Two γ2L subunit domains confer low Zn^{2+} sensitivity to ternary $GABA_A$ receptors

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1. The sensitivity of $GABA_A$ receptors (GABARs) to Zn^{2+} inhibition depends on subunit composition. The predominant neuronal forms of mammalian GABARs, $\alpha\beta\gamma$ and $\alpha\beta\delta$, are differentially sensitive to Zn^{2+} inhibition; $\alpha\beta\gamma$ receptors are substantially less sensitive than $\alpha\beta\delta$ receptors. Recently, functional domains involved in Zn^{2+} sensitivity have been identified in α and β subunits. Our aim in the present study was to localize functional domains of low Zn^{2+} sensitivity within γ2L subunits.

2. Chimeric subunits were constructed by progressively replacing the rat γ2L subunit sequence with that of the rat δ subunit sequence. Whole-cell currents were recorded from mouse L929 fibroblasts coexpressing wild-type rat α1 and β3 subunits with a chimeric δ-γ2L subunit.

3. Unlike α and β subunits, the γ2L subunit was found to contain a determinant of low Zn^{2+} sensitivity in the N-terminal extracellular region. In addition, we identified determinants in the M2 segment and the M2–M3 loop of the γ2L subunit that are homologous to those found in β and α subunits.

4. We postulate that the interface between the latter two domains, which may form the outer vestibule of the channel, represents a single functional domain modulating Zn^{2+} sensitivity. Thus, the Zn^{2+} sensitivity of ternary GABARs appears to be determined by two functional domains, one in the N-terminal extracellular region and one near the outer mouth of the channel.

The γ-aminobutyric acid type A receptor (GABAR) is the major inhibitory neurotransmitter receptor in the mammalian central nervous system. Functional mammalian GABARs are thought to be pentameric combinations of homologous subunits drawn from seven different families with multiple subtypes: α(1–6), β(1–3), γ(1–3), δ, ε, π and θ (Macdonald & Olsen, 1994; Davies *et al.* 1997; Hedblom & Kirkness, 1997; Bonnert *et al.* 1999). Like other members of the ligand-gated ion channel gene superfamily, GABAR subunits have a putative membrane topology consisting of a large N-terminus, four membrane-spanning segments (M1–M4), one extracellular and two intracellular loops connecting the membrane-spanning segments (M1–M2, M2–M3, M3–M4), and an extracellular C-terminus. It has been suggested that neuronal GABARs are predominantly ternary $\alpha\beta\gamma$ and $\alpha\beta\delta$ combinations (Angelotti *et al.* 1993; McKernan & Whiting, 1996).

The divalent cation Zn^{2+} has been proposed to be an endogenous modulator of synaptic transmission (Smart *et al.* 1994; Harrison & Gibbons, 1994). Its ability to inhibit native GABARs, however, varies with neuronal type, age and activity (Smart *et al.* 1994; Kapur & Macdonald, 1997). Studies of recombinant receptors indicate that the inhibitory activity of Zn^{2+} on GABAR currents depends on subunit composition (Draguhn *et al.* 1990; Smart *et al.* 1991). Binary $\alpha\beta$ GABARs have a high sensitivity to Zn^{2+} with IC_{50} values ranging from 0.1 to 1 μM (Draguhn *et al.* 1990; Smart *et al.* 1991; Wooltorton *et al.* 1997; Horenstein & Akabas, 1998). In ternary $\alpha\beta$X GABARs (where X is δ, γ, ε or π), Zn^{2+} sensitivity is decreased. Receptors containing π subunits have a Zn^{2+} IC_{50} of ~2 μM (Neelands & Macdonald, 1999), receptors containing δ subunits have Zn^{2+} IC_{50} values ranging from 5 to 16 μM (Saxena & Macdonald, 1996; Krishek *et al.* 1998), and receptors containing ε subunits have a Zn^{2+} IC_{50} of ~40 μM (Whiting *et al.* 1997; Neelands *et al.* 1999). Incorporation of a γ subunit, however, has an even greater effect on GABAR Zn^{2+} sensitivity, resulting in IC_{50} values that range from 20 to 600 μM (Saxena & Macdonald, 1996; Burgard *et al.* 1996; Krishek *et al.* 1998).

Functional domains involved in Zn^{2+} sensitivity have been identified in two GABAR subunit families, α and β. The extracellular end of the M2 segment in the β subunit has been shown to be a major determinant of Zn^{2+} sensitivity in β homomers and $\alpha\beta$ receptors (Wooltorton *et al.* 1997; Horenstein & Akabas, 1998). The M2–M3 extracellular loop of the α6 subtype has been shown to

confer higher Zn^{2+} sensitivity to $\alpha6\beta3\gamma2$L GABAR isoforms as compared to $\alpha1\beta3\gamma2$L receptors (Fisher & Macdonald, 1998). In addition, in $GABA_C$ receptors, which are structurally homologous to GABARs, the N-terminus has an essential role in conferring Zn^{2+} sensitivity to $\rho1$ homomers (Wang *et al.* 1995). Such functional domains, however, have yet to be identified for other GABAR subunit families including δ and γ.

To determine which domains of the $\gamma2$L subunit subtype are involved in conferring low Zn^{2+} sensitivity to GABARs, we constructed GABAR chimeras by replacing varying lengths of the rat $\gamma2$L subunit with the homologous sequence of the rat δ subunit. These chimeric subunits were coexpressed in mouse L929 cells with wild-type $\alpha1$ and $\beta3$ subunits, and whole-cell currents were elicited by application of GABA. The relative Zn^{2+} sensitivities of GABARs containing the chimeric subunits revealed that two different and non-contiguous $\gamma2$L subunit domains, one in the N-terminal extracellular region and one near the outer mouth of the channel, contributed to the regulation of Zn^{2+} sensitivity.

METHODS

Construction of expression vectors and mutagenesis

Full-length cDNAs for the rat GABAR $\alpha1$ (Dr A. Tobin, University of California, Los Angeles, CA, USA), $\beta3$ (Dr D. Pritchett, University of Pennsylvania, Philadelphia, PA, USA), δ (Dr K. Angelides, Baylor College of Medicine, Houston, TX, USA) and $\gamma2$L subunits (F. Tan, University of Michigan) were subcloned into the expression vector pCMVneo (Huggenvik *et al.* 1991). Chimeras were constructed using the splice overhang extension method. Point mutations were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA). Oligonucleotide primers were synthesized by the University of Michigan DNA synthesis core. Sequences of chimeras and point mutants were verified by fluorescent DNA sequencing (University of Michigan DNA sequencing core).

Cell culture and transient transfection

The mouse fibroblast cell line L929 (American Tissue Type Collection, Rockville, MD, USA) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all from Gibco-BRL, Grand Island, NY, USA). Cells were maintained in a 37 °C incubator with 95% air–5% CO_2 in 10 cm culture dishes. Cells were passaged every 3–4 days using 0.5% trypsin–0.2% EDTA (Boehringer-Mannheim, Indianapolis, IN, USA) in divalent-free, phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 0.15 mM NaCl, pH 7.3). Twenty-four hours prior to transfection, cells were seeded at a density of 300 000 in 60 mm culture dishes. Cells were transfected using a modified calcium phosphate precipitation method. Plasmids encoding GABAR subunits were mixed in a 1:1:1 ratio using 3–4 μg of each along with 2 μg of pHook-1 (Invitrogen, San Diego, CA, USA), which encodes the cell surface antibody sFv. Following addition of DNA, cells were incubated for 4–5 h in 3% CO_2 and shocked for 30 s with 15% glycerol in BBS buffer (50 mM Bes, 280 mM NaCl, 1.5 mM Na_2HPO_4). The following day, transfected cells were selected and concentrated using Capture-Tec beads (magnetic beads coated with hapten; Invitrogen). Selected cells were replated on 35 mm culture dishes and used for recording about 24 h later.

Electrophysiological recording solutions and techniques

For whole-cell recording, the external bath solution consisted of (mM): 142 NaCl, 8.1 KCl, 6 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 Hepes at pH 7.4 and osmolality between 311 and 325 mosmol kg^{-1}. The concentration of Mg^{2+} in the bath solution did not change the GABAR IC_{50} values for Zn^{2+}. The IC_{50} values for the wild-type receptors $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$L were similar to those reported previously in the literature (Gingrich & Burkat, 1998; Krishek *et al.* 1998). Recording electrodes were filled with an internal solution of (mM): 153 KCl, 1 $MgCl_2$, 5 K-EGTA, 10 Hepes and 2 Mg-ATP at pH 7.3 and osmolality adjusted to 295–300 mosmol kg^{-1}. These solutions provided a chloride equilibrium potential near 0 mV. Patch pipettes were pulled from microhaematocrit tubes made of soda-lime glass (i.d. = 1.1–1.2 mm, o.d. = 1.3–1.4 mm; Fisher Scientific, Pittsburgh, PA, USA) on a P-87 Flaming Brown puller (Sutter Instrument Co., San Rafael, CA, USA). Pipettes had resistances of 5–10 MΩ and were coated with polystyrene Q-Dope (GC Electronics, Rockfield, IL, USA) before use. Currents were recorded with an Axoclamp 200A patch clamp amplifier (Axon Instruments, Foster City, CA, USA), DigiData 1200 interface (Axon Instruments) and Zenith Pentium computer as well as on Beta videotape. Series resistance was compensated by 90%. GABA and $ZnCl_2$ were prepared as stock solutions of 100 mM in water. All working solutions were prepared on the day of the experiment by diluting stock solutions in external solution. Drugs were applied to cells using a modified U-tube delivery system with a 10–90% rise time of 70–150 ms (Greenfield & Macdonald, 1996). GABA-induced currents were recorded at a holding potential of −75 mV. All experiments were performed at room temperature.

Analysis of whole-cell currents

Whole-cell currents were analysed off-line using Axoscope (Axon Instruments) and Prism software (GraphPad, San Diego, CA, USA). Normalized concentration–response data for the different isoforms were fitted with a four-parameter logistic equation:

$$I = I_{max}/(1 + 10^{(\log EC_{50} - \log X)n_H}),$$

where X is the concentration of drug, n_H represents the Hill coefficient and I represents current expressed as a percentage of the maximum current elicited by saturating concentrations of GABA for each cell or in the case of Zn^{2+}, as a percentage of the current elicited by GABA alone (I_{max}). Data in the figures were derived from a single fit of averaged responses from all cells. Data in Tables 1 and 2 were derived from fits of individual cells and are reported as means ± S.E.M. Statistical tests were performed by one-way ANOVA and Newman-Keuls multiple comparisons tests where $P < 0.05$. Results of significance tests are presented in the relevant table legends.

