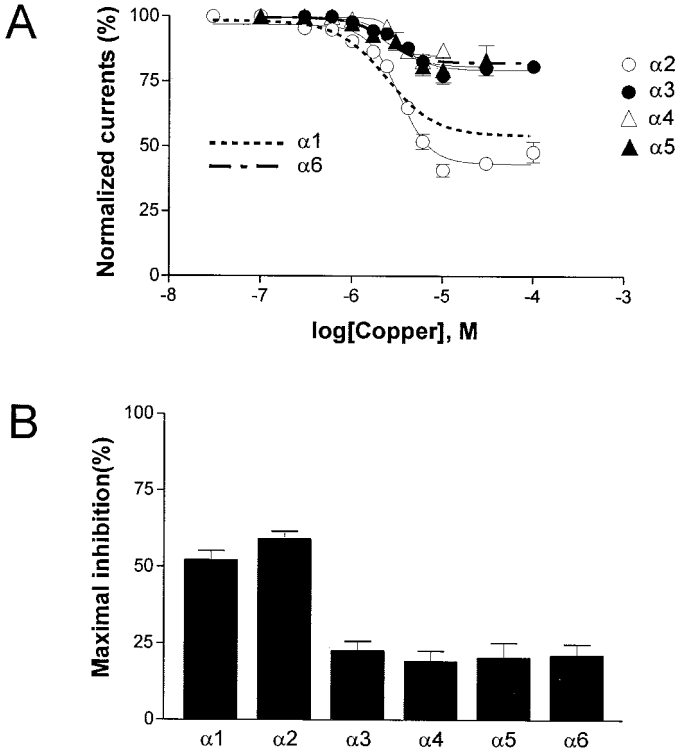

Fig. 2. Differential α subunit subtype dependent $Cu^{2[supi]+}$ modulation of $GABA_A$ receptor currents. A, the Cu^{2+} concentration-response relationship for α1, α2, α3, α4, α5, and α6 subtype-containing receptors were obtained by normalizing peak response to GABA (near EC_{50} value for each isoform) plus each Cu^{2+}concentration as a percentage of maximum current response to GABA alone. Symbols and vertical bars represent means and S.E.M. Data were fitted with a four-parameter logic equation. The average IC_{50} values for Cu^{2+} are presented in Table 1. B, comparison of maximal Cu^{2+} inhibition of six α subtype-containing receptors. Each bars represent mean ± S.E.M.

