Identification of a β Subunit TM2 Residue Mediating Proton Modulation of GABA Type A Receptors

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GABA type A (GABA_A) receptors are functionally regulated by external protons in a manner dependent on the receptor subunit composition. Although H^+ can regulate the open probability of single GABA ion channels, exactly what residues and receptor subunits are responsible for proton-induced modulation remain unknown. This study resolves this issue by using recombinant $\alpha 1\beta$ subunit GABA_A receptors expressed in human embryonic kidney cells. The potentiating effect of low external pH on GABA responses exhibited pK_a in accord with the involvement of histidine and/or cysteine residues. The exposure of GABA_A receptors to the histidine-modifying reagent DEPC ablated regulation by H^+ , implicating the involvement of histidine residues rather than cysteines in proton regulation. Site-specific substitution of all conserved external histidines to

alanine on the β subunits revealed that H267 alone, in the TM2 domain, is important for H^+ regulation. These results are interpreted as a direct protonation of H267 on $\alpha 1\beta$ receptors rather than an involvement in signal transduction. The opposing functional effects induced by Zn^{2+} and H^+ at this single histidine residue most likely reflect differences in charge delocalization on the imidazole rings in the mouth of the GABA_A receptor ion channel. Additional substitutions of H267 in β subunits with other residues possessing charged side chains (glutamate and lysine) reveal that this area of the ion channel can profoundly influence the functional properties of GABA_A receptors.

Key words: GABA_A receptor; pH modulation; β subunit; histidine, H^+ ; ion channel

GABA type A (GABA_A) receptors are the major mediators of inhibitory neurotransmission in the brain. Fast inhibition occurs via the synaptic GABA_A receptors (Smart, 1998), whereas extrasynaptic isoforms have been demonstrated to underlay continuous tonic inhibition (Otis et al., 1991). Those receptors expressed at inhibitory synapses will be regulated by endogenous processes, including trafficking, endocytosis, phosphorylation, redox reagents, and ions normally present *in vivo* (e.g., Zn^{2+} and H^+) (Kaila, 1994; Sieghart, 1995; Rabow et al., 1996; Moss and Smart, 2001). Variations in the level of endogenous ions in the CNS represent a rapid and effective method for regulating receptor function (Kaila, 1994; Smart et al., 1994). Previous studies have indicated that protons can differentially affect neuronal GABA_A receptors, resulting in potentiation, inhibition, or no effect on GABA-activated responses (Kaila, 1994; Robello et al., 1994; Pasternack et al., 1996; Zhai et al., 1998; Krishek and Smart, 2001). This variability to pH has been ascribed to differences in the neuronal receptor subunit composition that can be reproduced to some extent using recombinant GABA_A receptors (Krishek et al., 1996).

GABA_A receptors are presumed to be pentameric proteins (Nayeem et al., 1994) composed of combinations of the following subunits: $\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, δ , ϵ , π , and θ (Rabow et al., 1996; Mehta and Ticku, 1999). Notably, receptors composed of $\alpha 1\beta 1$, $\alpha 1\beta 1\delta$, and $\alpha 1\beta 1\gamma 2\delta$ subunits were differentially sensitive to external pH, whereas the $\alpha 1\beta 1\gamma 2$ subunit combinations (where

$i = 1, 2$) were primarily unaffected (Krishek et al., 1996). Subsequent single-channel studies in neurons indicated that raised H^+ concentrations caused inhibition of GABA-activated responses by reducing the single-channel open probability with no effect on channel conductance (Huang and Dillon, 1999; Krishek and Smart, 2001). Furthermore, for cerebellar granule neurons, developmental changes in GABA_A receptor $\alpha 1$ and $\alpha 6$ subunit expression (Laurie et al., 1992) occurred concurrently with changes in GABA_A receptor sensitivity to external pH, suggesting that protons may be a useful probe for detecting changes in receptor subunit composition (Krishek and Smart, 2001).