Expression of $\alpha\beta\delta$-$\gamma2$L chimeric GABARs

Although the δ and $\gamma2$L subunit families share only 35% homology with each other (Shivers *et al.* 1989), the four chimeras we constructed produced functional channels when coexpressed with wild-type α and β subunits. The GABA EC_{50} values for all of the chimeric isoforms were distinct from those observed for binary $\alpha\beta$ receptors (~1 μM; Angelotti *et al.* 1993), indicating that the chimeric subunits were incorporated and influenced channel properties.

RESULTS

Design of δ-$\gamma2$L chimeric subunits

To determine which domains of the $\gamma2$L subunit were involved in conferring low Zn^{2+} sensitivity to ternary GABARs, four chimeric subunits were created by

Table 1. GABA sensitivity of receptors containing wild-type, chimeric and mutant subunits

Receptor isoform	EC_{50} (μM)	Hill slope	Peak current (pA)	n
$\alpha\beta\delta$	4.4 ± 1.0	1.4 ± 0.1	136.0 ± 36.7	5
$\alpha\beta\gamma$	15.5 ± 3.6	1.5 ± 0.1	472.9 ± 145.9	5
$\alpha\beta\delta$-γ(M1e)	2.8 ± 0.9	1.5 ± 0.2	506.4 ± 142.9	5
$\alpha\beta\delta$-γ(M1i)	25.4 ± 4.8	1.1 ± 0.07	362.0 ± 250.9	3
$\alpha\beta\delta$-γ(M2e)	23.9 ± 3.4	0.9 ± 0.02	219.3 ± 50.8	5
$\alpha\beta\delta$-γ(M3e)	3.5 ± 0.4	1.3 ± 0.1	158.7 ± 62.8	6
$\alpha\beta\delta$(MVS→STI)	5.1 ± 1.1	1.2 ± 0.1	191.7 ± 44.0	3
$\alpha\beta\gamma$(STI→MVS)	19.1 ± 5.0	1.4 ± 0.3	2164 ± 1203	3
$\alpha\beta\delta$(M278S)	4.3 ± 1.7	1.2 ± 0.1	366.6 ± 129.2	3
$\alpha\beta\delta$(V279T)	3.0 ± 0.8	1.6 ± 0.2	137.4 ± 110.5	3
$\alpha\beta\delta$(S280I)	2.5 ± 0.3	1.0 ± 0.1	328.5 ± 134.0	3
$\alpha\beta\gamma$(I282S)	13.8 ± 1.4	1.7 ± 0.1	408.0 ± 115.6	4
$\alpha\beta\delta$(S283K)	3.0 ± 0.5	1.5 ± 0.1	197.2 ± 50.8	4
$\alpha\beta\gamma$(K285S)	13.2 ± 1.4	1.6 ± 0.2	636.6 ± 100.5	3

Values are means ± S.E.M. GABA log EC_{50} values for $\alpha\beta\delta$, $\alpha\beta\delta$(M278S), $\alpha\beta\delta$(V279T), $\alpha\beta\delta$(S280I), $\alpha\beta\delta$(S283K), $\alpha\beta\delta$(MVS→STI), $\alpha\beta\delta$-γ(M1e) and $\alpha\beta\delta$-γ(M3e) are all significantly different from those of $\alpha\beta\delta$-γ(M1i), $\alpha\beta\delta$-γ(M2e), $\alpha\beta\gamma$(STI→MVS), $\alpha\beta\gamma$(I282S), $\alpha\beta\gamma$(K285S) and $\alpha\beta\gamma$ (Newman-Keuls *post hoc*, one-way ANOVA). GABA Hill slope for $\alpha\beta\delta$-γ(M2e) is significantly different from that of $\alpha\beta\gamma$(I282S) (Newman-Keuls *post hoc*, one-way ANOVA).

Table 2. Zn^{2+} sensitivity of receptors containing wild-type, chimeric and mutant subunits

Receptor isoform	IC_{50} (μM)	Hill slope	n
$\alpha\beta\delta$	8.6 ± 1.8	−0.7 ± 0.1	5
$\alpha\beta\gamma$	307.6 ± 68.7	−0.9 ± 0.2	3
$\alpha\beta\delta$-γ(M1e)	23.9 ± 2.2	−0.9 ± 0.1	4
$\alpha\beta\delta$-γ(M1i)	19.8 ± 4.6	−0.9 ± 0.06	3
$\alpha\beta\delta$-γ(M2e)	11.3 ± 1.0	−0.8 ± 0.05	4
$\alpha\beta\delta$-γ(M3e)	5.4 ± 1.2	−0.9 ± 0.1	5
$\alpha\beta\delta$(MVS→STI)	15.8 ± 2.9	−0.9 ± 0.03	4
$\alpha\beta\gamma$(STI→MVS)	55.1 ± 6.0	−0.6 ± 0.03	3
$\alpha\beta\delta$(M278S)	7.2 ± 3.4	−0.9 ± 0.1	3
$\alpha\beta\delta$(V279T)	7.0 ± 1.9	−1.1 ± 0.3	2
$\alpha\beta\delta$(S280I)	5.3 ± 1.2	−1.1 ± 0.7	3
$\alpha\beta\gamma$(I282S)	345.7 ± 154	−0.9 ± 0.1	3
$\alpha\beta\delta$(S283K)	9.8 ± 2.3	−0.9 ± 0.1	5
$\alpha\beta\gamma$(K285S)	178.2 ± 26.7	−0.8 ± 0.05	4

Values are means ± S.E.M. Zn^{2+} log IC_{50} for $\alpha\beta\delta$-γ(M3e) is significantly different from those of $\alpha\beta\delta$-γ(M1e), $\alpha\beta\delta$-γ(M1i), $\alpha\beta\delta$(MVS→STI), $\alpha\beta\gamma$(STI→MVS), $\alpha\beta\gamma$(I282S), $\alpha\beta\gamma$(K285S) and $\alpha\beta\gamma$. Zn^{2+} log IC_{50} values for $\alpha\beta\delta$-γ(M1e), $\alpha\beta\delta$-γ(M1i), $\alpha\beta\delta$-γ(M2e), $\alpha\beta\delta$, $\alpha\beta\delta$(MVS→STI), $\alpha\beta\delta$(S283K), $\alpha\beta\delta$(M278S), $\alpha\beta\delta$(S280I) and $\alpha\beta\delta$(V279T) are significantly different from those of $\alpha\beta\gamma$ (STI→MVS), $\alpha\beta\gamma$(I282S), $\alpha\beta\gamma$(K285S) and $\alpha\beta\gamma$. Zn^{2+} log IC_{50} for $\alpha\beta\gamma$(STI→MVS) is significantly different from those of $\alpha\beta\gamma$(I282S), $\alpha\beta\gamma$(K285S) and $\alpha\beta\gamma$ (Newman-Keuls *post hoc*, one-way ANOVA). Zn^{2+} Hill slopes are not significantly different (Newman-Keuls *post hoc*, one-way ANOVA).

progressively replacing the wild-type γ2L subunit sequence from the N-terminus with wild-type δ sequence. Figure 1 depicts the putative membrane topology for each of the δ-γ2L chimeras including the large N-terminus, the four transmembrane segments (M1–M4) and their interconnecting loops, and the extracellular C-terminus. These chimeras divided the GABAR subunit sequence into five sections: (1) the N-terminus, (2) the M1 segment, (3) the M1–M2 loop and the M2 segment, (4) the M2–M3 loop and (5) the M3 and M4 segments, the M3–M4 loop and the C-terminus.

GABA sensitivity of GABARs containing wild-type and chimeric subunits

Wild-type and chimeric forms of the δ and γ2L subunits produced functional GABAR channels when coexpressed with wild-type α1 and β3 subunits in L929 fibroblasts. Cells were voltage clamped at −75 mV, and whole-cell currents were elicited by application of GABA. Comparison of concentration–response curves for GABARs containing wild-type subunits indicated that $\alpha1\beta3\gamma$2L receptors had a higher EC_{50} for GABA than $\alpha1\beta3\delta$ receptors (Fisher & Macdonald, 1997; $P < 0.001$; Table 1).

Currents from GABARs containing chimeric subunits were differentially sensitive to GABA (Fig. 2*A*). Two of the isoforms containing chimeric δ-γ2L subunits, $\alpha1\beta3\delta$-γ2L(M3e) and $\alpha1\beta3\delta$-γ2L(M1e), had GABA EC_{50} values similar to that of GABARs containing the wild-type δ subunit (Fig. 2*B*, Table 1). The other two chimeric receptors, $\alpha1\beta3\delta$-γ2L(M2e) and $\alpha1\beta3\delta$-γ2L(M1i), had higher GABA EC_{50} values that were similar to that of GABARs containing the wild-type γ2L subunit (Fig. 2*B*, Table 1). These results suggest that the juxtaposition of the γ subunit transmembrane and/or loop sequences with the δ subunit N-terminal extracellular sequence could influence GABA binding and/or channel gating such that a higher GABA EC_{50} value was observed.

Zn^{2+} sensitivity of GABARs containing wild-type and chimeric subunits

Currents evoked from wild-type $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma$2L receptors by EC_{50} concentrations of GABA were differentially inhibited by coapplication of 10 μM Zn^{2+} (Fig. 3*A*). The Zn^{2+} IC_{50} of $\alpha1\beta3\gamma$2L receptors was significantly higher than that of $\alpha1\beta3\delta$ receptors (Fig. 3*B*, $P < 0.001$; Table 2).

