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Protons inhibit Cl^- conductance by direct or allosteric interaction with the GABA-binding site in the rat recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptor

Ming-De Wang *, Mozibur Rahman, Di Zhu

Umeå Neurosteroid Research Center, Department of Clinical Science, Obstetrics and Gynecology, Umeå University, Bldg 5B, 5th floor, S-901 85 Umeå, Sweden

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Abstract

Functional roles of external pH on the Cl^- conductance were examined on *Xenopus* oocytes expressing rat recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptors. Acidic pH inhibited GABA-response in a reversible and concentration-dependent manner, significantly increasing the EC_{50} without appreciably changing the slope or maximal currents induced by GABA in the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ receptors. In contrast, protonation did not influence the pentobarbital-gated currents in the $\alpha_1\beta_2\gamma_{2L}$ receptors, suggesting that protons do not modulate channel activity by directly affecting the channel gating process. Protons competitively inhibited the bicuculline-induced antagonism on GABA in the $\alpha_1\beta_2\gamma_{2L}$ receptors. The data support the hypothesis that protons inhibit $GABA_A$ receptor function by direct or allosteric interaction with the GABA-binding site.

Keywords: Proton; GABA; Pentobarbital; $GABA_A$ receptor; *Xenopus* oocyte

1. Introduction

$GABA_A$ receptors are known to possess a variety of allosteric binding sites from which a number of drugs can modulate receptor function. Pharmacological and physiological properties observed for native neuronal $GABA_A$ receptors are most frequently reproduced by the expression of different combinations of α-, β- and γ-subunits. It is well-known that some pharmacological properties of recombinant $GABA_A$ receptors are critically dependent on the receptor subtypes present within the receptor complex.

Variations in the level of endogenous ions in the CNS (central nervous system) represent a rapid and effective method for regulating receptor function (Pasternack et al., 1996). Protons are naturally occurring in all tissues and thus constitute part of the physiological milieu to which receptors are normally exposed. 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; Krishek and Smart, 2001; Zhai et al., 1998). Recombinant $GABA_A$ receptors are differentially modulated by external pH depending on the subunit composition, which may partly explain why native $GABA_A$ receptors exhibit variable sensitivity to protons (Krishek et al., 1996). Subsequent single-channel studies in neurons indicated that raised H^+ concentrations caused an inhibition of GABA-activated responses by reducing the single-channel open probability with no effect on channel conductance (Krishek and Smart, 2001).

Evidently, protons modulate $GABA_A$ receptor by acting at a site (or sites) in the extracellular domain of the receptor, but the qualitative influences of H^+ is variable among different types of neurons (Kaila, 1994). In the present study we investigated the functional basis of pH sensitivity on the rat recombinant $GABA_A$ receptor over a pH range from 5.0 to 9.4 by studying the effect of proton on GABA- and pentobarbital-activation of channel openings. Protonation and subsequent determination of pK_a values may allow a broad identification of the key amino acid residues that are involved in the receptor function. We adopted a pH model developed by Krishek and co-workers (1996) which was derived by assuming that the receptor protein

* Corresponding author. Tel.: +46 90 785 3323; fax: +46 90 77 60 06.
E-mail address: mingde.wang@obgyn.umu.se (M.-D. Wang).

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can be represented as a weak diprotic acid and therefore possesses at least two sites for H^+ that influence the GABA-activated conductance. The present study provided evidence that protons inhibit $GABA_A$ receptor by influencing the GABA-binding site instead of modulating channel activity by directly affecting the channel gating process.