Modulating GABA_A receptor function with external pH may be important during CNS trauma and neurological pathologies by causing changes in neuronal excitability (Xiong and Stringer, 2000). However, the underlying amino acid residues on GABA_A receptors that are responsible for the proton effects remain unidentified. The present study investigates this question using heterologous expression of GABA_A receptors and resolves that a histidine residue at position 267 in the ion channel domain of the β subunit is not only completely responsible for proton regulation of $\alpha 1\beta$ receptor function, but also that after substitution, this location in the ion channel can profoundly influence the functional properties of the GABA_A receptor. To date, this histidine residue plays a unique role in the function of the GABA_A receptor's forming a potential ion binding site and significant transduction pathway. Some of these results have been reported previously in abstract form (Wilkins et al., 2001).

Received Jan. 31, 2002; revised April 18, 2002; accepted April 18, 2002.

This work was supported by the Medical Research Council. We thank Robert Harvey for providing $\beta 1/2^{\text{H267A}}$ clones.

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MATERIALS AND METHODS

cDNA constructs and site-specific mutagenesis. The murine GABA_A receptor $\alpha 1$, $\beta 1$, and $\beta 2$ subunit cDNAs were cloned into the vector pRK5. Site-specific mutagenesis was achieved by using oligonucleotides in conjunction with a primer-directed PCR method (Quickchange; Stratagene, La Jolla, CA), and purified DNAs were prepared using the Plasmid Maxi

Kit (Qiagen, Crawley, UK). The entire coding region of all mutants was sequenced using the BigDye ready reaction mix (PerkinElmer Life Sciences, Emeryville, CA/Applied Biosystems, Foster City, CA) and an ABI 310 automated DNA sequencer (Applied Biosystems).

Cell culture and electroporation. Human embryonic kidney (HEK) cells were cultured as described previously (Wooltorton et al., 1997b). The cells were transfected by electroporation (Gene Electropulser II, Hemel Hempstead, UK) using the following parameters: 0.4 kV, 125 μ F capacitance, and infinite resistance in the presence of 12 μ g of cDNA for the GABA_A receptor subunits, present in equal ratios, and 3 μ g of green fluorescent protein. Electroporated cells were plated onto poly-L-lysine-coated glass coverslips, which were used for electrophysiological recording 18–72 hr after transfection.

Patch-clamp electrophysiology. Membrane currents were recorded using a whole-cell patch-clamp technique from single HEK cells with an Axopatch 1-C amplifier (Axon Instruments, Foster City, CA). Patch pipettes (resistance, 3–5 M Ω) were filled with a solution containing (in mM): 120 KCl, 1 MgCl₂, 11 EGTA, 30 KOH, 10 HEPES, 1 CaCl₂, 2 adenosine triphosphate, and 12 creatine phosphate, pH 7.11. The cells were continuously perfused with Krebs' solution containing (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.52 CaCl₂, 11 glucose, and either 5 HEPES or 5 MES (see pH buffers). The Krebs' pH was finally adjusted to pH 5.4–8.4 with 1 or 5 N NaOH. Membrane currents were filtered at 5 kHz (–3 dB, sixth pole Bessel, 36 dB/octave) and analyzed with Clampex 8 (Axon Instruments). Any change of >10% in the membrane conductance/series resistance resulted in the cessation of recording. Drugs and solutions were rapidly applied to the cells using a modified Y-tube positioned ~300 μ m from the recorded cell. The response rise times were within 20–30 msec (Wooltorton et al., 1997b). All drugs were dissolved in external Krebs' solution at the appropriate pH. DEPC was added directly to the Krebs' solution, immediately before use, to yield final concentrations of 1 mM.

pH buffers. To ensure accurate control of the Krebs' pH over the range 5.4–8.4, two different pH buffers were used. For pH excursions between 6.8 and 8.4, HEPES (pK_a 7.5) was added to the Krebs' solution, and for the pH range 5.4–7, MES was used (pK_a 6.1), both at 5 mM final concentrations.