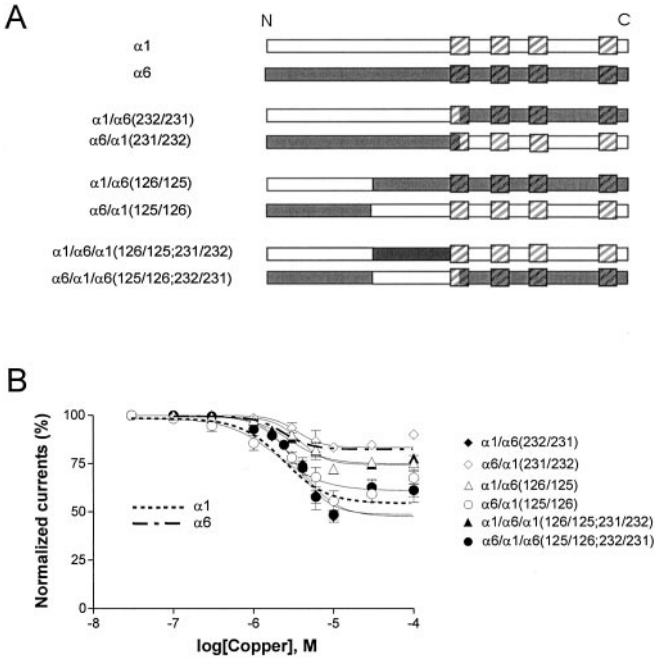

Fig. 3. Chimeric structures of α1 and α6 subunits of the $GABA_A$ receptor. A, the open regions represent α1 subtype sequence and the gray regions represent α6 subtype sequence. The four boxes represent putative transmembrane domains (TM1–TM4), and the numbers in parenthesis after the subunit represent N-terminal positions of amino acid residues at the splice site of each subunit. B, the Cu^{2+} concentration-response relationship for α1/α6 chimeric subunit-containing receptors, α1/α6(126/125), α6/α1(125/126), α1/α6(232/231), α6/α1(231/232), α1/α6/α1(126/125;231/232), and α6/α1/α6(125/126;232/231), and were obtained by normalizing peak response to GABA (near EC_{50} value for each isoform) plus each Cu^{2+} concentration as a percentage of maximum current response to GABA alone for each isoform. Symbols and vertical error bars represent mean and S.E.M., respectively. Data were fitted with a four-parameter logistic equation. The average IC_{50} values for Cu^{2+} are presented in Table 1.

232)-receptors with a single concentration 10 μM Cu^{2+}. The amount of Cu^{2+} inhibition increased from 28.0 to 41.4% ($p < 0.05$), a level of inhibition approaching that of α6/α1/α6(125/126;232/231)-receptors (51.4%) or α1-receptors (52.2%). These results are also consistent with the suggestion that the **V**(**R**,**Q**)AECPM**H** motif may constitute a major structure for conferring stronger Cu^{2+} sensitivity to α1- and α2-subtype containing GABA$_A$ receptors.

