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An N-terminal histidine regulates Zn^{2+} inhibition on the murine $GABA_A$ receptor $\beta3$ subunit

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1 Whole-cell currents were recorded from *Xenopus laevis* oocytes and human embryonic kidney cells expressing $GABA_A$ receptor $\beta3$ subunit homomers to search for additional residues affecting Zn^{2+} inhibition. These residues would complement the previously identified histidine (H267), present just within the external portal of the ion channel, which modulates Zn^{2+} inhibition.

2 Zinc inhibited the pentobarbitone-gated current on $\beta3^{H267A}$ homomers at pH 7.4, but this effect was abolished at pH 5.4. The Zn^{2+}-sensitive spontaneous $\beta3$ subunit-mediated conductance was also insensitive to block by Zn^{2+} at pH 5.4.

3 Changing external pH enabled the titration of the Zn^{2+} sensitive binding site or signal transduction domain. The pK_a was estimated at 6.8 ± 0.03 implying the involvement of histidine residues.

4 External histidine residues in the $\beta3$ receptor subunit were substituted with alanine, in addition to the background mutation, H267A, to assess their sensitivity to Zn^{2+} inhibition. The Zn^{2+} IC_{50} was unaffected by either the H119A or H191A mutations.

5 The remaining histidine, H107, the only other candidate likely to participate in Zn^{2+} inhibition, was substituted with various residues. Most mutants were expressed at the cell surface but they disrupted functional expression of $\beta3$ homomers. However, H107G was functional and demonstrated a marked reduction in sensitivity to Zn^{2+}.

6 $GABA_A$ receptor $\beta3$ subunits form functional ion channels that can be inhibited by Zn^{2+}. Two histidine residues are largely responsible for this effect, H267 in the pore lining region and H107 residing in the extracellular N-terminal domain.

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Keywords: $GABA_A$ receptor; Zn^{2+}; histidines; pH; β subunit; N-terminal domain; ion channel

Abbreviations: DEPC, diethylpyrocarbonate; $GABA_A$, γ-aminobutyric acid type A; $GABA_C$, γ-aminobutyric acid type C; GFP, green fluorescent protein; ΔG, conductance change; HEK, human embryonic kidney; H, histidine; MBM, modified Barth's medium; n_H, Hill coefficient; PB, pentobarbitone; TM2, second transmembrane domain

Introduction

The divalent cation, Zn^{2+}, is concentrated in specific neurones in the central nervous system and can be released following neural stimulation into the synaptic cleft where it can bind to numerous membrane proteins (Frederickson, 1989; Frederickson & Bush, 2001). In particular, Zn^{2+} can interact with a variety of ligand-gated and voltage-operated ion channels (Smart *et al*., 1994; Harrison & Gibbons, 1994). With respect to the γ-aminobutyric acid type A ($GABA_A$) and C ($GABA_C$) receptors, Zn^{2+} acts as a non-competitive or mixed inhibitor of GABA-activated Cl^- currents (Smart & Constanti, 1990; Dong & Werblin, 1995) and at least for the $GABA_A$ receptor, the potency of inhibition is largely dependent upon the receptor subunit composition (Smart *et al*., 1994). There has been considerable recent interest in determining likely binding sites for Zn^{2+} on $GABA_A$ receptors with histidines featuring prominently as the main targets on $GABA_A$ receptor $\beta3$ (Wooltorton *et al*., 1997a), $\beta1$ (Horenstein & Akabas, 1998), $\alpha6$ subunits (Fisher & Macdonald, 1998), and $GABA_C$ $\rho1$ subunits (Wang *et al*., 1995). Despite these studies, three problems have hampered the search for Zn^{2+} binding sites on $GABA_A$ receptors: these include the acknowledged heterogeneity of native $GABA_A$ receptors and their differential sensitivity to inhibition by Zn^{2+} (Smart *et al*., 1994; Rabow *et al*., 1995); the difficulty of determining Zn^{2+} binding sites on recombinant $GABA_A$ receptors composed of multiple subunits, e.g., α, β and γ subunits, which are generally accepted as the likeliest combination of subunits representing most native $GABA_A$ receptors; and the prospect of there being multiple discrete binding sites for Zn^{2+} on a single $GABA_A$ receptor.

In response to the preceding difficulties, two studies adopted a reductionist approach searching for Zn^{2+} binding sites by using homomeric $GABA_A$ receptors comprising $\beta3$ (Wooltorton *et al*., 1997a) or $\beta1$ (Horenstein & Akabas, 1998) subunits. The $\beta1$ and $\beta3$ subunits form functional homomeric receptors that exhibit spontaneous gating, which is inhibited by picrotoxin or Zn^{2+} (Sigel *et al*., 1989; Krishek

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et al., 1996; Wooltorton *et al*., 1997a). They can also be activated or modulated by pentobarbitone, whilst GABA is, for some species, largely ineffective (Sigel *et al*., 1989; Sanna *et al*., 1995; Krishek *et al*., 1996; Wooltorton *et al*., 1997b; Davies *et al*., 1997). Site-directed mutagenesis studies of β1 and β3 subunit receptors revealed that a single histidine (H) residue (H267) located in the presumed external portal of the ion channel (second transmembrane domain, TM2) was responsible for the binding and/or inhibitory effect of Zn^{2+} on spontaneous or pentobarbitone-gated β subunit ion channels (Wooltorton *et al*., 1997a; Horenstein & Akabas, 1998). The location of this 'site' (if indeed it represents a binding site) was unexpected since it suggested Zn^{2+} penetrated, to a limited extent, into the anion-selective channel of the $GABA_A$ receptor.

Although these experiments revealed a substantial reduction in the potency of Zn^{2+} as an inhibitor at homomeric β subunit $GABA_A$ receptors, application of higher concentrations of Zn^{2+} (exceeding 1–2 mM) caused a complete inhibition of the current gated by β3 homomeric subunits suggesting that additional residues, possibly forming a lower affinity binding site, were still present (Wooltorton *et al*., 1997a). Similar experiments were also performed on $\alpha1\beta$i (where i = 1 or 3) heteromers which were not spontaneously-gated and possessed a sensitivity to GABA unlike their homomeric counterparts. The inclusion of the mutant βi^{H267A} subunits in place of wild-type βi subunits also revealed a considerable increase in the IC_{50} for Zn^{2+}, but again, increasing the Zn^{2+} concentration resulted in substantial inhibition of the GABA-gated current (Wooltorton *et al*., 1997a; Horenstein & Akabas, 1998). Taken together, the results with homomeric and heteromeric $GABA_A$ receptors indicate that additional amino acid residues are contributing to Zn^{2+} inhibition by participating in signal transduction or by forming an extra binding site(s) for Zn^{2+}.

It is conceivable that residues affecting Zn^{2+} inhibition are not uniquely the preserve of the β subunits and may possibly be located on α subunits also, particularly since exchanging α1 for α3 isoforms in heteromeric γ-subunit-containing $GABA_A$ receptors resulted in altered sensitivity to Zn^{2+} (White & Gurley, 1995). For this reason, and because it is clear that an additional residue(s) mediating Zn^{2+} inhibition exists on β subunits, this study utilized site-directed mutagenesis and expression of recombinant β subunit $GABA_A$ receptors in combination with electrophysiology to locate those residues involved.

