Molecular Basis for Modulation of Recombinant $\alpha_1\beta_2\gamma_2$ GABA$_A$ Receptors by Protons

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Huang, Ren-Qi, Zhengl an Chen, and Glenn H. Dillon. Molecular basis for modulation of recombinant $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors by protons. J Neurophysiol 92: 883–894, 2004. First published March 17, 2004; 10.1152/jn.01040.2003. We have previously shown that extracellular protons inhibit recombinant and native GABA$_A$ receptors. In this report, we studied the site(s) and mechanism by which protons modulate the GABA$_A$ receptor. Whole cell GABA-activated currents were recorded from human embryonic kidney (HEK) 293 cells expressing recombinant $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors. Protons competitively inhibited the response to GABA and bicuculline. In contrast, change in pH did not influence direct gating of the channel by pentobarbital, and it did not influence spontaneous channel openings in $\alpha_1$($\beta_1,\delta_1)$//$\gamma_2$ receptors, suggesting pH does not modulate channel activity by affecting the channel gating process directly. To test the hypothesis that protons modulate GABA$_A$ receptors at the ligand binding site, we systemically mutated N-terminal residues known to be involved in GABA binding and assessed effects of pH on these mutant receptors. Site-specific mutation of $\beta_2$ Y265 to F or $\alpha_1$ F64 to A, both of which are known to influence GABA binding, significantly reduced pH sensitivity of the GABA response. These mutations did not affect Zn$^{2+}$ sensitivity, suggesting that H$^+$ and Zn$^{2+}$ do not share a common site of action. Additional experiments further tested this possibility. Treatment with the histidine-modifying reagent diethylpyrocarbonate (DEPC) reduced Zn$^{2+}$-mediated inhibition of GABA$_A$ receptors but had no effect on proton-induced inhibition of GABA currents. In addition, mutation of residues known to be involved in Zn$^{2+}$ modulation had no effect on pH modulation of GABA$_A$ receptors. Our results support the hypothesis that protons inhibit GABA$_A$ receptor function by direct or allosteric interaction with the GABA binding site. In addition, the sites of action of H$^+$ and Zn$^{2+}$ in GABA$_A$ receptors are distinct.

INTRODUCTION

GABA, the predominant fast inhibitory neurotransmitter of the adult CNS, acts principally via GABA$_A$ receptors. Activation of GABA$_A$ receptors results in membrane hyperpolarization and neuronal inhibition in adults. Inhibition is a fundamental process in brain activity and most neuronal brain cells express GABA$_A$ receptors. GABA$_A$ receptors are pentameric membrane proteins comprising seven different classes of subunits, many with multiple variants ($\alpha_1$-$\alpha_6$, $\beta_1$-$\beta_3$, $\gamma_1$-$\gamma_3$, $\rho_1$-$\rho_3$, $\delta$, $\epsilon$, and $\theta$) (Barnard et al. 1998; Whiting 1999). Within the GABA$_A$ receptor family, the combination of $\alpha_1\beta_2\gamma_2$ is the most abundantly expressed member in brain (Fritschy and Brüning 2003). GABA$_A$ receptors contain binding sites for modulators and therapeutic drugs such as benzodiazepines, barbiturates, and neurosteroids (Barnard et al. 1998).

GABA$_A$ receptor function is regulated by a host of endogenous ions including physiological concentration of protons. Protons regulate neuronal function with fluctuation at nanomolar levels. It has been known that pH in the brain changes with neural activity. In physiological conditions, brain pH of 6.5–8.0 may exist (Kaila and Ransom 1998). In certain pathological conditions such as stroke, seizure, and ischemia, brain pH can even shift up to 1 unit (Siesjö et al. 1985; Von Hanwehr et al. 1986). It is well known that protons modulate neuronal excitability, and this effect is partially mediated through pH modulation of GABA$_A$ receptors. Reports on proton modulation of mammalian GABA$_A$ receptors vary depending on the experimental conditions, animal species, brain regions, or subunit compositions (Huang and Dillon 1999; Krishke and Smart 2001; Krishke et al. 1996, 1998; Li et al. 2003; Mozrzymas et al. 2003; Pasternack et al. 1992, 1996; Robello et al. 1994; Smart 1992; Tang et al. 1990; Vyklický et al. 1993; Wilkins et al. 2002; Zhai et al. 1998). Moreover, the biophysical and molecular mechanisms for pH modulatory effects on GABA$_A$ receptor are not fully understood.

Wilkins et al. (2002) identified a histidine residue at 267 of the $\beta_2$ subunits that is necessary for proton modulation of binary $\alpha_1\beta_2$ GABA$_A$ receptors. They showed that mutation of H267 to A abolished modulation of GABA-activated currents at pH 5.4. However, this level of acidosis is unlikely to be encountered in vivo, and thus the role of H267 with regard to physiological regulation of the receptors by pH is unclear. Furthermore, receptors incorporating only $\alpha$ and $\beta$ subunits account for a minority of total GABA$_A$ receptors in the CNS (Barnard et al. 1998). Thus molecular determinants for pH modulation of a major GABA$_A$ subtype in CNS within the physiological pH range have not been investigated. We therefore studied the molecular basis for pH sensitivity of heterologously expressed $\alpha_1\beta_2\gamma_2$ receptors over a pH range that is encountered during physiological and pathological conditions. In this study, we provide evidence that protons inhibit GABA$_A$ receptors by alterations of the GABA binding site such that GABA affinity is decreased.