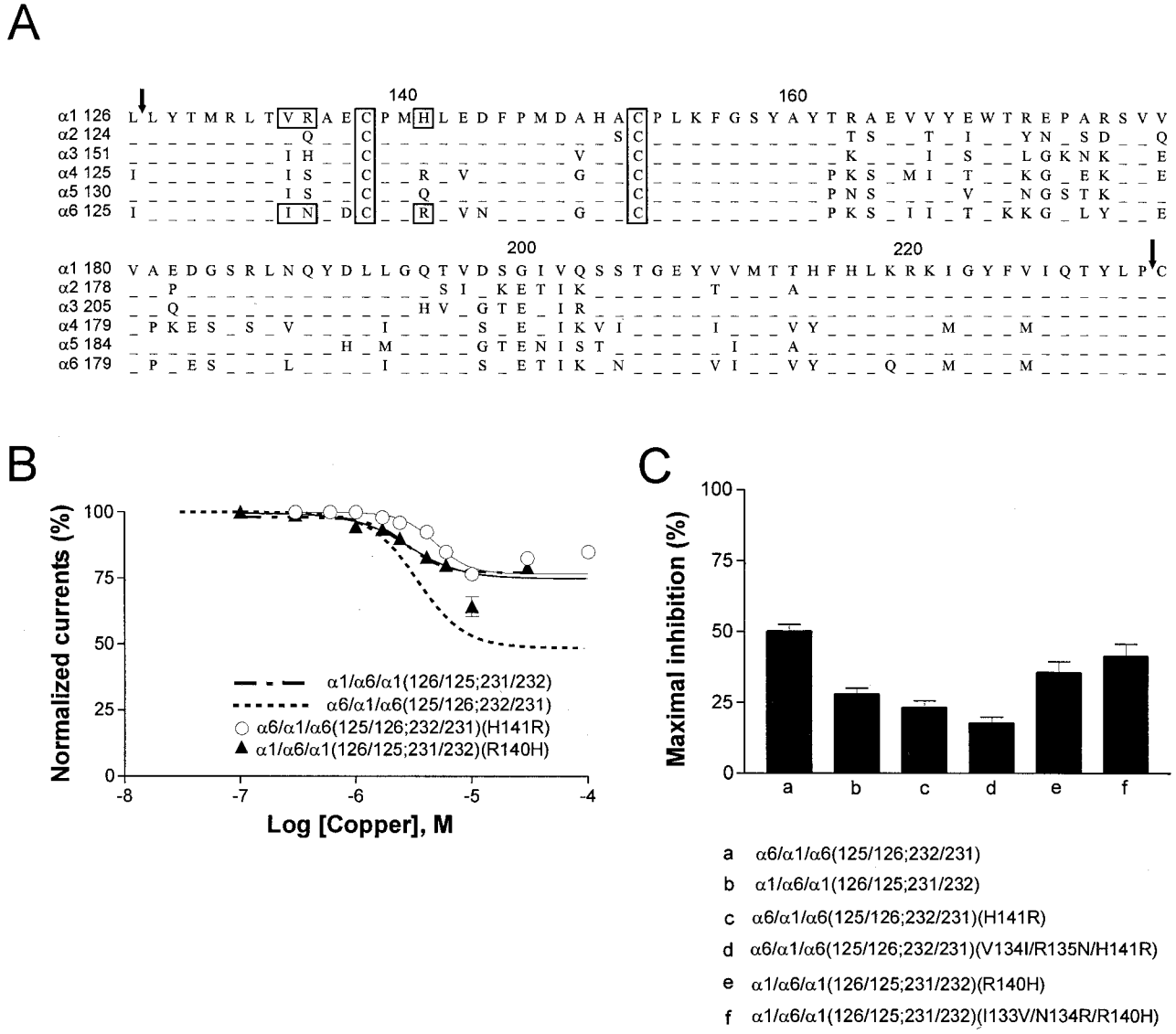

Fig. 4. The effect of residues in the N-terminal region (127–231) of α subunits on Cu^{2+} inhibition. A, sequence alignment of N-terminal regions between the front of the cysteine loop and TM1 of six wild-type α subtypes. Arrows indicate splice sites for α1/α6 chimeras at positions 126/125 and 232/231 of each α1 and α6 subtype. Dashes represent conserved amino acid residues as occurred in α1 subtype. The boxed residues represent amino acids mutated in wild-type and chimeric subunits. B, the Cu^{2+} concentration-response relationship for α1/α6 chimeric mutant subunit-containing α6/α1/α6(125/126;232/231) (H141R) and α1/α6/α1(126/125;231/232) (R140H) receptors were obtained by normalizing peak response to GABA (near EC_{50} value for each isoform) plus each Cu^{2+} concentration as a percentage of maximum current response to GABA alone for each isoform. Symbols and vertical error bars represent mean and S.E.M., respectively. Data were fitted with a four-parameter logistic equation. The average IC_{50} values for Cu^{2+} are presented in Table 2. C, comparison of maximal Cu^{2+} inhibition of α1/α6 chimeric mutant subunit-containing receptors. Each bar represents mean ± S.E.M.

TABLE 2
Cu^{2+} sensitivity of α subtype-mutated chimeric GABA$_A$ receptors

Receptor ($\alpha x\beta 3\gamma 2L$)	Max Inhibition	IC_{50}	n_H	n
	%	μM		
α6/**α1**/α6(125/126;232/231)(H141R)	23.3 ± 2.3	3.8 ± 0.5	−4.0 ± 0.3	3
α1/**α6**/α1(126/125;231/232)(R140H)	29.0 ± 4.5	2.9 ± 0.4	−2.8 ± 0.6	5
α6/**α1**/α6(125/126;232/231)(V134I/R135N)	43	2.3	−2.8	1
α6/α1/α6(125/126;232/231)(V134I/R135N/H141R)	17.8 ± 2.1	2.9 ± 0.2	−2.5 ± 0.2	5
α1/**α6/α1**(126/125;231/232)(I133V/N134R/R140H)	41.4 ± 4.2			5