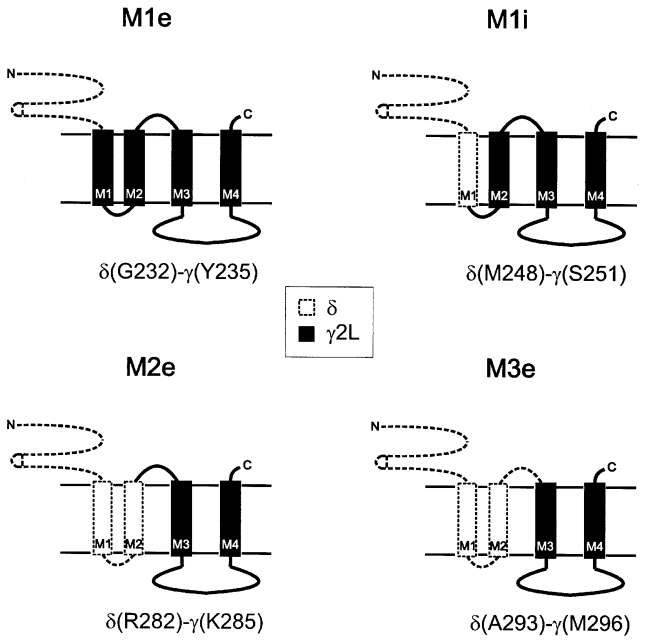

Figure 1. Schematic representation of four δ-γ2L chimeric subunits

The putative membrane topologies for four δ-γ2L chimeras are shown. Above each subunit, the chimera name is given, and below each subunit, the splice sites are given by amino acid and residue number. For each subunit, the extracellular N- and C-termini are indicated (N, C) and the four putative transmembrane segments labelled (M1–M4). The extent of rat δ subunit sequence is represented by dashed lines whereas the extent of rat γ2L subunit sequence is represented by continuous lines.

Currents evoked from chimeric GABARs by EC_{50} concentrations of GABA were also differentially inhibited by coapplication of 10 μM Zn^{2+} (Fig. 4*A*). Progressive replacement of the γ2L subunit from the N-terminus with the δ subunit sequence produced a progressive decrease in Zn^{2+} IC_{50} (Fig. 4*B*). The $\alpha1\beta3\delta$-γ2L(M1e) isoform had an average Zn^{2+} IC_{50} that was substantially lower than that of $\alpha1\beta3\gamma$2L receptors ($P < 0.001$; Table 2). Replacement of the wild-type γ2L subunit with this chimeric subunit accounted for 63 % of the log difference between Zn^{2+} IC_{50} values of $\alpha1\beta3\gamma$2L and $\alpha1\beta3\delta$-γ2L(M3e) receptors, suggesting that the N-terminal extracellular domain contained a critical determinant. Replacement of additional γ2L subunit sequence (the M1 segment) with the δ subunit sequence produced little change in Zn^{2+} sensitivity (Fig. 4*B*); the $\alpha1\beta3\delta$-γ2L(M1i) isoform had an average Zn^{2+} IC_{50} that was unchanged from that of the $\alpha1\beta3\delta$-γ2L(M1e) isoform (Table 2), suggesting that the M1 segment was not involved in conferring low Zn^{2+} sensitivity. Extending the δ subunit sequence through the γ2L subunit M1–M2 loop and the M2 segment, however, again decreased Zn^{2+} IC_{50} (Fig. 4*B*). The $\alpha1\beta3\delta$-γ2L(M2e) isoform had an average Zn^{2+} IC_{50} that was substantially lower than that of the $\alpha1\beta3\delta$-γ2L(M1e) isoform (Table 2). This chimeric subunit accounted for an additional 19 % of the log difference between Zn^{2+} IC_{50} values of $\alpha1\beta3\gamma$2L and $\alpha1\beta3\delta$-γ2L(M3e) receptors, suggesting that the M1–M2 loop and M2 segment contained another determinant of low Zn^{2+} sensitivity. Extending the δ subunit sequence through the γ2L subunit M2–M3 loop also decreased Zn^{2+} IC_{50} (Fig. 4*B*). The $\alpha1\beta3\delta$-γ2L(M3e) isoform had an average Zn^{2+} IC_{50} that was lower than that of the $\alpha1\beta3\delta$-γ2L(M2e) isoform (Table 2). This chimeric subunit accounted for an additional 18 % of the log difference between Zn^{2+} IC_{50} values of the $\alpha1\beta3\gamma$2L and $\alpha1\beta3\delta$-γ2L(M3e) receptors, suggesting that the M2–M3 loop contained a third determinant of low Zn^{2+} sensitivity.

Point mutations in the outer vestibule

Of the three structural determinants identified by the δ-γ2L chimeras, we proceeded to further delineate two adjacent domains: (1) the M1–M2 loop and the M2 segment and (2) the M2–M3 loop. We chose to focus on these adjacent regions since previous studies of GABARs had identified functional domains for Zn^{2+} in the M2 segment of β subunits and in the M2–M3 loop of α subunits, suggesting the presence of homologous domains in γ2L and δ subunits (Wooltorton *et al.* 1997; Fisher & Macdonald, 1998; Horenstein & Akabas, 1998). We targeted the extracellular end of the M2 segment and the proximal end of the M2–M3 extracellular loop, subunit regions putatively associated with the outer vestibule of the channel. Specific amino acid residues in the wild-type δ and γ2L subunits were targeted for site-directed mutagenesis based on studies of Zn^{2+} sensitivity involving other GABAR subunits.

The M2 segment. The putative channel-lining M2 segment is highly conserved across all GABAR subunits. There are, however, a few sequence differences among subunit families. Between the δ and γ2L subunits, there are differences at four positions in the M2 sequence (Fig. 5*A*). Three differences occur in a triplet of amino acids at the extracellular end of the M2 segment. Residues in the α1 subunit that are homologous to the second and third position of the triplet, I270 and S271, have been shown to be water accessible in $\alpha\beta\gamma$ receptors (Xu &

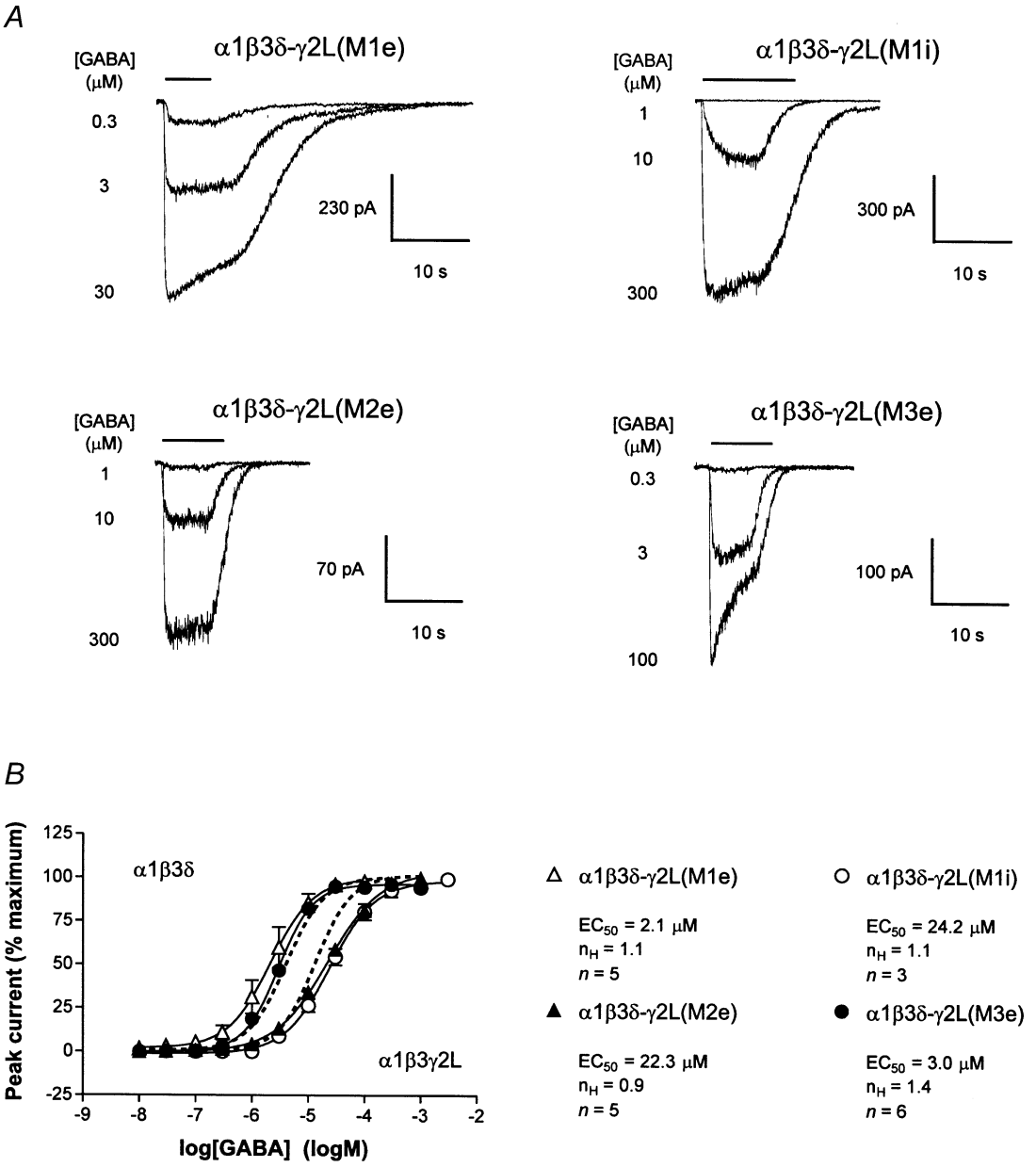

Figure 2. GABA sensitivity of GABARs containing chimeric δ-γ2L subunits

A, representative whole-cell currents from L929 fibroblasts expressing $\alpha1\beta3\delta$-γ2L(M1e) receptors (upper left), $\alpha1\beta3\delta$-γ2L(M1i) receptors (upper right), $\alpha1\beta3\delta$-γ2L(M2e) receptors (lower left), or $\alpha1\beta3\delta$-γ2L(M3e) receptors (lower right). The indicated concentrations of GABA were applied for 6–12 s (horizontal bars) to cells voltage clamped at −75 mV. *B*, concentration–response curves for cells expressing $\alpha1\beta3\delta$-γ2L(M1e) receptors (△), $\alpha1\beta3\delta$-γ2L(M1i) receptors (○), $\alpha1\beta3\delta$-γ2L(M2e) receptors (▲) and $\alpha1\beta3\delta$-γ2L(M3e) receptors (●) are shown by continuous lines. Concentration–response curves for wild-type $\alpha1\beta3\delta$ (left) and $\alpha1\beta3\gamma$2L (right) receptors are shown for comparison (dashed lines). The peak response to each concentration of GABA was normalized as a percentage of the maximum current response for each cell. Values are means ± S.E.M. Data for each isoform were fitted with a four-parameter logistic equation with the indicated EC_{50} values and Hill slopes (n_H).