METHODS

Cloned GABA$_A$ receptors

Wild-type or mutant receptor cDNA (in the vector pCDM8) was expressed in human embryonic kidney cells (HEK293). Human
GABA$_A$ receptor $\alpha_1$, $\beta_2$, and $\gamma_2L$ (long isoform of the $\gamma_2$ subunit) subunit cDNA were generously supplied by Dr. Nancy Leidenhermer (Louisiana State University). Cells were transfected using calcium phosphate precipitation technique to achieve transient expression of human $\alpha_1$$\beta_2$$\gamma_2L$, $\alpha_1$$\beta_2$, or the corresponding mutants (Chen and Okayama 1987). Briefly, HEK293 cells (CRL 1573, ATCC, Rockville, MD) were plated onto coverslips and transfected with wild-type or mutant subunits. Typically, 2 $\mu$g of plasmid cDNA per subunit was added to cells growing exponentially on one coverslip placed in a 35-mm culture dish. After 6–8 h, cells were washed and placed in fresh culture medium. Transfected cells were used for electrophysiological analysis 24–48 h after the transfection. Cells stably expressing rat $\alpha_1$$\beta_2$$\gamma_2$ receptors were also studied. A complete description of preparation of these cell lines has been published previously (Hamilton et al. 1993).

Site-directed mutagenesis

Mutations of receptor subunit cDNA were performed using commercially available QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA) with commercially produced mutagenic primers (Integrated DNA Technologies). All mutants were verified by DNA sequencing (Biotechnology Core Facility, Texas Tech University, Lubbock, TX).

Electrophysiology

Whole cell patch-clamp recordings were made at room temperature (22–25°C), with cells voltage-clamped at −60 mV. Patch pipettes of borosilicate glass (1B150F, World Precision Instruments, Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument, Novato, CA) to a tip resistance of 1–2.5 MΩ. The pipette solution contained (in mM) 140 CsCl, 10 EGTA, 10 HEPES, and 4 Mg-ATP (pH 7.2). Coverslips containing cultured cells were placed in a small chamber (~1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5–8 ml/min) with the following external solution containing (in mM) 125 NaCl, 5.5 KCl, 0.8 MgCl$_2$, 3.0 CaCl$_2$, 10 HEPES, and 10 d-glucose (pH 7.3). GABA-induced Cl$^-$ currents from the whole cell patch-clamp technique were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-201 headstage. Currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0, Axon Instruments) for subsequent analysis. Sixty to 80% series resistance compensation was applied at the amplifier.

Experimental protocol

pH of external solutions was altered by addition of NaOH or HCl and routinely checked before and during experiments. GABA was prepared in the extracellular solution and was applied from independent reservoirs by gravity flow for 10 s to cells using a Y-shaped tube positioned adjacent to the cell. With this system, the 10–90% rise time of the junction potential at the open tip averages ~30 ms (Huang and Dillon 1999). Once a control GABA response was determined, the effect of pH on the response was examined. To assess the pH effect, cells were first bathed in medium that was set to the test pH, and then GABA, dissolved in the same test pH solution, was applied to the cells. Because in the whole cell recordings, external solution of low pH elicited, as in other studies (Krishke et al. 1996; Zhai et al. 1998), a transient whole cell inward current via acid sensing ion channels (ASICs), GABA application at various pH test values was made after the transient current had recovered and stabilized. GABA applications were separated by ≥3-min intervals to ensure both adequate washout of GABA from the bath and recovery of receptors from desensitization, if present. In experiments using DEPC, it was added directly to the external solution immediately before use at a final concentration of 1 mM.

Chemicals

All drugs were obtained from Sigma. GABA and ZnCl$_2$ stocks were made in ddH$_2$O. Bicuculline, pentobarbital, and diazepam were made in DMSO and diluted in saline so that the final DMSO concentration (vol/vol) was <0.2%.

Data analysis

GABA concentration–response profiles were fitted to the following equation: $I_{max} = \frac{[\text{GABA}]^n}{(\text{EC}_{50}^n + [\text{GABA}]^n)}$, where $I$ and $I_{max}$ represent the normalized GABA-induced current at a given concentration and the maximal current induced by a saturating concentration of GABA, respectively. $\text{EC}_{50}$ is 50% effective GABA concentration, and $n$ is the Hill coefficient.

Concentration–response curves for bicuculline inhibitory effect were fitted to the following equation: $I_{max} = \frac{[\text{bicuculline}]^n}{([\text{bicuculline}]^n + IC_{50})}$, where $I$ is Cl$^-$ current amplitude at the end of drug application normalized to control at a given bicuculline concentration, $IC_{50}$ is the half-blocking concentration, and $n$ is the Hill coefficient.

Concentration–response profiles for protons were evaluated using approximately the EC$_{30}$ GABA concentration. A minimum of three individual experiments was conducted for each paradigm. All data are presented as means ± SE. Student’s t-test (paired or unpaired) or one-way ANOVA test was used to determine statistical significance ($P < 0.05$).