Methods

Vector construction and site-directed mutagenesis

The mouse $GABA_A$ receptor β3 subunit cDNA was cloned into the vector pRK5. Site-directed mutagenesis was achieved using 27-mer oligonucleotides and a primer-directed polymerase chain reaction method (Quikchange kit, Stratagene). DNAs for transfection were made using the Plasmid Maxi Kit (Qiagen) and all mutant constructs were completely sequenced using the BigDye ready reaction mix (Perkin-Elmer/Applied Biosystems) and an ABI 310 automated DNA sequencer (Applied Biosystems). In order to introduce multiple amino acid residues at position H107 in the $GABA_A$ receptor β3 subunit, we used a variation of this technique. The minimum degeneracy required to encode all amino acids at a given position in a degenerate oligonucleotide is 32-fold, i.e. the codon triplet NNS (where N = G, A, T or C and S = G + C). This triplet was then incorporated, at the equivalent position to H107, into two 27-mer degenerate oligonucleotides (H107X1 5′-AAGTCATTTGTCNNSGGA-GTGACAGTG-3′ and H107X2 5′-CACTGTCACTCCSNN-GACAAATGACTT-3′). PCR was performed using these primers for 18 cycles at 94°C for 1 min, 55°C for 1 min and 68°C for 15′ using 10 ng of the wild-type $GABA_A$ receptor β3 subunit construct as the template. One potential disadvantage of this method might be that *E. coli* transformed with the resultant PCR product could harbour two different plasmid types because the sense and antisense strands could differ at the degenerate position. However, DNA sequencing of the resultant mutants demonstrated that very few (2 from 32) templates were mixed. We were therefore able to easily generate multiple amino acid substitutions at the target locus, 13 out of a possible 20 amino acid substitutions (single amino acid code: C, G, I, K, L, P, Q, R, S, T, V, W, Y) were generated in the first 32 templates analysed. This method therefore offers a quick and very versatile approach for producing numerous amino acid substitutions at the same position.

Cell preparation: Oocyte extraction and microinjection

Oocytes were aseptically removed from anaesthetized *Xenopus laevis* after immersion in 0.5% tricaine and then stored in modified Barth's medium (MBM) containing (mM): NaCl 110, KCl 1, $NaHCO_3$ 2.4, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, and gentamycin 50 μg ml^{-1} at pH 7.6. Stage IV and V oocytes were separated and centrifuged (700–1100 *g* for 6 min at 10°C) for nuclear microinjection with 10 nl of 1 mg ml^{-1} DNA solution, encoding either the murine β3 wild-type, mutant $\beta3^{H267A}$, and further selected histidine mutant β3 $GABA_A$ receptor subunits incorporating H267A as a background. The injected oocytes were incubated at 18°C for 24 h then subsequently stored at 10°C and fed with fresh MBM every 2–3 days.

HEK cells and transfection

Human embryonic kidney (HEK) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM glutamine, 100 u ml^{-1} penicillin G and 100 mg ml^{-1} streptomycin at 37°C in 95% air–5% CO_2 (Smart *et al*., 1991). Exponentially growing cells were subject to electroporation (400 V, infinite resistance, 125 μF; BioRad Gene Electropulser II) with plasmids containing wild-type or mutant $GABA_A$ receptor subunit cDNAs together with a reporter plasmid expressing the S65T mutant of jellyfish green fluorescent protein (GFP; Heim *et al*., 1994).

Electrophysiology: intracellular recording

Membrane currents were recorded from cDNA-injected *Xenopus oocytes* using a two-electrode voltage clamp technique. Oocytes were superfused with an amphibian Ringer containing (mM): NaCl 110, KCl 2, HEPES 5 and

$CaCl_2$ 1.8 (pH 7.4) at 8–10 ml min^{-1} (bath volume, 0.5 ml). Voltage and current microelectrodes were filled with 0.6 M K_2SO_4 (1–2 MΩ). Currents were recorded using an Axoclamp 2B amplifier in conjunction with a Gould 2400S pen recorder.

Patch clamp recording

Whole-cell membrane currents were recorded from single HEK A293 cells using a List EPC7 amplifier. Thin-walled borosilicate patch electrodes (resistance 2–7 MΩ) were filled with electrolyte containing (mM): KCl 140, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, EGTA 11 and adenosine triphosphate 2, pH 7.2. Cells were continuously superfused with a Krebs solution containing (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10 and glucose 11, pH 7.4. For some of the low pH experiments, to check the stability of pH 5.4 Krebs, HEPES was replaced with MES. This exchange did not affect the pH sensitivity of Zn^{2+} inhibition. Recordings were performed 24–48 h after transfection by holding cells at −40 mV and filtering membrane currents at 10 kHz (−3dB, 6th pole Bessel, 36 dB per octave). Drugs and Krebs solution were rapidly applied (exchange rate 100 ms) to single cells using a modified U-tube (Wooltorton *et al*., 1997b). All drugs were constituted in Krebs solution and corrected to pH 5.4 or 7.4. Diethylpyrocarbonate was made fresh immediately prior to use and discarded after 30 min.

Analysis of ligand-modulated membrane conductances and currents

For *Xenopus* oocytes, membrane conductances were determined by applying hyperpolarizing voltage commands (1 s duration, −10 mV amplitude and 0.2 Hz frequency) from −25 mV holding potential in the absence and presence of a ligand. To construct equilibrium concentration–response relationships for GABA and pentobarbitone, the ligand-induced conductance change (ΔG) was calculated by subtracting the resting conductance from the conductance measured in the presence of each ligand. All the conductances, unless specified, were normalised (ΔG_N) to the maximum conductance change ($\Delta G_{N,\ max}$) and subsequently fitted with the following equation:

$$\Delta G_N/\Delta G_{N,max} = [1/1 + (EC_{50}/[A])^{n_H}] \quad (1)$$

where EC_{50} represents the concentration of ligand ([A]) inducing 50% of the maximal conductance evoked by a saturating concentration of ligand and n_H is the Hill coefficient. The reductions in the resting membrane conductance by picrotoxinin and Zn^{2+} were used to construct antagonist concentration–inhibition relationships. The antagonist-sensitive conductance (equivalent to the β3 subunit-gated spontaneous membrane conductance; Wooltorton *et al*., 1997b) was defined as 100% after the addition of a saturating concentration of picrotoxinin (10 μM, Wooltorton *et al*., 1997a,b). The inhibition of this conductance by intermediate concentrations of antagonists were fitted with the equation:

$$\Delta G_N^{/}/\Delta G_N = [1 - [B^{n_H}/(B^{n_H} + IC_{50}{}^{n_H})]] \quad (2)$$

where $\Delta G_N^{/}$ and ΔG_N represent the normalized GABA-induced (at a given GABA concentration) or β3 subunit-gated conductance in the presence and absence of antagonist respectively. B represents the antagonist concentration and IC_{50} defines the concentration of antagonist producing a 50% inhibition of the GABA-induced or β3 subunit-gated conductance. For the whole-cell recording from transfected HEK cells, peak currents induced by pentobarbitone in the absence and presence of Zn^{2+} were measured. Data were assessed for significance using unpaired *t*-tests or ANOVA with a Tukey *post-hoc* test. Only HEK cells transfected with β3 subunit cDNAs were sensitive to both Zn^{2+} and picrotoxinin at the concentrations used in this study. Moreover, spontaneously-gated β3 subunit channels were not observed in untransfected cells (Krishek *et al*., 1996).