Akabas, 1996). Residues in the $\beta1$ and $\beta3$ subunits homologous to the third position of the triplet, H292 and H267, respectively, were shown to be major determinants of Zn^{2+} inhibition in β homomers and binary $\alpha\beta$ receptors and also appeared to be water accessible (Wooltorton *et al.* 1997; Horenstein & Akabas, 1998). Therefore, we focused on the extracellular triplet of residues and made the mutations M278S, V279T, S280I (MVS→STI) and S280M, T281V, I282S (STI→MVS) in the δ and $\gamma2$L subunits, respectively.

Coexpression of δ or $\gamma2$L M2 mutant subunits with wild-type $\alpha1$ and $\beta3$ subunits resulted in functional GABARs. The GABA EC_{50} values of the $\alpha1\beta3\delta$(MVS→STI) and $\alpha1\beta3\gamma2$L(STI→MVS) isoforms were similar to those of their respective wild-type receptors (Table 1), suggesting that the triple mutations in the M2 segment did not affect GABA sensitivity.

The Zn^{2+} IC_{50} values of GABARs containing either of the M2 mutants differed from those of GABARs containing wild-type subunits (Fig. 5*B* and *C*). The $\alpha1\beta3\delta$(MVS→STI) isoform had an average Zn^{2+} IC_{50} that was substantially higher than that of the $\alpha1\beta3\delta$ isoform (Table 2). This mutant subunit accounted for 17 % of the log difference between Zn^{2+} IC_{50} values of $\alpha1\beta3\gamma2$L and $\alpha1\beta3\delta$ receptors. The $\alpha1\beta3\gamma2$L(STI→MVS) isoform had an average Zn^{2+} IC_{50} that was substantially lower than that of the $\alpha1\beta3\gamma2$L isoform ($P < 0.001$; Table 2). This mutant subunit accounted for 48 % of the log difference between Zn^{2+} IC_{50} values of $\alpha1\beta3\gamma2$L and $\alpha1\beta3\delta$ receptors. These results indicated that the extracellular end of the M2 segment plays an important role in determining the Zn^{2+} sensitivity of ternary GABARs. Introduction of the $\gamma2$L triplet into the δ subunit background decreased Zn^{2+} sensitivity whereas removal of the triplet from the $\gamma2$L subunit background increased Zn^{2+} sensitivity. The

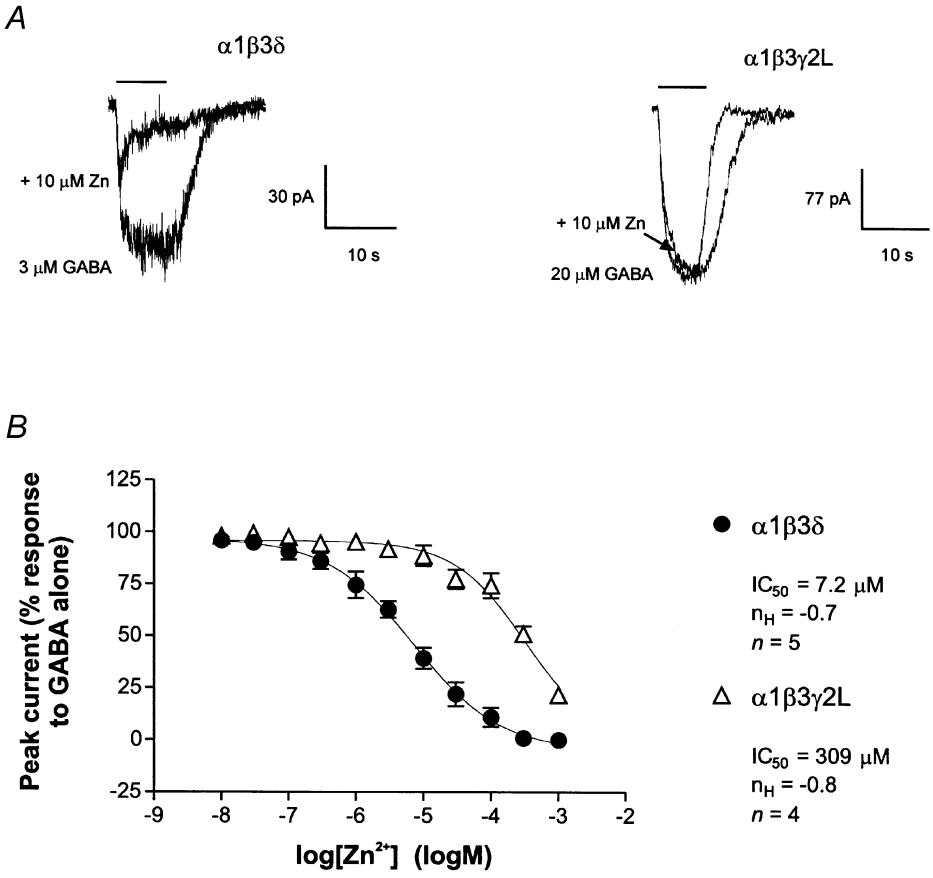

Figure 3. Zn^{2+} sensitivity of GABARs containing wild-type δ or $\gamma2$L subunits

A, representative whole-cell currents from L929 fibroblasts expressing $\alpha1\beta3\delta$ receptors (left) or $\alpha1\beta3\gamma2$L receptors (right). GABA or GABA plus 10 μM Zn^{2+} was applied for 7 s (horizontal bars) to cells voltage clamped at −75 mV. The concentration of GABA used was near the EC_{50} value for the given isoform. *B*, concentration–response curves for cells expressing $\alpha1\beta3\delta$ receptors (●) or $\alpha1\beta3\gamma2$L receptors (△). The peak response to each concentration of Zn^{2+} was normalized as a percentage of the maximum current response to GABA alone for each cell. Values are means ± S.E.M. Data for each isoform were fitted with a four-parameter logistic equation with the indicated IC_{50} values and Hill slopes (n_H).

effects of these mutations, however, were not equivalent for the γ2L and δ subunits (see Discussion).

To determine whether the influence of the M2 triplet on Zn^{2+} sensitivity was dependent on only one of the residues, we made a set of point mutations. Mutagenesis at the third position of the triplet in α and β subunits has been shown to influence the Zn^{2+} sensitivity of binary $\alpha\beta$ receptors (Wooltorton *et al.* 1997; Horenstein & Akabas, 1998). Therefore, point mutations were made at this position in the δ and γ2L subunits at S280 and I282, respectively. In addition, the contributions of the first and second residues of the triplet were tested by making the point mutations M278S and V279T in the δ subunit.

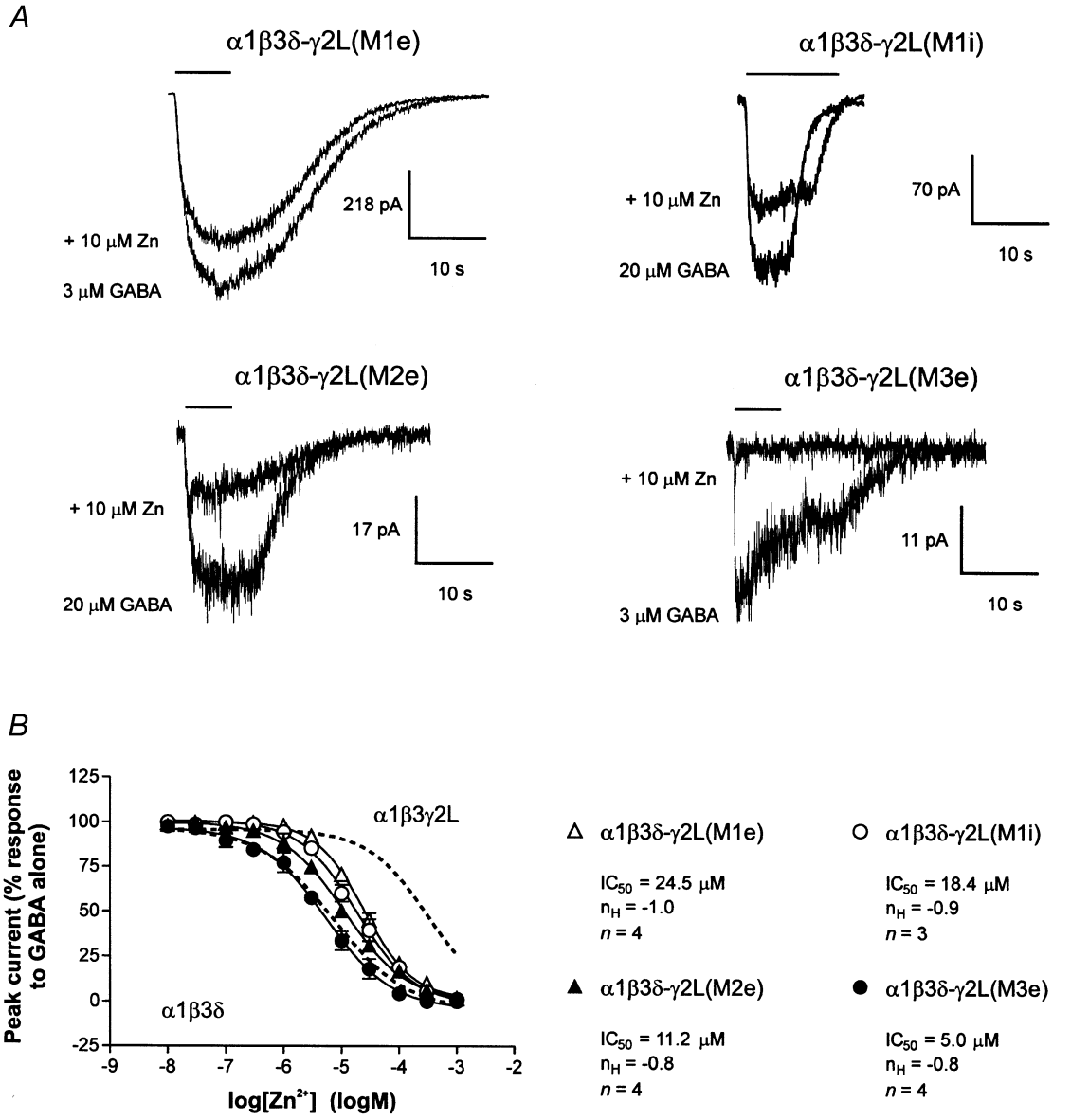

Figure 4. Zn^{2+} sensitivity of GABARs containing chimeric δ-γ2L subunits

A, representative whole-cell currents from L929 fibroblasts expressing $\alpha1\beta3\delta$-γ2L(M1e) receptors (upper left), $\alpha1\beta3\delta$-γ2L(M1i) receptors (upper right), $\alpha1\beta3\delta$-γ2L(M2e) receptors (lower left), or $\alpha1\beta3\delta$-γ2L(M3e) receptors (lower right). GABA or GABA plus 10 μM Zn^{2+} was applied for 6–12 s (horizontal bars) to cells voltage clamped at −75 mV. *B*, concentration–response curves for cells expressing $\alpha1\beta3\delta$-γ2L(M1e) receptors (△), $\alpha1\beta3\delta$-γ2L(M1i) receptors (○), $\alpha1\beta3\delta$-γ2L(M2e) receptors (▲), and $\alpha1\beta3\delta$-γ2L(M3e) receptors (●) are shown by continuous lines. Concentration–response curves for wild-type $\alpha1\beta3\delta$ (left) and $\alpha1\beta3\gamma$2L (right) receptors are shown for comparison (dashed lines). The peak response to each concentration of Zn^{2+} was normalized as a percentage of the maximum current response to GABA alone for each cell. Values are means ± S.E.M. Data for each isoform were fitted with a four-parameter logistic equation with the indicated IC_{50} values and Hill slopes (n_H).