The TM2 Zn^{2+} "Domain" Is Involved in Cu^{2+} Inhibition. Although the His in the **V**(**R**,**Q**)AECPM**H** motif seemed to be the primary determinant of the increased sensitivity of α1-receptor currents, α6-receptor currents were still sensitive to Cu^{2+} inhibition. Thus, we hypothesized that there may exist functional domains in α or other subunits responsible for Cu^{2+} sensitivity of $GABA_A$ receptor currents. It has been shown that the H267 residue in TM2 of β subtypes (Fig. 5A) is important for high-affinity Zn^{2+} inhibition of $\alpha1\beta1$-receptors (Horenstein and Akabas, 1998). H272 in the TM2–TM3 loop of the α6 subtype, close to the H267 residue of β subtypes, has also been shown to be a determinant of high Zn^{2+} sensitivity to α6-subtype receptors (Fisher and Macdonald, 1998). Recently, Sharonova et al. (2000) proposed that Cu^{2+} shares a binding site with Zn^{2+}. Therefore, we examined the effect of the His residues in TM2 of β subtypes and in the TM2–TM3 extracellular loop of α6 subtypes on Cu^{2+} inhibition to determine whether these residues are also involved in Cu^{2+} inhibition. These mutant subtypes produced functional $GABA_A$ receptors when coexpressed as $\alpha(1,6)\beta3\gamma2L$ receptors.

The maximal Cu^{2+} inhibition of $\alpha1\beta3$(H267S)$\gamma2L$ receptors (41.2%) was smaller than that of $\alpha1\beta3\gamma2L$ receptors (52.2%), but the reduction was not significant (Table 3). Cu^{2+} inhibited $\alpha1\beta3$(H267S)$\gamma2L$ receptors with an IC_{50} similar to that of $\alpha1\beta3\gamma2L$ receptors. $\alpha6\beta3$(H267S)$\gamma2L$ receptors showed less maximal Cu^{2+} inhibition (15%) than α6-receptors (23%); again, however, the reduction was not significant (Table 3). However, combination of β3(H267S) with α6(H273N) resulted in a virtually complete loss of Cu^{2+} inhibition (8%) ($P < 0.01$) of $\alpha6$(H273N)$\beta3$(H267S)$\gamma2L$ receptors (Fig. 5 and Table 3). These data suggested that the His residues in the β3 subtype TM2 and α6 subtype TM2–TM3 loop contributed partially to Cu^{2+} inhibition of $\alpha6\beta3\gamma2L$ receptors and that Cu^{2+} may interact with $GABA_A$ receptors at Zn^{2+} sites.

