0026-895X/00/070129-07$3.00/0
MOLECULAR PHARMACOLOGY

MOL 58:129–135, 2000 /13047/829463



# Molecular Basis for Proton Regulation of Glycine Transport by Glycine Transporter Subtype 1b

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Received October 25, 1999; accepted March 14, 2000

This paper is available online at http://www.molpharm.org

## ABSTRACT

In the central nervous system, glycine is a coagonist with glutamate at the *N*-methyl-D-aspartate subtype of ionotropic glutamate receptors. The GLYT1b subtype of glycine transporters is expressed in similar regions of the brain as the excitatory *N*-methyl-D-aspartate receptors and has been postulated to regulate glycine concentrations within excitatory synapses. We have expressed GLYT1b in *Xenopus laevis* oocytes and used electrophysiological techniques to investigate the pH regulation of glycine transporter function. We found that $H^+$ inhibits glycine transport by a noncompetitive mechanism, with half-maximal inhibition occurring at concentrations found in both physiological and pathological conditions. Charge-to-flux experiments revealed that the decreased current measured corresponds to a decreased influx of [$^3$H]glycine and that the proton inhibition of GLYT1b does not alter the coupling ratio of transport. The membrane potential does not affect proton inhibition of transport, suggesting that the site of action on GLYT1b is not within the electric field of the membrane. Mutation of histidine 421 to an alanine residue, in the fourth extracellular loop of GLYT1b, renders the transporter insensitive to regulation by pH, but does not seem to alter the kinetics of glycine transport. These results suggests that histidine 421 is responsible for mediating the inhibitory actions of protons. Proton modulation of GLYT1b may be an important factor in determining the dynamics of excitatory neurotransmission.





In the central nervous system, the amino acid glycine acts as a coagonist with glutamate on the *N*-methyl-D-aspartate (NMDA) subtype of ionotrophic glutamate receptors (Johnson and Ascher, 1987). One of the postulated mechanisms for regulation of synaptic glycine concentrations is by high affinity glycine transport [see below and Bergeron et al. (1998)]. Glycine transporters (GLYTs) are members of the $Na^+/Cl^-$-dependent neurotransmitter transporter family that includes the γ-aminobutyric acid, dopamine, norepinephrine, serotonin, choline, proline, betaine, and taurine transporters (Amara and Kuhar, 1993). Two high-affinity glycine transporters, both with multiple splice variants, have been identified (Guastella et al., 1992; Liu et al., 1992, 1993; Smith et al., 1992; Ponce et al., 1998; Hanley et al., 2000). The GLYT1 subtypes are expressed in the glial elements of the hippocampus, cortex, and cerebellum, as well as the brainstem and spinal cord. On the basis of tissue distribution studies, it has been suggested that the GLYT1 transporter subtypes are responsible for regulation of glycine levels at excitatory synapses (Smith et al., 1992). Glycine also acts as a classical inhibitory neurotransmitter in the spinal cord (Aprison and Werman, 1965). GLYT2 transporters are expressed in neurons of the spinal cord and brain stem directly associated with strychnine-sensitive glycine receptors and are likely to provide the principal uptake mechanism at inhibitory glycinergic synapses (Jursky and Nelson, 1995; Poyatos et al., 1997; Spike et al., 1997).

The concentration of glycine present within excitatory synapses is not well established, in particular whether or not the concentration in the immediate vicinity of NMDA receptors is sufficient to saturate the glycine-binding site on these receptors. The glycine concentration in the cerebrospinal fluid is estimated to be ~14 μM (Semba and Patsalos, 1993), which is well above the $EC_{50}$ value for glycine activation of NMDA receptors [0.2–1.7 μM (Hollmann and Heinemann, 1994)]. However, in a recent study using a novel glycine transport inhibitor, it was demonstrated that glycine concentrations within the synapse are not sufficient to saturate the glycine site on NMDA receptors (Bergeron et al., 1998). The concentrating capacity of glycine transporters is determined by the stoichiometry of ion flux coupling. The stoichiometry of glycine transport is likely to be 2 $Na^+$:1 $Cl^-$:1 glycine (Aragon et al., 1987); from this ratio, the *equilibrium* glycine concentration can be calculated. In standard ionic solutions, the equilibrium glycine concentration is calculated to be 149 nM (Attwell et al., 1993). The factors that will determine whether this concentration is reached within the time frame of excitatory neurotransmission are the discrete volume of the synapse, the number of transporters present within the immediate vicinity of the synaptic cleft, and the turnover rate for the transport process. If the resting concentration of

This work was supported by the National Health and Medical Research Council of Australia.

**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; GLYT, glycine transporter; GLYT1b, glycine transporter subtype 1b.

149 nM is achieved, it would be well within the dynamic response range of glycine activation of NMDA receptors. In this case, subtle changes in glycine transporter activity would be expected to greatly influence the occupancy of glycine at NMDA receptors. A reduction in the rate of glycine clearance from the synapse would transiently elevate glycine concentrations and increase NMDA receptor activity.

