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Mechanisms of H^+ modulation of glycinergic response in rat sacral dorsal commissural neurons

Yan-Fang Li *‡, Long-Jun Wu *‡, Yong Li *‡, Lin Xu † and Tian-Le Xu *‡

** Department of Neurobiology and Biophysics, University of Science and Technology of China, Hefei 230027, † Laboratory of Learning and Memory, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223 and ‡ Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*

Many ionotropic receptors are modulated by extracellular H^+. So far, few studies have directly addressed the role of such modulation at synapses. In the present study, we investigated the effects of changes in extracellular pH on glycinergic miniature inhibitory postsynaptic currents (mIPSCs) as well as glycine-evoked currents (I_{Gly}) in mechanically dissociated spinal neurons with native synaptic boutons preserved. H^+ modulated both the mIPSCs and I_{Gly} biphasically, although it activated an amiloride-sensitive inward current by itself. Decreasing extracellular pH reversibly inhibited the amplitude of the mIPSCs and I_{Gly}, while increasing external pH reversibly potentiated these parameters. Blockade of acid-sensing ion channels (ASICs) with amiloride, the selective antagonist of ASICs, or decreasing intracellular pH did not alter the modulatory effect of H^+ on either mIPSCs or I_{Gly}. H^+ shifted the EC_{50} of the glycine concentration–response curve from 49.3 ± 5.7 μM at external pH 7.4 to 131.5 ± 8.1 μM at pH 5.5, without altering the Cl^- selectivity of the glycine receptor (GlyR), the Hill coefficient and the maximal I_{Gly}, suggesting a competitive inhibition of I_{Gly} by H^+. Both Zn^{2+} and H^+ inhibited I_{Gly}. However, H^+ induced no further inhibition of I_{Gly} in the presence of a saturating concentration of Zn^{2+}. In addition, H^+ significantly affected the kinetics of glycinergic mIPSCs and I_{Gly}. It is proposed that H^+ and/or Zn^{2+} compete with glycine binding and inhibit the amplitude of glycinergic mIPSCs and I_{Gly}. Moreover, binding of H^+ induces a global conformational change in GlyR, which closes the GlyR Cl^- channel and results in the acceleration of the seeming desensitization of I_{Gly} as well as speeding up the decay time constant of glycinergic mIPSCs. However, the deprotonation rate is faster than the unbinding rate of glycine from the GlyR, leading to reactivation of the undesensitized GlyR after washout of agonist and the appearance of a rebound I_{Gly}. H^+ also modulated the glycine cotransmitter, GABA-activated current (I_{GABA}). Taken together, the results support a 'conformational coupling' model for H^+ modulation of the GlyR and suggest that H^+ may act as a novel modulator for inhibitory neurotransmission in the mammalian spinal cord.

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Corresponding author T.-L. Xu: Institute of Neuroscience, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, 320 Yue-yang Road, Shanghai 200031, China. Email: tlxu@ion.ac.cn

Tissue acidosis, which is a dominant factor in inflammation, tumours and ischaemia/hypoxia, contributes to pain sensing and hyperalgesia (Reeh & Steen, 1996). Under these conditions, interstitial fluid pH can decrease to 6.2 or even lower (Cobbe & Poole-Wilson, 1980). Neurons detect changes in extracellular pH by at least two distinct mechanisms: one is the activation of acid-gated ionic channels such as the vanilloid receptor (VR1) (Liu & Simon, 2000) and the acid-sensing ion channels (ASICs) (Waldmann & Lazdunski, 1998; Reeh & Kress; 2001); the other is the modulation of numerous voltage-gated and ligand-gated ion channels by extracellular H^+ (Deitmer & Rose, 1996). In regard to the latter, the NMDA receptor (Tang *et al.* 1990; Traynelis & Cull-Candy, 1990), the AMPA receptor (Ihle & Patneau, 2000), the $GABA_A$ receptor ($GABA_AR$) (Krishek & Smart, 2001; Wilkins *et al.* 2002), the glycine receptor (GlyR) α1 subunit (Harvey *et al.* 1999), and voltage-gated Ca^{2+} (Delisle & Satin, 2000) and Na^+ channels (Benitah *et al.* 1997) are all known targets. It has been proposed that ASICs play multiple roles in various functions, such as light touch, heavy touch, sour taste, pain, learning and memory (Bianchi & Driscoll, 2002). The functional significance of pH modulation of neuronal receptors, however, is still far from understood. In particular, few studies have directly addressed the role of such modulation on synaptic currents.

The GlyR and the $GABA_AR$ are the two major inhibitory receptors in the adult mammalian spinal cord and brainstem (Legendre, 2001; Watanabe *et al.* 2002) where they play important roles in the ultimate coordination of neuronal excitability and sensory activity. Whereas agents that enhance inhibitory neurotransmission, such as GlyR

and $GABA_AR$ agonists, have been explored as drugs to treat pain and stroke (Schwartz-Bloom & Sah, 2001; Ahmadi *et al*. 2002; Malan *et al*. 2002), the possible interaction between H^+ and the GlyR is not fully understood at the neuronal level. Furthermore, there is no direct evidence on whether and how extracellular pH modulates glycinergic neurotransmission. In this study, we demonstrate that acidification modulates glycinergic miniature inhibitory postsynaptic currents (mIPSCs) and the glycine-activated current (I_{Gly}) as well as inducing an amiloride-sensitive current in neurons mechanically dissociated from the rat sacral dorsal commissural nucleus (SDCN), which is implicated in pain perception (Lu *et al*. 1995). Both peak amplitude and the kinetics of glycinergic mIPSCs and the I_{Gly} are regulated. The H^+ modulation of the glycinergic response *in vitro* presumably reflects the pH sensitivity of the GlyR *in vivo*. Therefore, the present data provide novel insights into the pathology of such neuronal disorders as spinal injury and/or ischaemia and suggest the GlyR as a potential target of new pharmacological therapies.

METHODS

Preparation

The animal care and experimental protocols were approved by the Anhui Health Department, China. Neurons from the sacral dorsal commissural nucleus (SDCN) were mechanically dissociated as described previously (Xu, 1999; Wang *et al.* 2001). In brief, 2-week-old Wistar rats were anaesthetized with pentobarbital sodium (45–50 mg kg^{-1}, I.P.) and a laminectomy was performed to expose the lower lumbar and sacral spinal cord. A segment of the lumbosacral (L5–S3) cord was quickly dissected out and immersed in an ice-cold incubation solution (see below for composition). The animal was then decapitated. After removal of the attached dorsal rootlets and the pia mater, the spinal segment was sectioned at 400 μm with a vibratome tissue slicer (LEICA VT1000S, Leica Instruments Ltd, Wetzlar, Germany). The slices were preincubated in a well-oxygenated incubation solution for 50 min at room temperature (22–25 °C). A vibration-isolation system (Kay & Wong, 1986; Wu *et al.* 2001) was then used to mechanically dissociate the SDCN neurons. Briefly, a fire-polished glass pipette mounted on a vibrator was placed lightly on the surface of the SDCN region of the slice and vibrated horizontally at 5–10 Hz for about 5 min under the control of a pulse generator. After 20 min of dissociation, the mechanically isolated neurons attached to the bottom of the culture dish and were ready for electrophysiological experiments.

Solutions

The ionic composition of the incubation solution was (mM): 124 NaCl, 24 $NaHCO_3$, 5 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose, aerated with 95 % O_2–5 % CO_2 to a final pH of 7.4. The standard external solution contained (mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes or Mes, and 10 glucose. The pH was adjusted to 7.4 with tris(hydroxymethyl)aminomethane (Tris-base). The osmolarity of all external solutions was adjusted to 325–330 mosmol l^{-1} with sucrose. The patch pipette solution for whole-cell patch recording was (mM): 120 KCl, 30 NaCl, 0.5 $CaCl_2.2H_2O$, 1 $MgCl_2.6H_2O$, 5 EGTA, 2 MgATP and 10 Hepes; pH was adjusted to 7.2 with Tris-base. The low-Cl^- pipette solution contained (mM): 113 potassium-D-gluconate, 30 sodium-D-gluconate, 7 KCl, 0.5 $CaCl_2.2H_2O$, 1 $MgCl_2.6H_2O$, 5 EGTA, 2 MgATP and 10 Hepes with pH adjusted to 7.2 with Tris-base. The osmolarity of both pipette solutions was adjusted to 280–300 mosmol l^{-1} with sucrose. The external pH was adjusted to different values by addition of 1 N NaOH or 1 N HCl and was routinely checked before and during experiments. When the current–voltage (*I–V*) relationships for the glycine response at different extracellular pH were examined, 0.3 μM tetrodotoxin (TTX) and 100 μM $CdCl_2$ were added to the standard external solution, and KCl was replaced with equimolar CsCl in the internal solution.

Drugs and application system

Drugs used in the present experiments were from Sigma. They were first dissolved in ion-free water and then diluted to final concentrations in the standard external solution just before use. Drugs were applied using a rapid application technique termed the 'Y-tube' method, which allows a complete exchange of external solution surrounding a neuron within 20 ms (Murase *et al*. 1990).

Electrophysiological recordings

The electrophysiological recordings were performed in the conventional whole-cell patch-clamp recording configuration under voltage-clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm (Narishige, Tokyo, Japan) on a two-stage puller (PP-830, Narishige). The resistance between the recording electrode filled with pipette solution and the reference electrode was 5–7 MΩ. The liquid junction potential was 3–4 mV, and it was used to calibrate the holding potential (V_H). Membrane currents were measured using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA, USA), filtered at 1 kHz, sampled and analysed using a DigiData 1320A interface and a computer with the pCLAMP system (Version 8.0, Axon Instruments). The series resistance, estimated from optical cancellation of the capacity transient, was 10–30 MΩ and in most experiments, 70–90 % series resistance was compensated. The membrane potential (V_H) was held at −50 mV throughout the experiment except when the current–voltage (*I–V*) relationships were examined. All experiments were carried out at room temperature (22~25 °C).