Analysis of whole-cell current data. Peak amplitude membrane currents activated by GABA (I) were determined at –50 mV holding potential. GABA equilibrium concentration–response relationships were constructed by measuring the peak GABA currents, which were normalized to the response induced by 10 μ M GABA in control Krebs' solution at pH 7.4 (I_{10}) and subsequently fitted with the Hill equation:

$$I/I_{10} = [1 / (1 + (EC_{50}/[A])^n)], \quad (1)$$

where EC_{50} represents the concentration of GABA ($[A]$) inducing 50% of the maximal current evoked by a saturating concentration of GABA and n is the Hill coefficient.

When the maxima of the curves demonstrated a clear depression with incrementing GABA concentration, the data were fitted with the following equation, accounting for the reduced maximum response by assuming that the ligand binds to two distinct sites, one producing potentiation and the other producing inhibition (or desensitization) of the GABA-activated current:

$$I/I_{10} = [1 / (1 + (EC_{50}/A)^n)] - [1 / (1 + (IC_{50}/A)^m)], \quad (2)$$

where I , I_{10} , EC_{50} , and n are as defined, IC_{50} is the GABA concentration producing a 50% inhibition of the current, and m represents the corresponding Hill coefficient. To ensure a fit to the inhibitory component, occasionally the IC_{50} was constrained where limited numbers of data points were available.

The GABA concentration–response curve data were analyzed using ANOVA with a Bonferroni *post hoc* test. Significance was determined at the $p < 0.05$ level.

The Zn²⁺ inhibition concentration relationships were fitted with the following equation:

$$I'_N/I_N = [1 - (B^n/(B^n + IC_{50}^n))], \quad (3)$$

where I'_N and I_N represent the normalized GABA-induced current in the presence and absence of Zn²⁺ at concentration B , respectively, and IC_{50} defines the concentration of Zn²⁺ producing 50% inhibition of the GABA-induced current.

Figure 1. External pH modulates the GABA concentration–response relationships. For $\alpha 1\beta 1$ (*A*) and $\alpha 1\beta 2$ (*B*) GABA_A receptors, peak amplitude responses were measured at pH 7.4 ($\circ$) and pH 5.4 ($\blacksquare$) and were normalized to the responses induced by 10 μ M GABA at pH 7.4 (= 1). In this and comparable figures, the curves were generated according to Equations 1 and/or 2, presented in Materials and Methods. All points in this and comparable figures represent the mean $\pm$ SEM from $n = 3$ –5 cells. The insets illustrate sample GABA-activated currents induced by 10 μ M GABA applied for the duration of the solid line at the indicated external pH. Calibration: 200 pA, 2 sec.

RESULTS

External pH and recombinant $\alpha 1\beta i$ GABA_A receptors

To assess whether the identity of the βi subunit was important for the pH-induced regulation of GABA_A receptor function, recombinant GABA_A receptors composed of $\alpha 1\beta 1$ and $\alpha 1\beta 2$ were expressed in HEK cells and used to construct full GABA concentration–response curves at normal physiological pH 7.4 and after a pH excursion to 5.4 (Fig. 1). For either construct, increasing the total H⁺ concentration by 100-fold resulted in potentiated responses to GABA and an increased maxima for the concentration–response curves ($p < 0.05$ for the range of 5 μ M to 1 mM GABA). The GABA EC_{50} s for $\alpha 1\beta 1$ (pH 7.4, 5.0 ± 0.7 μ M; pH 5.4, 3.3 ± 0.6 μ M; $n = 3$ –5) and $\alpha 1\beta 2$ (pH 7.4, 3.2 ± 0.4 μ M; pH 5.4, 4.9 ± 0.7 μ M; $n = 3$ –5) displayed no significant change, suggesting that GABA potency was primarily unaffected by the change in external pH (Fig. 1*A,B*). A direct comparison between the concentration–response curves for the two GABA_A receptor heteromers indicated that H⁺ induced a slightly larger potentiation of GABA-activated responses on $\alpha 1\beta 1$ receptors compared with $\alpha 1\beta 2$ receptors for 10 μ M GABA-activated responses (potentiated by 197.6 ± 37.5 and $120.1 \pm 15.5\%$, respectively; $n = 5$ –7). An additional distinction between $\alpha 1\beta 1$ and $\alpha 1\beta 2$ GABA_A receptors was that at high GABA concentrations (>100 μ M) in pH 5.4, the peak of the GABA concentration–response curve was depressed only for the $\alpha 1\beta 1$ receptors, possibly indicative of inhibition or increased desensitization. Overall, these data suggested that the identity of the β subunit in $\alpha 1\beta i$ constructs, although partially influential, did not have a major effect on the extent and type of modulation by H⁺.