Coexpression of the δ or γ2L M2 triplet mutants with wild-type α1 and β3 subunits resulted in functional GABARs. The GABA EC_{50} values of the $\alpha1\beta3\delta$(M278S), $\alpha1\beta3\delta$(V279T), $\alpha1\beta3\delta$(S280I) and $\alpha1\beta3\gamma$2L(I282S) isoforms were similar to those of their respective wild-type receptors (Table 1), suggesting that these point mutations in the M2 triplet had little effect on GABA sensitivity.

The Zn^{2+} IC_{50} values of GABARs containing any of the M2 triplet mutants were similar to those of GABARs containing wild-type subunits (Fig. 6*A* and *B*). The $\alpha1\beta3\delta$(M278S), $\alpha1\beta3\delta$(V279T) and $\alpha1\beta3\delta$(S280I) isoforms had average IC_{50} values that were similar to that of $\alpha1\beta3\delta$ receptors (Table 2). The $\alpha1\beta3\gamma$2L(I282S) isoform had an average IC_{50} that was similar to that of $\alpha1\beta3\gamma$2L

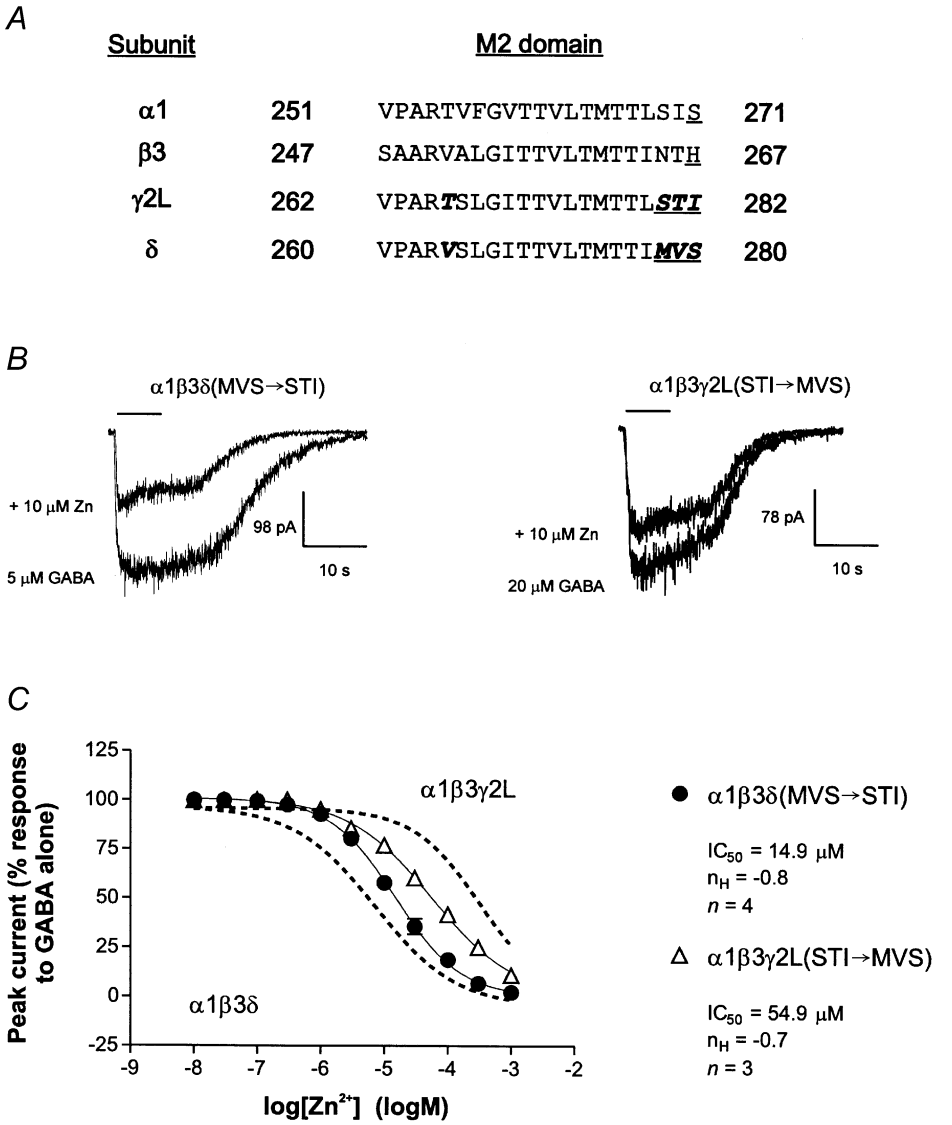

Figure 5. Zn^{2+} sensitivity of GABARs containing M2 mutant subunits

A, M2 segment sequences for the rat α1, β3, γ2L and δ subunits are shown. Residue numbers are based on the mature protein amino acid sequences. Residues in the α1 and β3 subunits previously shown to be water accessible are underlined (Xu & Akabas, 1996; Horenstein & Akabas, 1998). The four sequence differences between the M2 segments of the γ2L and δ subunits are in bold and italicized. The triplets at the extracellular ends of the γ2L and δ M2 segments that were targeted for mutagenesis are underlined. *B*, representative whole-cell currents from L929 fibroblasts expressing $\alpha1\beta3\delta$(MVS→STI) receptors (left) and $\alpha1\beta3\gamma$2L(STI→MVS) receptors (right). GABA or GABA plus 10 μM Zn^{2+} was applied for 7 s (horizontal bars) to cells voltage clamped at −75 mV. *C*, concentration–response curves for cells expressing $\alpha1\beta3\delta$(MVS→STI) receptors (●) and $\alpha1\beta3\gamma$2L(STI→MVS) receptors (△). Concentration–response curves for wild-type $\alpha1\beta3\delta$ (left) and $\alpha1\beta3\gamma$2L (right) receptors are shown for comparison (dashed lines). The peak response to each concentration of Zn^{2+} was normalized as a percentage of the maximum current response to GABA alone for each cell. Values are means ± S.E.M. Data for each isoform were fitted with a four-parameter logistic equation with the indicated IC_{50} values and Hill slopes (n_H).

receptors (Table 2). These results indicated that mutations of the individual residues in the M2 triplet did not replicate the effect of the triple mutation on the Zn^{2+} sensitivity of ternary GABARs.

The M2–M3 loop. The M2–M3 loop is involved in modulating the Zn^{2+} sensitivity of $\alpha\beta\gamma$ receptors containing the α1 or α6 subunit subtypes (Fisher & Macdonald, 1998). α6β3γ2L receptors are more sensitive to Zn^{2+} than α1β3γ2L receptors. Residue H273 of the α6 subunit confers increased Zn^{2+} sensitivity to α6 subunit-containing receptors whereas the homologous residue in the α1 subunit, N274, confers decreased Zn^{2+} sensitivity

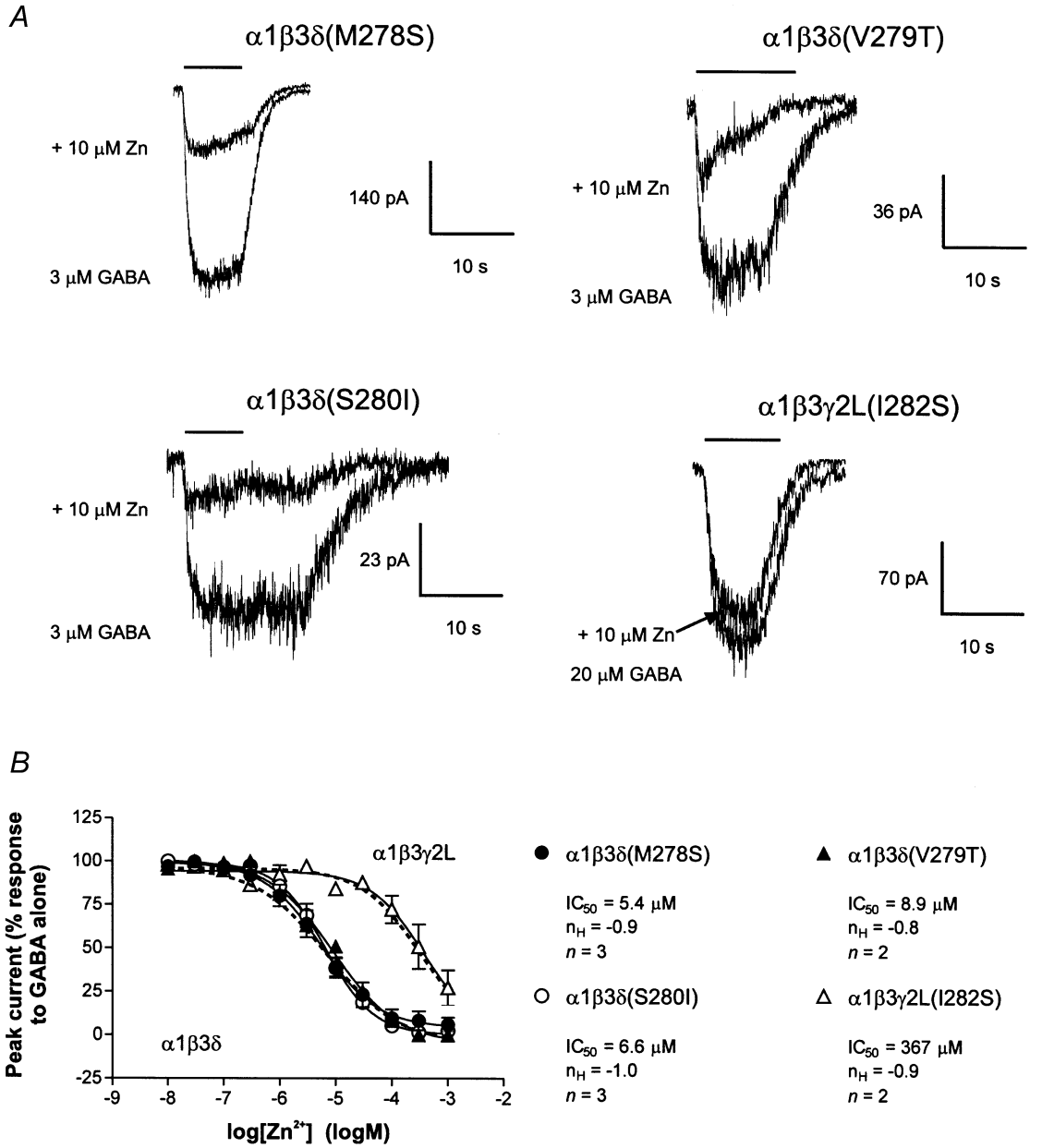

Figure 6. Zn^{2+} sensitivity of GABARs containing subunits with M2 triplet mutations