Data analysis

Clampfit software was used for data analysis. The continuous theoretical curves for concentration–response relationships of glycine at pH 7.4 and 5.5 were drawn according to a modified Michaelis–Menten equation by the method of least-squares (the Newton–Raphson method) after normalizing the amplitude of the response:

$$I = I_{max}C^h/(C^h + EC_{50}{}^h), \quad (1)$$

where I is the normalized value of the current, I_{max} the maximal response, C the drug concentration, EC_{50} the concentration that induced the half-maximal response and h the apparent Hill coefficient. The curve for the effect of H^+ on I_{Gly} was also fitted using the following equation:

$$I = I_{max}(IC_{50})^h/(C^h + IC_{50}{}^h), \quad (2)$$

where IC_{50} represents the concentration that induced the half-maximal inhibitory effect and the other abbreviations are as defined above. All data were calculated as the means ± S.E.M. When examining evoked currents, statistical comparison was carried out using Student's *t* test for two-groups comparisons and one-way analysis of variance (ANOVA) for multiple comparisons. While examining the H^+ effect on mIPSCs, events were counted

and analysed using the Mini Analysis Program (Version 4.3.3, Synaptosoft, Inc., Leonia, NJ, USA) and a detection threshold of 5–15 pA was applied, depending on the noise level. Analysis of mIPSCs was performed with cumulative probability plots and the cumulative histograms were compared using the Kolmogorov–Smirnov test for significant differences. Statistically significant differences were assumed when $P < 0.05$ for all data. P and n represent the level of significance and the number of neurons, respectively.

RESULTS

H^+-gated currents in rat sacral dorsal commissural nucleus (SDCN) neurons

In keeping with histological observations, the dissociated SDCN neurons were morphologically heterogeneous (Sasek *et al.* 1984; Lu *et al.* 1995; Xu *et al.* 1998). Most of the neurons were medium sized (10–15 μm in diameter) with oval or triangular somata and one to three apical stem dendrites. Neurons were randomly selected for recording. Fast drops of extracellular pH evoked rapidly desensitizing inward currents in most neurons examined at a holding potential (V_H) of −50 mV, while an increase of extracellular pH did not induce detectable current. Figure 1*A* shows the concentration dependence of the H^+-evoked current, the amplitude of which increased with decreasing extracellular pH. The H^+-evoked current could be reversibly inhibited by 100 μM amiloride (Fig. 1*B*), the selective antagonist for cloned acid-sensing ion channels (ASICs) (Tang *et al.* 1988; Waldmann *et al.* 1997), in a dose-dependent manner. Moreover, a Na^+-free extracellular solution markedly depressed the amplitude of the proton-evoked current, indicating that the current was mainly carried by Na^+ (data not shown).

H^+ modulation of glycinergic mIPSCs

In the presence of 0.3 μM tetrodotoxin (TTX), 10 μM DL-2-amino-5-phosphovaleric acid (APV), 3 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 3 μM bicuculline, mIPSCs were recorded from neurons with intact glycinergic presynaptic terminals attached; namely a 'synaptic bouton preparation' (Akaike *et al.* 1992; Xu, 1999). The mIPSCs were completely and reversibly blocked by 0.3 μM strychnine, the selective glycine receptor (GlyR) antagonist (Fig. 2*A*), indicating the presence of glycinergic mIPSCs. As shown in Fig. 2, increasing extracellular pH from 7.4 to 8.5 potentiated the amplitude and frequency of glycinergic mIPSCs to 146.9 ± 18.9 % ($P < 0.05$, $n = 8$) and 212.8 ± 26.3 % ($P < 0.02$, $n = 8$), respectively, while lowering the external pH to 6.5 inhibited the amplitude and frequency of mIPSCs to 49.4 ± 5.8 % ($P < 0.002$, $n = 8$) and 68.7 ± 6.7 % ($P < 0.01$, $n = 8$) of control, respectively. Changing external pH also modulated the kinetics of glycinergic mIPSCs. An external pH of 6.5 significantly accelerated the decay time constant of mIPSCs (68.7 ± 0.7 % of control, $P < 0.0001$, $n = 8$), whereas pH 8.5 had no significant effect.

Modulation of glycine-evoked current (I_{Gly}) by H^+

To clarify the mechanism of H^+ modulation of glycinergic mIPSCs, the effect of H^+ on the I_{Gly} was examined. Under our experimental conditions (153 mM Cl^- in the recording pipette), 30 μM glycine-activated inward currents at a V_H of −50 mV were completely inhibited by 1 μM strychnine, the selective inhibitor of the glycine receptor (GlyR) (data not shown). To eliminate possible interference from a direct H^+-induced response, different pH solutions were pre-perfused for 15 s to allow for the complete desensitization of H^+-activated currents. As shown in Fig. 3, the peak amplitude of I_{Gly} in response to 100 μM glycine was attenuated when extracellular pH decreased and enhanced when extracellular pH increased. The IC_{50} (concentration inducing half-maximal inhibition) was calculated to be pH 6.18 ± 0.17 (Fig. 3*Ba*).

We also examined the H^+ modulation of I_{Gly} with 10 mM intracellular Cl^- concentration ($[Cl^-]_i$). As shown in Fig. 3*C*, the decreased $[Cl^-]_i$ did not alter the inhibitory effect on the I_{Gly} induced by 100 μM glycine. At

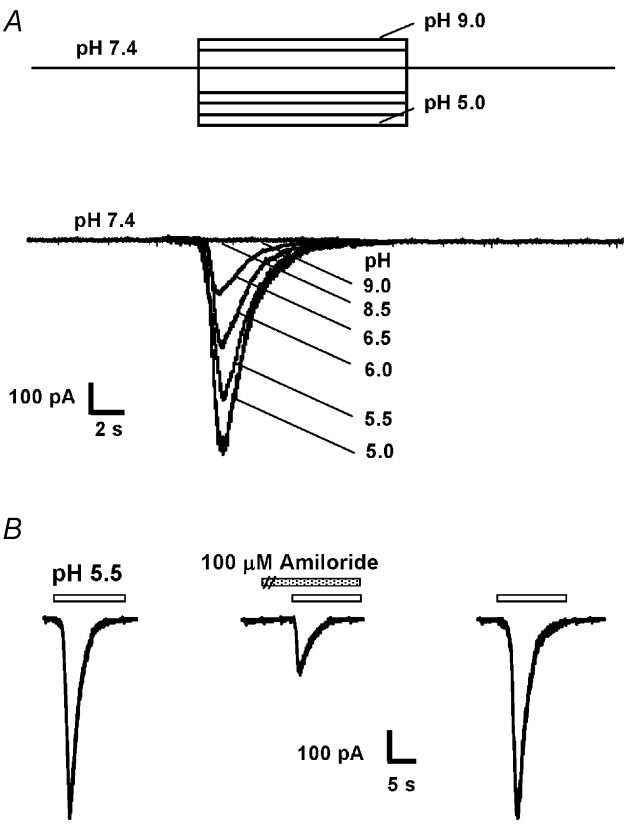

Figure 1. H^+-activated currents in rat sacral dorsal commissural nucleus (SDCN) neurons

A, currents activated by changes of extracellular pH in the same neuron. Only decreased extracellular pH induced visible currents in the neurons tested. *B*, amiloride (100 μM) reversibly inhibited the H^+-activated current induced by extracellular pH 5.5. Results similar to those in *A* and *B* were obtained from 10 neurons in each case. In this and subsequent figures, the bars above each current trace indicate the period of drug application.