2. Materials and methods

2.1. Drug preparation

Unless otherwise stated, chemicals used were from RBI/Sigma (St. Louis, MO, USA). 2-[*N*-Morpholino]ethanesulfonic acid (MES, pK_a=6.1), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pK_a=7.5) and tris (hydroxylmethyl) aminomethane (PK_a=8.5) were used to buffer the external solution (ND96) with various pH. The composition of the standard ND96 solution was (in mM): 96 NaCl, 1 KCl, 1 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES at pH 7.4. HEPES was used to buffer the external solution with pH in the range of 6.8–8.0. For external solution with pH between 5.0 and 6.8, MES was used at concentration of 15 mM. In experiments performed by using external solutions with pH higher than 8.0, Tris was used at concentration of 20 mM. pH of the final external solutions was altered by addition of NaOH or HCl and routinely checked before and during experiments. When drugs were to be applied at different pH values, the cell was first superfused with external solution at the new pH for at least 1 min to inactivate proton-evoked currents. Pentobarbital with a constant pK_a at 8.0 was dissolved in 0.1 M NaOH as a stock solution of 25 mM, and subsequently diluted in external solution (ND96) with adjusted pH. All chemicals were then diluted in external solution for experiments.

2.2. In vitro transcription and expression in the Xenopus oocyte

Sexually mature female *Xenopus* laevis (Horst Kähler; Bedarf für Forschung and Lehre, Hamburg, Germany) were kept in optimal condition by authorized animal keepers in local animal house and fed with standard frog food. Experimental protocols were approved by the Animal Experimentation Ethical Committee in Umeå. Stage V–VI oocytes were harvested under 0.1% tricaine (3-aminobenzoic acid ethyl ester) anaesthesia. Oocytes were defolliculated by shaking for 20 min at 37 °C in collagenase (2 mg/ml) dissolved in calcium-free solution containing (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES at pH 7.4. Capped mRNAs, encoding rat $GABA_A$ receptor subunits were transcribed in vitro using the mMESSAGE mMachine ™ T7 kit (Ambion, Austin, TX, USA) according to manufacturer's instructions from linearized pBluescript vectors containing receptor coding regions. Subunit transcripts were injected in equimolar ratios (20–40 ng total RNA) to construct the binary (1:1) and ternary receptor (1:1:1) at 24 h following defolliculation. A Nanoject II auto nanoliter injector (Drummond scientific company, Broomall, PA, USA) was used in mRNA microinjection. Oocytes were incubated up to 3 days at 18 °C in the standard ND96 at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (50 μg/ml).

2.3. Oocyte electrophysiology

Two-electrode voltage-clamp whole-cell recording were performed with a Warner OC725 amplifier (Warner instrument corporation, Hamden, CT, USA) 2–3 days following RNA injection. The extracellular recording solution was ND96 medium with no supplements. Intracellular recording pipettes were filled with 3 M KCl and had open tip resistances of ~1 MΩ. Drugs were bath applied from a common tip via a gravity-driven multibarrel drug-delivery system—ValveLink 16 pinch valve perfusion system which was controlled by a Valvelink 16 controller (AutoMate Scientific, Inc, San Francisco, CA, USA). Drugs were always co-applied with GABA or pentobarbital and were not pre-applied in the absence of GABA or pentobarbital. Cells were clamped at −70 mV for all experiments, and the steady-state current at the end of 20 s drug applications was measured for quantification of current amplitudes.

2.4. Data analysis

Data acquisition and analysis were performed with pClamp software (Axon Instruments, San Francisco, CA, USA). Data plotting and curve fitting were done with Sigma Plot software (SPSS, Chicago, IL, USA). Data are presented in the text and figures as mean±S.E.M. Statistical differences were determined using a two-tailed Student's *t*-test. The percentage of modulation of GABA- and pentobarbital-activated current was calculated as $(I_M - I_N)/I_N$, where I_N (normalizing current) and I_M (measured current) are the amplitudes of the GABA- or pentobarbital-activated current in the absence and presence of the test substance, respectively. Fitting of the dose–response relationships were performed using the Hill equation as follows: $I = I_{max} \times \frac{C^n}{(EC_{50}^n + C^n)}$, where C is the concentration of drug, I_{max} is the maximum current amplitude, EC_{50} is the concentration of drug that produces 50% of I_{max}, and n is the Hill coefficient.