RESULTS

Effect of protons on recombinant GABA$_A$ $\alpha_1$$\beta_2$$\gamma_2$ receptors

The modulatory effects of pH on human $\alpha_1$$\beta_2$$\gamma_2$ GABA$_A$ receptors is shown in Fig. 1. As we reported previously (Huang and Dillon 1999), the amplitude of current activated by EC$_{30}$ GABA (6 $\mu$M) was increased when pH was increased from 7.3 to 7.8 and 8.4, and markedly attenuated when pH was decreased from 7.3 to 6.4 and 5.4 (Fig. 1, A and B). The effect of pH on GABA current was rapid and completely reversible (Fig. 1A). Figure 1B shows the average sensitivity of GABA-activated current to protons over the range of pH 5.4–8.4. The GABA current activated by EC$_{30}$ GABA was enhanced to 184 ± 15% of control at pH 8.4 and inhibited to 64 ± 4.8% at pH 6.4 and 53 ± 6% of control at pH 5.4, respectively ($n = 5$–10). The influence of pH on concentration–response relationship for GABA-activated currents was also studied (Fig. 1, C and D). Figure 1C shows that acidic pH (6.4) inhibited the response to low concentration of GABA (10 $\mu$M) but had little effect on the response to saturating GABA concentration (1,000 $\mu$M) in the same cell. As reported in Fig. 1D, the acidic pH caused a significant increase in EC$_{50}$ values for GABA from 10 ± 3.0 $\mu$M at pH 7.3 to 19 ± 5.5 $\mu$M at pH 6.4 ($P < 0.05$, paired t-test, $n = 14$). In contrast, the maximal GABA current and Hill coefficient values were not significantly changed by acidic pH. Our data suggest that protons inhibit GABA-activated current in human $\alpha_1$$\beta_2$$\gamma_2$ GABA$_A$ receptors.
in a competitive manner, which is consistent with the previous studies on rat GABA<sub>\alpha</sub> receptors in recombinant (Huang and Dillon 1999) and native preparations (Li et al. 2003; Zhai et al. 1998).

**Effect of protons on direct gating of GABA<sub>\alpha</sub> receptors by pentobarbital**

The characterized site for pentobarbital (PB) binding is distinct from the GABA-binding site (Amin 1999; Amin and Weiss 1993). In addition, although the transitions that initiate gating are by default distinct for GABA-gated versus PB-gated channel openings, the final gating event is similar when the receptor is opened by PB or GABA (Akk and Steinbach 2000; Rho et al. 1996). Eighty-nine to 98% of pentobarbital molecules remain in the nonionized form within the pH 6.8–7.3, based on Henderson-Hasselbach equation. Therefore it is possible to distinguish pH effect on GABA binding from gating by examining acidic pH effect on direct gating by PB in the absence of GABA. The EC<sub>50</sub> for PB direct activation was estimated to be 347 ± 1100 μM with PB at concentrations from 30 to 1000 μM (EC<sub>20</sub> to EC<sub>95</sub>; n = 6, data not shown). Because high concentrations of pentobarbital produce “autoinhibition” (Akaike et al. 1987), PB concentrations >1000 μM were not tested. Figure 2 shows the proton effect on gating elicited by GABA or by PB recorded from the same cell stably expressing rat α1β2γ2 GABA<sub>\alpha</sub> receptors. Acidic pH (6.8) inhibited the currents activated by EC<sub>30</sub> GABA (5 μM) to 72.6 ± 3.6% of the control (n = 12, P < 0.01, paired t-test) but did not affect direct gating by PB at the concentration from 100 to 1000 μM (EC<sub>50</sub> to EC<sub>95+</sub>; n = 12, P > 0.05 for all concentrations of PB tested, paired t-test, compared with control at pH 7.3). This lack of pH effect on pentobarbital-mediated gating strongly argues against the possibility that pH modulates GABA<sub>\alpha</sub>

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**FIG. 1.** Effect of pH on GABA-activated currents in α1β2γ2 receptors transiently expressed in HEK293 cells. A: typical recording of current activated by EC<sub>30</sub> GABA (6 μM) over a pH range of 5.4–8.4. The GABA current was potentiated by diazepam (DZ, 1 μM), indicating the expression of γ2 subunits. B: summary of relative current activated by EC<sub>30</sub> GABA plotted as a function of pH. GABA currents over the pH range of 8.4–5.4 were normalized to the current recorded at control condition (pH 7.3, assigned as 100%). Data plotted are means ± SE. Each data point is the average of 4 cells at a holding potential of −60 mV. C: current activated by the application of 10 and 1,000 μM GABA at pH 7.3 and 6.4 in the same cell. Note that acidic pH inhibited the response to a low concentration of GABA but did not affect the response to saturating GABA. D: concentration–response relationship of GABA current at pH 7.3 and 6.4. Each data point is the average current from 14 cells. Curves shown are the best fits of the data to the logistic function. Note that acidic pH significantly shifts the EC<sub>50</sub> value for GABA without significantly affecting the maximal currents or Hill coefficient. EC<sub>50</sub> and Hill coefficients (in parentheses) were pH 7.3, 10 ± 3.0 μM (1.06 ± 0.07); pH 6.4, 19 ± 5.5 μM (1.09 ± 0.09). EC<sub>50</sub> value at pH 6.4 is significantly different from the control (P < 0.05, n = 14, paired t-test).
receptor function by alternating the gating mechanism at the level of the channel pore.