Confocal microscopy

The expression patterns of mutant $\beta3^{H107X}$ subunits (where X = substituting amino acid) were labelled with a murine bd17 antibody (Boehringer Mannheim, 5 μg ml^{-1}) and resolved with a secondary anti-mouse IgG conjugated with a tetramethylrhodamine analog (TRITC, excitation, 568 nm, emission 580 nm wavelengths). The expression of the receptor subunits was localized using a Leica DMRE fluorescence microscope with both 40X and 63X oil immersion objectives and confocal microscopy was performed with the Leica TCS SP multi-band spectrophotometer with the krypton laser line at 18°C. Images were processed off-line by using Corel Photopaint.

Results

Strategy for site-directed mutagenesis

Additional residues, possibly forming a presumed low affinity Zn^{2+} binding site, were assumed to exist on $GABA_A$ receptors after a histidine residue, H267, located in the second transmembrane domain of the β subunits was substituted for alanine. This residue is critical for Zn^{2+} inhibition of both the spontaneous and pentobarbitone-gated currents that characterize β3 and β1 homomeric $GABA_A$ receptors (Wooltorton *et al*., 1997a; Horenstein & Akabas, 1998). Substitution of H267 greatly reduced the sensitivity of the receptor to Zn^{2+} (Figure 1A,B), causing a large rightward displacement of the Zn^{2+} inhibition curve, increasing the Zn^{2+} IC_{50} by over 1000-fold (β3, 0.15±0.03 μM; $\beta3^{H267A}$, 199±13 μM; mean±s.e.mean, $n=7$ oocytes). Although very effective, this mutation did not abolish inhibition indicating the likely presence of an additional lower affinity Zn^{2+} binding site, or conceivably, Zn^{2+} binding was not completely disrupted around the environment of H267.

There are numerous residues that could participate in forming a Zn^{2+} binding site or in contributing to the Zn^{2+} inhibitory signal transduction. These include, other external histidines, glutamates, aspartates and cysteines. The latter were discounted since if the two N-terminal cysteines participate in disulphide bridge formation (Barnard *et al*., 1987; Amato *et al*., 1999), then they would be unavailable to coordinate with Zn^{2+}. Tentative identification of the residues likely to be involved was obtained by using H^+ and diethylpyrocarbonate (DEPC) as probes.

Sensitivity of Zn^{2+} inhibition to H^+ and diethylpyrocarbonate

The Zn^{2+} sensitivity of the mutant $\beta3^{H267A}$ subunit homomer was assessed in the presence of low pH and also after exposing the receptor to DEPC. Both experimental protocols would ascertain whether histidine residues were important for the additional Zn^{2+} inhibitory effect since H^+ will compete with Zn^{2+} for binding to imidazole groups on histidines and DEPC will covalently and irreversibly modify histidines (Miles, 1977; Lundblad & Noyes, 1984) preventing any potential Zn^{2+} binding. Using an external pH 7.4, pentobarbitone (1 μM – 3 mM; PB), used since these homomers are insensitive to GABA, increased the membrane conductance for $\beta3^{H267A}$ subunit expressing oocytes. Zinc (300 μM) laterally displaced the PB equilibrium concentration – response curve, increasing the EC_{50} from 46 ± 10 μM to 96 ± 10 μM ($P < 0.05$, $n = 5$), and reduced the maximum response (Figure 2A).