The pH titration data were fitted according to the following function reported earlier (Krishek et al., 1996): $I = f\left(\frac{K_{a1}(nK_{a2} + 2m[H^+]) + 1[H^+]^2}{[H^+]^2 + K_{a1}(2[H^+] + K_{a2})}\right)$. The normalized GABA-activated conductance is most simply related to the three states of the receptor (uncharged, mono- and divalent anions) as a function of the relative titration of the 'diprotic' receptor protein. The terms l, m and n are the relative contributions each form of the receptor protein (P) makes to the overall titration curve, where l weights the unassociated receptor protein (PH_2), m the monovalent anion (PH^-) and n the divalent anion (P^{2-}). This function provided estimates of pK_a values (pK_{a1} and pK_{a2}, where pK_{aj}=−log K_{aj}, and K_{aj} represents an acid dissociation constant). The experimental data were fitted with this function using a non-linear least-squares routine.

3. Results

To illustrate the sensitivity of the recombinant $GABA_A$ receptors to external pH and to assess the appropriateness of pH model used to fit the experimental data in the pH titration plots, GABA-activated currents were recorded from the oocyte expressing $\alpha_1\beta_2\gamma_{2L}$ receptor. Deviation in external pH on either side of 7.4 resulted in a reversible (Fig. 1A) and $[H^+]$-dependent inhibition of steady-state response to GABA (Fig. 2A). Acidic pH decreased GABA sensitivity by shifting its dose–response curve to the right, significantly increasing the GABA EC_{50} from 12.9 μM at pH 7.4 to 458.6 μM at pH 5.0 ($P<0.001$). The slope of GABA dose–response curve was not significantly changed (2.3 at pH 7.4 and 2.5 at pH 5.0, Fig. 2C) and the maximal currents induced by GABA were not altered between pH 7.4 and pH 5.0 (Fig. 3A and B). When plotting the steady-state GABA dose–response according to Hill equation at pH 7.4 and 5.0, a competitive antagonism between GABA and H^+ was revealed (Fig. 2C) and the corresponding pK_B value determined by Schild analysis was 3.70. From these data it was reasonable to draw the conclusion that H^+either interacted with GABA binding site as a specific antagonist or there was a significant decrease of active GABA molecules when lowering external pH from 7.4 to 5.0.

On the other hand, increasing the external pH from 7.4 to 9.4 resulted in a reduction of the steady-state GABA response (Fig. 1A). The change in the dose–response curve–a decrease in the slope from 2.3 at pH 7.4 to 1.6 at pH 9.4 and maximal current induced by GABA, but no significant change in the EC_{50} values from 12.9 μM at pH 7.4 to 11.7 μM at pH 9.4–is not consistent with a competitive antagonism by H^+ (Fig. 2C). This line of evidence argues strongly against the view that H^+ interacts with GABA binding site as an competitive antagonist since an expected parallel rightward shift of GABA–dose response curve was absent as $[H^+]$ was increased by 0.01 M from pH 9.4 to 7.4. The GABA molecule has two potentially charged centers ($–NH_2$ and –COOH) which are affected by external pH. With the amino acid and carboxyl groups, GABA analogues can exist in up to three different charged forms: anionic, zwitterionic and cationic. The pK_a values for the two charged centers are 4.0 (–COOH, pK_{a1}) and 10.55 ($–NH_2$, pK_{a2}). Based on Henderson–Hasselbach equation: $K_{a1}=\frac{[H^+][A^-]}{[A]}$, where $\frac{[A^-]}{[A]}$ represents the ratio between zwitterionic/cationic molecules, it is possible to predict the charge behavior of the GABA molecule at different external pH values and estimate the corresponding percentage of the three molecules in solution. Over the range of external pH 5.0–9.4, between 92% and 100% of GABA molecules exist as zwitterions (Krishek et al., 1996; Roberts and Sherman, 1993).