Effect of protons on spontaneous opening of α1(L264T)β2γ2 receptors

To further distinguish whether pH modulation is primarily dependent on GABA binding or channel gating, we examined the pH effect on spontaneous receptor activation that is independent of GABA binding. A conserved TM2 leucine (α1L264) plays a key role in channel gating and substitution of this residue with threonine or alanine results in spontaneous opening in GABA_A receptors (Daiziel et al. 2000; Scheller and Forman 2002). When the membrane potential was voltage clamped at −60 mV, cells expressing rat α1(L264T)β2γ2 receptors consistently displayed large holding currents and application of the open channel blocker picrotoxin resulted in an outward current (Fig. 3A). Picrotoxin is a nontitratable compound and its charge is not affected by change of pH. We thus estimated the effect of pH on spontaneous opening by measuring picrotoxin-induced current at holding voltage of −60 mV in the absence of GABA. As shown in Fig. 3, change of pH from 8.4 to 5.4 had little effect on the spontaneous opening measured with 1 mM picrotoxin (PTX). To determine whether the loss of proton sensitivity of spontaneous currents was due to mutation of L264T, we examined the effect of pH on GABA-activated current in the same mutant receptors. In agreement
Effect of protons on bicuculline inhibition of GABA response

The above results indicate that protons inhibit the GABA-activated currents in a competitive manner and might not be involved in alteration of channel gating. This leads to the possibility that protons may influence the GABA response at GABA binding sites. Bicuculline has been characterized as a competitive inhibitor of GABA binding to the GABA receptor. If protons act on the GABA binding site, the protons would be predicted to also competitively inhibit the bicuculline effect, i.e., shift the bicuculline concentration-inhibition curve to the right. As shown in Fig. 4, the IC50 for bicuculline in blocking GABA-activated currents increased as extracellular pH decreased. Bicuculline IC50 values were 1.22 ± 0.24 μM at pH 7.8, 1.95 ± 0.15 μM at pH 7.3, and 2.96 ± 0.24 μM at pH 6.8 (P < 0.01, 1-way ANOVA test). The inhibition was complete at higher bicuculline concentration (30 μM), and Hill coefficients were close to 1 (P > 0.05). Ninety-two to 100% of GABA molecules are in the electronneutral zwitterionic form over the pH range 5–9.4 (Krishek et al. 1996; Roberts and Sherman 1993). Bicuculline methiodide is a weak base with a pKa value of 4.84. Over the range of pH range tested here, 97–100% of bicuculline exists as the unionized form in solution, according to the Henderson-Hasselbach equation. Thus the observed effect of pH on bicuculline inhibition of GABA current is unlikely due to an effect on either the GABA or bicuculline molecules, and instead is attributable to an action of pH on the receptor.

Effect of mutation of GABA binding residues on proton sensitivity

To further test the hypothesis that protons may interact with GABA at GABA binding sites, we systemically mutated residues in the N-terminal extracellular region that are involved in GABA binding. With the knowledge of GABA binding sites from previous investigations (Amin and Weiss 1993; Boileau et al. 1999, 2002; Holden and Czajkowski 2001, 2002; Newell and Czajkowski 2003; Newell et al. 2000; Wager and Czajkowski 2001), we initially made eight mutants in the human β2 subunit (Y62, D95, Y97, Y157, T160, T202, Y205, and R207) and two mutants in the human α1 subunit (F64 and R66). These mutations cover the key residues that have been identified so far to form the ligand-binding pocket, based on the six polypeptide loop model (Corringer et al. 2000). The mutant α1 or β2 subunits were co-expressed with wild-type β2 and γ2 or α1 and γ2 subunits, respectively, in HEK293 cells. Functional expression of ternary receptors was confirmed by the presence of both diazepam (1 μM) potentiation and modest inhibition (~30%) by Zn2+ (100 μM). GABA concentration-response curves were constructed for each configuration, and pH effects on the mutants were tested.

FIG. 4. Effect of pH on bicuculline inhibition of GABA currents in α1β2γ2 receptors stably expressed in HEK293 cells. Currents are normalized to the amplitude at pH 7.3. Bicuculline was co-applied for 10 s with equal potency of [GABA](EC50). Inset: equal potency of [GABA] at different pH in the absence of bicuculline. Note that increasing [H+] shifted the bicuculline inhibition curve to the right. P < 0.05, unpaired t-test, n = 6–20.
All of the mutant subunits assembled into functional ternary receptors. GABA concentration–response analysis of the mutant receptors revealed mutations of residues on the β2 subunit of loop B (Y157F, T202S) and loop C (T202S, Y205F, R207C) and on the α1 subunit of loop D (F64A, R66L) caused significant shifts in GABA EC$_{50}$ value (Table 1). The Y157F, T160S, T202S, Y205F, and R207C mutations on the β2 subunits caused 9- to 47-fold shifts in GABA EC$_{50}$ compared with wild-type, whereas the mutation of F64A and R66L in the α1 subunits caused 400- and 1,142-fold increases in GABA EC$_{50}$ (Table 1). Residues Y62 of the β2 subunit is a determinant of high affinity GABA binding, and the Y62F mutation resulted in modest increase in GABA EC50 in GABA EC50, in Xenopus oocytes (Newell et al. 2000). In this study, the Y62F mutation did not significantly alter the GABA EC$_{50}$ (Table 1). Residues D95 and Y97 of the β2 subunit influence GABA sensitivity when mutated to cysteine (Boileau et al. 2002). In this report, mutation of D95 and Y97 to V and F, respectively, did not drastically alter the GABA EC$_{50}$ (Table 1).