Identifying the residues underlying H⁺ modulation of $\alpha 1\beta i$ GABA_A receptors

To establish the residue(s) that is important for the H⁺ modulation of $\alpha 1\beta i$ GABA_A receptors, we titrated the H⁺-induced potentiation for $\alpha 1\beta 1$ and $\alpha 1\beta 2$ GABA_A receptors against the external Krebs' pH; this yielded pK_as of 6.73 ± 0.09 and $6.91 \pm$

Figure 2. Modification of histidine residues by DEPC prevents proton-induced modulation of $\alpha 1\beta 1$ GABA_A receptors. Normalized GABA concentration–response relationships obtained at pH 7.4 (○) and pH 5.4 (●) after exposure to DEPC (1 mM) are shown. For comparison, the GABA concentration–response curves at pH 7.4 (dotted line) and pH 5.4 (dashed line) were taken from Figure 1*A*. Data are from $n = 3$ –5 cells.

0.13, respectively ($n = 5$). We therefore deduced that histidines (pK_a 6) are the most likely candidates to be involved in proton modulation of GABA_A receptors. Although cysteine residues also have a comparable pK_a (~8) they were considered unlikely to be involved, considering that the only two external cysteine residues, located within the N terminal, are postulated to form a disulfide bridge (Barnard et al., 1987; Pan et al., 1995, 2000; Amato et al., 1999), precluding their involvement with H^+ -induced modulation.

To investigate a potential role for histidine residues in H^+ regulation of GABA_A receptor function, we used DEPC, a reagent that irreversibly converts neutral imidazole groups into *N*-carboxyhistidyl derivatives (Miles, 1977). HEK cells expressing $\alpha 1\beta 1$ subunit constructs were exposed to 1 mM DEPC at pH 7.4. A brief application (4 min) of DEPC to $\alpha 1\beta 1$ receptors irreversibly abolished the potentiation of GABA-activated responses observed at pH 5.4 in the absence of DEPC. Inspection of GABA concentration–response curves revealed that after DEPC treatment, lowering the external pH to 5.4 induced a small lateral shift in the GABA concentration–response relationship, causing a reduction in the potency of GABA (EC_{50} s for $\alpha 1\beta 1$: pH 7.4, $5.1 \pm 1.3 \mu M$; pH 5.4, $18.8 \pm 4.6 \mu M$; $p < 0.05$) (Fig. 2). These data were in accord with one or more histidines underlying the H^+ -induced potentiation of GABA-activated responses, and this effect appeared to be masking a weak inhibitory action of H^+ that was clearly manifest only when the histidines had been covalently modified.