Fluctuations in pH, under physiological and pathological conditions, modulate the activity of a number of proteins involved in neurotransmission. Glutamate transport is thermodynamically coupled to the pH gradient, with 1 $H^+$ transported with each glutamate molecule (Zerangue and Kavanaugh, 1996). However, under pathological conditions of elevated extracellular $K^+$, reduced pH inhibits reverse glutamate transport (Billups and Attwell, 1996). The activity of NMDA receptors is also modulated by fluctuations in pH. Protons act as noncompetitive inhibitors of NMDA receptors with half-maximal inhibition occurring at pH 7.3 (Traynelis and Cull-Candy, 1990). Thus, under physiological conditions, the activity of NMDA receptors may be dramatically altered by subtle fluctuations in pH. Another important factor that may influence the dynamics of excitatory neurotransmission is that pH may regulate the activity of glycine transport. Aragon et al. (1987) reported that glycine uptake by C6 glioma cells, which contain a mixture of high- and low-affinity transporters, is inhibited by reduced pH. In the light of recent studies suggesting that glycine transporters may play a dynamic role in regulating glycine concentrations within excitatory synapses, we have investigated in more detail the mechanism and site of action of protons on the glycine transporter, GLYT1b, expressed in *Xenopus laevis* oocytes. The results presented suggest that protons regulate the activity of GLYT1b, which may provide an important regulatory mechanism in excitatory neurotransmission.

## Experimental Procedures

**Materials.** All chemicals were obtained from Sigma Chemical Co. (Sydney, Australia) unless otherwise stated. Restriction enzymes were obtained from Bresatec (Adelaide, Australia). Dr. Marc Caron kindly supplied the plasmid containing the human GLYT1b cDNA.

**Expression of GLYT1b in *Xenopus laevis* Oocytes and Electrophysiological Recordings.** cDNAs encoding human GLYT1b were subcloned into pOTV (oocyte transcription vector). The GLYT1b-OTV plasmid was linearized with *Spe*I and cRNA transcribed from the cDNA construct with T7 RNA polymerase and capped with 5′7-methyl guanosine using the mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX). Mutations in GLYT1b were generated using the Quickchange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) and used according to the manufacturer's instructions. Oocytes were harvested from *Xenopus laevis* as described previously (Vandenberg et al., 1997). cRNA (50 nl) was injected into defoliculated, stage V *Xenopus laevis* oocytes and incubated at 16°C in standard frog Ringer's solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.55), supplemented with 2.5 mM sodium-pyruvate, 0.5 mM theophylline, and 50 $\mu$g/ml gentamicin. Two to eight days later, current recordings were made using the two-electrode voltage clamp technique with a Geneclamp 500 (Axon Instruments, Foster City, CA) interfaced with an MacLab 2e chart recorder (ADInstruments, Sydney, Australia). In experiments concerning the voltage dependence of glycine transport, the Geneclamp was interfaced with a Digidata 1200 (Axon Instruments) controlled by an IBM-compatible computer using the pCLAMP software (version 7; Axon Instruments).

Recordings were made using the standard frog Ringer's solution and, in experiments investigating the effects of pH, HCl and NaOH were used to adjust pH of the extracellular recording solutions. In experiments in which the sodium concentration was altered, equimolar choline was substituted for sodium and KOH was used to adjust pH. The current-voltage relationships for glycine transport at different pH values were determined by subtraction of steady-state current measurements in the absence of glycine, obtained during 300-ms voltage pulses to potentials between −100 mV and +60 mV in 10-mV steps, from corresponding current measurements in the presence of glycine as described previously (Vandenberg et al., 1995).

**[$^3$H]Glycine Uptake Studies.** [$^3$H]Glycine uptake by oocytes expressing GLYT1b and uninjected oocytes was measured under voltage-clamp conditions. Oocytes were voltage-clamped at −60 mV and 30 $\mu$M [$^3$H]glycine applied for 1 min with constant flow followed by a 2-min wash out. The oocyte was removed from the recording chamber and the [$^3$H]glycine flux for each oocyte was measured by scintillation counting. Estimates of the net charge generated by [$^3$H]glycine transport were calculated by integrating the transport current over 1 min using the Chart software (Version 3.5; ADInstruments).

**Analysis of Kinetic Data.** Current (I) as a function of glycine concentration ([Gly]) was fitted by least-squares analysis to $I/I_{max} = [Gly] / (EC_{50} + [Gly])$, where $I_{max}$ is the maximal current and $EC_{50}$ is the concentration of glycine that generates a half-maximal current. $I_{max}$ for glycine dose responses at different pH values were normalized to a maximal glycine dose at pH 7.5. Current as a function of proton concentration ($[H^+]$) was also fitted by least-squares analysis to $I/I_{max} = (1 - [H^+]) / (IC_{50} + [H^+]) + C / I_{max}$, where $IC_{50}$ is the $[H^+]$ at half-maximal reduction in transport current and C is the residual transport current at maximal proton inhibition of transport. Sodium dose responses at pH values of 7.5 and 5.5 were fitted to $I/I_{max} = [Na^+]^n / (EC_{50})^n + [Na^+]^n)$, where $n$ is the Hill coefficient.

ANOVA in Excel 5 (Microsoft Corp., Redmond, WA) was used to test for significant differences between measurements, and $P$ values less than .05 were taken as being significant.