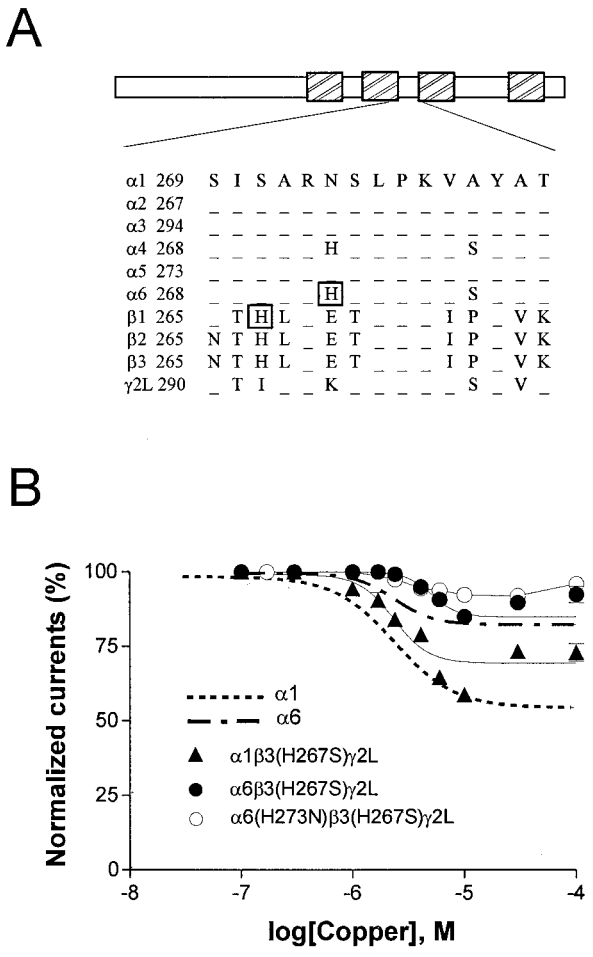

Fig. 5. The role of TM2 and the TM2–TM3 loop in Cu^{2+} inhibition of $GABA_A$ receptors. A, comparison of amino acid sequences in TM2–TM3 loop of α, β, and γ subunits. Dashes indicate conserved amino acids as occurred in the α1 subtype. The boxed His residues represent amino acids that are known to be involved in Zn^{2+} inhibition (Fisher and Macdonald, 1998; Horenstein and Akabas, 1998). B, Cu^{2+} concentration-response relationships from L929 fibroblast expressing $\alpha1\beta3$(H267S)$\gamma2L$, $\alpha6\beta3$(H267S)$\gamma2L$, and $\alpha6$(H273N)$\beta3$(H267S)$\gamma2L$ mutant-containing receptors were obtained by normalizing peak response to GABA plus each Cu^{2+} concentration as a percentage of maximum current response to GABA alone. Symbols and vertical error bars represent mean and S.E.M., respectively. Data were fitted with a four-parameter logistic equation. The average IC_{50} values for Cu^{2+} were presented in Table 3.

Discussion

An N-Terminal His in the Cys-Loop Is the Primary Determinant of a Subtype-Dependent Sensitivity to Cu^{2+} Inhibition. In this study, we have demonstrated that Cu^{2+} produces α subtype-dependent block of $GABA_A$ receptor currents and that the α subtype-dependent Cu^{2+} antagonism is caused by regulation of maximal inhibition rather than IC_{50}. Cu^{2+} produces a greater maximal inhibition of α1- and α2-receptors and lesser maximal inhibition of α3-, α4-, α5-, and α6-receptors. We have identified the structural bases for α subtype-dependent Cu^{2+} inhibition of $GABA_A$ receptors. The chimera and mutagenesis analysis suggested that an α1 subtype N-terminal functional domain (127–232 region) was important for greater Cu^{2+} inhibition of α1 subtype than of α6 subtype currents. We also demonstrated that α1 subtype residues Val134, Arg135, and His141 in a **VR**AECPM**H** motif confer higher Cu^{2+} sensitivity to $\alpha1\beta3\gamma2L$ receptors and that His141 is the major determinant in the motif. The **IN**ADCPM**R** sequence confers the lower Cu^{2+} sensitivity of $\alpha6\beta3\gamma2L$ receptors. Interestingly, Hosie et al. (2003) have shown recently that Glu137 and His141 residues in the N terminus of the α1 subtype are part of critical Zn^{2+}-coordinating domains. Interestingly, these two residues occurred in the VRAECPMH motif, which is responsible for increased Cu^{2+} inhibition. $\alpha2\beta3\gamma2L$ receptors have a Cu^{2+} sensitivity that is similar to that of $\alpha1\beta3\gamma2L$ receptors, and the α2 subtype has a sequence (**VQ**AECPM**H**) similar to that of the α1 subtype, with only a single exchange of a Gln for an Arg residue in the 2′ position. The $\alpha3\beta3\gamma2L$, $\alpha4\beta3\gamma2L$, and $\alpha5\beta3\gamma2L$ receptors have lower Cu^{2+} sensitivities, similar to that of $\alpha6\beta3\gamma2L$ receptors, and α3, α4, and α5 subtypes have sequences that are similar to that of the α6 subtype (Fig. 4A). In the 2′ position the α3, α4, α5, and α6 subtypes have His, Asn, or Ser residues rather than the α1 subtype Arg residue. In the 8′ position, the α5 subtype has an exchange of a Gln for an Arg residue. The α3 subtype, however, has a His in the 8′ position, as does the α1 subtype. A comparison of the α1 subtype sequence (**VR**AECPM**H**) with the α3 subtype sequence (**IH**AECPM**H**) is consistent with the observation that, although the 8′ His is the major determinant of Cu^{2+} sensitivity, the Val-Arg residues play an important permissive role that the Ile-His residues cannot and is

consistent with the conclusion that the entire 8-amino acid motif is important, not simply a single residue.