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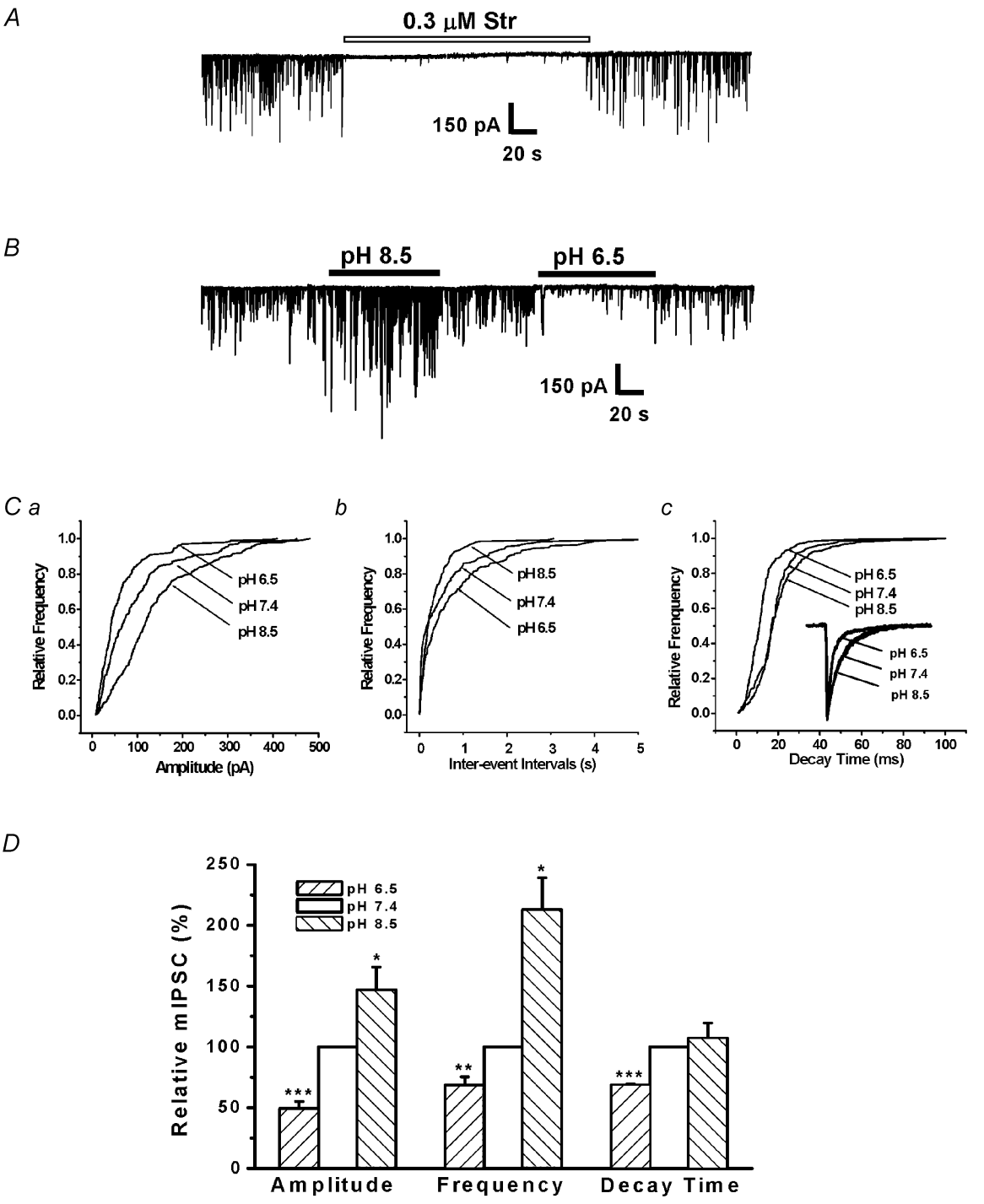

Figure 2. Effect of H^+ on glycinergic miniature inhibitory postsynaptic currents (mIPSCs)

A, the mIPSCs were completely and reversibly blocked by 0.3 μM strychnine (Str) in the presence of 0.3 μM TTX, 3 μM bicuculline, 3 μM CNQX and 10 μM APV, indicating they were glycinergic mIPSCs. *B*, sample recording showing the effect of external pH 8.5 and 6.5 on the glycinergic mIPSCs in a single neuron. *C*, normalized cumulative curves show the modulatory effect of extracellular pH on amplitude (*Ca*), frequency (*Cb*) and decay time (*Cc*) of mIPSCs from a sample neuron. Inset in *Cc* shows the normalized mIPSC at extracellular pH 6.5, 7.4 and 8.5. The Kolmogorov–Smirnov test was used to determine significant differences between cumulative distributions. *D*, statistical results demonstrating that extracellular pH 8.5 potentiated whereas pH 6.5 inhibited both the amplitude and frequency of the glycinergic mIPSCs. An acidic external pH also significantly decreased the decay time of glycinergic mIPSCs. Each column represents the average from eight neurons. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control.

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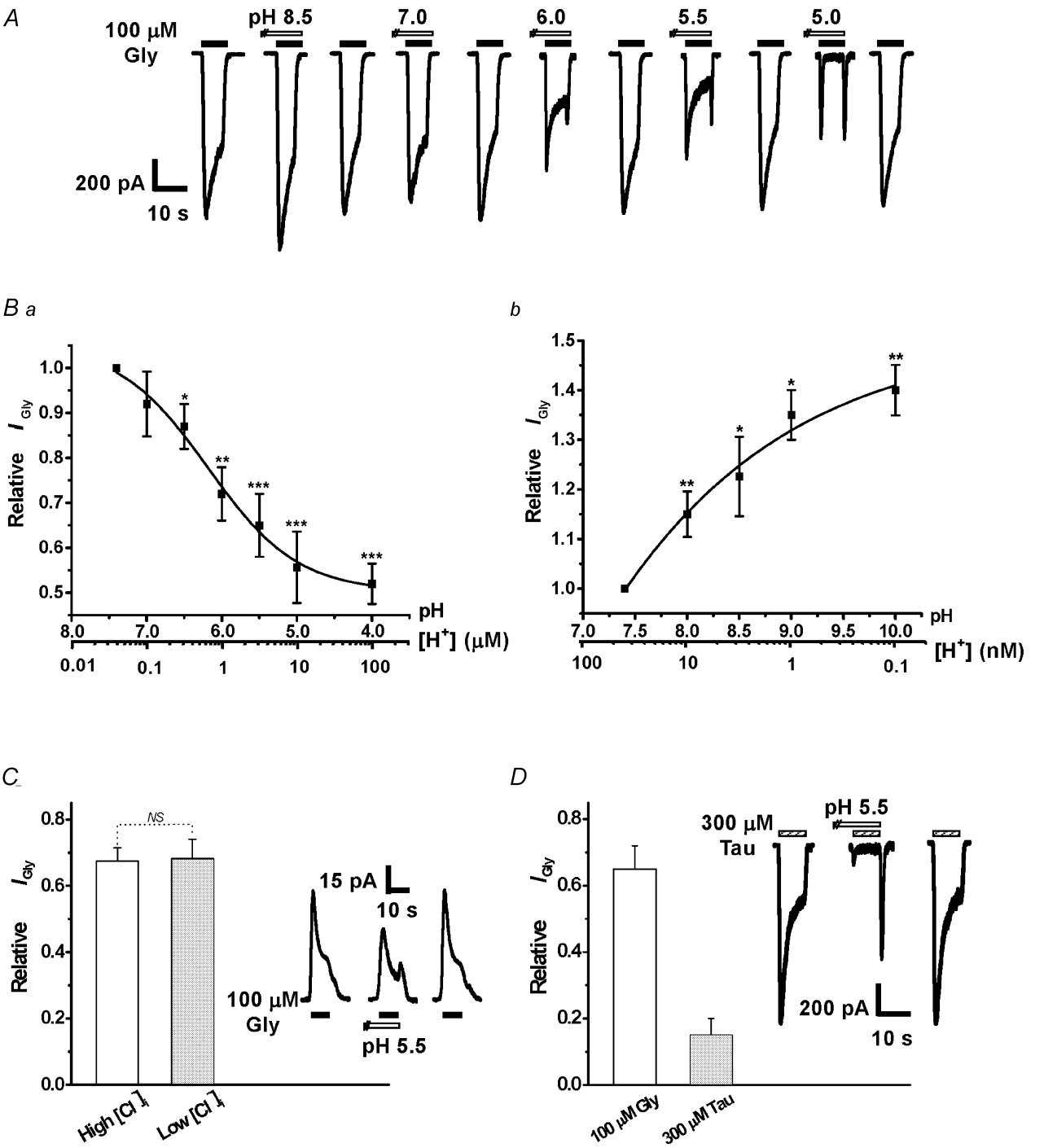

Figure 3. Biphasic effect of extracellular pH on glycine-activated current (I_{Gly})

A, sample recording illustrating the modulating effects of extracellular pH on the I_{Gly} induced by 100 μM glycine. In order to exclude interference from H^+-activated currents, different pH solutions were pre-perfused for about 10 s to fully desensitize acid-sensing ion channels (ASICs). *B*, summarized results showing the inhibition of I_{Gly} by acidic extracellular pH (n = 6–14) (*Ba*) and its potentiation by alkaline pH (n = 6–11) (*Bb*). Relative responses at different extracellular pH were normalized to the I_{Gly} at pH 7.4. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control. In this and subsequent figures the vertical bars show means ± S.E.M. *C*, graph showing the effect of extracellular pH 5.5 on the I_{Gly} induced by 100 μM glycine under the conditions of 10 mM intracellular Cl^- concentration ($[Cl^-]_i$) compared with 150 mM $[Cl^-]_i$. Each column represents the average response from six neurons. In this and subsequent figures *NS* indicates no significant statistical difference. *D*, comparison between the inhibition of the I_{Gly} induced by 100 μM glycine and the I_{Tau} induced by 300 μM taurine by acidic extracellular pH 5.5 (n = 5).

extracellular pH 5.5, the responses were 67.1 ± 4.5 % and 68.5 ± 6.7 % (of control I_{Gly} at extracellular pH 7.4) with 153 mM and 10 mM $[Cl^-]_i$, respectively ($P > 0.9$, $n = 6$).

A similar effect of external pH on the current activated by taurine (I_{Tau}), a low affinity glycine receptor (GlyR) agonist, was found, although H^+ inhibited I_{Tau} more completely (15.2 ± 6.1 % at pH 5.5, Fig. 3*D*).

H^+ modulation of glycinergic response is not due to the activation of ASICs or the change of intracellular pH

Several members of the ASIC family have been cloned and most of them have been found to be expressed throughout the peripheral and central nervous systems (Konnerth *et al.* 1987; Davies *et al.* 1988; Waldmann & Lazdunski, 1998; Reeh & Kress, 2001). Although we attempted to minimize any influence of ASICs in the present experiments by applying glycine after the complete desensitization of H^+-activated currents, the possible involvement of ASICs in the inhibition of I_{Gly} by H^+ remained. However, in the presence of 100 μM amiloride, the selective antagonist of ASICs (Tang *et al.* 1988; Waldmann *et al.* 1997), an extracellular pH of 5.5 induced a level of inhibition indistinguishable from that in the absence of amiloride (69.4 ± 9.5 % *versus* 66.0 ± 6.6 % of control I_{Gly} with or without amiloride, respectively; $P > 0.1$, $n = 7$; Fig. 4*A*). In addition, replacement of external Na^+ with Li^+ did not significantly alter the inhibition at extracellular pH 5.5 (data not shown), further reducing the likelihood that ASICs are involved.