## Results

Glycine transport is coupled to the $Na^+$ and $Cl^-$ gradients across the cell membrane with a probable stoichiometry of 2 $Na^+$:1 $Cl^-$:1 glycine (Aragon et al., 1987), generating a net transfer of one positive charge per transport cycle. The electrogenic nature of glycine transport allows the use of electrophysiology techniques to characterize the transport process (Supplisson and Bergman, 1997; Lopez-Corcuera et al., 1998). Application of glycine to oocytes expressing GLYT1b, voltage clamped at −60 mV, generates dose-dependent inward currents with an $EC_{50}$ value of 20 ± 4 $\mu$M ($n$ = 11) (Fig. 1).

**Proton Modulation of Glycine Transport.** Dose-dependent glycine transport currents were measured at pH values of 5.5, 6.0, and 7.5 for each oocyte. The maximal transport currents were reduced at pH values of 5.5 and 6.0 compared with pH 7.5. However, there was no significant difference in the $EC_{50}$ values for glycine at the different pH levels [$EC_{50}$ at pH 5.5 = 20 ± 5 $\mu$M ($n$ = 8); $EC_{50}$ at pH 6.0 = 17 ± 4 $\mu$M ($n$ = 5); $EC_{50}$ at pH 7.5 = 20 ± 4 $\mu$M ($n$ = 11)] (Fig. 2A). The reduction in maximal current amplitude with no change in $EC_{50}$ values suggests that proton inhibition is noncompetitive with respect to glycine transport. Glycine transport was measured, using a fixed glycine concentration of 30 $\mu$M, at pH levels varying from 5.0 to 8.0 to get an indication of the pH range at which inhibition occurs and also the extent of maximal inhibition (Fig. 2B). The pH at which inhibition was

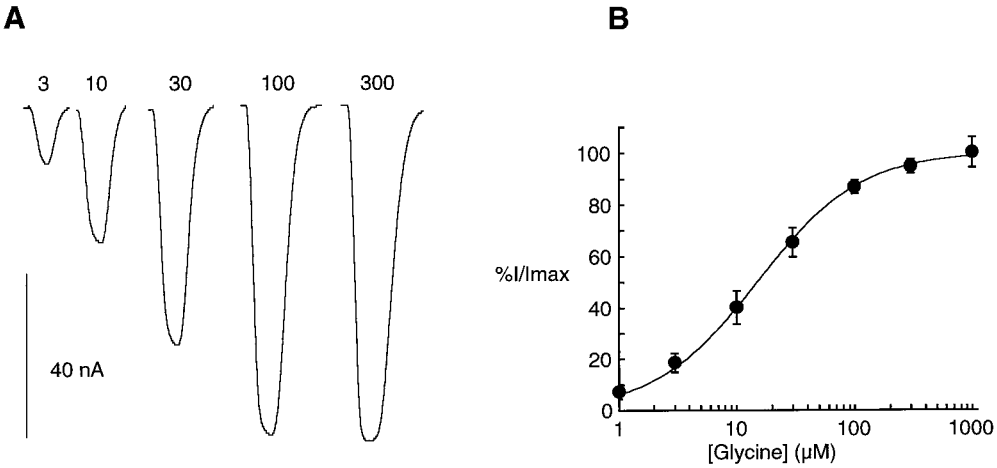


**Fig. 1.** Glycine transport by GLYT1b is electrogenic. Application of glycine to oocytes expressing GLYT1b generates a dose-dependent inward current when voltage-clamped at −60 mV. A, current trace from a representative oocyte in response to application of increasing doses of glycine (μM). B, a normalized dose-response curve. Results presented represent the mean ± S.E., $n$ = 11 oocytes.

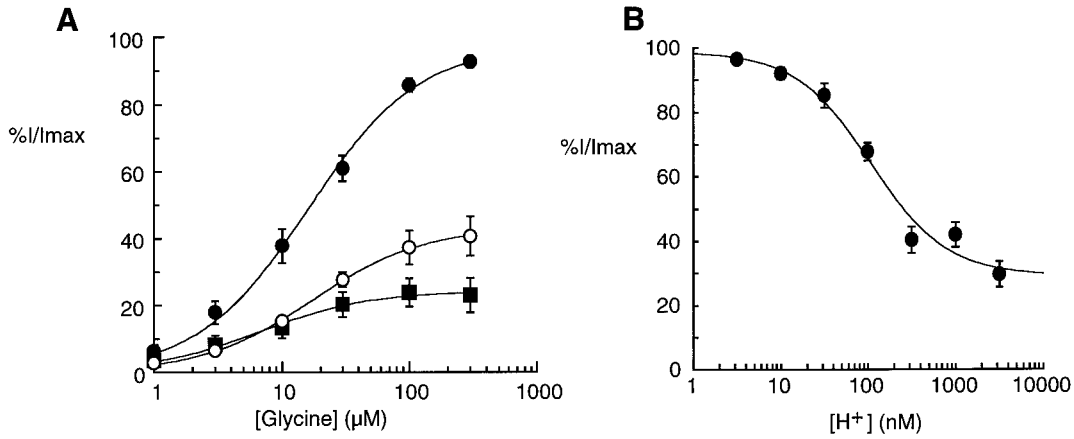


**Fig. 2.** Protons inhibit glycine transport without interfering with glycine binding. Oocytes expressing GLYT1b were voltage-clamped at −60 mV. A, glycine dose-dependent transport currents were measured in buffers at pH 7.5 (●; $EC_{50}$ = 20 ± 4 μM; $n$ = 11); pH 6.0 (○; $EC_{50}$ = 17 ± 4 μM; $n$ = 5), and pH 5.5 (■; $EC_{50}$ = 20 ± 5 μM; $n$ = 8). B, transport of 30 μM glycine in frog Ringer's solution was measured at the indicated pH values ($n$ = 7). Current measurements were normalized to the maximal current at pH 7.5 and fit to $I/I_{max} = (1 - [H^+]) / (IC_{50} + [H^+]) + c/I_{max}$ (see *Experimental Procedures)*. All data represent mean ± S.E. for the number of oocytes indicated.