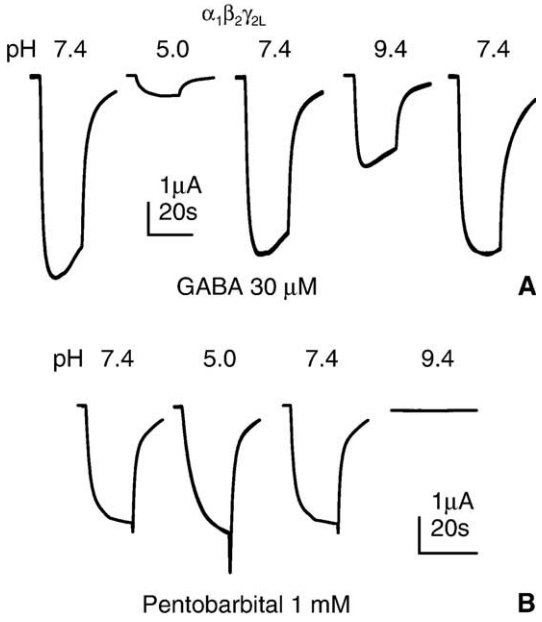

Fig. 1. Deviation in external pH from 7.4 caused a reversible change of GABA-activated and pentobarbital-gated currents in the $\alpha_1\beta_2\gamma_{2L}$ receptor. (A) Sample traces showing consecutive application of 30 μM GABA at pH 5.0, 7.4 and 9.4 on one oocyte expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor. The interval between GABA applications was 120 s. Note that the effect of external pH on GABA-activated currents was reversible. (B) Sample traces showing consecutive application of 1 mM pentobarbital at pH 5.0, 7.4 and 9.5 on another oocyte expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor. The interval between pentobarbital applications was 120 s. Note that lowering external pH from 7.4 to 5.0 caused a reversible change in the steady-state currents gated by pentobarbital. Voltage was clamped at −70 mV in all tested cells.

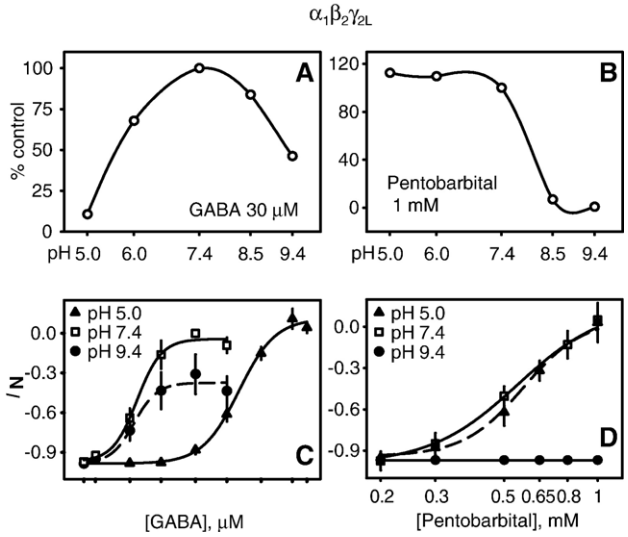

Fig. 2. The influence of external pH on GABA-activated and pentobarbital-gated currents in the rat recombinant $\alpha_1\beta_2\gamma_{2L}$ receptor. (A) Normalized pH titration relationships for the currents activated by 30 μM GABA in the $\alpha_1\beta_2\gamma_{2L}$ receptor ($n=5$). Each data point was normalized to the GABA-activated current at pH 7.4. The curve was generated according to the pH model. (B) Normalized pH titration relationships for the current directly gated by 1 mM pentobarbital in the $\alpha_1\beta_2\gamma_{2L}$ receptor ($n=5$). Each data point was normalized to the pentobarbital-gated current at pH 7.4. (C) Normalized GABA concentration–response curves in the $\alpha_1\beta_2\gamma_{2L}$ receptor at external pH 7.4; pH 5.0 and pH 9.4. Each *point* is calculated relative to the normalizing current response (I_N) activated by 100 μM GABA at pH 7.4. Curves shown are fits to the Hill equation, with EC_{50} values and Hill slopes of 12.9 μM/2.3 ($N=6$) at pH 7.4; 458.6 μM/2.5 ($N=4$) at pH 5.0 and 11.7 μM/1.6, ($N=6$) at pH 9.4, respectively. (D) Normalized concentration–response curves of pentobarbital-gating effect in the $\alpha_1\beta_2\gamma_{2L}$ receptor at pH 7.4; pH 5.0 and pH 9.4. Each *point* is calculated relative to the normalizing current response (I_N) gated by 1 mM pentobarbital at pH 7.4. Curves shown are fits to the Hill equation, with EC_{50} values and Hill slopes of 552 μM/3.1 ($N=5$) at pH 7.4 and 578 μM/4.5 ($N=6$) at pH 5.0, respectively. Note that no pentobarbital-gated currents were recorded at external pH 9.4.