Site-specific mutagenesis of external histidines on $\alpha 1\beta 1$ receptors

Considering the ablation of H^+ modulation by DEPC, the exact molecular determinants of this potentiation were sought using site-specific mutagenesis. The $\alpha 1\beta 1$ heteromers possess numerous external histidine residues, eight on the $\alpha 1$ subunit and four on the $\beta 1$ subunits. External residues were selected because internal pH changes have little effect on GABA_A receptor function (Krishek et al., 1996). The β subunit was examined primarily because of the pivotal role this subunit family plays in the expres-

Figure 3. Mutating histidines 107 or 119 in the $\beta 2$ subunit do not alter proton-induced modulation of $\alpha 1\beta 2$ GABA_A receptors. Normalized GABA concentration–response relationships for $\alpha 1\beta 2^{H107A}$ (*A*) and $\alpha 1\beta 2^{H119A}$ (*B*) at pH 7.4 (□) and pH 5.4 (■). The comparative concentration–response curves for the wild-type $\alpha 1\beta 2$ receptor at pH 7.4 (dotted line) and pH 5.4 (dashed line) were taken from Figure 1*B*. Data are from $n = 3$ –5 cells.

sion and function of GABA_A receptors (Connolly et al., 1996). The number of histidines considered to be potentially involved in the H^+ modulation on the β subunit was reduced to three by including only those that are conserved between all β subunits, because H^+ modulation of GABA_A receptor function is not critically dependent on the identity of the β subunit. The three candidate histidines on the $\beta 2$ subunit were H107 and H119, located in the N terminal, and H267, located in TM2, the ion channel domain. Each histidine in the $\beta 2$ subunit was sequentially substituted, initially to alanine, and coexpressed with wild-type $\alpha 1$ subunits in HEK cells.

Neither the mutant $\alpha 1\beta 2^{H107A}$ nor $\alpha 1\beta 2^{H119A}$ GABA_A receptors differed in their response to changing the external pH to 5.4, which still potentiated responses to GABA to levels similar to those observed with the wild-type $\alpha 1\beta 2$ receptors (EC_{50} s: $\alpha 1\beta 2^{H107A}$: pH 7.4, $4.1 \pm 0.6 \mu M$; pH 5.4, $4.8 \pm 0.9 \mu M$; $\alpha 1\beta 2^{H119A}$: pH 7.4, $3.0 \pm 0.4 \mu M$; pH 5.4, $4.9 \pm 0.5 \mu M$; $p > 0.05$), compared with $\alpha 1\beta 2$ wild type for both mutants (Fig. 3*A,B*). However, substituting H267 with alanine, previously identified to play a major role in Zn^{2+} inhibition of GABA-activated responses (Wooltorton et al., 1997a; Horenstein and Akabas, 1998) on $\alpha 1\beta 1$ receptors, ablated the potentiating effect of H^+ (Fig. 4). Although the GABA concentration–response curve for the mutant $\alpha 1\beta 1^{H267A}$ did not exhibit an enhanced maximum response at pH 5.4, it was evident for both $\alpha 1\beta 1^{H267A}$ and $\alpha 1\beta 2^{H267A}$ receptors that low pH caused a small reduction in GABA potency (EC_{50} s for $\alpha 1\beta 2^{H267A}$: pH 7.4, $3.2 \pm 0.4 \mu M$; pH 5.4, $7.1 \pm 1.9 \mu M$; $p > 0.05$) (Fig. 4). This weak inhibitory effect of H^+ was manifest between GABA concentrations of 1 and 30 μM and appeared analogous to the effect of DEPC on the GABA concentration–response curve for the wild-type $\alpha 1\beta 1$ (Fig. 2) or $\alpha 1\beta 2$ receptors.

The lack of any potentiating effect of H^+ on the GABA_A receptor incorporating the mutant H267A suggested that this histidine residue may form a key protonation site for H^+ ions and is likely to be the only histidine involved. This latter point was reassessed by exposing $\alpha 1\beta 1^{H267A}$ GABA_A receptors to 1 mM DEPC. This modifying agent did not further affect the GABA concentration–response curves, nor did it affect the weak inhibitory effect revealed once the H^+ -induced potentiating effect had been abolished (data not shown).