half-maximal was 7.0 ± 0.1 ($n$ = 7) with a maximal inhibition of 63 ± 8% ($n$ = 7). Thus, at a physiological pH of 7.3, the rate of glycine transport by GLYT1b is at 79% of maximal activity.

Protons modulate the activity of a number of sodium channels and sodium-dependent transporters by competing with sodium in binding to the protein (Woodhull, 1973; Tolner et al., 1995). We investigated this idea as a possible mechanism for proton inhibition of glycine transport by measuring glycine transport currents using a fixed glycine concentration and varying the sodium concentration (Fig. 3). At pH 7.5, the $EC_{50}$ value for sodium dependence of glycine transport was 24 ± 3 mM ($n$ = 8) with a Hill coefficient of 1.7 ± 0.3 ($n$ = 8). Although the glycine transport current at a maximal dose of sodium was reduced at pH 5.5 compared with pH 7.5, the $EC_{50}$ value for sodium was not significantly different ($EC_{50}$ = 33 ± 3 mM; Hill coefficient = 2.3 ± 0.2; $n$ = 8; single-factor ANOVA). These results suggest that sodium and protons do not compete for the same binding site on the transporter. A small difference in Hill coefficients for sodium were observed at the two pH levels, but the significance of this difference in terms of a kinetic mechanism for inhibition of transport is not clear (Colquhoun, 1998). Although glycine transport currents are voltage-dependent, the extent of inhibition of glycine transport was constant over the membrane potential range of

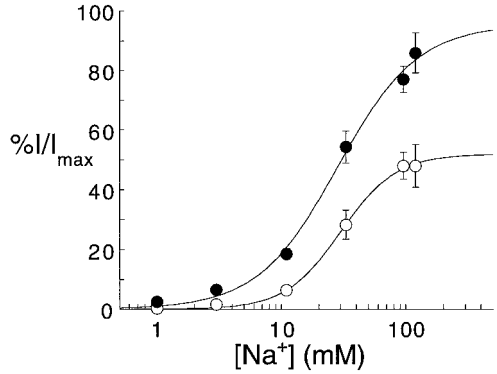


**Fig. 3.** Protons reduce maximal transport, but do not change the $EC_{50}$ value for $[Na^+]$. Glycine (30 μM) was applied to oocytes expressing GLYT1b with varying extracellular $[Na^+]$ at pH 7.5 (●; $EC_{50}$ $(Na^+)$ = 24 ± 3 mM; $n$ = 8) and pH 5.5 (○; $EC_{50}$ $(Na^+)$ = 33 ± 3 mM; $n$ = 8). Transport currents were normalized to the maximal current at pH 7.5 and fitted to $I/I_{max} = ([Na^+]^n)/(EC_{50}{}^n + [Na^+]^n)$. Data represent mean ± S.E. from the indicated number of oocytes.

−100 mV to + 60 mV (Fig. 4). This indicates that the proton-binding site on GLYT1b is unlikely to be within the electric field of the membrane. These results, together with the non-competitive interaction between glycine and protons and so-

dium and protons, suggest that protons are unlikely to directly interfere with ion coupling of glycine transport.

In other members of the $Na^+/Cl^-$-dependent neurotransmitter transporter family, protons have been shown to potentiate substrate activated conductances without altering the influx of substrate (Cao et al., 1997). We investigated the relationship between glycine transport currents and [$^3$H]glycine flux measurements and the effects of pH on this relationship. [$^3$H]glycine (30 $\mu$M) was applied to oocytes voltage-clamped at −60 mV in frog Ringer's buffer at pH 5.5 or 7.5 for 1 min. The rate of [$^3$H]glycine transport was calculated after subtraction of [$^3$H]glycine uptake of nude oocytes from oocytes expressing GLYT1b. At pH 5.5, the transport currents and [$^3$H]glycine uptake were reduced by 64 ± 7 and 55 ± 8%, respectively, compared with pH 7.5 (Fig. 5). The total charge transferred across the membrane was calculated by integrating the current measured during the 1 min application of 30 $\mu$M [$^3$H]glycine and from this value the charge-to-flux ratio was calculated. At pH 5.5, the charge to flux ratio of 1.0 ± 0.3 was not significantly different from the value obtained at pH 7.5 of 1.2 ± 0.3 (single-factor ANOVA) (Fig. 5). These results demonstrate that the reduction in transport *current* at reduced pH is caused by a reduction in transport *rate*. The charge-to-flux ratios obtained are consistent with the stoichiometry of 2 $Na^+$:1 $Cl^-$:1 glycine, as suggested by Aragon et al. (1987).