As described above in Fig. 1, in cells transiently expressing human GABA$_{A}$ α1β2γ2 wild-type receptors, GABA current activated by EC$_{50}$ GABA (6 μM) was potentiated to 183 ± 15% of the control at pH 8.4 and inhibited to 64 ± 4.8% of the control at pH 6.4 (Table 1; Fig. 1). Expression of the α1 F64A or β2 Y205F mutants resulted in a significant reduction in pH sensitivity at both acidic and alkaline condition (P < 0.05, unpaired t-test) compared with wild-type receptors (Table 1; Figs. 5 and 6). Receptors expressing the mutants β2(Y62F), β2(D95Y), β2(Y97F), β2(Y157F), β2(T160S), β2(T202S), β2(R207C), or α1(R66L) failed to alter proton sensitivity (Table 1; Fig. 5). Figure 5 shows the effect of protons on GABA-activated current in wild type and mutant α1β2(Y205F)γ2 receptors. The Y205F mutation greatly reduced modulatory effects of pH on the currents activated by EC$_{50}$ GABA over the pH range 5.4–9.4 compared with wild-type α1β2γ2 receptors. Whereas the GABA EC$_{50}$ was shifted by protons in wild-type receptors (Fig. 1, C and D), the EC$_{50}$ was not shifted by protons in α1β2(Y205F)γ2 receptors (pH 7.3: 480 ± 80 μM; pH 6.4: 550 ± 59 μM, n = 4, P > 0.05, paired t-test, Fig. 5C). As reported previously (Amin and Weiss 1993), no current was detected when β2(Y205) was mutated to S or A. Thus we could not test proton sensitivity in these mutant receptors.

The recognition site for GABA and the antagonist bicuculline appears to be located at the interface between α and β subunits. We investigated whether coexpression of mutated α1(F64A) with mutated β2(Y205F) would further influence proton sensitivity. As shown in Table 1 and Fig. 6, double point mutations of α1(F64A)β2(Y205F)γ2 produced a similar magnitude of effect on proton sensitivity of GABA response compared with single point-mutation receptors, although EC$_{50}$ values for GABA were further increased.

### Effect of modification of Zn$^{2+}$ action sites on proton sensitivity

Histidine (pK$_{a}$ = 6.0) is a likely candidate for providing a proton binding site. Recently, Wilkins et al. (2002) showed that H267, which plays a major role in Zn$^{2+}$-induced inhibition of the GABA$_{A}$ receptor, is also responsible for potentiating effect of acidity on binary murine α1β1/2 GABA$_{A}$ receptors. Exposure of receptors to a histidine-modifying reagent, or substitution of H267 with alanine, abolished the potentiation of GABA current by pH 5.4 (Wilkins et al. 2002). We therefore attempted to examine whether histidine residues are also involved in proton-induced inhibition of GABA currents in ternary α1β2γ2 receptors.

DEPC selectively reacts with histidine residues at pH < 7 (Miles 1977). As shown in Fig. 7, A and B, treatment of α1β2γ2 receptors with DEPC (1 mM, 5 min via bath application) significantly attenuated Zn$^{2+}$-mediated inhibition of GABA$_{A}$ receptors (the percentage of inhibition by 500 μM Zn$^{2+}$: 70 ± 2.7% without DEPC, 39 ± 7% in DEPC, n = 3, P < 0.05, paired t-test). In contrast, DEPC treatment did not alter pH-mediated inhibition of these receptors (the currents activated by 5 μM GABA at pH 5.4: 74 ± 3% of control without DEPC, 82.2 ± 1.9% of the control in DEPC, n = 3, P > 0.05, paired t-test). Next, we mutated H267 at the β2 subunit to alanine and co-expressed it with wild-type α1 and γ2 subunits. As shown in Fig. 7C, proton sensitivity of the GABA response in the mutant α1β2(H267A)γ2 receptors was not different from wild-type over the pH range 5.4–8.4 (Fig. 7C). Zn$^{2+}$ sensitivity tested with 500 μM Zn$^{2+}$ co-applied with EC$_{50}$ GABA was essentially abolished in α1β2(H267A)γ2 receptors (101 ± 6.7% of control, n = 3).