A, representative whole-cell currents from L929 fibroblasts expressing α1β3δ(M278S) receptors (upper left), α1β3δ(V279T) receptors (upper right), α1β3δ(S280I) receptors (lower left), or α1β3γ2L(I282S) receptors (lower right). GABA or GABA plus 10 μM Zn^{2+} was applied for 7–12 s (horizontal bars) to cells voltage clamped at −75 mV. *B*, concentration–response curves for cells expressing α1β3δ(M278S) receptors (●), α1β3δ(V279T) receptors (▲), α1β3δ(S280I) receptors (○), or α1β3γ2L(I282S) receptors (△) are shown by continuous lines. Concentration–response curves for wild-type α1β3δ (left) and α1β3γ2L (right) receptors are shown for comparison (dashed lines). The peak response to each concentration of Zn^{2+} was normalized as a percentage of the maximum current response to GABA alone for each cell. Values are means ± S.E.M. Data for each isoform were fitted with a four-parameter logistic equation with the indicated IC_{50} values and Hill slopes (n_H).

to α1 subunit-containing receptors. In α1β1γ2 receptors, α1(N274) has been shown to be water accessible (Xu & Akabas, 1996), and sequence alignment indicates that the position occupied by α6(H273) differs between the δ and γ subunit families (Fig. 7*A*). We hypothesized that the positively charged K285 in the γ2L subunit might electrostatically repulse Zn^{2+} from the outer mouth of the channel whereas the polar, uncharged S283 residue in the δ subunit might interact with Zn^{2+} (Karlin & Zhu, 1997) and thereby stabilize the cation in the channel. It has

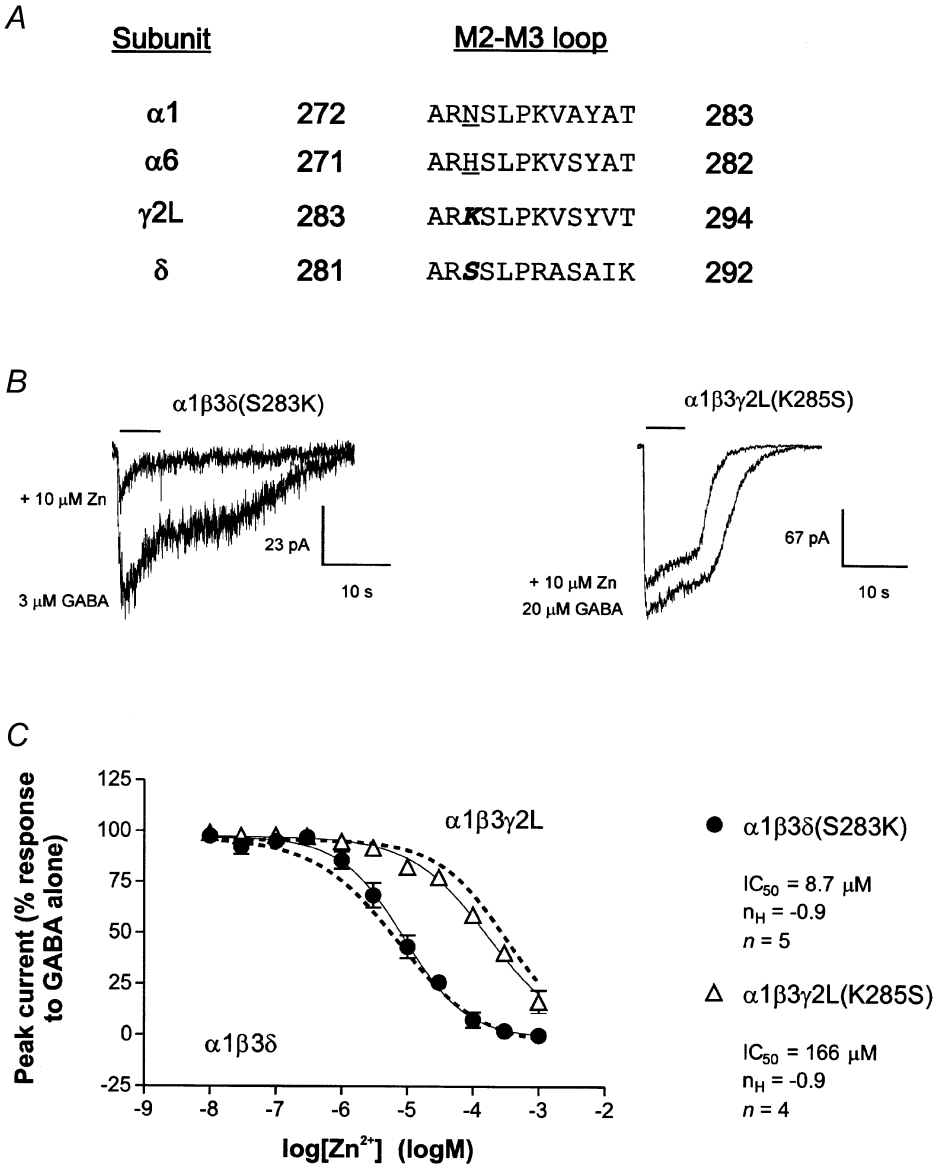

Figure 7. Zn^{2+} sensitivity of GABARs containing M2–M3 mutant subunits

A, M2–M3 loop sequences for the rat α1, α6, γ2L and δ subunits are shown. Residue numbers are based on the mature protein amino acid sequences. Residues in the α1 and α6 subunits previously shown to influence Zn^{2+} sensitivities of α1β3γ2L and α6β3γ2L receptors are underlined (Fisher & Macdonald, 1998). The homologous residues in the γ2L and δ subunits that were targeted for mutagenesis are in bold and italicized. *B*, representative whole-cell currents from L929 fibroblasts expressing α1β3δ(S283K) receptors (left) and α1β3γ2L(K285S) receptors (right). The indicated concentrations of GABA were applied for 6–8 s (horizontal bars) to cells voltage clamped at −75 mV. *C*, concentration–response curves for cells expressing α1β3δ(S283K) receptors (●) and α1β3γ2L(K285S) receptors (△). Concentration–response curves for wild-type α1β3δ (left) and α1β3γ2L (right) receptors are shown for comparison (dashed lines). The peak response to each concentration of Zn^{2+} was normalized as a percentage of the maximum current response to GABA alone for each cell. Values are means ± S.E.M. Data for each isoform were fitted with a four-parameter logistic equation with the indicated IC_{50} values and Hill slopes (n_H).

been previously reported that replacement of K285 with an alanine residue did not change the Zn^{2+} insensitivity of ternary receptors (Smart *et al.* 1994). To assess the role of this M2–M3 residue in determining Zn^{2+} sensitivity, the point mutations S283K and K285S were made in the δ and γ2L subunits, respectively.

Coexpression of δ or γ2L M2–M3 mutant subunits with wild-type α1 and β3 subunits resulted in functional GABARs. The GABA EC_{50} values of the two mutant subunit-containing isoforms, $\alpha1\beta3\delta$(S283K) and $\alpha1\beta3\gamma$2L(K285S), were similar to those of their respective wild-type receptors (Table 1). These results indicated that these point mutations in the M2–M3 loop had little effect on GABA sensitivity.

Mutations in the M2–M3 loop of δ or γ2L subunits had different effects on the Zn^{2+} sensitivities of ternary GABARs (Fig. 7*B* and *C*). The $\alpha1\beta3\delta$(S283K) isoform had an average IC_{50} that was slightly higher than that of $\alpha1\beta3\delta$ receptors (Table 2), suggesting that substitution of a lysine residue at S283 in the wild-type δ background had minimal effect on Zn^{2+} sensitivity. However, the $\alpha1\beta3\gamma$2L(K285S) isoform had an average IC_{50} that was lower than that of $\alpha1\beta3\gamma$2L receptors (Table 2). This mutant subunit resulted in a reduction in Zn^{2+} sensitivity that was 15% of the log difference between Zn^{2+} IC_{50} values of $\alpha1\beta3\gamma$2L and $\alpha1\beta3\delta$ receptors, suggesting that substitution of a serine residue at K285 in the wild-type γ2L subunit caused an increase in Zn^{2+} sensitivity. Similar to the M2 triple mutants, these point mutations in the M2–M3 loop of δ and γ2L subunits did not have equivalent effects on Zn^{2+} sensitivity, suggesting that the subunit context was an added factor (see Discussion).

DISCUSSION

For the purposes of this discussion, we defined a functional domain as one or more amino acids that determined the effectiveness of a modulatory compound (e.g. Zn^{2+}). The functional domain could represent the actual binding site for the compound, a transduction device between binding and drug effect, or a structural feature that could indirectly influence binding and/or transduction. In our study, the functional domain was defined by functional differences (e.g. IC_{50}) that were introduced by chimera construction and mutagenesis. Using this approach we identified novel structural determinants of low Zn^{2+} sensitivity for γ2L subunit-containing ternary GABARs (Fig. 8). One functional domain in the γ2L subunit for low Zn^{2+} sensitivity was localized to a subunit region forming the outer vestibule of the channel and was composed of residues in the M2 segment and the M2–M3 loop. The other functional domain in the γ2L subunit for low Zn^{2+} sensitivity was localized to the N-terminal extracellular region. Together, these two functional domains appear to form the basis for differences in Zn^{2+} sensitivity between $\alpha\beta\delta$ and $\alpha\beta\gamma$ receptors.