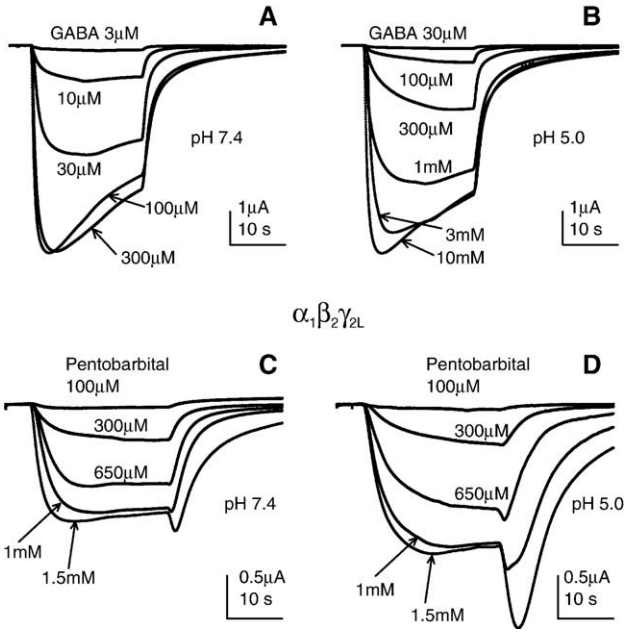

Fig. 3. Protons inhibited GABA-activated currents, but not pentobarbital-gated currents in the $\alpha_1\beta_2\gamma_{2L}$ receptor. Sample traces showing GABA-activated currents at pH 7.4 (A) and pH 5.0 (B) in the same cell. Note that the no appreciable change in the maximal currents by GABA. Sample traces showing pentobarbital-gated currents at pH 7.4 (C) and pH 5.0 (D) in another cell. Note that the steady-state current amplitude increased less significantly than the "rebound" response of pentobarbital-gated current. Voltage was clamped at −70 mV in all tested cells.

Therefore we concluded that the antagonism of H^+ on GABA response when external pH deviated from neutral value of 7.4 was not dependent on the reduction of GABA molecules as zwitterions.

The GABA (30 μM) titration curve obtained in the $\alpha_1\beta_2\gamma_{2L}$ receptor (Fig. 2A) agreed well with that reported earlier (Krishek et al., 1996). According to the pH model, pH titration predicted the existence of two sites with pK_a values of 5.2 and 9.4. The present study revealed that both extracellular alkaline and acid media suppressed GABA-activated currents in a rapid and reversible manner (Fig. 1A). Acidification did not affect the maximal currents induced by GABA, whereas alkalization reduced the efficacy of GABA (reduced maximal response) without significant change in its potency (EC_{50}). It is likely, however, that the effect of H^+ is not due to alteration of the GABA molecule, but instead due to protonation of the GABA-binding site.