### Table 1. Concentration–response data for human recombinant GABA$_{A}$ receptors and pH sensitivity of GABA response

<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA Sensitivity</th>
<th>pH Sensitivity (% of Control)</th>
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<tr>
<td></td>
<td>EC$_{50}$ (μM)</td>
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<tr>
<td>α1β2γ2</td>
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<tr>
<td>α1(F64A)β2(Y205F)γ2</td>
<td>60,000 ± 8,900</td>
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Values are means ± SE. GABA EC$_{50}$ values were determined by fitting GABA concentration–response profiles (peak current amplitudes) to the logistic function. pH sensitivity was examined using a GABA concentration corresponding to the respective EC$_{50}$ value for all receptors. The currents are normalized to the current at control (pH 7.3). The statistical significance of proton sensitivity was displayed in Fig. 6.
compared with wild-type receptors (32 ± 2.5% of control, n = 4). Finally, we examined whether substitution of H267 in β2 subunit in the binary α1β2 configuration would influence proton effect on GABA current. In wild-type human α1β2 receptors, the current activated by EC₅₀ GABA was potentiated by alkalinization and inhibited by acidification, although the magnitude of proton modulatory effect was smaller than that in α1β2γ2 receptors (Fig. 7, C and D). In the mutant α1β2(H267A) receptors, Zn²⁺ inhibition tested with 100 μM Zn²⁺ was considerably reduced (47.5 ± 4.5% of control, n = 4) compared with wild-type α1β2 (97.9 ± 1.3% of control, n = 5) while the EC₅₀ for GABA in mutant was similar to wild-type [2.8 ± 0.4 μM in α1β2(H267A) vs. 3.0 ± 0.1 μM in wild-type α1β2]. As shown in Fig. 7D, proton sensitivity in the mutant receptor was not different from the wild-type over pH 6.4–8.4. However, at pH 5.4, the GABA current was inhibited to 58 ± 3.1% of control (n = 10) in mutant receptors, a level that was significantly lower than observed in wild-type receptors (81 ± 5.5% of control, n = 8, P < 0.01, unpaired t-test). Thus H267 does play some role in pH sensitivity, but this is observed only at very extreme levels of acidosis, and only in binary (not the more ubiquitous ternary) receptors.

In addition, Hosie et al. (2003) recently identified additional residues that define Zn²⁺ inhibition in GABAₐ receptors. The negatively charged residues α1(E138), β2(E182), and β2(E270), along with β2(H267), have been shown to confer full sensitivity to Zn²⁺ in GABAₐ receptors (Hosie et al. 2003). We individually mutated α1 E138, β2 E182, or β2 E270 to the neutral residue alanine. In the ternary receptors incorporating the mutant subunits [i.e., α1(E138A)β2γ2


α1β2(E182A)γ2, or α1β2(E270A)γ2, the Zn2+-induced inhibition was greatly diminished as tested with 500 μM Zn2+ (data not shown), which confirmed previous observation by Hosie et al. (2003). However, the proton sensitivity was not affected by these mutations (data not shown).

To further examine the possibility that protons and Zn2+ interact with GABA receptors at the same site, we did a reciprocal experiment to test the Zn2+ sensitivity in the receptors containing α1F64A or β2Y205F, which display reduced sensitivity to protons (Figs. 5 and 6; Table 1). α1β2(Y205F)γ2 or α1(F64A)β2γ2 receptors exhibited Zn2+ sensitivity similar to that observed in wild-type α1β2γ2 receptors (Fig. 8). Collectively, our data indicate that protons and Zn2+ do not share the same binding site in GABA receptors.

Discussion

Evidence for proton interaction with GABA binding site

In this report, we sought to define the mechanism by which pH influences GABA receptors. We show that pH modulation occurs in part by influencing GABA binding, either directly or allosterically. Several lines of evidence support this conclusion. Our previous report (Huang and Dillon 1999) and that of Zhai et al. (1998) and Li et al. (2003) showed that the proton effect is competitive with respect to GABA. Our current studies showing that protons also competitively inhibit the actions of bicuculline, a competitive antagonist of GABA, further substantiate the contention that protons are influencing the GABA binding pocket. This conclusion is also supported by early studies that showed [3H]GABA binding in mammalian brain membranes is decreased with a decrease in pH over the range 5.8–8.0 (Olson et al. 1981). More recently, Mozrzymas et al. (2003) employed model simulation to quantitatively analyze proton modulation of GABA-activated currents from hippocampus neurons and suggest that part of the proton effect is mediated by a reduction of GABA binding rate.

Our data do not support the hypothesis that protons inhibit GABA receptor function by directly altering channel gating at the channel pore. We conclude this based on the results of two separate sets of experiments. First, acidic pH did not affect pentobarbital-mediated channel gating. Although the binding site for barbiturates has not been defined, it is clearly distinct from the GABA binding site, since mutations that drastically alter GABA sensitivity do not alter barbiturate sensitivity (Amin and Weiss 1993). Single-channel studies show the conductance and open time of pentobarbital-activated single-channel currents are indistinguishable from single-channel events gated by GABA, which suggests the fundamental gating process is similar for both ligands (Jackson et al. 1982; Mathers and Barker 1980; Rho et al. 1996). Second, in the absence of GABA, protons did not influence spontaneous channel openings in α1(L264T)β2γ2 receptors. In contrast, the sensitivity of GABA-activated currents to protons remained intact in these mutant receptors. While not definitive, when considered with the above data, the fact that protons inhibit GABA-gated current without impairing pentobarbital-gated current or spontaneous currents is also consistent with the possibility that protons may interact with the GABA binding site.