**Site-Directed Mutagenesis of Histidine Residues in the Extracellular Domains of GLYT1b.** The p$K_a$ value for proton inhibition of glycine transport by GLYT1b is 7.0 ± 0.1 (Fig. 2B), which is within the range of reported p$K_a$ values for the titration of histidine residues. We tested the hypothesis that a histidine residue in an extracellular domain of GLYT1b is responsible for conferring pH sensitivity using a site-directed mutagenesis strategy. Histidine residues at positions 199, 239, 410, 421, and 588 in the extracellular loops of GLYT1b were mutated to alanine, and histidine 213 was mutated to proline because in the closely related GLYT2 subtype of glycine transporters, a proline is found at this site. Application of glycine to oocytes expressing the six mutant transporters generated inward currents of similar magnitude to wild-type GLYT1b, which demonstrates that the functional integrity of the transporters was not compromised. The activity of the mutants was initially compared with wild-type GLYT1b by measuring glycine transport currents at pH 6.0 and 7.5; only the H421A mutant showed activity significantly different from wild type (Fig. 6A). In wild-type GLYT1b, the amplitude of the transport current at pH 6.0 was 44 ± 6% ($n$ = 8) of the current measured at pH 7.5,

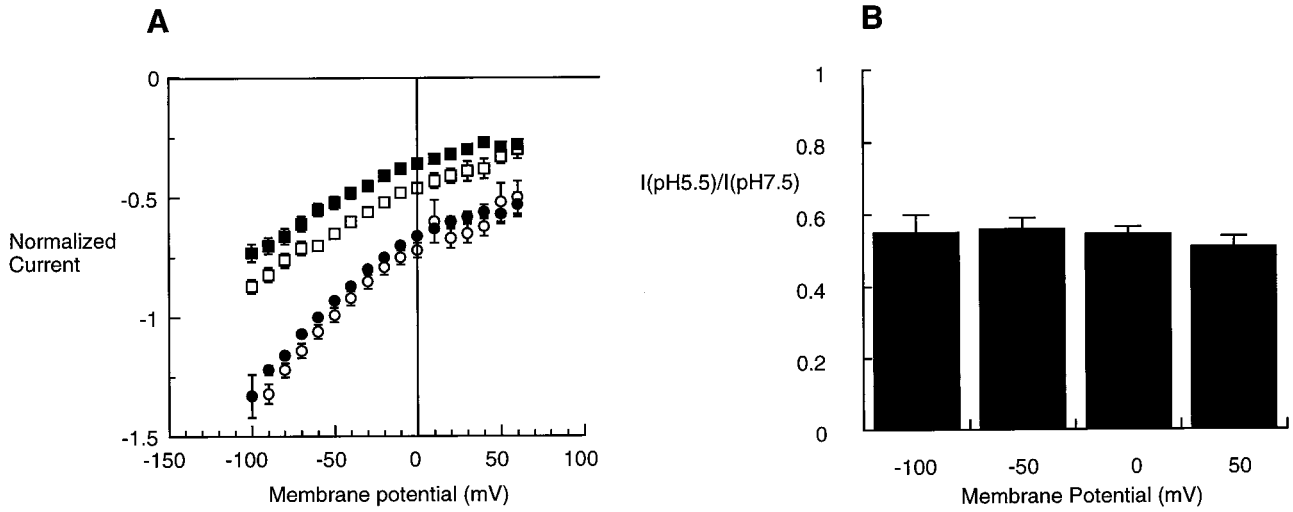


**Fig. 4.** Proton inhibition of glycine transport is independent of membrane potential. Glycine (100 $\mu$M) was applied to oocytes expressing GLYT1b at pH 8.0 (○; $n$ = 7), pH 7.5 (●; $n$ = 13), pH 6.5 (□; $n$ = 8), and pH 5.5 (■; $n$ = 12) and transport currents measured by the voltage step protocol outlined under *Experimental Procedures*. Data was normalized to the transport current generated at −60 mV at pH 7.5 for each oocyte.

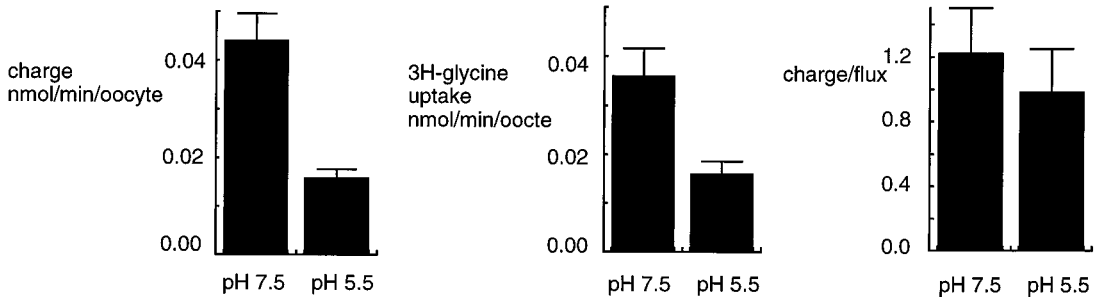


**Fig. 5.** Charge, flux and charge-to-flux ratio measurements for [$^3$H]glycine uptake at pH 5.5 and 7.5. Oocytes expressing GLYT1b were voltage-clamped at −60 mV and 30 $\mu$M [$^3$H]glycine applied for 1 min, followed by a 2-min washout. The total charge transfer was calculated from the current-time integral (A) and [$^3$H]glycine flux was measured by liquid scintillation counting (B). Values represent mean ± S.E. The charge-to-flux ratios were obtained by dividing the charge transfer by the flux of [$^3$H]glycine and adding proportional errors (C). Although there were significant differences in charge transfer and [$^3$H]glycine flux values at pH 5.5 ($n$ = 10) compared with pH 7.5 ($n$ = 12) ($P < 0.05$; single-factor ANOVA), the charge-to-flux ratio at the two pH values was not significantly different (single-factor ANOVA).