Figure 4. Mutation of the TM2 histidine 267 in the $\beta 2$ subunit ablates proton sensitivity of $\alpha 1\beta 2$ GABA_A receptors. A normalized GABA concentration–response relationship for $\alpha 1\beta 2^{\text{H267A}}$ GABA_A receptors at pH 7.4 (□) and pH 5.4 (■) is shown, including the comparison with the curve fits for $\alpha 1\beta 2$ receptors at pH 7.4 (dotted line) and pH 5.4 (dashed line). The inset demonstrates typical responses to 10 μM GABA recorded at pH 7.4 and pH 5.4 for $\alpha 1\beta 2^{\text{H267A}}$ receptors. Calibration: 200 pA, 2 sec.

Charged mutations at H267 in the β subunit

To further investigate whether H^+ could be binding to H267, the residue that also has a pivotal role in Zn^{2+} inhibition, two additional substitutions were made in the $\beta 2$ subunit for subsequent coexpression with wild-type $\alpha 1$ subunits. These substitutions introduced amino acids with charged side chains: glutamate (E, negative) and lysine (K, positive) at position H267. Glutamate was selected because it has a projected pK_a for the side chain carboxyl group of ≤ 4 and probably less in the GABA_A receptor protein and would not be protonated at pH 7.4 (100% anionic) and only minimally at pH 5.4 (96.2% anionic). Thus, we predict that H^+ will be ineffective at potentiating GABA-activated responses if they do indeed bind to H267. However, for Zn^{2+} inhibition, the ability of the glutamate carboxyl group to act as an electron donor suggests that it will be capable of binding Zn^{2+} , a role that it fulfills in Zn^{2+} -containing metalloenzymes (Vallee and Auld, 1990). Thus, if Zn^{2+} binds to H267, we would expect the retention of some inhibitory activity at the mutant $\alpha 1\beta 2^{\text{H267E}}$ receptor but possibly at a reduced level compared with the wild-type receptor containing $\beta 2^{\text{H267}}$.

Exposure of $\alpha 1\beta 2^{\text{H267E}}$ receptors to low external pH 5.4 abolished the effect of H^+ on GABA-activated responses, as predicted if this residue forms a site for protonation (EC_{50} : pH 7.4, $3.4 \pm 0.5 \mu\text{M}$; pH 5.4, $4.8 \pm 1 \mu\text{M}$; $p > 0.05$ compared with $\alpha 1\beta 2$ wild type) (Fig. 5A). However, the level of Zn^{2+} inhibition over the concentration range of 1 μM to 10 mM for the $\beta 2^{\text{H267E}}$ substitution was less than expected (Fig. 6). The mutation disrupted antagonism, but the Zn^{2+} IC_{50} was $30.6 \pm 7.3 \mu\text{M}$, almost identical to that determined previously for GABA_A receptors incorporating the mutation $\beta 2^{\text{H267A}}$ (Wooltorton et al., 1997a) but greater than that for the $\alpha 1\beta 2$ wild type (IC_{50} : $0.65 \pm 0.03 \mu\text{M}$; $n = 4$) (Fig. 6).

The lysine substitution at position 267 was performed because the side chain, with a projected pK_a of 10, is positively charged at pH 7.4 (99.7% cationic) and also at pH 5.4 (100%). Therefore, this residue would already be protonated and could not partici-

Figure 5. Effect of charged mutations at position H267 in the β subunit on $\alpha 1\beta 2$ GABA_A receptor sensitivity to protons. Normalized GABA concentration–response relationships for $\alpha 1\beta 2^{\text{H267E}}$ (A) and $\alpha 1\beta 2^{\text{H267K}}$ (B) GABA_A receptors at pH 7.4 (○) and pH 5.4 (●) are shown, together with curve fits for the $\alpha 1\beta 2$ wild-type receptor at pH 7.4 (dotted line) and pH 5.4 (dashed line). Data are from $n = 3$ –5 cells. The inset illustrates 10 μM GABA-activated currents at pH 7.4 and pH 5.4 obtained from $\alpha 1\beta 2^{\text{H267K}}$ receptors. Note the increased rate and extent of receptor desensitization. Calibration: 400 pA, 2 sec.