Role of the outer vestibule in regulating Zn^{2+} sensitivity

One functional domain in the γ2L subunit conferring low Zn^{2+} sensitivity was localized to the region of the outer vestibule of the channel. It was composed of the extracellular end of the M2 segment and the proximal end of the M2–M3 loop. The γ2L subunit determinant in the M2 segment for reduced Zn^{2+} sensitivity consisted of three amino acid residues rather than a single one as was found for the β subunits (Wooltorton *et al.* 1997; Horenstein & Akabas, 1998). Individual substitutions within the triplet based on γ2L and δ sequence differences did not change the Zn^{2+} sensitivity of ternary receptors. As in the α1 and α6 subunit subtypes (Fisher & Macdonald, 1998), the γ2L subunit M2–M3 loop was also found to make a contribution to Zn^{2+} sensitivity. Although these two γ2L subunit regions were identified as separate determinants, it is likely that they form a single functional domain that was inadvertently divided by the design of our chimeric subunits.

It is likely that the M2 triplet of γ2L and δ subunits is not directly involved in Zn^{2+} binding. In the β subunit family, the homologous residue to the first position in the triplet has been shown to influence sensitivity to a variety of modulatory compounds. Replacement of the β1 subunit subtype with either the β2 or β3 subunit subtype

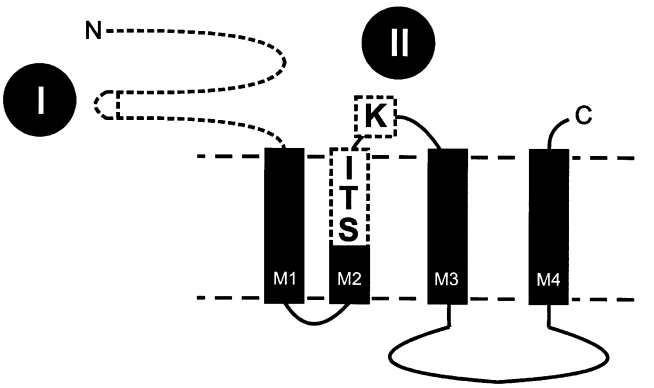

Figure 8. Schematic representation of two domains of the γ2L subunit involved in conferring low Zn^{2+} sensitivity

The putative membrane topology of a γ2L subunit is shown. The extracellular N- and C-termini are indicated (N, C) and the four putative transmembrane segments labelled (M1–M4). The subunit domains involved in conferring low Zn^{2+} sensitivity to ternary GABARs are indicated by dashed lines with identified key residues (boxed text) shown in their approximate locations. The first domain (I) is composed of the N-terminal extracellular region. The second domain (II) is composed of a triplet of amino acid residues (STI) at the extracellular end of the M2 segment and a single residue (K) at the proximal end of the M2–M3 loop.

confers greater receptor sensitivity to furosemide inhibition as well as to loreclezole, ethanol, enflurane, etomidate and β-carboline potentiation (Wingrove *et al.* 1994; Stevenson *et al.* 1995; Belelli *et al.* 1997; Mihic *et al.* 1997; Thompson *et al.* 1999). The differences in drug sensitivities have been attributed to N265 (rat β3 subunit) and the homologous S265 (rat β1 subunit). These observations suggest that the first position in the M2 segment of the γ2L and δ GABAR subunits has a role in transduction of modulator effects rather than in direct binding. Transduction of Zn^{2+} binding may be a role subserved by the second and third positions of the M2 triplet as well as by γ2L(K285) in the M2–M3 loop. Residues γ2(T281) and γ2(I282) have recently been identified as two of the three transduction elements required to couple benzodiazepine binding to GABA current potentiation (Boileau & Czajkowski, 1999). The third element that was identified is γ2(S291), which is six amino acids C-terminal to γ2(K285) (Boileau & Czajkowski, 1999).

The third position of the M2 triplet has been shown to largely determine the Zn^{2+} sensitivity of β homomers and binary αβ receptors (Wooltorton *et al.* 1997; Horenstein & Akabas, 1998). Substitution of a serine (as in the δ subunit) or an isoleucine (as in the γ2L subunit) for the histidine in this β subunit position was found to decrease the Zn^{2+} sensitivity of α1β1 GABARs (Horenstein & Akabas, 1998). Substitution of a histidine at the homologous position of the α1 subunit (S271) was found to increase the Zn^{2+} sensitivity of α1β1 receptors (Horenstein & Akabas, 1998). Thus, this position appears to be available for Zn^{2+} binding in α and β subunits. In γ2L subunits, however, Zn^{2+} interaction does not seem plausible as substitution of a histidine at γ2(I282) was not found to increase the Zn^{2+} sensitivity of α1β1γ2 receptors (Horenstein & Akabas, 1998).

Role of the N-terminus in regulating Zn^{2+} sensitivity

Another determinant of the low Zn^{2+} sensitivity of γ2L subunits was localized to the N-terminus. This result was somewhat unexpected in the light of recent studies pointing to the extracellular portion of the M2 segment and the M2–M3 loop as important determinants of GABAR Zn^{2+} sensitivity (Wooltorton *et al.* 1997; Fisher & Macdonald, 1998; Horenstein & Akabas, 1998). However, it was shown that recombinant $GABA_C$ receptor ρ1 subunits possessed a determinant of Zn^{2+} sensitivity in the homologous extracellular region (Wang *et al.* 1995). A single residue, H156, was found to be critical for Zn^{2+} sensitivity. Residue H156 is homologous to a highly conserved asparagine, which is found among all GABAR subunit families except π. Interestingly, this residue is adjacent to H101 (rat sequence) in the α1 subunit, which is required for benzodiazepine sensitivity in ternary αβγ receptors (Smith & Olsen, 1995). The amino acid sequence in the vicinity of this residue is highlighted by two tryptophan residues (W69 and W94 in the rat GABAR α1 subunit) that are conserved in all members of the ligand-gated ion channel superfamily and in GABARs demarcate GABA and benzodiazepine binding regions (Smith & Olsen, 1995). In fact, mutation of the ρ1(H156) has also been found to influence the GABA sensitivity of ρ1 homomers (Kusama *et al.* 1994).

Functional domains determining the Zn^{2+} sensitivity of another member of the ligand-gated ion channel superfamily, the glycine receptors (GlyRs), have also been localized to the extracellular N-terminus. Mutagenesis based on chimeric subunit analysis has identified amino acid residues influencing potentiation and inhibition of human α1 homomers by Zn^{2+} (Lynch *et al.* 1998; Laube *et al.* 2000). Replacement of D80 resulted in a loss of potentiation and replacement of T112 resulted in a loss of inhibition. These two residues are located near the conserved tryptophan residues. Mutagenesis based on histidine targeting has also identified residues influencing potentiation and inhibition of GlyR currents by Zn^{2+} (Harvey *et al.* 1999). Mutation of H107 abolished Zn^{2+}-mediated inhibition and mutation of H215 abolished Zn^{2+}-mediated potentiation. Mutation of H109, however, abolished both inhibition and potentiation by Zn^{2+}. Histidines 107 and 109 are proximal to the conserved tryptophan residues whereas H215 is not.

The complex nature of functional domains for Zn^{2+} in the N-terminal extracellular region of GlyRs suggests that such domains may also be complex in the homologous region of the GABAR γ2L and δ subunits. If the tertiary subunit of ternary GABARs participates in Zn^{2+} binding, then one might expect that the Zn^{2+} functional domains could be identified by looking for the presence or absence of common Zn^{2+} co-ordinating residues (H, C, D and E; Karlin & Zhu, 1997). This approach will not work, however, if the functional domains are involved in a role other than Zn^{2+} binding. Thus, chimeric subunit analysis would prove more fruitful.

Subunit context of mutations and shifts in Zn^{2+} sensitivity

The shifts in Zn^{2+} sensitivity conferred by our site-directed mutant subunits were asymmetric. The triple mutations in the M2 segment of the γ2L subunit induced a 48% shift towards δ subunit-like sensitivity whereas in the δ subunit, the mutations induced only a 17% shift towards γ2L subunit-like sensitivity. The point mutation in the M2–M3 loop of the γ2L subunit induced a 15% shift towards δ subunit-like sensitivity whereas in the δ subunit, the mutation induced only a 4% shift towards γ2L subunit-like sensitivity. Although it is tempting to presume that asymmetry is an indicator of irrelevance of a given residue or residues, we would argue that, in fact, it points to the importance of context (i.e. tertiary structure) for functional domains. In the 'native' context of the γ2L subunit, removal of the M2 triplet and the M2–M3 loop residue can be interpreted as a loss of elements necessary for low Zn^{2+} sensitivity. In the 'non-native' context of the δ subunit, introduction of the γ2L

subunit M2 triplet and M2–M3 loop residue cannot, however, be interpreted as acquisition of these same elements because the appropriate subunit context is not available. Therefore, these mutations would not be expected to have the symmetrical effect on Zn^{2+} sensitivity in an otherwise 'non-native' δ subunit context.

The importance of subunit context for functional domain properties also was demonstrated by the shifts of Zn^{2+} sensitivity induced by the δ-γ2L chimeric subunits. Removal of the γ2L subunit N-terminus (δ-γ2L(M1e)) resulted in a 63 % shift in sensitivity from γ2L subunit-like to δ subunit-like. The estimated contribution of the N-terminus to the low Zn^{2+} sensitivity of γ2L subunits based on the site-directed mutant data would indicate a shift of ~40 % (see above). Sequential removal of the M2 triplet (δ-γ2L(M2e)) and the M2–M3 loop residue (δ-γ2L(M3e)) resulted in shifts of ~20 % each from γ2L subunit-like to δ subunit-like. The sum of these shifts (~40 %) is somewhat lower than the sum of those induced by the site-directed mutations involving these subunit segments where the subunit context included the γ2L N-terminus (~60 %). Thus, the potency of the γ2L subunit outer vestibule in lowering Zn^{2+} sensitivity appears to be reduced in the context of the δ subunit N-terminus.