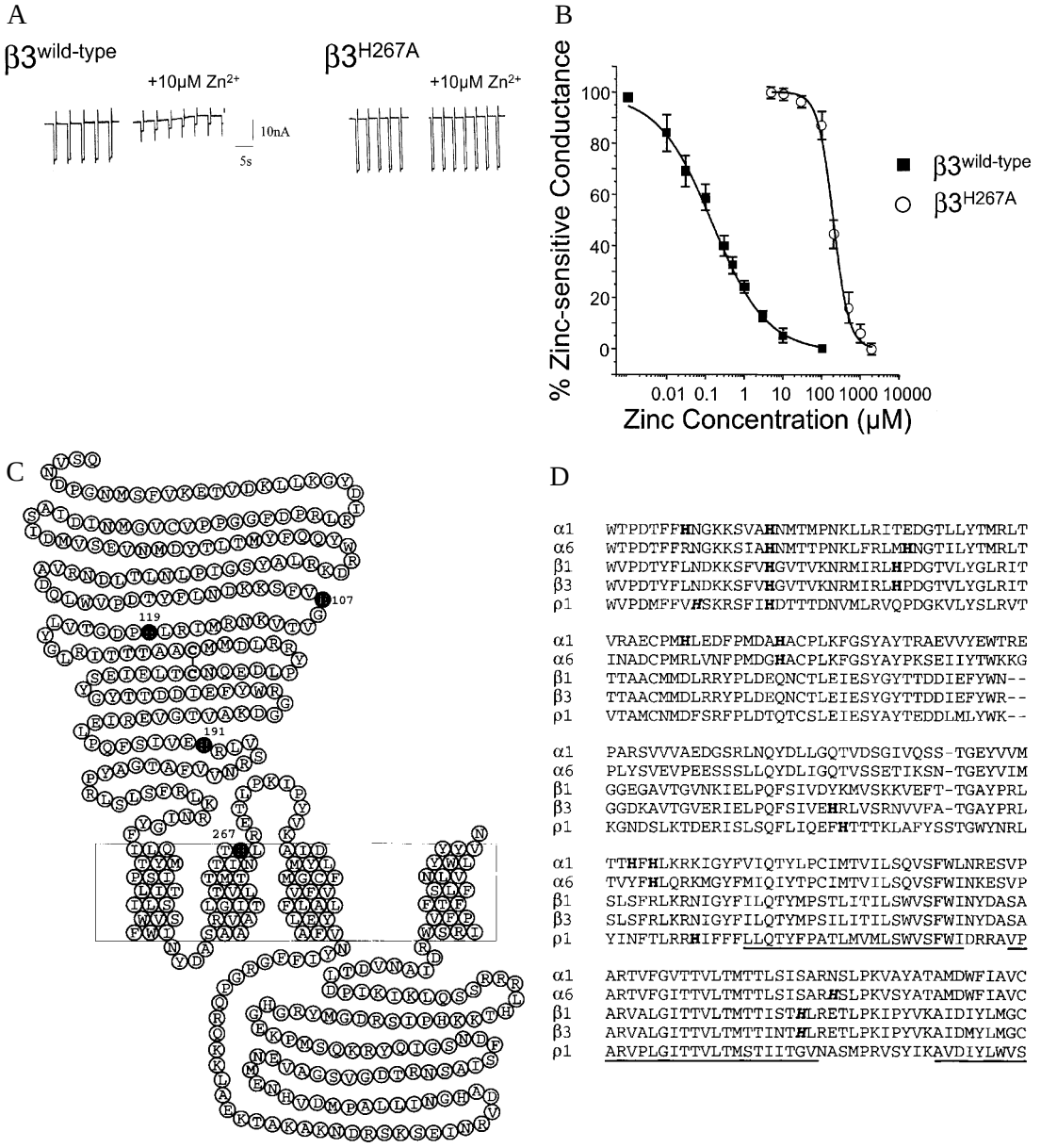

Figure 1 Inhibition of spontaneous Cl^- currents through $\beta3$ subunit $GABA_A$ receptors by Zn^{2+} and sequence alignments of external histidine residues for $GABA_A$ and $GABA_C$ receptor subunits. (A) Membrane currents recorded from wild-type $\beta3$ and mutant $\beta3$(H267A) subunits expressed in oocytes held at -40 mV. Membrane conductances in the presence and absence of Zn^{2+} were assessed using hyperpolarizing voltage commands (-10 mV, 1 s, 0.2 Hz). (B) Zinc concentration inhibition relationships for blocking the spontaneous Cl^- current transduced by $\beta3$ wild-type and $\beta3^{H267A}$ mutant $GABA_A$ receptors. The IC_{50}s were 0.15 ± 0.01 μM ($\beta3$) and 199 ± 13 μM ($\beta3^{H267A}$). Data points represent the mean $\pm$ s.e. from seven cells. (C) Schematic diagram of the $GABA_A$ receptor $\beta3$ subunit illustrating the transmembrane domains (TM) and external N- and C-termini and large intracellular loop between TM3 and TM4. The external histidine residues and single histidine in TM2 are highlighted. (D) sequence alignments of part of the N-terminal extracellular domain and TM1 – TM3 for $GABA_A$ receptor $\alpha1$, $\alpha6$, $\beta1$ and $\beta3$ subunits, and the $GABA_C$ receptor subunit, $\rho1$. Histidines are depicted as bold characters and those previously demonstrated to affect Zn^{2+} modulation are also italicized.

Reducing the external pH to 5.4 resulted in a small increase in the potency of PB. However, low pH ablated the residual Zn^{2+} inhibition of the PB-modulated conductance causing the PB concentration-response curves in the absence and presence of Zn^{2+} to be coincident (EC_{50}, PB control, 21 ± 4 μM; $+Zn^{2+}$, 19 ± 4 μM; $P > 0.05$, $n = 5$, Figure 2B).

$\beta 3$ subunit homomers possess the property of spontaneous gating since both Zn^{2+} and picrotoxin can induce reductions in membrane Cl^- conductance that is evident only in receptor expressing cells (Wooltorton *et al*., 1997b). Similar to the PB conductance, the Zn^{2+} sensitivity (10 μM – 2 mM) of the spontaneous conductance for $\beta 3^{H267A}$ subunit homomers was also abolished by reducing the external pH from 7.4 to 5.4 (Figure 2C) suggesting that H^+ and Zn^{2+} are probably competing for similar amino acid residues. Furthermore, by increasing H^+ concentration 100-fold and completely preventing inhibition by Zn^{2+}, it is likely that such a pH change traversed the pK_a for those amino acid(s) involved in Zn^{2+} inhibition. As histidine is the only amino acid with a pK_a that would be affected by this pH change (pK_a 6.1) it became the primary candidate on $\beta 3$ homomers.

Further evidence for the involvement of histidine(s) in the inhibitory effect of Zn^{2+} was obtained by using DEPC. Application of 1 mM PB to HEK cells expressing the $\beta 3^{H267A}$ receptor caused an inward current and associated rebound current characteristic of these homomers (Wooltorton *et al*., 1997b). Zinc (100 μM) reduced the PB-modulated currents in a reversible manner (Figure 3); however, subsequent exposure of the cell to 1 mM DEPC for 5 min, whilst not affecting the PB-induced response (Figure 3B), resulted in the complete and irreversible loss of the inhibitory action of Zn^{2+} (Figure 3).

External histidine residues in the $\beta 3^{H267A}$ subunit: candidates for coordinating Zn^{2+} inhibition

A review of the histidine residues postulated to be accessible to external Zn^{2+} in the $\beta 3$ subunit revealed H107, H119 and H191 in the N-terminal extracellular domain in addition to H267 contained in the ion channel pore forming region, TM2

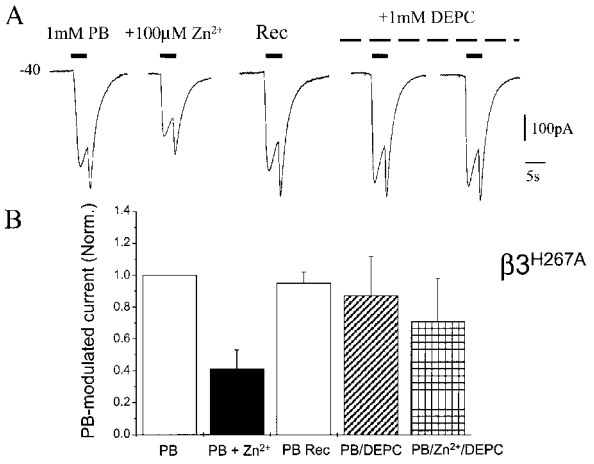

Figure 3 Modification of histidine residues in $\beta 3$ subunits by diethylpyrocarbonate affects Zn^{2+} inhibition. (A) Membrane currents modulated by PB and Zn^{2+} in HEK cells expressing $\beta 3^{H267A}$ mutants in the absence and presence of diethylpyrocarbonate (DEPC). Drugs were applied for the duration indicated by the lines. (B) Bargraph of PB-modulated current for $\beta 3^{H267A}$ mutants following exposure to 1 mM PB; $+100$ μM Zn^{2+}; after recovery; following continuous application of 1 mM DEPC with either PB or PB + Zn^{2+} ($n = 3$).

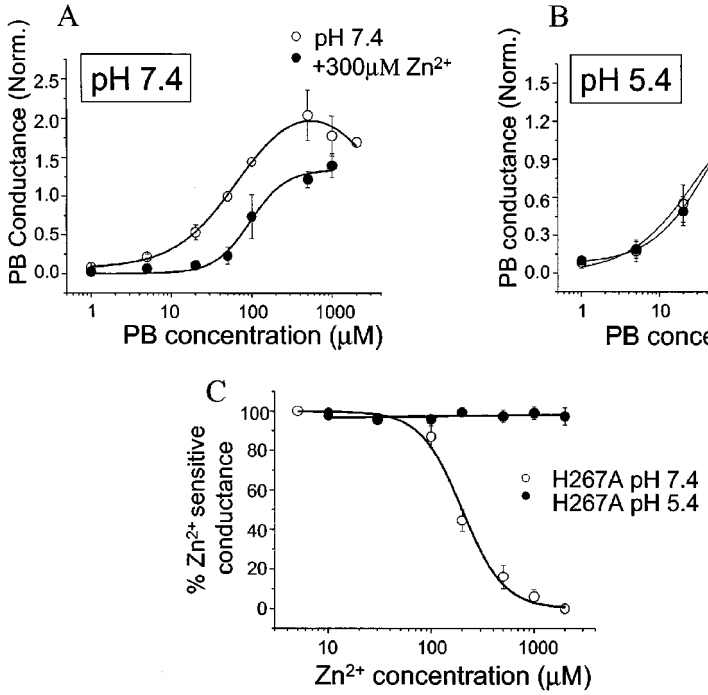

Figure 2 Sensitivity of Zn^{2+} regulation of $\beta 3^{H267A}$ $GABA_A$ receptor subunits expressed in oocytes to external pH. Concentration response curves were constructed for pentobarbitone (PB) modulated currents and normalized to the response evoked by 50 μM PB, in the absence and presence of 300 μM Zn^{2+} recorded from oocytes expressing $\beta 3^{H267A}$ homomers in Ringer at pH 7.4 (A) and 5.4 (B). Curves were fit to the data as described in the methods ($n = 3$). (C) Zinc concentration inhibition relationship for spontaneous currents mediated by $\beta 3^{H267A}$ homomers in oocytes exposed to Ringer at pH 7.4 and 5.4. Data were obtained from $n = 5$ cells.