It is widely accepted that GABA binding involves multiple residues at the N-terminus. Based on the nicotinic acetylcholine receptor (nAChR), the ligand binding site is formed by loops (designated loops A–F) at the intersubunit interfaces (Corringer et al. 2000). Conventional mutagenesis and the substituted cysteine accessibility method (SCAM) have implicated at least 10 residues in GABA binding, most of which exist in the β subunit. Aromatic [α1F64, β2(Y62, Y97, Y157, Y205)] and hydroxylated [β2(T160, T202, S205, S209)] residues are particularly important in forming acidic pH 6.4

FIG. 6. Effect of mutations of residues involved in GABA binding on proton sensitivity in human α1β2γ2 subunit configuration transiently expressed in HEK293 cells. Current activated by EC50 GABA was recorded in control (pH 7.3), acidic (pH 6.4), and alkaline pH (pH 8.4). The percentage of current inhibition (potentiation) = [current amplitude (I) at pH 6.4 or 8.4 – I at pH 7.3]/I at pH 7.3 × 100. Results represent the means ± SE of ≥4 cells (see Table 1). *P < 0.05, unpaired t-test, compared with wild-type.
the GABA binding site (Amin and Weiss 1993; Boileau et al. 1999, 2002; Holden and Czajkowski 2002; Newell and Czajkowski 2003; Newell et al. 2000; Wager and Czajkowski 2001). We systematically mutated all of these residues (and nonaromatics 1R66, 2R207, and 2H9252) and found effects on GABA sensitivity that are generally consistent with published reports. We identified two residues (2Y205 of loop C and 1F64 of loop D) that also decrease pH sensitivity. In this study, mutation of 2Y205F or 1F64A produced 47- and 1,143-fold shifts in GABA EC50, respectively. Substitution of Y205 with F, which has similar volume but lacks a hydroxyl group, significantly reduced proton sensitivity. Interestingly, a similar magnitude (~50%) of reduction of proton sensitivity was observed in receptors expressing a single mutation (1F64A or 2Y205F) compared with those expressing the double mutation [1F64A]/2Y205F/γ2], while apparent affinity for GABA was further reduced in the double mutation receptors. This may be interpreted with the model of GABA binding proposed by Newell and Czajkowski (2003), which shows that 2Y205 residue at loop C is facing 1F64 residue of loop D at the β-α subunit interface. We speculate that protons may influence the GABA binding at these two residues via a common mechanism. Mutation of both resi-

![Graph showing effect of diethylpyrocarbonate treatment (A and B) and β2(H267A) mutation (C and D) on proton sensitivity.](image)

**Fig. 7.** Effect of diethylpyrocarbonate treatment (A and B) and β2(H267A) mutation (C and D) on proton sensitivity. A: representative records show that diethylpyrocarbonate (DEPC), a histidine modifying reagent, did not prevent the effect of pH at rat α1β2γ2 receptors, but did decrease the extent to which Zn2+ inhibited GABA-gated current. The sensitivity to 500 μM Zn2+ and acidic pH (pH 5.4) was tested on the same cell in the absence or in the presence of 1 mM DEPC in the bath. B: summary of effect of DEPC treatment on GABA current inhibition induced by Zn2+ or pH 5.4. DEPC treatment significantly decreased the inhibitory effect on GABA-activated current by Zn2+ at pH 7.3 but did not affect pH sensitivity of the GABAα receptor. *P < 0.05, paired t-test, compared with control, n = 3. C and D: graph of relative responses induced by EC50 GABA plotted as a function of pH in wild-type and mutant ternary (C) and binary (D) GABAα receptors. Current activated by EC50 GABA was recorded in wild-type or mutant receptors at different pH (pH 5.4–8.4). The currents are normalized to the amplitude at pH 7.3. Each data point contains ≥8 cells. β2(H267A) mutation did not influence pH sensitivity of ternary receptors or binary receptors at pH above 6.4. However, inhibition of GABA current by pH 5.4 was significantly enhanced in α1β2(H267A) compared with wild-type α1β2 receptors. **P < 0.01, unpaired t-test, compared with wild-type.
Contrast to the report of Wilkins et al., we observed that acidic levels of acidosis (pH 5.4, Wilkins et al. 2002) for a potentiating effect of GABA currents. Additionally, the fact that we did not fully abolish inhibition of GABA currents by Zn\textsuperscript{2+} receptors. In this study, treatment with the histidine-modifying agent DEPC treatment on pH sensitivity presented here does not support the hypothesis that protons and Zn\textsuperscript{2+} share a common site of action in GABA\textsubscript{A} receptors.