In order to determine whether changes in intracellular pH contributed to the H^+ modulation of I_{Gly}, the effect of extracellular pH 5.5 on I_{Gly} under acidic intracellular conditions was examined (Fig. 4*B*). Decreased intracellular pH did not significantly alter the inhibitory effect of extracellular pH 5.5 on I_{Gly} (67.4 ± 4.0 % and 67.1 ± 5.7 % of control at intracellular pH 7.2 and 6.5, respectively; $P > 0.9$, $n = 6$–9).

Decreasing intracellular pH or changing the pH of extracellular solutions in the presence of 100 μM amiloride did not affect the H^+ modulation of glycinergic mIPSCs

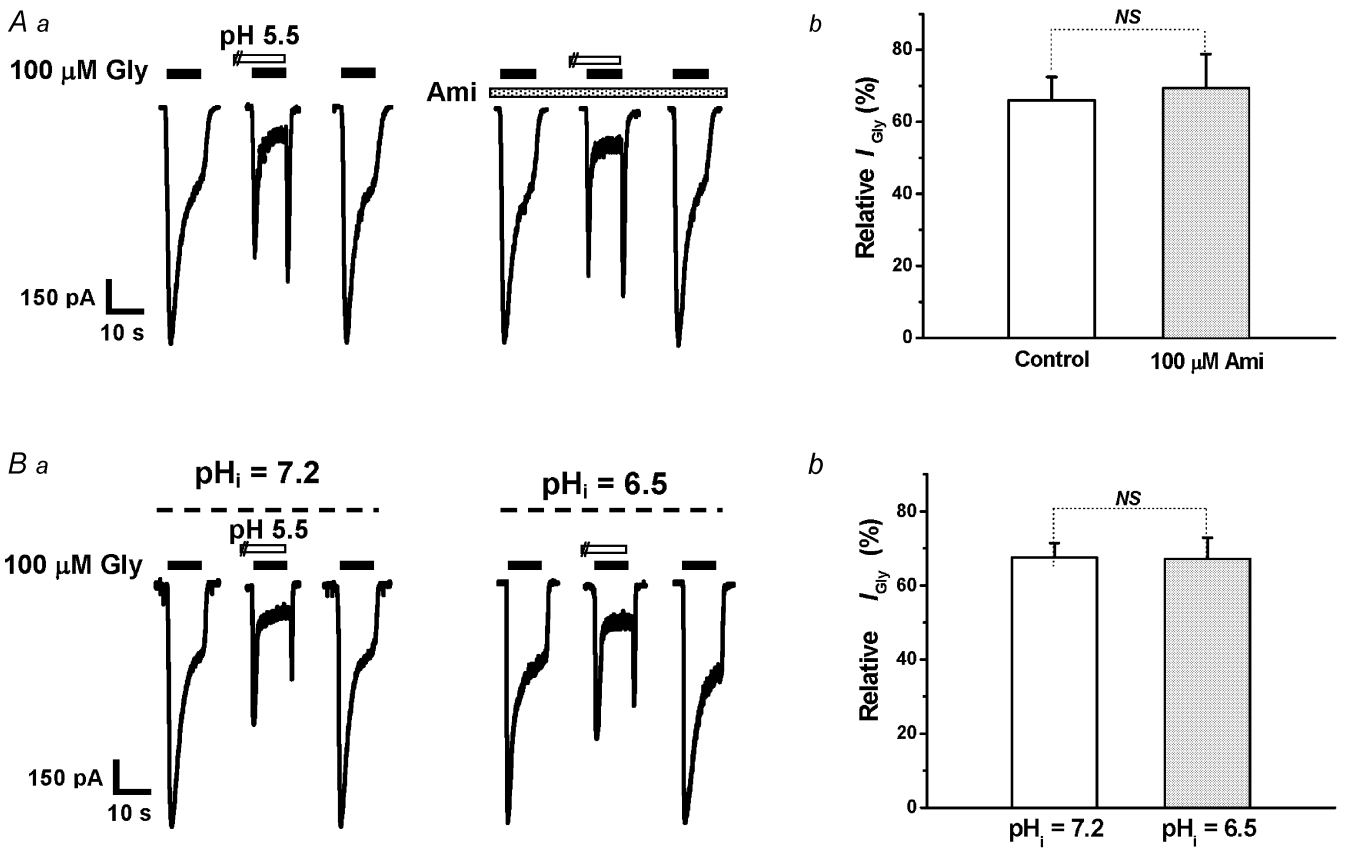

Figure 4. Blocking acid-sensing ion channels (ASICs) with amiloride or decreasing the intracellular pH (pH_i) did not affect the inhibition of I_{Gly} by H^+

A, sample recordings (*Aa*) and statistical results (*Ab*) illustrating the inhibitory effect of extracellular pH 5.5 on the I_{Gly} induced by 100 μM glycine with or without 100 μM amiloride, the selective antagonist of ASICs. Each column in *Ab* represents the average of seven neurons. *B*, sample recordings (*Ba*) and statistical results (*Bb*) showing the inhibitory effect of extracellular pH 5.5 on the I_{Gly} at intracellular pH 7.2 and 6.5. Each column in *Bb* represents the average of six neurons.

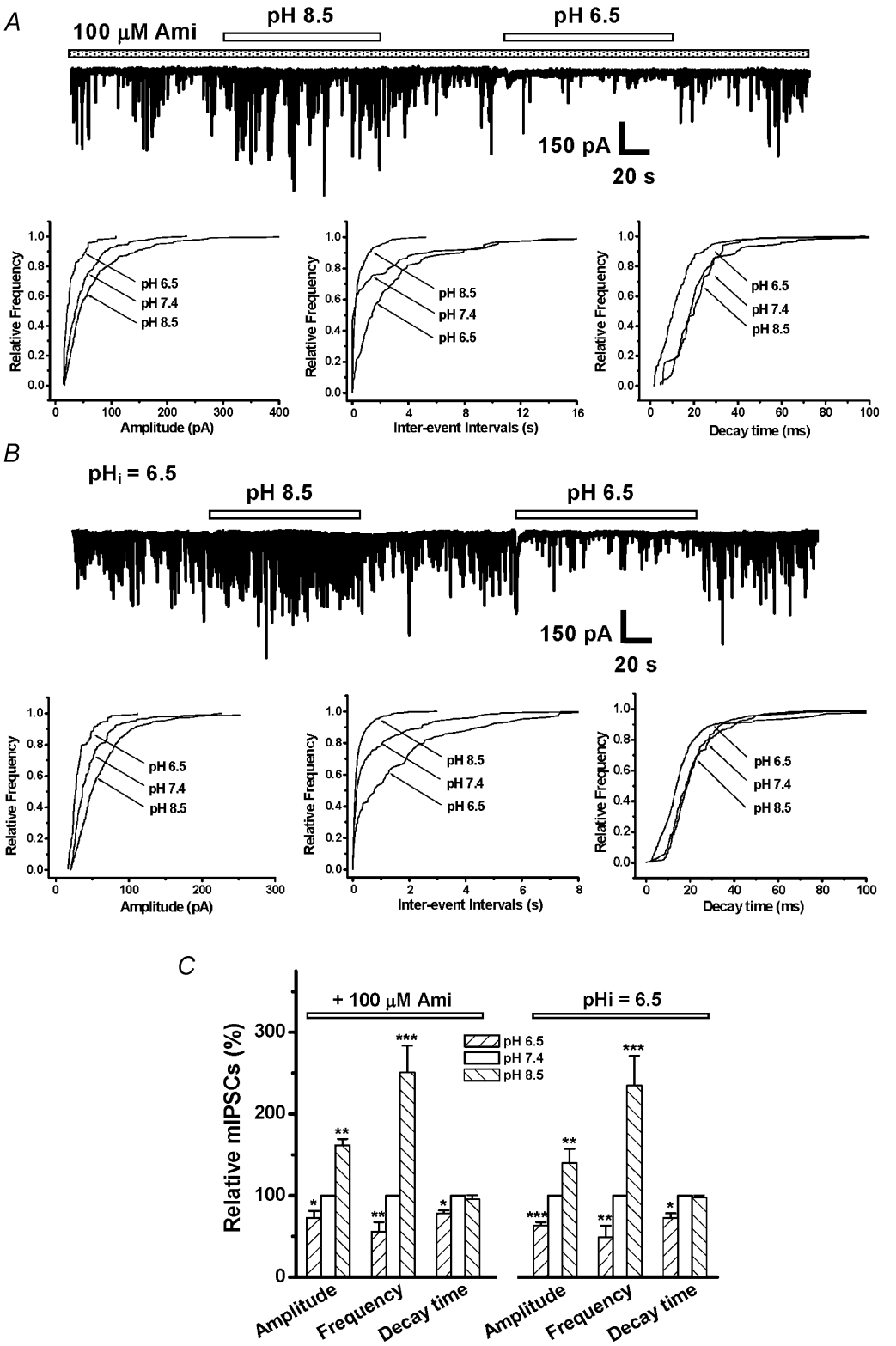

Figure 5. Blocking ASICs with amiloride or decreasing the pH_i did not affect the H^+ modulation of glycinergic mIPSCs

Sample recordings and normalized cumulative curves showing the modulatory effect of extracellular pH on glycinergic mIPSCs in the presence of 100 mM amiloride (Ami) (*A*) or an acidic pH_i of 6.5 (*B*). *C*, statistics derived from *A* and *B*. Each column represents the average of five neurons. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control.

either (Fig. 5). With an acidic pH_i of 6.5, lowering the external pH to 6.5 still inhibited the amplitude and frequency of mIPSCs to 63.2 ± 4.2 % ($P < 0.02$, $n = 5$) and 48.7 ± 14.4 % ($P < 0.008$, $n = 5$) of control, a level of inhibition comparable to that induced by pH 6.5 with a pH_i of 7.2 (Fig. 2). Similarly, in the presence of 100 μM amiloride, an extracellular pH of 6.5 induced a level of inhibition on the amplitude and frequency of mIPSCs (72.6 ± 8.6 %, $P < 0.04$ and 55.7 ± 11.7 %, $P < 0.01$ of control, respectively; $n = 6$), which was indistinguishable from that in the absence of amiloride (Fig. 2). In the presence of 100 μM amiloride or an acidic pH_i of 6.5, the decay time constant of mIPSCs was 78.2 ± 3.6 % ($P < 0.05$, $n = 6$) or 72.3 ± 6.1 % ($P < 0.03$, $n = 5$), respectively, of control external pH, values comparable to that observed in Fig. 2.