(Figure 1C). Notably, the histidine at position 107 is highly conserved throughout $GABA_A$, $GABA_C$, glycine and nicotinic acetylcholine receptor subunits. However, H119 is conserved only within the $GABA_A$ receptor β subunit family and H191 is unique to the $GABA_A$ receptor $\beta 3$ subunit (Figure 1D). Both H107 and H119 were considered as primary candidates for coordinating or modulating Zn^{2+} inhibition because H191 was not present in the $\beta 1$ subunit which also exhibited a similar low potency Zn^{2+} inhibition following substitution of H267. The external histidines were systematically substituted for alanines leaving the background mutation H267A intact since this mutation removed the high potency effect of Zn^{2+}. The mutations were assessed individually and then, if appropriate, also in multiples.

Expression of $\beta 3^{H119A,\ H267A}$ in *Xenopus* oocytes resulted in functional receptors exhibiting both spontaneous and PB-modulated currents. The H119A substitution caused a small reduction in PB potency compared to the H267A mutant but not when compared to the wild-type (Figure 4, Table 1); however, the sensitivity to Zn^{2+} was not further reduced with the Zn^{2+} inhibition curves yielding similar IC_{50}s of approximately 200 μM (Figure 4B, Table 1). Similarly, oocytes injected with $\beta 3^{H191A,\ H267A}$ cDNA also resulted in functional receptor expression. As for H119A, the PB equilibrium concentration response curves displayed a small reduction in PB potency only when compared to the H267A mutant (Figure 4C, Table 1). The H191A substitution did not, however, reduce the potency of Zn^{2+} (Figure 4D, Table 1). The Hill coefficients for the H119A and H191A $\beta 3$ subunit receptors were significantly increased compared to the $\beta 3$ wild-type receptor when the H267A mutation was included, suggesting that within the Zn^{2+} inhibition curve for $\beta 3$ wild-type subunits, there is more than one component to the inhibition. Thus other residues are important for Zn^{2+}

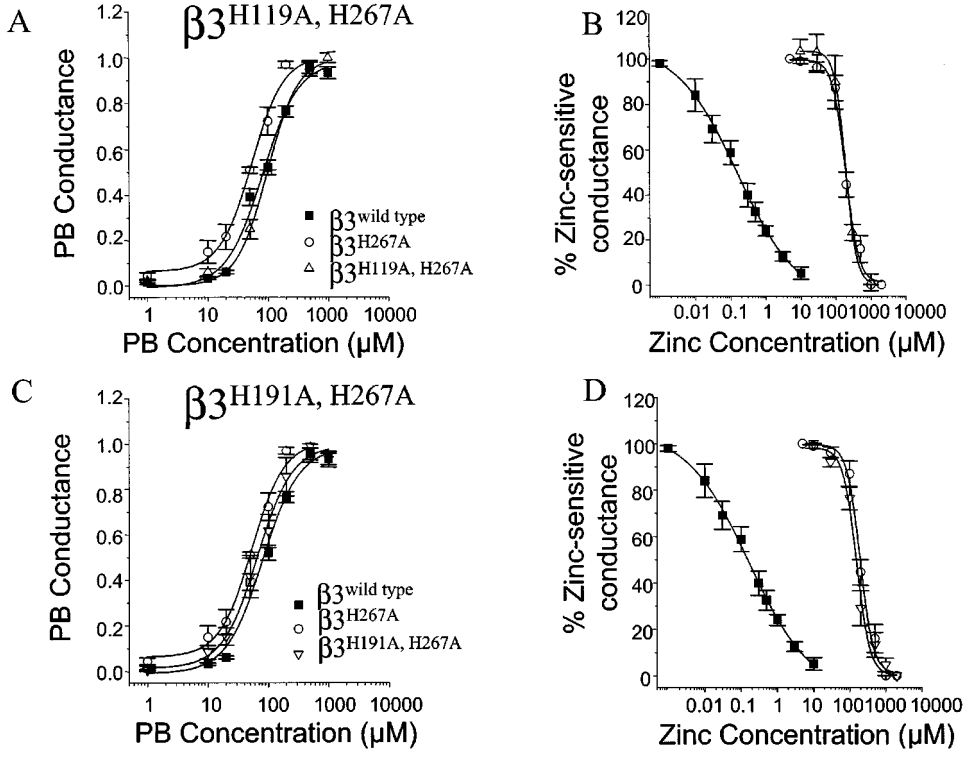

Figure 4 Effect of mutating histidine residues H119 and H191 in $GABA_A$ receptor $\beta 3^{H267A}$ homomers on Zn^{2+} inhibition. Concentration response curves for PB were established for $\beta 3$, $\beta 3^{H267A}$ and either $\beta 3^{H119A,\ H267A}$ (A) or $\beta 3^{H191A,\ H267A}$ (C) $GABA_A$ receptor constructs expressed in oocytes. Data were normalised to the maximum response for each construct and obtained from $n=11$ cells. Concentration inhibition relationships for Zn^{2+} antagonizing the spontaneous Cl^- current was determined for $\beta 3$, $\beta 3^{H267A}$ and either $\beta 3^{H119A,\ H267A}$ (B) or $\beta 3^{H191A,\ H267A}$ (D) constructs. Data are mean$\pm$s.e.mean from $n=8$ cells.

Table 1 PB and Zn^{2+} potencies on homomeric β subunit receptors

Receptor subunit	*PB EC_{50} (μM)*	n_H	*Zn^{2+} IC_{50} (μM)*	n_H
$\beta 3$	81$\pm$14	1.44$\pm$0.32	0.15$\pm$0.03	0.84$\pm$0.06
$\beta 3^{H267A}$	55$\pm$12	1.62$\pm$0.35	199$\pm$15	2.2$\pm$0.08#
$\beta 3^{H119A,\ H267A}$	107$\pm$4*	1.3$\pm$0.4	203$\pm$9	2.84$\pm$0.1#
$\beta 3^{H191A,\ H267A}$	81$\pm$5*	1.2$\pm$0.34	155$\pm$19	2.35$\pm$0.06#
$\beta 3^{H119A,\ H191A,\ H267A}$	131$\pm$7*	1.42$\pm$0.44	nd	nd

Pentobarbitone and Zn^{2+} potencies determined in HEK cells expressing $\beta 3$ wild-type or mutant subunits. All EC_{50}s and IC_{50}s were ascertained as described in the Methods. The * signifies $P<0.05$ compared with $\beta 3^{H267A}$ and # signifies $P<0.05$ compared with $\beta 3$. All values determined from $n=8-11$ cells.

inhibition and that the smaller, lower potency Zn^{2+} inhibitory component can only be clearly observed after substituting H267.