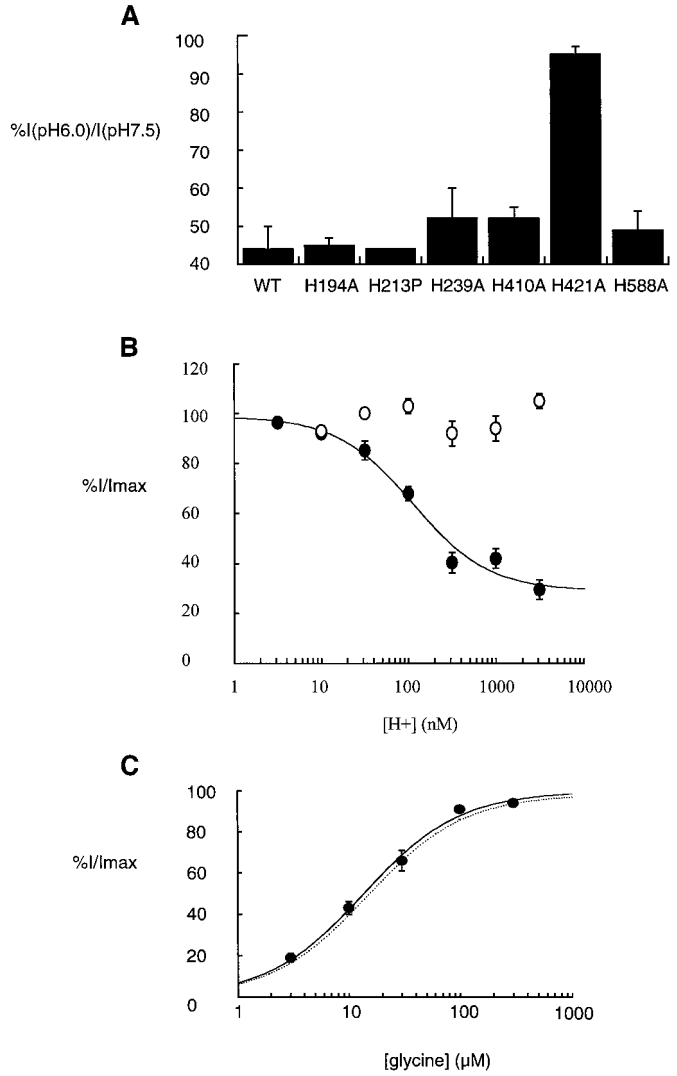


**Fig. 6.** Proton inhibition of glycine transport currents for mutant and wild-type GLYT1b. A, glycine (100 $\mu$M) was applied to oocytes expressing the various mutant and wild-type GLYT1b transporters that were voltage clamped at −60 mV and at pH 7.5 and 6.0. Current measurements are normalized to the transport current measured at pH 7.5 for each transporter. Results presented are the mean ± S.E. from three cells expressing the H199A, H213P, H239A, or H588A mutants and five cells expressing the H421A mutant. The data for the wild-type GLYT1b are derived from Fig. 2. B, pH titration curves for 100 $\mu$M glycine transport currents measured over the pH range of 5.5 to 8.0 for the wild-type and H421A GLYT1b transporters. The data are normalized to the transport currents measured at pH 7.5 and are mean ± S.E. from five cells. C, glycine dose response curves for transport currents by wild-type and H421A GLYT1b. The data presented for the H421A GLYT1b are mean ± S.E. from five cells and the fitted curve for the wild-type from Fig. 1 are replotted (dotted line) to assist comparison with the mutant.

whereas for H421A, the transport current at pH 6.0 was 95 ± 2% of the value at pH 7.5 (Fig. 6, A and B). The $EC_{50}$ value for glycine transport by the H421A-GLYT1b was 14 ± 3 $\mu$M (Fig. 6C), which is not significantly different from wild-type GLYT1b ($EC_{50}$ = 20 ± 4), which suggests that the mutation has disrupted the proton regulatory site on GLYT1b without affecting the glycine transport function.