Figure 6. Zn^{2+} concentration inhibition curves for $\alpha 1\beta 2$ (■), $\alpha 1\beta 2^{\text{H267E}}$ (●), and $\alpha 1\beta 2^{\text{H267K}}$ (▲) receptors determined by coapplication of Zn^{2+} with 10 μM GABA. The ordinate describes the response amplitude to GABA in Zn^{2+} as a percentage of the control response to GABA in the absence of Zn^{2+} . After each Zn^{2+} application, full recoveries from inhibition were obtained. The data were obtained from $n = 3$ –5 cells and were fitted using Equation 3, presented in Materials and Methods.

pate in the modulation of GABA-activated currents between pH 5.4 and pH 7.4. In addition, Zn^{2+} inhibition should be markedly reduced, as observed with the $\beta 2^{\text{H267A}}$ substitution. However, the charged side chain presented by the lysine residue conferred unusual and profoundly different functional properties on the GABA_A receptor. First, this mutation did not ablate the H^+ sensitivity of 10 μM GABA-activated currents, with potentiation by $52 \pm 7\%$ observed at pH 5.4 compared with pH 7.4 (EC_{50} : pH 7.4, $17.2 \pm 7.4 \mu\text{M}$; pH 5.4, $11 \pm 1.9 \mu\text{M}$; $p < 0.05$ compared with $\alpha 1\beta 2$ wild type; $n = 5$) (Fig. 5B). Second, the lysine mutation further reduced the sensitivity to Zn^{2+} , with the inhibition curve then defined by an IC_{50} of $990 \pm 116 \mu\text{M}$ (Fig. 6). Finally, the

desensitization profile of the GABA-induced currents for $\alpha 1\beta 2^{H267K}$ receptors was markedly affected, with increased rates of desensitization and peak currents declining back to the baseline holding current, even during GABA application (Fig. 5B, *inset*). These results clearly emphasized the crucial nature of the identity of the residue at position 267 in the ion channel lining of the β subunit in shaping the response profile of the GABA_A receptor.

DISCUSSION

This study concludes that for $\alpha 1\beta 1$ GABA_A receptors, H⁺-induced potentiation completely relies on a single histidine residue, H267, in β subunits; this residue resides at the external portal of the ion channel. Previous site-specific mutagenesis revealed that H267 also underpinned a substantial component of Zn²⁺ inhibition of GABA-activated responses, suggesting that it is important for the allosteric modulation of GABA_A receptor function.

The involvement of a histidine residue, conserved between the β subunit isoforms, in the regulation of GABA_A receptor function was suggested from the similar pH titration profiles for $\alpha 1\beta 1$ and $\alpha 1\beta 2$ receptors. This was also supported by the unequivocal effect of DEPCs completely removing any potentiation by H⁺ on $\alpha 1\beta 1$ and $\alpha 1\beta 2$ receptors. Interestingly, this action unveiled a small inhibitory effect of H⁺ that was observed only at low pH values with a projected pK_a approaching 4, indicating the involvement of aspartate and/or glutamate residues. Mutagenesis subsequently confirmed that only H267 in the β subunits was critically involved in H⁺-induced potentiation. Because H⁺ can compete and prevent the inhibitory effect of Zn²⁺ on recombinant and native GABA_A receptors (Krishek et al., 1998) and because H267 has already been suggested to be an important coordinating residue for Zn²⁺ inhibition at $\alpha 1\beta 1$ subunit GABA_A receptors (Woollorton et al., 1997a; Horenstein and Akabas, 1998), we suggest that protonation of this residue is likely to underlie the potentiating effect of H⁺ on $\alpha 1\beta 1$ GABA_A receptors. Furthermore, mutating H267 does not affect GABA activation of the receptor, thus suggesting that this residue is not part of the main agonist signal transduction process.