Barbiturates have previously been shown to have three actions on $GABA_A$ receptors: a potentiation of GABA responses (Evans, 1979; Study and Barker, 1981), a direct activation of $GABA_A$ receptors (Franks and Lieb, 1994; Robertson, 1989) and, at high concentrations, a block of the $GABA_A$ receptor-coupled chloride channel (Kirkness and Turner, 1988; Peters et al., 1988; Robertson, 1989). The characterized site for pentobarbital binding is distinct from the GABA-binding site (Amin and Weiss, 1993). In order to verify if protons act primarily by affecting channel gating, we continued to examine the action of external pH on current directly gated by pentobarbital in the $\alpha_1\beta_2\gamma_{2L}$ receptor.

Lowering pH from 7.4 to 5.0 caused a reversible (Fig. 1B) and marginal increase of steady-state current directly gated by pentobarbital (Fig. 3C and D). The potency of pentobarbital to gate open the Cl^- channel was not changed significantly (EC_{50}=552 μM at pH 7.4; EC_{50}=578 μM at pH 5.0, Fig. 2D). On the other hand, increasing external pH to 9.4 was associated with a complete loss of gating effect by pentobarbital (Figs. 1B and 2B and D). It is known that barbiturates carry a negative charge on the oxygen at 4-position (O^4) of the six-member heterocyclic ring at a pH of 9.0–10, making this ionized form of pentobarbital totally inert on the $GABA_A$ receptor. Based on the ionization constant pK_a=8, it can be calculated that pentobarbital is 75% and 50% ionized at pH 8.5 and 8.0 respectively, whereas at physiological pH 7.4 it is approximately 25% ionized and 75% in the uncharged form. At pH 5.0 more than 99% of pentobarbital molecule is in uncharged form (Robertson, 1989). The titration curve of current directly gated by pentobarbital supported the view that uncharged pentobarbital molecule is the active form that directly activated ion channels. Lack of proton effect on pentobarbital-gated channel opening in acidic media strongly argues against the possibility that protons modulate $GABA_A$ receptor by alternating the gating mechanism at the level of the channel pore.

These findings reveal that the competitive inhibition of protons on the GABA-activated current did not involve channel gating, which leads us to believe that protons may alter the sensitivity of the GABA-binding site. In a series of studies in the binary $\alpha_1\beta_2$ receptor, we observed that the potency to open the Cl^- channel by GABA and pentobarbital increased significantly ($P<0.01$ and $P<0.05$, respectively) as γ_2-subunit was deleted from the GABA-binding site (Fig. 4A and B). This line of evidence indicated that the γ_2-subunit plays an important role on the apparent efficacy of GABA and pentobarbital to activate Cl^- channel. Therefore, we continued to investigate the effect of protons on the GABA response in the γ_2-less $GABA_A$ receptor. Acidic pH induced a similar parallel rightward shift of GABA–dose curve in the $\alpha_1\beta_2$ receptor (Fig. 4C). However, the pK_B was reduced to 3.0 from 3.70 obtained in the $\alpha_1\beta_2\gamma_{2L}$ receptor. Increasing the external pH from 7.4 to 9.4 caused an inhibition of maximal GABA response in the $\alpha_1\beta_2$ receptor compatible to that seen in the $\alpha_1\beta_2\gamma_{2L}$ receptor (Figs. 2C and 4C). Despite a significant increase in channel sensitivity to GABA and pentobarbital in the $\alpha_1\beta_2$ receptor, the effect of external pH on GABA-mediated current was significantly reduced in the $\alpha_1\beta_2$ receptor in comparison with the $\alpha_1\beta_2\gamma_{2L}$ receptor. It is possible that the degree of protonation linked to the GABA-binding site was lower in the γ_2-less $GABA_A$ receptor.