Cations Have Multiple Interactions with GABA$_A$ Receptors. GABA$_A$ receptor currents are modified by several cations. It has been suggested that functional domains for cation modulation are partially shared among them (Celentano et al., 1991; Ma and Narahashi, 1993; Fisher and Macdonald, 1998). Ma and Narahashi (1993) suggested that Cu^{2+} and Zn^{2+} may share a common binding site on the GABA$_A$ receptor. The α6 subtype H273 residue in the TM2–TM3 extracellular loop has been shown to play a role in conferring high Zn^{2+} sensitivity to α6 subtype-containing receptors (Fisher and Macdonald, 1998). This His residue in the TM2–TM3 loop was reported to regulate α subunit dependent Ni^{2+}-, Cd^{2+}-, and La^{3+}-inhibition but not Cu^{2+}-inhibition of GABA$_A$ receptor currents (Fisher and Macdonald, 1998; H. Kim and R. L. Macdonald, unpublished observations). The functional domain for Cd^{2+} inhibition has been suggested also to be localized to the N-terminal region of α1 subtypes (Fisher and Macdonald, 1998). Although we did not explore further structural determinants of Cd^{2+} inhibition in this study, it is possible that Cd^{2+} may also interact with the α1 subtype N-terminal functional domain (127–232) that determines Cu^{2+} sensitivity.

We also provide evidence that the H267 residue in the β3 subunit TM2, which is known to be associated with Zn^{2+} modulation (Fisher and Macdonald, 1998; Horenstein and Akabas, 1998), is involved in Cu^{2+} inhibition. The β3 subtype H267S mutation alone minimally affected maximal Cu^{2+} inhibition in α1-receptors and α6-receptors. However, combination β3 H267S with α6 H273N (i.e., removal of two His residues from α6-receptors) virtually abolished Cu^{2+} inhibition but not in $\alpha1\beta3$(H267S)-receptors, which also lack these two His residues. These results suggest that Cu^{2+} shares a portion of the functional domain with Zn^{2+} and confirms that N-terminal determinants of α1 subtypes are important for Cu^{2+} inhibition. Taken together, these data indicate multiple cation sites in GABA$_A$ receptor complex, and the **VR**AECPM**H** motif provides a novel cation regulatory site for polyvalent cations.

Multiple α- and β-Subtype His Residues Contribute to Cu^{2+} Inhibition of GABA$_A$ Receptor Currents. The relevant β and α subtype residues that regulate Cu^{2+} sensitivity are quite close in the distal regions of the GABA$_A$ receptor channel, being located in the distal TM2 (His267) of the β subunit and in the TM2–TM3 loop (His273) of the α subunit. The residues that control the α-subtype dependent Cu^{2+} sensitivity of the GABA$_A$ receptor channel (the **VR**AECPM**H** motif) are remote from this location in the N terminus and flank the N-terminal cysteine of the cysteine pair that forms the signature cys loop (loop 7) of all GABA$_A$, glycine, nicotinic cholinergic, and serotonin 5HT3 receptor channels. The basis for this remote interaction may be inferred from the crystal structure of the ACh binding protein (Brejc et al., 2001). The structure of this protein revealed that the cys loop in the ACh binding protein is hydrophilic and projects toward the side of the protein, which would interface with the membrane in ACh receptors. Furthermore, Kash et al. (2003) provided evidence that a GABA$_A$ receptor α1 subtype cys loop residue Asp149 interacts with Lys279 in the middle portion of the TM2–TM3 loop of GABA$_A$ receptors. Both of these residues are conserved in all GABA$_A$ receptor subunits. The interaction has been shown to be electrostatic because charge reversal of the cys loop Asp and TM2–TM3 Lys residues maintained receptor function (Kash et al., 2003). The TM2–TM3 Lys residue is quite near the β subunit TM2 His and the α6 subtype TM2–TM3 His, and the cys loop Asp is near the α1 subtype cys loop His is in the **VR**AECPM**H** motif. In addition, the β subunit E182 has been proposed to interact with the α1 E137 and H141 residues in the **VR**AECPM**H** motif to contribute to the Zn^{2+} binding site (Hosie et al., 2003), supporting the proposal that this motif could be a critical structural determinant for Cu^{2+} inhibition. Therefore, there is a cluster of His residues in the β subunit TM2, the α6 subtype TM2–TM3 loop, and the cys loop, and it is likely that Cu^{2+} interacts with these α and β subunit His residues to inhibit GABA$_A$ receptor channels.