Effect of H^+ on the concentration–response relationship of I_{Gly}

Figure 6*A* shows the currents activated by 30 μM glycine (left series) and 1 mM glycine (right series) at control pH 7.4 and acid pH 5.5 in a SDCN neuron. At a glycine concentration near the EC_{50} (30 μM), extracellular pH 5.5 reduced the peak I_{Gly} to 40.4 ± 7.5 % ($n = 7$) of control. At the saturating concentration of 1 mM glycine, dropping the pH to 5.5 had no significant effect on the peak I_{Gly} amplitude (105.6 ± 9.1 % of control, $P > 0.5$, $n = 9$). Figure 6*B* shows the two concentration–response relationships at pH 7.4 and 5.5. An external pH of 5.5 shifted the glycine concentration–response curve to the right and significantly increased the EC_{50} from 49.3 ± 5.7 μM (pH 7.4) to 131.5 ± 8.1 μM (pH 5.5) without changing the Hill coefficient (1.1 and 1.3 at pH 7.4 and 5.5, respectively) or the maximum value.

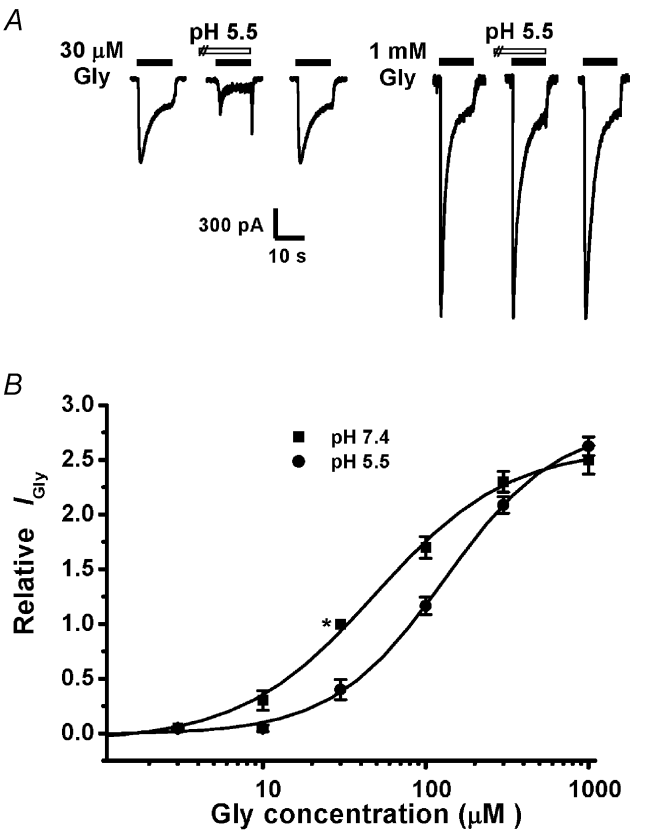

Figure 6. Effect of H^+ on the concentration–response relationships for I_{Gly}

A, typical recordings illustrating the effect of pH 5.5 on I_{Gly} activated by a concentration of glycine near the EC_{50} (30 μM) and a saturating concentration (1 mM). *B*, concentration–response relationships for I_{Gly} at pH 7.4 (■) and 5.5 (●). All currents were normalized to the peak amplitude of the I_{Gly} induced by 30 μM glycine at pH 7.4 (*) and each point represents the average of 6–14 neurons. The V_H was −50 mV.

Interaction of H^+ and Zn^{2+}

In transfected HEK cells expressing the human GlyR α1 subunit, Harvey *et al.* (1999) demonstrated that H^+ and Zn^{2+} competitively bind to the same site on the GlyR and allosterically modulate its function. Because this finding suggests that H^+ modulates I_{Gly} through an allosteric mechanism, the effects of H^+ and Zn^{2+} on I_{Gly} were investigated. As found previously in the same preparation (Wang *et al.* 1998), 100 μM Zn^{2+} significantly inhibited the I_{Gly} induced by 100 μM glycine (68.4 ± 5.7 % of control, $P < 0.001$, $n = 7$), a level of inhibition comparable to that induced by pH 5.5 (70.5 ± 5.5 %, $P < 0.001$, $n = 7$) (Fig. 7). In this preparation, the saturating concentration of Zn^{2+} for blocking I_{Gly} is 1 mM (Wang *et al.* 1998). In the presence of 1 mM Zn^{2+}, pH 5.5 did not further inhibit the I_{Gly} induced by 100 μM glycine (30.8 ± 2.2 % *versus* 33.1 ± 2.6 % in control pH 7.4 and pH 5.5, respectively, $n = 7$, $P > 0.3$), which indicates that H^+ and Zn^{2+} share common binding sites on the GlyR.

Effect of H^+ on I_{Gly} kinetics

H^+ modulated not only the amplitude, but also the kinetics of I_{Gly} (Fig. 8*A*). In most neurons tested, H^+ produced a transient inward rebound current immediately after the washout of glycine when the extracellular pH had been decreased. The relative amplitude of the rebound current (current immediately after washout normalized to the peak I_{Gly} at pH 7.4) increased as the extracellular pH dropped (Fig. 8*A* and *B*).

The I_{t4} response is defined as the whole-cell current routinely measured 4 s after the response onset normalized to its own peak current, reflecting the desensitization of the I_{Gly} (Harvey *et al.* 1999). In the present study, lowering the extracellular pH decreased the I_{t4} value in a H^+ concentration-dependent manner (Fig. 8*C*), which indicates the accelerated desensitization of I_{Gly} in the presence of H^+.

Decreased extracellular pH does not change the Cl^- permeability of the GlyR

Previous workers have reported that a channel blocking mechanism often accompanies a rebound current

(Legendre *et al.* 2000; Fisher, 2002). In this study, acidification was found to reduce the duration of glycinergic synaptic currents (Fig. 2), which might be a result of blocking or closure of the GlyR Cl^- channel. To test whether the rebound current observed results from open-channel block, the current–voltage relationship for H^+ inhibition of I_{Gly} was examined. Glycine (100 μM) at extracellular pH 5.5 was applied at V_H values ranging between +30 and −70 mV (Fig. 9*A*). Figure 9 shows the current–voltage relationships of the peak and rebound I_{Gly} currents induced by 100 μM glycine at extracellular pH 7.4 or 5.5. The membrane potential significantly influenced the action of H^+ on I_{Gly} and the rectification properties of I_{Gly} were pronounced in the presence of low pH. However, the rebound current occurred at both negative and positive holding potentials. In addition, varying the external pH did not change the reversal potentials of either peak or rebound I_{Gly} currents (−1.4 ± 1.7 mV at pH 7.4 *versus* −2.2 ± 2.0 mV and −2.1 ± 1.9 mV of peak and rebound current at pH 5.5, respectively; ANOVA, $P > 0.2$, $n = 5$) and they were all close to the theoretical Cl^- equilibrium potential (E_{Cl}) of −1.3 mV calculated from the

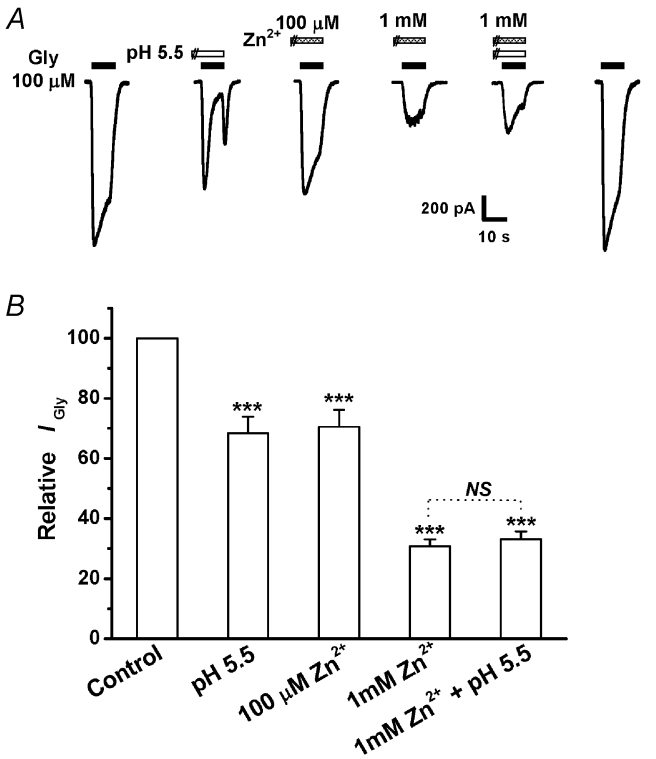

Figure 7. Interactions between H^+ and Zn^{2+} and the modulation of I_{Gly}

A, sample recordings illustrating the modulatory effect of pH 5.5 and Zn^{2+} on the I_{Gly} induced by 100 μM glycine. *B*, statistical results derived from *A*. Extracellular application of pH 5.5, 10 μM Zn^{2+}, 1 mM Zn^{2+} and 1 mM Zn^{2+} plus pH 5.5 all significantly inhibited the I_{Gly}. In the presence of 1 mM Zn^{2+}, pH 5.5 induced no further inhibition. Each column represents the averaged value of seven neurons. *** $P < 0.001$.