## Discussion

It has been assumed that glycine concentrations within excitatory synapses are sufficient to saturate glycine binding sites on NMDA receptors. However, in a recent study, glycine concentrations of up to 100 $\mu$M (2 orders of magnitude greater than the $EC_{50}$ value for NMDA receptors) were required to get maximal glycine enhancement of NMDA-mediated synaptic currents in neonatal hypoglossal motoneurons of the rat (Berger et al., 1998). This study concluded that glycine concentrations within certain synapses are not sufficient to saturate the NMDA receptor and are tightly regulated by glycine transporters. In a separate study, employing a novel GLYT1-selective blocker, *N*[3-(4′-flurophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine, it was demonstrated that blocking glycine transport enhanced NMDA-mediated synaptic currents in slices from the brainstem of the rat (Bergeron et al., 1998). This observation also suggests that resting glycine concentrations within the synapse are not sufficient to saturate NMDA receptors. These conclusions are in contrast to those of earlier studies, which in most cases concluded that the resting glycine concentration within the synapse was sufficient to saturate the binding site for glycine on NMDA receptors (Kemp and Leeson, 1993; Westergren et al., 1994). An explanation that accounts for both these findings is that glycine transporters may be abundantly expressed near NMDA receptors and are highly efficient at controlling glycine concentrations within a confined space (Supplisson and Bergman, 1997). Therefore, exogenous application of glycine, at concentrations well above the $EC_{50}$ value for glycine activation of NMDA receptors, is required to potentiate the response. From this argument, it follows that processes modulating the rate of glycine clearance from the synapse may be important determinants of the extent of glycine occupancy of NMDA receptors. In addition to the requirement for glycine as a coagonist of NMDA receptors, glycine also slows the rate of desensitization of NMDA receptors (Vyklicky et al., 1990). Therefore alterations in glycine concentrations in the synapse caused by modulation of glycine transport would modulate both amplitude and time course of NMDA receptor-mediated currents. NMDA receptors bind glycine with an affinity of 0.2 to 2 $\mu$M; therefore, a transient elevation in glycine concentrations from 150 nM to, say, 1 $\mu$M, caused by inhibition of glycine transport, would cause a 2- to 5-fold increase in occupancy of NMDA receptors. Thus, endogenous or exogenous modulators of glycine transporters may be expected to influence the dynamics of excitatory neurotransmission mediated by NMDA receptors. Protons may be such modulators. In this study we have used electrophysiological techniques to investigate in detail the mechanism of proton regulation of glycine transport.

**Protons Regulate the Rate of Glycine Transport.** Glycine transport has been demonstrated to be sensitive to changes in pH in C6 glioma cells (Zafra and Gimenez, 1989). We have further characterized this process for the GLYT1b subtype expressed in *X. laevis* oocytes. Protons inhibit glycine transport by GLYT1b with half-maximal inhibition at pH 7.0 ± 0.1 (Fig. 2B). Lowering the pH did not alter the $EC_{50}$ values for either glycine or sodium transport but did reduce the maximal transport rate (Figs. 2 and 3), suggesting that proton inhibition is noncompetitive with respect to both glycine and sodium. Proton inhibition of glycine transport is independent of the membrane potential (Fig. 4), indicating that the proton inhibition site is unlikely to be within the electrical field of the membrane and that protons are unlikely to influence any voltage-dependent steps in the transport

process. Finally, the reduction in transport currents at low pH values directly correlates with a reduction in the rate of glycine transport, which demonstrates that protons do not alter the ion flux-coupling ratio of glycine transport (Fig. 5). In light of these findings, we propose that protons modulate glycine transport by binding to an extracellular site on the transporter that is distinct from the sites that bind and transport glycine and sodium through the pore of the protein.

**The $H^+$ Modulatory Site on GLYT1b and Comparisons with Other Neurotransmitter Transporters.** Mutation of histidine 421 in the fourth extracellular loop to an alanine residue selectively removes pH sensitivity of GLTY1b without altering glycine transport. Thus, this residue seems to confer the pH sensitivity of GLYT1b. This residue is not conserved in the GLYT2 subtypes of glycine transporters or other members of the $Na^+/Cl^-$-dependent neurotransmitter transporter family; although an exhaustive study has not been performed, of the transporters investigated to date, only the GLYT1 transporters seem to be noncompetitively inhibited by protons. The structurally related serotonin transporter is also modulated by pH, but in a very different manner. Protons increase the amplitude of the transport conductance, but do not seem to alter the rate of serotonin transport (Cao et al., 1997). A serine residue at position 490 and a glutamate residue at position 493 in extracellular loop 5 have been identified as conferring this property (Cao et al., 1998). It is interesting to note that this proton regulatory site is in a different extracellular loop of the transporter than the proton regulatory site of GLYT1b, which suggests that the different loops may play different functional roles in regulating transporter function. The dopamine transporter (DAT) is noncompetitively inhibited by zinc ions by a mechanism that shows a number of parallels with the mechanism of proton inhibition of glycine transport (Norregaard et al., 1998). Two of the amino acid residues that form part of the zinc binding site are located at the beginning and end of the fourth extracellular loop (Loland et al., 1999). The location of the proton regulatory site on GLYT1b and the zinc-binding site on DAT to the fourth extracellular loop of this family of structurally related transporters suggests that this loop may form part of an important regulatory domain. The interaction of zinc with DAT, or $H^+$ with GLYT1b, may alter the conformation of this loop and transmit this change to transmembrane domains 7 and 8 to regulate the rate of transport. It would be of considerable interest if mutations of residues in other members of the $Na^+/Cl^-$-dependent neurotransmitter transporter family in this extracellular domain that correspond to the zinc and $H^+$ binding sites of DAT and GLYT1b are capable of altering the regulatory properties of these other transporters. The identification of an extracellular regulatory domain may also be exploited in attempts to develop compounds designed to mimic the actions of $H^+$ or $Zn^{2+}$ ions. Such compounds may be of therapeutic value in treating various neurological disorders in which altered transporter function have been implicated.