To further examine the role of H267, we substituted this histidine for glutamate and lysine to vary the charge in the side chain. Although inclusion of the carboxyl side chain for $\alpha 1\beta 2^{H267E}$ abolished H⁺-induced potentiation, Zn²⁺ inhibition was reduced to an extent similar to that observed after alanine substitution. This suggested that the orientation of the side chain in this position must be important, because glutamate, by virtue of electron donation, would be expected to bind Zn²⁺ and thus substitute, at least partly, for histidine. However, it is clear that this did not occur. The lysine substitution, however, yielded unexpected results, with the H⁺-induced potentiation only partly reduced when we might have expected abolition; however, the level of Zn²⁺ inhibition was markedly reduced, far more than expected from the results with the neutral alanine substitution at position 267. We had predicted that the H⁺-induced potentiation would be ablated because of the positively charged lysine side chain (pK_a 10); however, if H⁺ is protonating the lysine residue at position 267, this residue cannot be 100% charged, and the pK_a must be reduced by at least 2000- to 3000-fold (2–3 pH units) to ~7 for this to occur. Such a shift in pK_a has been reported in the inward rectifier potassium channel, K_{ir} 1.1, where an intracellular pH-sensitive lysine can be protonated at pH 5.4 (Schulte et al., 1999). Such an effect can be achieved only if other positively

charged residues (e.g., arginines and lysines) electrostatically shield the proton-sensitive lysine from basal H⁺ (Schulte et al., 1999). Indeed, both R269 and K274 (conserved throughout the GABA_A receptor α , β , and γ subunit families) lie close, at least in primary sequence order, to H267K. In the β subunits, such proximity might act to shield the lysine at 267 from H⁺. The results with Zn²⁺ and H267K could be explained by the action this residue has in further disrupting, beyond that achieved with H267A, the allosteric mechanism for Zn²⁺ inhibition.

Although mutagenesis cannot unequivocally indicate the presence of a binding site on a receptor, the lack of any relatively comparable effects on H⁺ and Zn²⁺ modulation by E267 and K267 suggests that this position is probably not involved in a common signal transduction pathway that is “downstream” of ion binding sites. Furthermore, because H⁺ and Zn²⁺ have opposite effects on GABA_A receptor function, it is also unlikely that H267 is an element in part of a common signal transduction mechanism.

The identification of H267 as a potential binding site for H⁺ and Zn²⁺ presented a curious problem. If these positively charged ions are capable of binding to the same residue, how can they induce completely opposite effects of inhibition (Zn²⁺) and potentiation (H⁺) on GABA_A receptor function? The key to this question may involve the different ways in which H⁺ and Zn²⁺ interact with the imidazole groups of histidines. The addition of H⁺ will cause protonation of individual imidazole rings, independent of any other residues. This protonation and subsequent potentiation of the GABA response might involve electrostatic repulsion between adjacent imidazole groups on the β subunits in the channel (two to three depending on subunit stoichiometry), causing a local conformational change in the receptor. In contrast, zinc ions will form a coordinated bond with one of the imidazole ring nitrogens and at least three other residues (which could include other imidazole rings from additional β subunits in the receptor) and an activated water molecule. This would be typical of many Zn²⁺ binding sites in metalloenzymes (Vallee and Auld, 1990). The coordination of Zn²⁺ with H267 would inhibit receptor function allosterically rather than by a physical channel block mechanism (Legendre and Westbrook, 1991; Smart, 1992; Gingrich and Burkat, 1998). This coordination, compared with protonation, would not polarize adjacent imidazole rings. Thus, mechanistically, at the level of amino acid side chains, it is conceivable that H⁺ and Zn²⁺ could cause differential effects on channel function, but the precise movements of amino acids will need to await x-ray crystallographic study. A similar scenario accounts for the differential effects of Zn²⁺ and H⁺ on the M2 ion channel from influenza A virus (Okada et al., 2001).

Overall, these data indicate the importance of a single TM2 residue in the ion channel lining of the β subunits for H⁺-induced modulation of GABA_A receptor function and also suggest that H267 could be a potential binding site for at least two types of cations.

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