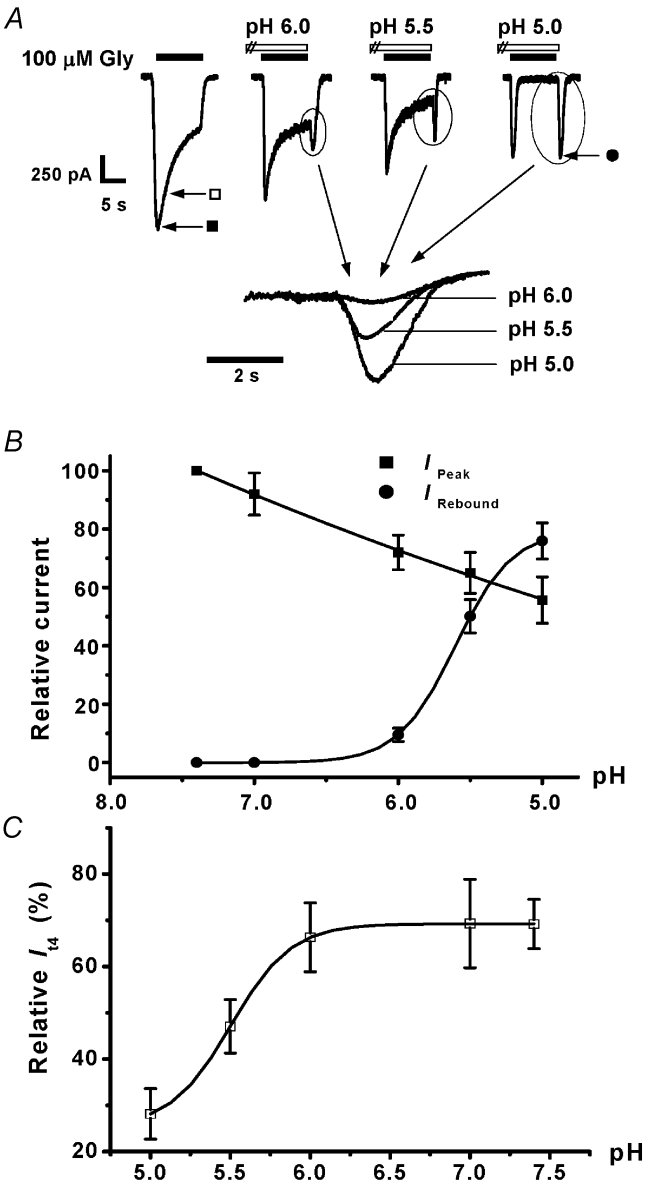

Figure 8. Effect of H^+ on the kinetics of I_{Gly}

A, sample recordings illustrating the effect of H^+ on the kinetics of the I_{Gly} induced by 100 μM glycine (upper traces). Rebound currents induced by the drop in extracellular pH immediately after washout are shown on an expanded scale (lower traces). The filled (■) and open (□) squares show the peak and I_{t4} (whole-cell current routinely measured 4 s after the response onset normalized to its own peak current) current levels, respectively. Filled circles (●) show the I_{Gly} rebound current. *B*, statistical data illustrating the relative peak (I_{Peak}, ■) and rebound ($I_{Rebound}$, ●) I_{Gly} currents (normalized to the peak I_{Gly} amplitude at pH 7.4) at different acidic external pH values. The amplitude of $I_{Rebound}$ increased whereas the I_{Peak} decreased with decreasing extracellular pH. Each column represents data from 6–8 neurons. *C*, the relationship of relative I_{t4} responses and extracellular pH. The amplitude of the I_{t4} response decreased at acidic external pH, indicating accelerated desensitization as external pH dropped. Each point represents data from 9–17 neurons.

given extra- and intracellular Cl^- concentrations with the Nernst equation, indicating that the peak and rebound currents at pH 5.5 were mediated by the GlyR Cl^- channel.

Effects of sequence of glycine application and acidification

The minimal voltage dependence of H^+ action makes an open-channel block mechanism unlikely. Another possibility is that during the washout of agonists, the deprotonation rate is faster than the unbinding rate of glycine from GlyR. If H^+ unbinds first, the unbinding glycine may further activate the undesensitized GlyR, hence inducing an apparent rebound current. To test this possibility, the H^+ effect on I_{Gly} with different protocols for drug application was examined in the presence of 100 μM amiloride. As shown in Fig. 10, pH 5.5 altered the amplitude and kinetics of I_{Gly} significantly only when the acidic solution and the glycine were applied together. Sequential application of acidic solution and glycine did not significantly change either the amplitude or kinetics of I_{Gly} (Fig. 10*Ae* and *Be*). The degree of amplitude reduction with three protocols was greatest (about 66 %) when glycine application followed the pH reduction and both were terminated at the same time (Fig. 10*Ab*), then about 69 % when glycine was applied and removed during the continuous perfusion of low pH (Fig. 10*Ad*) and about 82 % when the glycine and pH reduction were applied and removed simultaneously (Fig. 10*Ac*). Similarly, co-application of pH 5.5 and glycine in three protocols all decreased the I_{t4} values significantly (Fig. 10*B: b*, 43.2 ± 4.0 %; *c*, 49.5 ± 7.1 %; *d*, 45.6 ± 8.3 % compared with control of 65.4 ± 8.3 %, $n = 5$). In addition, the termination of acidification was important for the appearance of the rebound current. When both acid and glycine were terminated at the same time, a rebound current occurred (Fig. 10*Ab* and *c*). However, continuous application of pH 5.5 before, during and after the application of glycine eliminated the rebound current (Fig. 10*Ad*).

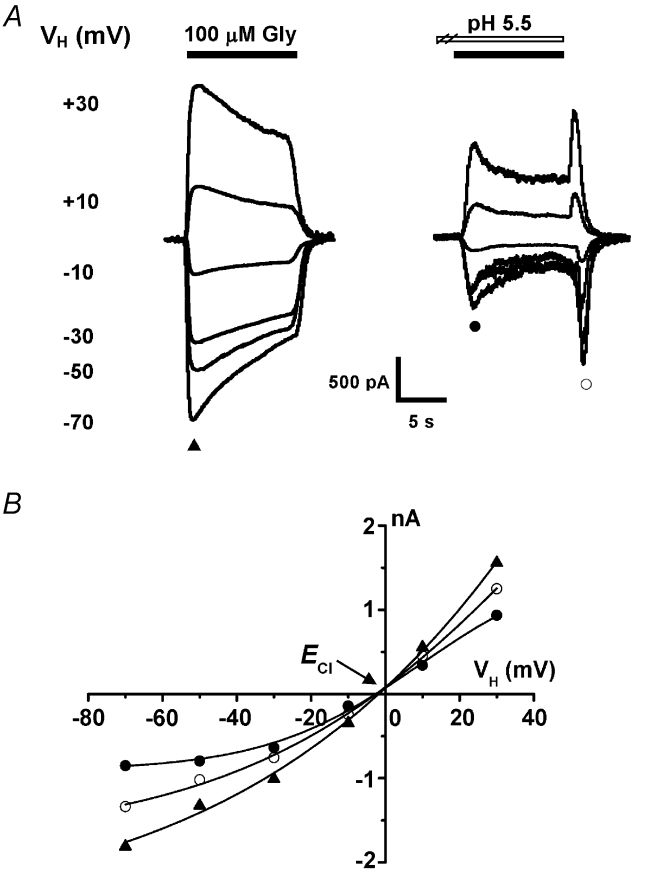

Figure 9. Current–voltage relationships of the I_{Gly} induced by 100 μM glycine

A, typical I_{Gly} at extracellular pH 7.4 (left series) and 5.5 (right series) at different V_H in the same neuron. *B*, three current–voltage relationships derived from *A* for the peak I_{Gly} at extracellular pH 7.4 (▲) and 5.5 (●) and rebound current at pH 5.5 (○). Altering the external pH values did not change the reversal potential of I_{Gly}; E_{Cl} indicates the theoretical Cl^- equilibrium potential. Similar results were obtained from four other neurons.

Effect of H^+ on GABA-activated currents

As functional GABAergic and glycinergic cotransmission has been demonstrated in the rat SDCN (Wu *et al.* 2002), the effect of H^+ on GABA-activated currents (I_{GABA}) was also studied. Figure 11*A* shows the effect of changing extracellular pH on I_{GABA} induced by 10 μM and 300 μM GABA. The amplitude of I_{GABA} in response to 10 μM GABA decreased to 33.2 ± 3.4 % of control ($P < 0.005$, $n = 4$) when extracellular pH was dropped to 5.5, and increased to 154.1 ± 6.1 % ($P < 0.01$, $n = 5$) when extracellular pH was raised to 8.5. However, the shift of extracellular pH from the normal value of 7.4 to 5.5 or 8.5 did not alter the amplitude of I_{GABA} in response to the saturating concentration of 300 μM GABA ($P > 0.1$, $n = 4$–7) (Fig. 11*B*), indicating the action of H^+ is to decrease the affinity of the $GABA_A$ receptor for the agonist. Unlike the effect of H^+ on I_{Gly}, acidic pH did not cause an apparent rebound current after washout of GABA.