**Physiological and Pathological Implications of Proton Regulation of Glycine Transport.** The $IC_{50}$ value for proton modulation of glycine transport is close to the standard physiological extracellular pH of 7.3; therefore, small fluctuations in pH will significantly alter the rate of glycine clearance from the synapse. An acidic shift in the extracellular pH reduces the rate of glycine transport and may cause a transient elevation in synaptic glycine concentrations. An alkaline shift in extracellular pH increases the rate of glycine transport and will bring the resting glycine concentration closer to the theoretical equilibrium concentration of 149 nM. The physiological consequences of changes in the rate of glycine transport on the dynamics of excitatory neurotransmission are complex because protons also modulate the activity of a number of other proteins involved in neurotransmission. Protons inhibit NMDA receptors by binding to an extracellular site on the receptor with an $IC_{50}$ value of pH 7.3, but do not seem to influence the activity of the AMPA and kainate subtypes of ionotropic glutamate receptors (Tang et al., 1990; Traynelis and Cull-Candy, 1990). Protons play a dual role in determining the activity of glutamate transporters. First, the proton gradient across the cell membrane is coupled to the transport process and will influence the concentrating capacity of the transporter (Zerangue and Kavanaugh, 1996; Levy et al., 1998) Second, protons also inhibit reverse glutamate transport into the synapse (Billups and Attwell, 1996). Thus, under mild acidic conditions, glycine concentrations may be elevated, glutamate concentrations may initially be unchanged, but may increase with prolonged acidic conditions (see below), and NMDA receptor activity will be reduced. The inhibition of NMDA receptors under these conditions has generally been thought of as being the overriding factor in determining the dynamics of excitatory neurotransmission (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Billups and Attwell, 1996).

However, splice variants of the NR1 subunit of NMDA receptors containing exon 5 are less sensitive to proton inhibition (Traynelis et al., 1995). Exon 5 is spliced into NR1 subunits expressed in the brainstem, thalamus, cerebellum, colliculi, CA3 region of the hippocampus, pontine nucleus, sensory motor cortex, and the subthalamic nucleus (Standaert et al., 1993; Laurie and Seeburg, 1994); therefore, NMDA receptors in these regions, which contain exon 5 in NR1 subunits, will not be significantly modulated by acidic shifts in pH. Thus, in these regions, elevations of glycine concentrations under mild acidic conditions, because of inhibition of glycine transport, may play an important role in increasing the activity of NMDA receptors.

Fluctuations in extracellular pH occur as a consequence of normal excitatory neurotransmission (Chesler and Kaila, 1992) and also under pathological conditions, such as an ischemic insult (von Hanwehr et al., 1986). The time frame for these fluctuations is an important factor in determining which processes may be modulated. Transport rates for the γ-aminobutyric acid and glutamate transporters have been estimated to be of the order of 10 $s^{-1}$ (Mager et al., 1993; Wadiche et al., 1995). If we assume that the rate of transport by GLYT1b is similar, then if pH fluctuations are to influence the rate of glycine transport, they must occur over a comparable time frame. Prolonged acidic pH shifts, measured using extracellular pH microelectrodes, have been measured in a number of brain regions after repetitive stimulation and epileptiform activity [reviewed by Chesler and Kaila (1992)]. Changes in pH of up to 0.3 pH units develop in a matter of seconds and may be maintained for several minutes. If such changes occur near glycine transporters, then modulation of transporter activity may be an important factor in determining the dynamics of neurotransmission. During the first minute of cerebral ischemia, extracellular pH steadily declines from 7.3 to approximately 6.7, which would cause significant inhibition of glycine transport. With prolonged ischemia, there are large changes in sodium and potassium

gradients across the cell membrane and elevations in extracellular glutamate concentrations caused by reverse glutamate transport (Rossi et al., 2000) and also elevated glycine concentrations (Globus et al., 1991). Excessive stimulation of NMDA receptors during prolonged ischemia is thought to be largely responsible for triggering cell death. The results presented in this study suggest that the elevation in glycine concentrations may be caused in part by pH inhibition of glycine transport. Although glycine is a necessary factor in the pathological processes of ischemia (McNamara et al., 1990; Lazarewicz et al., 1997), its impact may be most critical in the early phase of ischemia, before large changes in the $Na^+$ and $K^+$ gradients develop and glutamate concentrations become elevated. The elevation in glycine concentrations within the synapse caused by pH inhibition of glycine transport may lead to increased activity of NMDA receptors containing the exon 5 in the NR-1 subunits and may be one of the factors triggering the subsequent destructive phase of ischemia.

In light of the results presented here and those of Berger et al. (1998) and Bergeron et al. (1998), investigations into the effects endogenous and exogenous compounds on glycine transporters may lead to a more complete understanding of the role of glycine transporters in the regulation of excitatory neurotransmission. This information may also provide the basis for the development of novel therapeutic targets for the treatment of neurological disorders.

**Acknowledgment**

We thank Drs. Mark Connor and Mac Christie for critical review of the manuscript.

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