Bicuculline methiodide (BMI) is a specific antagonist on the GABA binding site and we expected that protons would also competitively inhibit the BMI effect if protons alter the sensitivity of the GABA-binding site. As external pH dropped from 7.4 to 6.0 (Fig. 4D), the dose–response curve of BMI to block 10 μM GABA-activated currents in the $\alpha_1\beta_2\gamma_{2L}$ receptor shifted to the right and the IC_{50} of BMI increased significantly by 3.4-fold from 1.1 μM at pH 7.4 to 3.6 μM at pH 6.0 ($P<0.05$). The block was complete at [BMI]>30 μM and Hill coefficients were close to 1, suggesting that the block can be

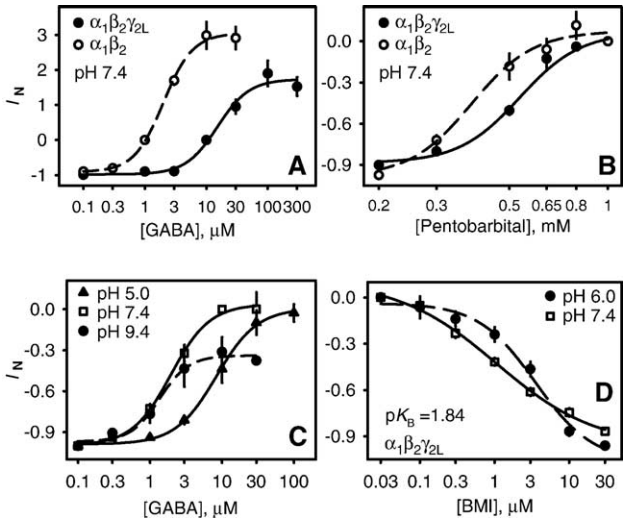

Fig. 4. The effect of γ_2-subunit on the GABA-activated and pentobarbital-gated Cl^- conductance and the effect of protons on the biccuculine-binding. (A) Concentration–response curves of GABA in the $\alpha_1\beta_2\gamma_{2L}$ receptor and $\alpha_1\beta_2$ receptor at pH 7.4. Each *point* is calculated relative to the normalizing current (I_N) activated by 10 μM GABA in the $\alpha_1\beta_2\gamma_{2L}$ receptor and 1 μM GABA in the $\alpha_1\beta_2$ receptor, respectively. Curves shown are best fits to the Hill equation with EC_{50} values of 15.2 μM (N=6) and 2.0 μM (N=5) for the $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ receptor, respectively. The Hill slopes for the two curves were 1.7 and 1.8, respectively. (B) Concentration–response curves of pentobarbital-gated current in the $\alpha_1\beta_2\gamma_{2L}$ receptor and $\alpha_1\beta_2$ receptor at pH 7.4. I_N represents currents directly gated by 1 mM pentobarbital in the respective subtype. Curves shown are fits to the Hill equation with EC_{50} values and Hill slopes of 538 μM/4.7 (N=6) in the $\alpha_1\beta_2\gamma_{2L}$ receptor and 384 μM/4.6 (N=7) in the $\alpha_1\beta_2$ receptor. (C) Normalized GABA concentration–response curves in the $\alpha_1\beta_2$ receptor at pH 7.4, pH 5.0 and pH 9.4. Each *point* is calculated relative to the normalizing response (I_N) activated by 10 μM GABA at pH 7.4. Curves shown are fits to the Hill equation, with EC_{50} values and Hill slopes of 1.9 μM/1.6 (N=5) at pH 7.4, 8.4 μM/1.5 (N=9) at pH 5.0 and 1.4 μM/2.1 (N=7) at pH 9.4, respectively. (D) Normalized concentration–response curves of bicuculline methiodide (BMI) to block currents activated by 10 μM GABA at pH 7.4 and pH 6.0 in the $\alpha_1\beta_2\gamma_{2L}$ receptor. Each *point* is calculated relative to the normalizing response (I_N) activated by 10 μM GABA at pH 7.4 or pH 6.0, respectively. Curves shown are fits to the Hill equation, with IC_{50} values and Hill slopes of 1.1 μM/−0.9 (N=6) at pH 7.4 and 3.6 μM/−1.1 (N=6) at pH 6.0, respectively.

produced after the binding of a single antagonist molecule. BMI is a weak base with pK_a value of 4.8. At the pH range between 6.0 and 7.4, 97% to 100% of BMI exists as the unionized form based on the Henderson–Hasselbach equation. Thus we concluded that protons are weak competitive inhibitors to bicuculline-binding (pK_B=1.84). The reduced potency of bicuculline at acidic pH is likely caused by protonation of the GABA-binding site.