To ensure that H119 and H191 were not acting in tandem, a combined mutant receptor, $\beta3^{H119A,\ H191A,\ H267A}$, was expressed in oocytes. However, despite the reduction in spontaneous gating and a rightward shift in the PB equilibrium concentration response curve compared to the $\beta3^{H267A}$ mutant (Table 1), Zn^{2+} still inhibited PB-modulated responses. The maximum responses to PB (1 mM) were inhibited non-competitively by $60\pm5\%$ ($\beta3^{H267A}$) compared to $58\pm10\%$ ($\beta3^{H119A,\ H191A,\ H267A}$) in the presence of 200 μM Zn^{2+} ($P>0.05$, $n=8$). Clearly Zn^{2+} was still exerting a considerable inhibitory effect on the mutant $\beta3$ subunit despite ablating two of the three external histidine residues. To ascertain the pH sensitivity of the residual Zn^{2+} inhibition on the $\beta3^{H119A,\ H191A,\ H267A}$ receptor, the external pH was varied between 5.4 and 8.4. Zinc inhibition was reduced by lowering the external pH to 5.4 with pH titration yielding a pK_a of 6.8 ± 0.1 (Figure 5). These results implied that H107 is probably important for Zn^{2+} inhibition since this was the last external histidine.

Importance of H107 in β3 subunits for the inhibition by Zn^{2+}

The expression of our initial substitution of H107 for alanine, in the background mutation of H267A in $\beta3$ subunits ($\beta3^{H107A,\ H267A}$), did not result in the formation of functional $\beta3$ subunit receptors ($n=8$). Similarly, the single mutant $\beta3^{H107A}$ subunits also failed to form functional receptors ($n=10$). To assess whether the expression of the H107A mutant was dependent upon the expression system, human embryonic kidney (HEK) cells were separately transfected with either $\beta3^{H107A}$ or $\beta3^{H107A,\ H267A}$ subunit cDNAs. On each occasion these cells failed to express functional receptors ($n=5$) although co-transfection of the reporter DNA encoding for GFP validated the transfection technique and expression competency of the HEK cells.

The highly conserved nature of H107 amongst members of the ligand-gated ion channel superfamily initially suggested an important role for this amino acid in subunit assembly, function, protein folding and/or transport to the cell surface. To enhance the prospect that one or more particular $\beta3^{H107}$ variant subunits may be expressed, a series of mutations were made of the form, $\beta3^{H107X}$, where X was A, R, K, G, I and L. Mutant $\beta3$ subunit expression was examined using confocal microscopy to assess their ability to access the cell surface membrane (Figure 6). All of these mutants demonstrated clear cell surface expression; however, transfection of HEK cells resulted in functional ion channels for only $\beta3^{H107G}$ and to a lesser extent, with $\beta3^{H107I}$, although expression levels varied with cell morphology. Curiously, the small, rounded, intensely fluorescent (due to GFP co-expression) transfected HEK cells expressed robust PB-modulated currents (>100 pA) for the $\beta3^{H107G,\ H267A}$ mutant homomers. In contrast, the flattened slightly fluorescent transfected HEK cells had poor almost unusable expression levels with PB-modulated currents less than 10pA. The $\beta3^{H107G}$ mutant was examined for sensitivity to Zn^{2+} with the background mutation H267A. Membrane currents were activated by 1 mM PB and the co-application of 1 mM Zn^{2+} demonstrated only slight inhibition of either the primary inward current or rebound current characteristic of these β subunit homomers (Figure 7A; Wooltorton *et al*., 1997b). Inhibition curves for Zn^{2+} continuing up to 2 mM caused less than 10% inhibition in the PB-modulated current suggesting this residue is critically important for transducing or coordinating Zn^{2+} inhibition of $\beta3$ homomers (Figure 7B). If the background mutation, H267A, was omitted, expression levels of $\beta3^{H107G}$, even in small rounded HEK cells was quite marginal and difficult to use quantitatively (peak currents to

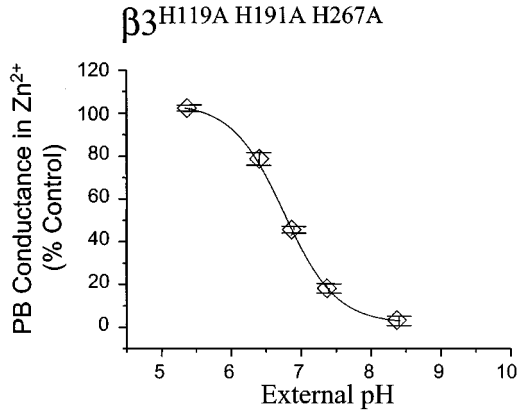

Figure 5 Zn^{2+} sensitivity of the $\beta3^{H119A,H191A,H267A}$ $GABA_A$ receptor is affected by external pH. In expressing oocytes, control responses to 100 μM PB were recorded in the presence of 200 μM Zn^{2+} over the external Ringer pH range 5.4 to 8.4 and presented as a pH titration. The reduced inhibitory effect of Zn^{2+} as the external pH increased was determined as a percentage of the control PB response at each pH in the absence of Zn^{2+}. The curve was generated according to the inhibition model described in the methods. The pK_a determined from the curve fit was 6.8 ± 0.1 ($n=3$).

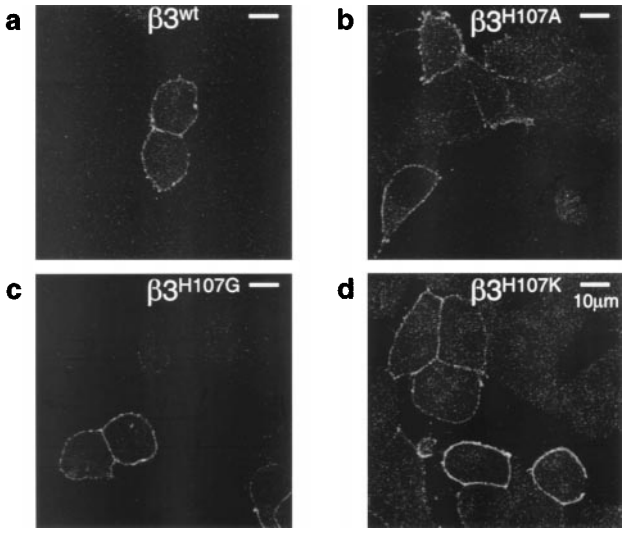

Figure 6 Confocal microscopy of the cell surface expression of H107X mutants. The panels illustrate HEK cells expressing wild-type $\beta3$ homomers (A) and $\beta3$ receptor mutants: H107A (B), H107G (C), H107K (D) after exposure to bd17 antisera. Staining for the $\beta3$(H107X) mutants revealed their relative location between cell surface membrane and intracellular compartments. Scale bar represents 10 μm.