Although our study demonstrated that the N-terminus of the γ2L and δ GABAR subunits contributed to Zn^{2+} sensitivity, it would not be surprising if the homologous regions of α and β subunits were also found to play a role. The alignment of various N-terminal residues implicated in agonist and benzodiazepine binding among the different subunit families suggests that residues involved in Zn^{2+} inhibition are also aligned (Sigel & Buhr, 1997). Indeed, differences in the mechanism of Zn^{2+} antagonism (non-competitive, competitive, mixed) among various receptor isoforms (Legendre & Westbrook, 1991; Gingrich & Burkat, 1998; Krishek *et al.* 1998) might be explained by the availability of Zn^{2+} functional domains, not only near the channel mouth but also at subunit interfaces whereby agonist binding could be influenced.

Angelotti, T. P., Uhler, M. D. & Macdonald, R. L. (1993). Assembly of $GABA_A$ receptor subunits: analysis of transient single cell expression utilizing a fluorescent substrate/marker gene combination. *Journal of Neuroscience* **13**, 1418–1428.

Belelli, D., Lambert, J. J., Peters, J. A., Wafford, K. & Whiting, P. J. (1997). The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. *Proceedings of the National Academy of Sciences of the USA* **94**, 11031–11036.

Boileau, A. J. & Czajkowski, C. (1999). Identification of transduction elements for benzodiazepine modulation of the $GABA_A$ receptors: three residues are required for allosteric coupling. *Journal of Neuroscience* **19**, 10213–10220.

Bonnert, T. P., McKernan, R. M., Farrar, S., le Bourdelle, B., Heavens, R. P., Smith, D. W., Hewson, L., Rigby, M. R., Sirinathsinghji, D. J., Brown, N., Wafford, K. A. & Whiting, P. J. (1999). Theta, a novel gamma-aminobutyric acid type A receptor subunit. *Proceedings of the National Academy of Sciences of the USA* **96**, 9891–9896.

Burgard, E. C., Tietz, E. I., Neelands, T. R. & Macdonald, R. L. (1996). Properties of recombinant γ-aminobutyric $acid_A$ receptor isoforms containing the α5 subunit subtype. *Molecular Pharmacology* **50**, 119–127.

Davies, P. A., Hanna, M. C., Hales, T. G. & Kirkness, E. F. (1997). Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunit. *Nature* **385**, 820–823.

Draguhn, A., Verdorn, T. A., Ewert, M., Seeburg, P. H. & Sakmann, B. (1990). Functional and molecular distinction between recombinant rat $GABA_A$ receptor subtypes by Zn^{2+}. *Neuron* **5**, 781–788.

Fisher, J. L. & Macdonald, R. L. (1997). Single channel properties of recombinant $GABA_A$ receptors containing γ2 or δ subtypes expressed with α1 and β3 subtypes in mouse L929 cells. *Journal of Physiology* **505**, 283–297.

Fisher, J. L. & Macdonald, R. L. (1998). The role of an α subtype M_2-M_3 His in regulating inhibition of $GABA_A$ receptor current by zinc and other divalent cations. *Journal of Neuroscience* **18**, 2944–2953.

Gingrich, K. J. & Burkat, P. M. (1998). Zn^{2+} inhibition of recombinant $GABA_A$ receptors: an allosteric, state-dependent mechanism determined by the γ-subunit. *Journal of Physiology* **506**, 609–625.

Greenfield, L. J. Jr & Macdonald, R. L. (1996). Whole cell and single channel $\alpha1\beta1\gamma2S$ $GABA_A$ receptor currents elicited by a 'multipuffer' drug application device. *Pflügers Archiv* **432**, 1080–1090.

Harrison, N. L. & Gibbons, S. J. (1994). Zn^{2+}: an endogenous modulator of ligand- and voltage-gated ion channels. *Neuropharmacology* **33**, 935–952.

Harvey, R. J., Thomas, P., James, C. H., Wilderspin, A. & Smart, T. G. (1999). Identification of an inhibitory Zn^{2+} binding site on the human glycine receptor α1 subunit. *Journal of Physiology* **520**, 53–64.

Hedblom, E. & Kirkness, E. F. (1997). A novel class of $GABA_A$ receptor subunit in tissues of the reproductive system. *Journal of Biological Chemistry* **272**, 15346–15350.

Horenstein, J. & Akabas, M. H. (1998). Location of a high affinity Zn^{2+} binding site in the channel of $\alpha1\beta1\gamma$-aminobutyric $acid_A$ receptors. *Molecular Pharmacology* **53**, 870–877.

Huggenvik, J. I., Collard, M. W., Stofko, R. E., Seasholtz, A. F. & Uhler, M. D. (1991). Regulation of the human enkephalin promoter by two isoforms of the catalytic subunit of cyclic adenosine 3′5′-monophosphate-dependent protein kinase. *Molecular Endocrinology* **5**, 921–930.

Kapur, J. & Macdonald, R. L. (1997). Rapid seizure-induced reduction of benzodiazepine and Zn^{2+} sensitivity of hippocampal dentate granule cell $GABA_A$ receptors. *Journal of Neuroscience* **17**, 7532–7540.

Karlin, S. & Zhu, Z.-Y. (1997). Classification of mononuclear zinc metal sites in protein structures. *Proceedings of the National Academy of Sciences of the USA* **94**, 14231–14236.

Krishek, B. J., Moss, S. J. & Smart, T. G. (1998). Interaction of H^+ and Zn^{2+} on recombinant and native rat neuronal $GABA_A$ receptors. *Journal of Physiology* **507**, 639–652.

Kusama, T., Wang, J.-B., Spivak, C. E. & Uhl, G. R. (1994). Mutagenesis of the GABA ρ1 receptor alters agonist affinity and channel gating. *NeuroReport* **5**, 1209–1212.

Laube, B., Kuhse, J. & Betz, H. (2000). Kinetic and mutational analysis of Zn^{2+} modulation of recombinant human inhibitory glycine receptors. *Journal of Physiology* **522**, 215–230.

Legendre, P. & Westbrook, G. L. (1991). Noncompetitive inhibition of γ-aminobutyric $acid_A$ channels by Zn. *Molecular Pharmacology* **39**, 267–274.

Lynch, J. W., Jacques, P., Pierce, K. D. & Schofield, P. R. (1998). Zinc potentiation of the glycine receptor chloride channel is mediated by allosteric pathways. *Journal of Neurochemistry* **71**, 2159–2168.

Macdonald, R. L. & Olsen, R. W. (1994). $GABA_A$ receptor channels. *Annual Review of Neuroscience* **17**, 569–602.

McKernan, R. M. & Whiting, P. J. (1996). Which $GABA_A$-receptor subtypes really occur in the brain? *Trends in Neurosciences* **19**, 139–143.

Mihic, S. J., Ye, Q., Wick, M. J., Koltchine, V. V., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., Harris, R. A. & Harrison, N. L. (1997). Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature* **389**, 385–389.

Neelands, T. R., Fisher, J. L., Bianchi, M. & Macdonald, R. L. (1999). Spontaneous and γ-aminobutyric acid (GABA)-activated $GABA_A$ receptor channels formed by ϵ subunit-containing isoforms. *Molecular Pharmacology* **55**, 168–178.

Neelands, T. R. & Macdonald, R. L. (1999). Incorporation of the π subunit into functional $GABA_A$ receptor channels. *Molecular Pharmacology* **56**, 598–610.

Saxena, N. C. & Macdonald, R. L. (1996). Properties of putative cerebellar γ-aminobutyric $acid_A$ receptor isoforms. *Molecular Pharmacology* **49**, 567–579.

Shivers, B. D., Killisch, I., Sprengel, R., Sontheimer, H., Köhler, M., Schofield, P. R. & Seeburg, P. H. (1989). Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3**, 327–337.

Sigel, E. & Buhr, A. (1997). The benzodiazepine binding site of $GABA_A$ receptors. *Trends in Pharmacological Sciences* **18**, 425–429.

Smart, T. G., Moss, S. L., Xie, X. & Huganir, R. L. (1991). $GABA_A$ receptors are differentially sensitive to zinc: dependence on subunit composition. *British Journal of Pharmacology* **99**, 643–654.

Smart, T. G., Xie, X. & Krishek, B. J. (1994). Modulation of inhibitory and excitatory amino acid receptor ion channels by zinc. *Progress in Neurobiology* **42**, 393–441.

Smith, G. B. & Olsen, R. W. (1995). Functional domains of $GABA_A$ receptors. *Trends in Pharmacological Sciences* **16**, 162–168.

Stevenson, A., Wingrove, P. B., Whiting, P. J. & Wafford, K. A. (1995). β-carboline γ-aminobutyric $acid_A$ receptor inverse agonists modulate γ-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. *Molecular Pharmacology* **48**, 965–969.

Thompson, S. A., Arden, S. A., Marshall, G., Wingrove, P. B., Whiting, P. J. & Wafford, K. A. (1999). Residues in transmembrane domains I and II determine γ-aminobutyric acid type A receptor subtype-selective antagonism by furosemide. *Molecular Pharmacology* **55**, 993–999.

Wang, T.-L., Hackam, A., Guggino, W. B. & Cutting, G. R. (1995). A single histidine residue is essential for zinc inhibition of GABA ρ1 receptors. *Journal of Neuroscience* **15**, 7684–7691.

Whiting, P. J., McAllister, G., Vasilatis, D., Bonnert, T. P., Heavens, R. P., Smith, D. W., Hewson, L., O'Donnell, R., Rigby, M. R., Sirinathsinghji, D. J. S., Marshall, G., Thompson, S. A. & Wafford, K. A. (1997). Neuronally restricted RNA splicing regulates the expression of a novel $GABA_A$ receptor subunit conferring atypical functional properties. *Journal of Neuroscience* **17**, 5027–5037.

Wingrove, P. B., Wafford, K. A., Bain, C. & Whiting, P. J. (1994) The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the β2 and β3 subunit. *Proceedings of the National Academy of Sciences of the USA* **91**, 4569–4573.

Wooltorton, J. R. A., McDonald, B. J., Moss, S. J. & Smart, T. G. (1997). Identification of a Zn^{2+} binding site on the murine $GABA_A$ receptor complex: dependence on the second transmembrane domain of β subunits. *Journal of Physiology* **505**, 633–640.

Xu, M. & Akabas, M. H. (1996). Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor α1 subunit. *Journal of General Physiology* **107**, 195–205.

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