4. Discussion

In the present study, we want to define whether the proton-induced rightward shift in the GABA dose–response curve is due to a competitive inhibition on the GABA-binding site or an allosteric modulation of the Cl^- channel. The directly gated current by pentobarbital was not sensitive to acidic pH, which argues against the view that protons inhibit $GABA_A$ receptor function by directly altering channel gating at the channel pore. One important piece of evidence supporting protonation of the GABA-binding site is that protons also competitively inhibit the action of bicuculline. The potency of bicuculline to block GABA response decreased at acidic pH in the $\alpha_1\beta_2\gamma_{2L}$ receptor. This leads to the view that protons can inhibit GABA activation by influencing the GABA binding site. In a recent study employing model stimulation to quantitatively analyze proton modulation of GABA-activated current from hippocampus neurons, it was shown that part of proton effect is mediated by a reduction of GABA-binding rate (Mozrzymas et al., 2003). Wilkins et al. (2002) identified a histidine residue at 267 of the β_2 subunits that is accountable for a potentiating effect of acidic pH on GABA response recorded in murine $\alpha_1\beta_{1/2}$ $GABA_A$ receptors expressed in human embryonic kinkey cells. They demonstrated that site-specific substitution of H267A abolished modulation of GABA-activated current at pH 5.4 and interpreted proton modulation of the $GABA_A$ receptor as a direct protonation of H267 on $\alpha_1\beta_{1/2}$ receptors rather than involvement in signal transduction (Wilkins et al., 2002). However, a recent report by Huang and co-authors (Abraham et al., 1970; Huang et al., 2004) revealed that acidic pH has an inhibitory action on GABA response recorded from recombinant human $\alpha_1\beta_2\gamma_{2L}$ receptors. The authors observed that proton-induced inhibition of GABA response was not blocked by DEPC (diethyl pyrocarbonate) treatment or the H267A mutation as Wilkins et al. (2002) reported earlier. In agreement with our results from *rat* recombinant $GABA_A$ receptor expressed in *Xenopus* oocyte, the report by Huang et al. (2004) from *human* recombinant $GABA_A$ receptor transfected in human embryonic kidney (HEK) 293 cells supports the view that protons inhibit $GABA_A$ receptor function by direct or allosteric interaction with the GABA binding site.

Previous studies reported that GABA response is inhibited by extracelluar pH (Huang and Dillon, 1999; Smart, 1992; Zhai et al., 1998). However some other reports suggest that an enhancement of GABA response by pH (Feng and Macdonald, 2004; Pasternack et al., 1996). One explanation of these different results is due to the difference in the GABA application kinetics. Bath application of GABA is much slower than channel activation and thereby the effect of modulators on steady-state response can be resolved. It cannot detect the peak current amplitude. This was noted in the report of Krishek and co-workers (1996). Another factor that may be involved in discrepancies is that the pH effect on the $GABA_A$ receptor changed with substitution of γ-subunit to δ subunit (Feng and Macdonald, 2004). An earlier report by Pasternack and co-workers (1996) also mentioned that the presence of more than one population of $GABA_A$ subunit in hippocampal CA1 area revealed different affinity for $GABA_A$ receptor.

In summary, results from the present study indicate that protons inhibit the recombinant $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ $GABA_A$ receptor in a reversible and dose-dependant manner. Pentobarbital-gated currents remain less influenced when external pH was lowered from the physiological level, suggesting that protons do not affect the channel opening process. Our results clearly indicated that the uncharged form of molecule is essential to confer the sensitivity of pentobarbital to directly

gate the ion channel in the $GABA_A$ receptor. Bicuculline-induced GABA antagonism is also affected by external pH, which supports the view that protons directly or allosterically interact with the GABA-binding site.

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