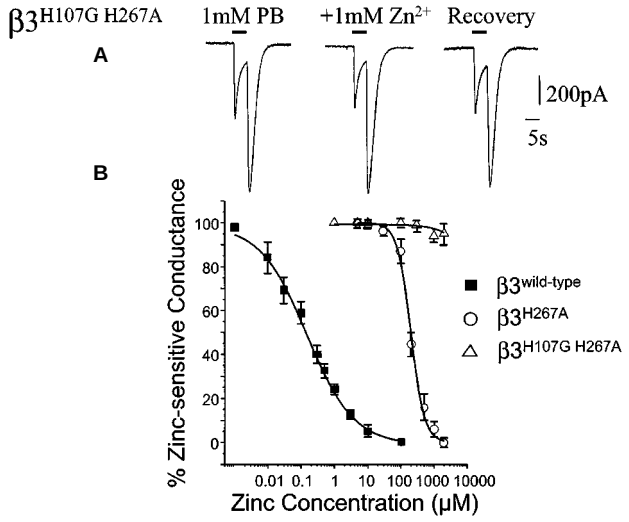

Figure 7 Ablation of inhibition by Zn^{2+} on H107G $\beta3$ subunit mutants. (A) Membrane currents recorded from $\beta3^{H107G,\ H267A}$ expressing HEK cells modulated by 1 mM PB in the absence and presence of 1 mM Zn^{2+}. (B) Zn^{2+} inhibition concentration response curves for $\beta3$ wild-type, $\beta3^{H267A}$ and $\beta3^{H107G,\ H267A}$ subunit receptors. All data were fitted according to the methods ($n=5$).

1 mM PB, <20 pA) but 1 mM Zn^{2+} effectively blocked the PB-induced currents (data not shown).

To analyse whether the H107G mutation prevented Zn^{2+} inhibition of heteromeric GABA-sensitive receptors, the H107G substitution was incorporated into an $\alpha1\beta3^{H267A}$ subunit background forming $\alpha1\beta3^{H107G,\ H267A}$. This receptor retained its sensitivity to Zn^{2+} inhibition typical of an $\alpha1\beta3^{H267A}$ receptor (IC_{50}s: 21 ± 3 μM, $\alpha1\beta3^{H267A}$; 24 ± 6 μM, $\alpha1\beta3^{H107G,\ H267A}$, $n=6$), suggesting that for $\alpha\beta$ heteromers, H107 was not a critical determinant.

Discussion

The previous examination of H267 as a major determinant of the inhibitory effect of Zn^{2+} on the $GABA_A$ receptor also revealed the likely existence of additional residues that can form another Zn^{2+} sensitive site or are involved in signal transduction. This became apparent from constructing Zn^{2+} inhibition curves for the spontaneous current mediated by wild-type and mutant $\beta3$ subunits. Although the H267A mutation caused a clear 1326-fold increase in the Zn^{2+} IC_{50}, it was also apparent that complete inhibition of the conductance gated by $\beta3$ subunit receptors was achieved at high Zn^{2+} concentrations. The use of homomeric $\beta3$ subunit ion channels simplified the strategy for site-directed mutagenesis since these channels are expected to form near symmetrical arrangements of amino acids unaffected by subunit-subunit interactions with α and γ subunits. Thus single mutations would be expected to have maximum impact on the Zn^{2+} inhibitory effect and/or binding site. To date, whether homomeric β subunits actually exist *in vivo* remains unclear, but if so, they would constitute a membrane conductance capable of supporting tonic inhibition by virtue of their ability to spontaneously gate (Sigel *et al*., 1989; Krishek *et al*., 1996). In addition, their sensitivity to external Zn^{2+} is within the range predicted to exist in the CNS following stimulation, increasing from the suggested basal levels of 1 pM – 1 nM to approximately several micromolar (Frederickson & Bush, 2001).

IThe search for the additional residues influencing the residual Zn^{2+} inhibition centred on histidines since lowering the Ringer pH appeared quite effective at abolishing all Zn^{2+}-induced inhibition at concentrations up to 1 mM. The pH reduction from 7.4 to 5.4 would encompass the pK_a for histidine with pK_as for other amino acids (assuming no changes are imparted by the local protein microenvironment) lying outside this range. It was assumed that H^+ and Zn^{2+} are possibly competing for similar binding sites involving histidine residues. At pH 5.4, raising the Zn^{2+} concentration to 2 mM failed to reveal any inhibition of PB-gated currents on $\beta3^{H267A}$ constructs. In addition, prevention of Zn^{2+} inhibition by the histidine-modifying reagent, DEPC, provided additional evidence suggesting the involvement of histidine residues.

Evidence for the involvement of H107 in Zn^{2+} inhibition

The search for additional residues affecting Zn^{2+} inhibition on the $GABA_A$ receptor $\beta3$ subunit resulted in the identification of histidine residue, H107. The importance of this residue was highlighted by the failure of separate mutation of residues H119 and H191 with H267 to diminish the residual Zn^{2+} inhibition. A number of H107 mutations ablated functional expression of $\beta3$ subunit ion channels. A role for H107 in subunit-subunit assembly was thought to be unlikely since H^+, DEPC and presumably Zn^{2+} can all access this residue in the mature $\beta3$ subunit homomer thus affecting receptor function. This degree of accessibility would appear incompatible with H107 being involved in close binding proximity to the interface of another juxtaposed subunit, for example, forming part of an assembly box. Moreover, in the studies of $GABA_A$ receptor assembly boxes to date, H107 has not been identified as a contributory amino acid (Taylor *et al*., 1999; Klausberger *et al*., 2000). Taken together with the confocal microscopy data, this suggested that H107 was not important for receptor assembly, protein folding or transport of receptors to the cell surface, but more likely to be interfering with ion channel gating as spontaneous gating, a feature associated with both $\beta1$ and $\beta3$ homomers, and also PB-gated currents were absent for $\beta3^{H107A}$ receptors. This histidine is highly conserved across $GABA_A$, $GABA_C$ and glycine receptor subunits and is also found in the comparable position in members of the nicotinic acetylcholine receptor family suggesting it plays a vital role that cannot simply be provided by substituting amino acids. Interestingly, more conservative substitutions of H107 to lysine or arginine ($\beta3^{H107R}$, $\beta3^{H107K}$), both residues with positively charged side chains, failed to result in functioning mutant $\beta3$ receptors despite cell surface expression.

The use of three different approaches to affect histidines, namely H^+, DEPC and Zn^{2+} provided compelling evidence for the involvement of a histidine residue in Zn^{2+} inhibition for which H107 is the remaining candidate. Interestingly, the ability of Zn^{2+} to inhibit PB-modulated currents mediated by $\beta3^{H107G}$ receptors suggested that in the absence of H267A, substitution of H107 alone has little individual effect on Zn^{2+} sensitivity in accordance with its participation with a presumed low affinity site or inhibitory signal transduction.

However, given the clear disparity between the Zn^{2+} IC_{50}s for wild-type $\beta3$ and mutant $\beta3^{H267A}$ receptors (1326-fold), we might expect a biphasic inhibition concentration curve for Zn^{2+} unless the low affinity Zn^{2+} site only becomes available after mutation of H267A.

It is unclear whether H107 constitutes a discrete binding site for Zn^{2+} on the N-terminus from the previously identified residue H267 in the pore-lining region, or whether both residues are participating in signal transduction. The tertiary structure of the $\beta3$ homomeric receptor is unknown and thus it may be possible that H107 and associated parts of the N-terminus are localised near the mouth of the ion channel such that H107 and H267 may participate in the formation of a single Zn^{2+} binding site. Although this cannot be discounted at present, the number of intervening amino acid residues (160) do not conform to known Zn^{2+} binding site consensus sequences such as those contained in metalloenzymes. For the latter, the number of intervening amino acid residues (X) between three coordinating histidines are classified into the short (1–3 residues) and long (18–123 residues) spacers ($HX_{1-3}HX_{18-123}H$) (Vallee & Falchuk, 1993). Furthermore, if H107 and H267 do form part of the same site/transduction mechanism, mutating H107 alone should have produced some effect on Zn^{2+} inhibition which was not observed, albeit by measuring quite small PB-modulated currents in HEK cells.