Physiological Significance of Cation Modulation of GABA$_A$ Receptors. In dorsal root ganglion neurons, Cu^{2+} blocked 100% of GABA-induced currents with about a 16 μM IC_{50} with a single population block pattern (Ma and Narahashi, 1993), which is different from our finding in recombinant receptors ($\alpha x\beta3\gamma2L$) that currents were not completely blocked (maximally about 50%) by Cu^{2+} and that the Cu^{2+} inhibition curves had two populations of block. Cu^{2+} inhibition of olfactory bulb neurons was similar to that of dorsal root ganglion neurons (Trombley and Shepherd, 1996). However, in cerebellar Purkinje cell GABA$_A$ receptor currents, maximal Cu^{2+} block was only 60% and the IC_{50} was lower (35 nM) (Sharonova et al., 1998) than in dorsal root ganglion neurons. Variations in Cu^{2+} pharmacology may come from different region-specific GABA$_A$ receptor subunit subtype combinations. It has been shown that α2, α3, β3, γ1, γ2, and γ3 transcripts were expressed in dorsal root ganglia and α1, β2, β3, and γ2 mRNAs were detected in cerebella Purkinje cells (Laurie et al., 1992b). In olfactory bulb, although expression pattern and intensity were different depending on the region, except for the α6 subtype, most GABA$_A$ receptor subunits were detected (Laurie et al., 1992a).

Although Zn^{2+} showed an inhibition pattern that was similar to that of Cu^{2+} in dorsal root ganglion and olfactory bulb neurons with similar IC_{50} values (10 μM) and almost 100% maximal inhibition (Ma and Narahashi, 1993; Trombley and Shepherd, 1996), Zn^{2+} inhibited GABA currents with lower

TABLE 3
Cu^{2+} sensitivity of β subtype-mutated GABA$_A$ receptors

Receptor ($\alpha x\beta3\gamma2L$)	Max Inhibition	IC_{50}	n_H	n
	%	μM		
$\alpha1\beta3$(H267S)$\gamma2L$	41.2 ± 1.4	2.7 ± 0.3	−3.3 ± 0.3	4
$\alpha6\beta3$(H267S)$\gamma2L$	15.0 ± 1.2	5.0 ± 0.3	−4.1 ± 0.3	4
$\alpha6$(H273N)$\beta3$(H267S)$\gamma2L$	8.0 ± 1.7			5

IC_{50} values (35 μM) and similar maximal inhibition (66%) compared with Cu^{2+} in cerebellar Purkinje cells. Although Cu^{2+} and Zn^{2+} are released from nerve terminals during synaptic activity (Assaf and Chung, 1984; Kardos et al., 1989), the fact Cu and Zn^{2+} are coreleased at synaptic cleft is unclear. However, the data from the present and other studies (Sharonova et al., 2000) study strongly suggest that they might interact with each other with $GABA_A$ receptor complex and participate in modulation of synaptic transmission. Further mechanistic study will be needed to understand the interaction between Cu^{2+} and Zn^{2+} in neurotransmitter receptors.

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