DISCUSSION

We report, for the first time, how H^+ modulates glycinergic mIPSCs and the glycine receptor (GlyR) in acutely dissociated sacral dorsal commissural nucleus (SDCN) neurons. The SDCN, which is located in the grey matter dorsal to the central canal in the lower lumbar and sacral spinal cord, receives primary inputs from pelvic organs and descending inputs from several regions of the brain (Honda, 1985). It has been demonstrated that somatic and visceral afferents converge onto SDCN neurons, which are involved in the central processing of nociceptive information (Lu *et al.* 2001). These characteristics thus make the SDCN an ideal place to study the modulation of nociceptive signals, especially visceral nociception. The H^+ modulation of the glycinergic response *in vitro*

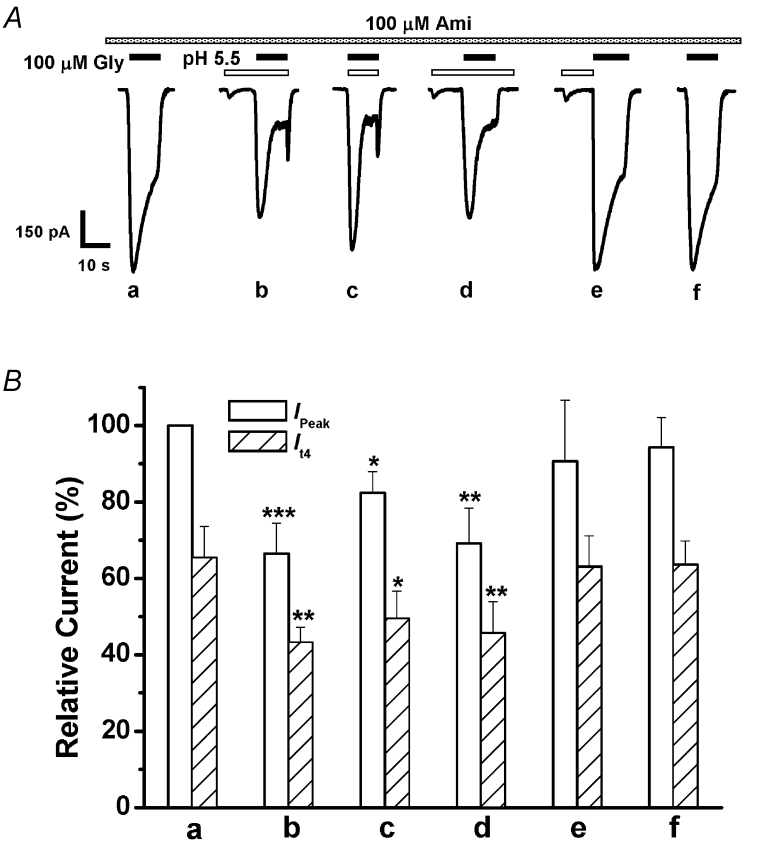

Figure 10. Modulation by extracellular pH 5.5 of I_{Gly} with different sequences of drug application

A, sample recordings demonstrating H^+ modulation of the I_{Gly} induced by 100 μM glycine with four different sequences of drug application (*b, c, d, e*) in the presence of 100 μM amiloride. *B*, statistical results of peak I_{Gly} and I_{t4} values with sequence of application of pH 5.5 solution and glycine illustrated in *A* ($n = 6$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control.

presumably reflects the pH sensitivity of the GlyR *in vivo*. Therefore, the modulatory effect of H^+ on glycinergic mIPSCs and the GlyR may operate under certain pathological conditions such as inflammation, tumours and ischaemia, making the present study significant for the understanding of pain and other neurological signs.

Mechanisms by which H^+ modulates the glycinergic response: allosteric modulation

Several possibilities for explaining the H^+ inhibition of the glycinergic response can be immediately excluded. First, the half-maximal value (IC_{50}) for the H^+ inhibition of I_{Gly} induced by 100 μM glycine was about pH 6.18, which

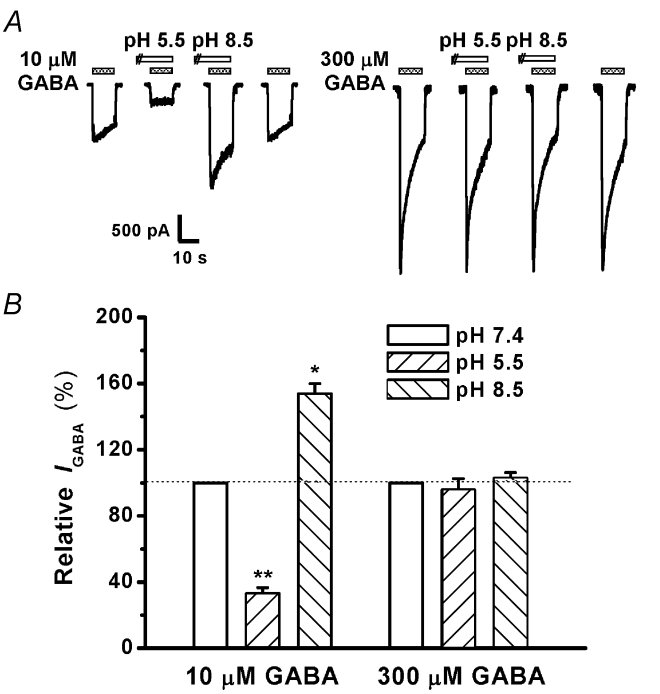

Figure 11. Effect of H^+ on GABA-activated current (I_{GABA}) in rat SDCN neurons

A, typical recordings illustrating the effect of extracellular pH of 5.5 and 8.5 on the I_{GABA} induced by 10 μM and 300 μM GABA. *B*, statistical data derived from the results shown in *A*. The changes of extracellular pH had little effect on the I_{GABA} induced by 300 μM GABA, while it significantly inhibited the I_{GABA} induced by 10 μM GABA at pH 5.5 and enhanced it at pH 8.5. Each column represents the averaged value from 4–7 neurons. * $P < 0.05$ and ** $P < 0.01$ compared with control.

differs by more than three pH units from the pK_a (acid dissociation constants) of glycine in aqueous solutions (2.34 and 9.60 for the carboxyl and amino moieties, respectively) (Reich, 1995). Therefore, it seems unlikely that the H^+ inhibition was due to a change in the ionization of the glycine molecule itself. Second, blockade of acid-sensing ion channels (ASICs) with 100 μM amiloride did not alter the H^+ inhibition of either I_{Gly} or glycinergic mIPSCs. Moreover, replacement of Na^+ with Li^+, another cation that has a much lower permeability than Na^+ (data not shown), had no significant effect on the inhibition of I_{Gly} by H^+, which together exclude the possible involvement of ASICs and the indirect action of Na^+ influx on the H^+ inhibition of the glycinergic response. Third, according to a previous report, challenging *Xenopus* oocytes with Ringer solution or perfusing HEK cells with Krebs solution at pH 5.4 or 9.4 evoked a very small shift in the intracellular pH (Kaila, 1994). In the present experiment, decreasing intracellular pH to 6.5 did not significantly alter the inhibition of either I_{Gly} or glycinergic mIPSCs by H^+. Therefore, it appears unlikely that the small variations in internal pH could account for the inhibition of I_{Gly} by external pH changes.

A more feasible mechanism would be that H^+ modulates the function of the GlyR allosterically or as a competitive inhibitor. The decrease of external pH significantly shifted the I_{Gly} concentration–response curve to the right and increased the EC_{50} without changing the Hill coefficient or the maximum value, which strongly indicates that H^+ allosterically inhibits I_{Gly} by decreasing the apparent affinity of glycine for its receptor. In addition, as evident in Fig. 3*D*, H^+ inhibited the taurine-activated current more completely than I_{Gly}. If H^+ acts as a competitive inhibitor of the GlyR, this result is expected given that taurine is a low-affinity GlyR agonist. Furthermore, H^+ could not induce further inhibition of I_{Gly} in the presence of a saturating concentration of Zn^{2+}, suggesting that H^+ may compete with Zn^{2+} on the GlyR. It is known that GlyR function is allosterically modulated by Zn^{2+} acting on its histidine residues (Harvey *et al.* 1999). Therefore it is possible, although speculative, that H^+ binds to the histidine residues on GlyR since the IC_{50} value for H^+ inhibition of I_{Gly} is pH 6.18, which is close to the pK_a of histidine. In support of this assumption, H^+ has been reported to act as a modulator of several ion channel receptors, including the $P2X_2$ purinoceptor (Clyne *et al.* 2002), the $GABA_A$ receptor (Wilkins *et al.* 2002) and the glycine transporter 1b (Aubrey *et al.* 2000), by acting on the amino acid residues involving histidine. However, the precise binding sites of H^+ and Zn^{2+} on GlyR still need to be elucidated with