Variability of pH effects on GABA\textsubscript{A} receptors

It should be noted that reported effects of pH on GABA\textsubscript{A} receptor function have varied. We have consistently observed that protons inhibit GABA\textsubscript{A} receptors; this effect has been seen in different preparations (recombinant receptors in HEK293 cells and brain slices), different species (rat, mouse, and human receptors) and different brain regions (hypothalamus, brain stem, and cortex) (this study; Huang and Dillon 1999; unpublished observations). Whereas many studies are consistent with our observations (Krishk et al. 2001; Li et al. 2003; Smart 1992; Zhai et al. 1998), others have reported no effect (Tang et al. 1990; Vylický et al. 1993) or stimulatory effects of protons on GABA\textsubscript{A} receptor function (Krishek and Smart, 1996; Pasternack et al. 1992; Robello et al. 1994; Wilkins et al. 2002). The discrepancy may be attributable to different experimental conditions, subunit compositions, or animal species. For example, experiments conducted using bath application of ligands (Krishk et al. 1996, 1998; Robello et al. 1994) cannot resolve the true peak amplitude or possible kinetic alterations of GABA-activated currents because of the slow kinetics of solution exchange. Indeed, Krishk et al. (1996) showed differential effects of pH on GABA currents using bath perfusion or U-tube application. Additionally, some studies concluded insensitivity of GABA\textsubscript{A} receptors to protons based on observations using a near saturating GABA concentration (Tang et al. 1990; Vylický et al. 1993). Considering that the pH effect is competitive with respect to GABA (Huang and Dillon 1999; Li et al. 2003; Zhai et al. 1998), this experimental approach is inadequate to fully address the question. Finally, proton sensitivity has been shown to be GABA\textsubscript{A} receptor subunit dependent (Krishk et al. 1996); a developmental effect of pH sensitivity (Krishek and Smart, 2001) is consistent with this hypothesis. Differential proton modulatory effects on currents activated by low or high concentrations of GABA in hippocampal neurons also indicate this possibility (Mozzynas et al. 2003; Pasternack et al. 1996). Thus differences in subunit combinations may also underlie the conflicting reports of proton sensitivity of GABA\textsubscript{A} receptor.

Distinct sites for proton and Zn\textsuperscript{2+} action

H267 of the β2 subunit has been reported to be accountable for a potentiating effect of GABA α1β channels by extreme levels of acidosis (pH 5.4, Wilkins et al. 2002). However, in contrast to the report of Wilkins et al., we observed that acidic pH has an inhibitory action on α1β2 receptors. Our data do not suggest that H267 of the β2 subunit plays a critical role in proton modulation of GABA\textsubscript{A} ternary α1β2y2 or binary α1β2 receptors. In this study, treatment with the histidine-modifying reagent DEPC or mutation of H267 to A greatly diminished inhibition of GABA currents by Zn\textsuperscript{2+}, confirming the role of H267 in Zn\textsuperscript{2+}-induced inhibition of the receptor. However, proton-induced inhibition of the GABA response was more strikingly inhibited in α1β2(H267A) receptors compared with wild-type α1β2 receptors. We do not have a clear explanation for the discrepancy between our results and those of Wilkins et al. (2002). However, considering this level of acidosis is unlikely to be achievable in an intact organism, the degree to which these studies relate to proton modulation of the GABA\textsubscript{A} receptor is unclear.

Our results also do not support the hypothesis that protons and Zn\textsuperscript{2+} share the same binding site on the GABA\textsubscript{A} receptor. Residues α1(E138), β2(E182), and β2(E270), along with β2(H267), have been shown to confer full sensitivity to Zn\textsuperscript{2+} in GABA\textsubscript{A} receptors (Hosie et al. 2003). However, as with the β2(H267A) mutation, we observed no change in proton sensitivity in receptors expressing α1(E138A), β2(E182A), or β2(E270A) mutations. The lack of effect of mutations or DEPC treatment on pH sensitivity presented here does not support the hypothesis that protons and Zn\textsuperscript{2+} share a common site of action in GABA\textsubscript{A} receptors.
Functional implications of proton modulation of GABA<sub>A</sub> receptors

Changes in brain pH exert profound effects on neuronal excitability. Almost all ligand-gated ion channels studied so far are modulated by changes in pH. The α1β2γ2 combination represents the largest population of native GABA<sub>A</sub> receptors and are highly expressed synthetically (soma and dendrites) and extrasynaptically in all neurons (Fritschy and Brüning 2003). Synthetic GABA<sub>A</sub> receptors are gated by near saturating GABA concentrations at the synaptic cleft and mediate fast inhibitory neurotransmission (Clements 1996). Extrasynaptic GABA<sub>A</sub> receptors are likely activated by low GABA concentration in the extracellular space and mediate tonic inhibition (see Fritschy and Brüning 2003). Based on our findings that the effect of pH is GABA concentration-dependent, changes in extracellular pH would likely have the greatest effect on extrasynaptic GABA<sub>A</sub> receptors, with resulting changes in tonic inhibition. Protons may also alter kinetics of GABAergic IPSPs during the clearance of GABA from synaptic cleft. It has been reported that, despite an increase in extracellular GABA seen in ischemic tissue, GABA<sub>A</sub> receptor function is actually depressed following an ischemic insult (see review of Green et al. 2000; Schwartz-Bloom and Sah 2001). Down-regulation of GABA<sub>A</sub> receptor function by acidic pH might contribute to the development of neuronal overexcitation elicited by pathologically conditioned conditions associated with marked acidosis. It has been shown in animal models that enhancing GABAergic inhibitory mechanisms can serve a neuroprotective role against excitotoxic insults and therefore may be a logical pharmacologic approach for the therapy of acute ischemic stroke (Green et al. 2000).

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