Potential Zn^{2+} binding domains on $GABA_A$, $GABA_C$ and glycine receptors

A comparison of previous studies reveals some interesting facets to Zn^{2+}-induced modulation of inhibitory amino acid receptors. For $GABA_A$, $GABA_C$ and glycine receptor subunits, histidine residues have been identified as major elements of the inhibitory effect of Zn^{2+} on GABA- and glycine-activated responses. Homomeric receptors formed from either $GABA_A$ receptor $\beta1$ or $\beta3$ subunits were dependent upon H267 in the pore-lining region, TM2, for Zn^{2+} inhibition (Wooltorton *et al*., 1997a; Horenstein & Akabas, 1998). The position of this residue and the prospect of Zn^{2+} penetrating into the anion-selective ion channel suggested that, as for the nicotinic acetylcholine receptor, the ion channel gate and selectivity filter reside deep within the ion channel (Wooltorton *et al*., 1997a; Horenstein & Akabas, 1998). In both studies, however, a residual effect of Zn^{2+} was evident suggesting alternative binding sites capable of modulating the β subunit homomers.

IAn alternative homomeric receptor formed from $GABA_C$ $\rho1$ subunits is also sensitive to inhibition by Zn^{2+} (Calvo *et al*., 1994; Wang *et al*., 1994, 1995; Chang *et al*., 1995). Mutation of an N-terminal histidine residue, H156, to tyrosine resulted in abolition of Zn^{2+} inhibition up to 1 mM and only slight (<10%) inhibition at 10 mM, contrasting with the IC_{50} for Zn^{2+} on wild-type $\rho1$ subunit receptors of between 10–50 μM (estimated from Figure 4B, Wang *et al*., 1995). Interestingly, even in this study, mutation of the highly conserved H163 (equivalent to H107 for the $GABA_A$ receptor $\beta3$ subunit) to either asparagine or tyrosine failed to produce functional ion channels. Moreover, H156 was also sensitive to the substituting amino acid with asparagine failing to produce functional ion channels.

A different homomeric receptor formed from glycine receptor $\alpha1$ subunits also displays sensitivity to Zn^{2+} (Bloomenthal *et al*., 1994; Laube *et al*., 1995). Zinc has a dual regulatory role causing potentiation of glycine-gated currents at low Zn^{2+} concentrations (<10 μM) and then inhibition at higher concentrations (>10 μM). Recent site-directed mutagenesis experiments have noted that two histidines in the glycine receptor $\alpha1$ subunit, H107 and H109, forming part of a recognized Zn^{2+} binding motif conserved in selected metalloenzymes (Vallee & Falchuk, 1993), 'H-phenylalanine (F)-H', are involved in the inhibitory effects of Zn^{2+} (Harvey *et al*., 1999). Substitution of either or both residues to alanine is sufficient to reduce Zn^{2+} inhibition, an effect that also occurred when the flanking residue, T112, was substituted (Laube *et al*., 2000), suggesting localized involvement of these three residues in a Zn^{2+} binding site. The potentiating effect of Zn^{2+} is more complex and only H109A mutation appeared to abolish this effect; however, several other point mutations can also abolish Zn^{2+} induced potentiation (Laube *et al*., 1995; Lynch *et al*., 1998) suggesting that the H109A mutation may be affecting this aspect of Zn^{2+} action by allosteric mechanisms (Harvey *et al*., 1999). The relative positions of these histidines in the N-terminal portion of the glycine receptor $\alpha1$ subunit are of interest. Histidine 107 is unique to the glycine receptor $\alpha1$ subunit and only 5 residues C-terminal to the relative position of the Zn^{2+}-sensitive H156 in the $\rho1$ subunit. Histidine 109 is the highly conserved histidine found throughout $GABA_A$, $GABA_C$ and glycine receptor subunits (and is equivalent to H107 in the $GABA_A$ receptor $\beta3$ subunit). Curiously, mutation of H109 to alanine was tolerated in glycine receptor $\alpha1$ subunits allowing the formation of functional ion channels.

Examination of heteromeric $GABA_A$ receptors revealed that for $\alpha1\beta1$ and $\alpha1\beta3$ constructs, mutation of H267 caused a profound reduction in the Zn^{2+} sensitivity of these receptors (Wooltorton *et al*., 1997a; Horenstein & Akabas, 1998). Abolition of Zn^{2+}-induced inhibition was not evident since alternative sites clearly exist on the β subunits and potentially also on the $\alpha1$ subunit. Interestingly, H107 in the $\beta3$ subunit, whilst clearly vital for Zn^{2+} inhibition on the $\beta3$ homomers does not appear to play any such role in the $\alpha1\beta3$ heteromeric $GABA_A$ receptor. This suggests that this residue can have markedly different roles depending upon the co-expressed subunits and may indicate that the homomeric and heteromeric forms of the $GABA_A$ receptor have quite different quarternary structures markedly affecting the function, in this case, of H107. Whether this is simply a matter of access to H107 or a more complex change in signal transduction mechanisms between the receptors will require crystallographic structural data.

Previous studies have also identified some differences in Zn^{2+} inhibition of recombinant $GABA_A$ receptors comprising different α subunits. Notably, receptors expressed with $\alpha6\beta\gamma$ subunits are more sensitive to inhibition than $\alpha1\beta\gamma$ counterparts (Knoflach *et al*., 1996; Fisher *et al*., 1997). These $GABA_A$ receptor α subunits differ in the TM2–TM3 region of 12 amino acids at only two locations where histidine and serine residues in $\alpha6$ replace asparagine and alanine residues in $\alpha1$ respectively. Substitution of H273 to asparagine in the $\alpha6$ subunit and co-expression with $\beta3$ and $\gamma2$L subunits, resulted in expressed $GABA_A$ receptors with similar Zn^{2+} sensitivity to $\alpha1\beta3\gamma2$L receptors. In comparison, conversion of N273 to histidine in the $GABA_A$ receptor $\alpha1$ subunit

increased the Zn^{2+} inhibition of GABA-activated currents recorded from $\alpha1^{N273H}\beta3\gamma2L$ receptors to levels similar to that of $\alpha6\beta3\gamma2L$ receptors (Fisher & Macdonald, 1998). Thus this external histidine residue in $\alpha6$ subunits appears to be responsible for the enhanced sensitivity to Zn^{2+} typified by $\alpha6$ subunit-containing $GABA_A$ receptors.

In conclusion, Zn^{2+} clearly has complex actions on inhibitory amino acid neurotransmitter receptors, but overall, histidine appears to be a favoured amino acid involved in the inhibitory action of this divalent cation. Moreover, in contrast to zinc-containing metalloenzymes (Vallee & Falchuk, 1993), a unique consensus motif does not appear, from the available evidence, to be important for coordinating Zn^{2+} inhibition between these highly homologous neurotransmitter receptors, since residues on the N-terminal, within TM2 and between TM2 and TM3 domains are clearly vital to support receptor regulation by Zn^{2+}.

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