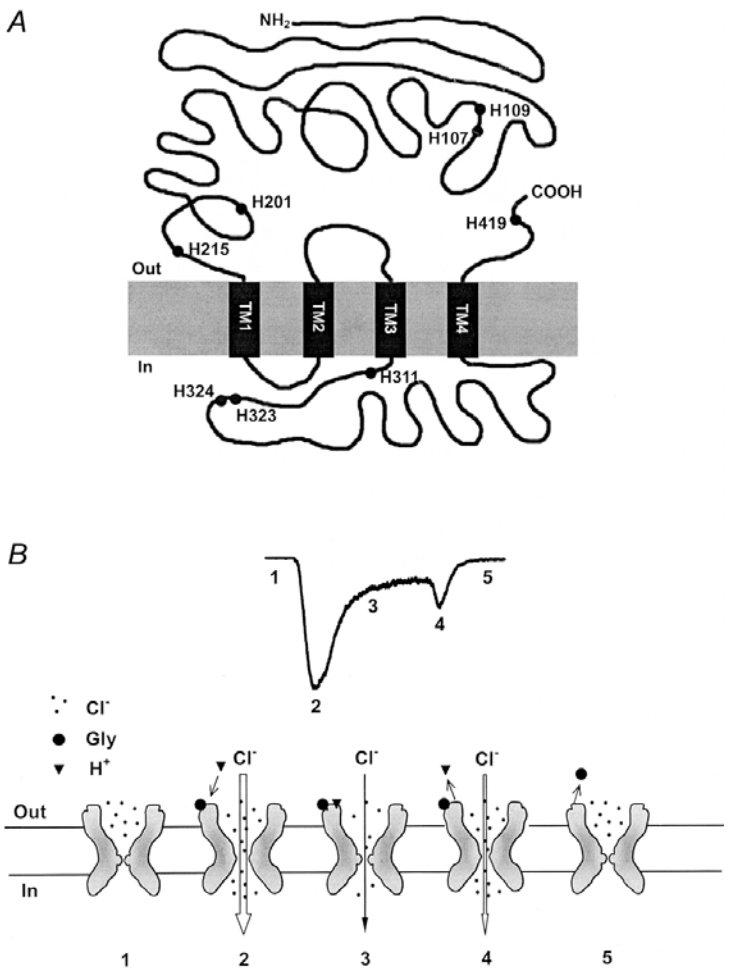

Figure 12. A possible model of glycine receptor (GlyR) modulation by H^+

A, schematic representation of the membrane-spanning topology of the GlyR α1 subunit. The histidine residues are indicated. *B*, schematic diagram illustrating the possible mechanism by which H^+ modulates the GlyR. H^+ competitively binds to the GlyR (2), induces a global conformational change of the GlyR, and closes the pore domain (3). After washout, the remaining glycine activates the channel again and induces a rebound current because the unbinding rate of H^+ is faster than that of glycine (4).

molecular biological methods such as site-specific mutagenesis.

H^+ appeared to modulate not only the amplitude, but also the kinetics of I_{Gly}. A large transient inward rebound current was produced immediately after the washout of glycine at extracellular pH lower than 6.0 (H^+ concentration higher than 1 μM). The amplitude of this rebound current increased with decreasing extracellular pH, indicating that other mechanisms might be involved besides allosteric modulation. One possibility is that H^+ acts as an open-channel blocker of the GlyR. However, the rebound current occurred at both negative and positive holding potentials. If the H^+ cation binds to a site near the pore of the GlyR channel and acts as an open-channel blocker, it would be expected that the rebound current should be inhibited at positive membrane voltages.

Recently, Chang & Weiss (2002) provided direct evidence, using $GABA_A$ receptor ($GABA_AR$) modulation by positively charged picrotoxin, that a competitive antagonist does not merely sterically hinder ligand binding as usually assumed, but rather induces conformational changes in receptor proteins. The global conformational rearrangement induced by the binding of picrotoxin to the $GABA_AR$ can close the channel pore rapidly (Chang & Weiss, 2002). Their study may provide insights into the mechanism by which positive allosteric modulators of anion channels exert their effects. We thus assume that H^+ modulation of the GlyR is similar to the mechanism revealed by Chang & Weiss (2002). Accordingly, H^+ competitively inhibited I_{Gly} by decreasing the affinity of glycine for the GlyR. However, H^+ does not necessarily bind to the same site as glycine, since the binding of glycine involves several specific amino acid residues at the disulphide loop motif of the extracellular N-terminal of the GlyRα subunit, including alanine 52, phenylalanine 159 to tyrosine 161 and lysine 200 to lysine 206 (Vandenberg *et al.* 1992; Ryan *et al.* 1994; Saul *et al.* 1994), whereas H^+ mainly binds to histidine residues on receptors (Harvey *et al.* 1999; Aubrey *et al.* 2000; Clyne *et al.* 2002; Wilkins *et al.* 2002). Nevertheless, the binding of H^+ to the GlyR definitely affects the binding of glycine, probably by a global conformational change (Chang & Weiss, 2002). Therefore, we propose that H^+ binds competitively with glycine and the binding of H^+ induces a global conformational change of the GlyR which effectively closes the GlyR Cl^- channel, resulting in acceleration of the apparent GlyR 'desensitization'. Consequently, lowering the external pH accelerated the decay time constant of glycinergic mIPSCs and significantly decreased the I_{t4} values of I_{Gly}. However, the deprotonation rate is faster than the rate of glycine unbinding from the GlyR. Because H^+ unbinds first during the washout of drugs, the unbinding glycine further activates the undesensitized GlyR, hence inducing an apparent rebound current (Fig. 12*B*). This is evident in Fig. 10; H^+ altered the amplitude and kinetics of I_{Gly} only when acid solution and glycine were co-applied simultaneously, whereas sequential application of acidic solution and glycine did not change either the peak amplitude or the I_{t4} value. In addition, continuous application of low pH before, during and after the application of glycine eliminated the rebound current. According to this model, it is reasonable that the rebound current existed only at low concentrations of glycine. At saturating concentrations of glycine, H^+ can no longer efficiently compete with glycine. In addition, the H^+ binding site is usually thought to involve the amino acid histidine. There is no histidine in the pore region but four histidine residues occur near the N-terminal of the α1 subunit of GlyR (Fig. 12*A*), suggesting that H^+ may bind to the motif near the N-terminal but not to the pore domain of the GlyR, which makes the observed voltage independence of the rebound current understandable. Finally, this model is logical because glycine also binds to the N-terminal of the GlyRα1 subunit, which provides a relatively short spatial distance for the interaction between glycine and H^+.

It is interesting to note that the apparent effect of H^+ on I_{Gly} kinetics differs from a previous report (Harvey *et al.* 1999). This discrepancy might be due to the different subunit composition of the GlyR examined. In the present study, we used spinal neurons dissociated from 14-day-old rats. At this age, neurons express the mature form of GlyR, which is heterogeneously composed of α1- and β-subunits (Malosio *et al.* 1991). In the study reported by Harvey *et al.* (1999) only the human α1 homomeric GlyR was tested. This difference in the effect of H^+ on I_{Gly} kinetics also hints at the importance of the GlyR β-subunit, which may be associated with the global conformational change of the GlyR induced by H^+.

The modulatory effect of H^+ on GABA-induced current (I_{GABA})

We also studied H^+ modulation of I_{GABA} in SDCN neurons. We showed that increasing extracellular pH potentiated, whereas decreasing extracellular pH inhibited the I_{GABA} induced by 10 μM GABA. At a near-saturating concentration of 300 μM GABA, however, H^+ had no significant effect on I_{GABA}. These results suggest that H^+ decreases the affinity of the $GABA_AR$ for its agonist. Many reports have demonstrated the H^+ sensitivity of the $GABA_AR$ in different preparations. Some reports are consistent with the present results (Gruol *et al.* 1980; Zhai *et al.* 1998) whereas the others differ or even contradict the present study (Tang *et al.* 1990; Pasternack *et al.* 1992). These apparent differences in sensitivity of I_{GABA} to extracellular H^+ might depend on the developmental stage, which probably reflects the subunit composition of the $GABA_AR$ in the central nervous system (Krishek & Smart, 2001).

Pathophysiological implications

Transient changes in extracellular pH occur in both pathophysiological conditions such as inflammation and ischaemia (Chesler, 1990; Reeh & Steen, 1996) and under physiological conditions including synaptic transmission where the acidic contents of transmitter vesicles cause an extracellular acid shift within the synaptic cleft (Krishtal *et al*. 1987). Changes in H^+ concentration have been clearly shown to serve important roles by regulating several types of voltage-gated and ligand-gated ion channels. In the present study, H^+ inhibited the function of the inhibitory GlyR. This H^+ modulation of the GlyR in acutely dissociated SDCN neurons *in vitro* presumably reflects the pH sensitivity of the GlyR *in vivo*. The H^+ inhibition of glycinergic miniature IPSCs (mIPSCs) we observed further supports this notion at the level of synaptic transmission. Increased external pH potentiated, whereas its decrease inhibited both the amplitude and frequency of glycinergic mIPSCs, suggesting the involvement of both presynaptic and postsynaptic mechanisms. However, the changes in mIPSCs frequency could also result indirectly from the effect of H^+ on mIPSCs amplitude (Li *et al*. 2003). Increasing extracellular pH to 8.5 potentiated the function of postsynaptic GlyR and therefore the average amplitude of mIPSCs, making the originally negligible mIPSCs detectable at pH 8.5. Conversely, dropping extracellular pH reduced some originally detectable mIPSCs to a negligible size.

Ischaemia is a prevalent and serious problem, and a leading cause of death and disability. It is well known that excitotoxicity is the major cause of neuronal death following transient cerebral ischaemia. Thus the H^+ sensitivity of the GlyR may contribute to the modulation of neural excitability after ischaemia. The present study provides useful and important evidence relevant to protection against neural damage in ischaemia. In addition, local decreases in pH under such pathophysiological conditions as inflammation and ischaemia can elicit pain, although the precise mechanism is not clear. The present results therefore add to our understanding of the mechanisms underlying the pain that occurs in inflammatory and/or ischaemic conditions.

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