Asymmetric Cross-inhibition between GABA$_A$ and Glycine Receptors in Rat Spinal Dorsal Horn Neurons*

Received for publication, April 9, 2003, and in revised form, June 16, 2003
Published, JBC Papers in Press, July 28, 2003, DOI 10.1074/jbc.M303735200

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Presynaptic nerve terminals of inhibitory synapses in the dorsal horn of the spinal cord and brain stem can release both GABA and glycine, leading to coactivation of postsynaptic GABA$_A$ and glycine receptors. In the present study we have analyzed functional interactions between GABA$_A$ and glycine receptors in acutely dissociated neurons from rat sacral dorsal commissural nucleus. Although the application of GABA and glycine activates pharmacologically distinct receptors, the current induced by a simultaneous application of these two transmitters was less than the sum of currents induced by applying two transmitters separately. Sequential application of glycine and GABA revealed that the GABA-evoked current is more affected by glycine than glycine-evoked responses by GABA. Activation of glycine receptors decreased the amplitude and accelerated the rate of desensitization of GABA-induced currents. This asymmetric cross-inhibition is reversible, dependent on the agonist concentration applied, but independent of both membrane potential and intracellular calcium concentration or changes in the chloride equilibrium potential. During sequential applications, the asymmetric cross-inhibition was prevented by selective GABA$_A$ or glycine receptor antagonists, suggesting that occupation of binding sites that are not necessary to induce glycine and GABA$_A$ receptors functional interaction, and receptor channel activation is required. Furthermore, inhibition of phosphatase 2B, but not phosphatase 1 or 2A, prevented GABA$_A$ receptor inhibition by glycine receptor activation, whereas inhibition of phosphorylation pathways rendered cross-talk irreversible. Taken together, our results demonstrated that there is an asymmetric cross-inhibition between glycine and GABA$_A$ receptors and that a selective modulation of the state of phosphorylation of GABA$_A$ receptor and/or mediator proteins underlies the asymmetry in the cross-inhibition.

Although neurotransmission involves specific activation of receptor channels by distinct neurotransmitters, different classes of receptor can be colocalized at the same postsynaptic site and may be activated by the corelease of more than one type of neurotransmitter from the same presynaptic nerve terminal. Recently it has been demonstrated that simultaneous activation of different postsynaptic receptors by the coapplication of their specific neurotransmitter induces cross-modulation of their activation properties. This cross-talk phenomenon has been proposed to represent a fast adaptive process in controlling signal transmission (1). Negative cross-talk was demonstrated between ATP P2X and nicotinic acetylcholine receptors (2–5), between dopamine and adenosine receptors (6), between γ-aminobutyric acid type A (GABA$_A$) and dopamine (1) or P2X receptors (7), as well as between dopamine and N-methyl-D-aspartic acid receptors (8). In general, the cross-talk between these receptors is characterized by a partial occlusion of transmitter-evoked currents, i.e. the sum of the amplitudes of responses evoked by each agonist is larger than the amplitude of responses evoked by coapplication of the two neurotransmitters. The molecular mechanism involved in the interaction between P2X and nicotinic acetylcholine receptors is unknown. Negative cross-talk between dopamine and adenosine receptors, between GABA$_A$ and dopamine receptors, and between dopamine and N-methyl-D-aspartic acid receptors involves direct intramembranous protein-protein interaction (1, 6, 8), whereas cross-talk between P2X receptor and GABA$_A$ receptors (GABA$_A$R) is modulated by chloride efflux and intracellular Ca$^{2+}$ (7).

In the spinal cord, brain stem (9–12), and cerebellum (13), GABA and glycine can be coreleased by the same synaptic terminal, whereas GABA$_A$R and glycine receptors (GlyR) can be coaggregated at the same postsynaptic site (13, 14). There is evidence suggesting that GABA$_A$ and GlyR both gate chloride-permeable channels but have distinct structures, resulting from oligomerization of specific subunits (15, 16). There is evidence suggesting that GABA$_A$R and GlyR can interact negatively in mammalian (17, 18) or lamprey (19) spinal cord neurons, in olfactory bulb cells (20), and in the hippocampus (21), i.e. the effects of GABA and glycine are less than additive when they are coupled. The mechanisms responsible for this cross-inhibition between GABA and glycine remain poorly understood. One possibility is that it reflects the presence, at least in the olfac-

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* This work was supported by Knowledge Innovation Project Grant KSCX 2-2-04 from the Chinese Academy of Sciences, National Basic Research Program of China Grant G1999054000, and National Natural Science Foundation of China Grants 30170247 and 30125015 (to T.-L. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: GABA$_A$, γ-aminobutyric acid type A; ATP$_S$, adenosine 5′-O-(3-thiotriophosphate); BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CSPN, cyclosporin A; $E_{Cl}^T$, theoretical CI$^-$ equilibrium potential; $E_{GABA}$ and $E_{Gly}$, GABA- and glycine-induced currents, respectively; GABA$_A$R, GABA$_A$ receptor(s); GlyR, glycine receptor(s); $I_{Cl,\text{GABA}}$ and $I_{Cl,\text{Gly}}$, GABA- and glycine-induced CI$^-$ currents, respectively; OA, okadaic acid; $V_h$, holding potential.
and subsequent figures, unless otherwise noted, the \( V_h \) was 10 mV. Normally) Wistar rats (2 weeks old) were decapitated. A segment about 15 mm long of lumbosacral (L5-S3) spinal cord was quickly dissected at room temperature (22–25 °C). Culture dishes (Corning 430165, Corning, Inc., Corning, NY) were used as recording chambers and were perfused at 0.5–2.0 ml/min with the standard external solution. 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), and had a resistance of 4–6 megohms. Membrane potentials were corrected for the liquid junction potential, which had a measured value of 3–4 mV. The series resistance (\( R_s \)) estimated from visual cancellation of the capacity transient, was 5–15 megohms. In most experiments, 80–90% series resistance compensation was applied. Artifacts caused by inadequate voltage clamp and space clamp were minimized by selecting for experiment neurons bearing no or short processes. Transmitter-evoked currents recorded from the cell soma normally did not exceed 5 nA. Unless otherwise noted, CsCl solution was used in the recording pipette. After taking these precautions, the largest recorded currents did not differ in time course from the smaller ones. To ensure cell dialysis, data for measurements were obtained at

EXPERIMENTAL PROCEDURES

Cell Preparation—Rat sacral dorsal commissural nucleus neurons were acutely dissociated according to the method of Wu et al. (11). In brief, pentobarbital-sodium-anesthetized (45–50 mg kg \(^{-1}\), intraperitoneally) Wistar rats (2 weeks old) were decapitated. A segment about 10–15 mm long of lumbosacral (L5-S3) spinal cord was quickly dissected out and immersed in the standard external solution at freezing temperatures. After removing attached dorsal rootlets and the pia matter with O2 was subsequently placed in the chamber to immerse the tissue block. The tissue block was then placed in the cutting chamber of a vibratome tissue slicer (LEICA VT1000S, Leica Instruments Ltd., Wetzlar, Germany). A cold standard external solution (about 4 °C) bubbled with O2 was subsequently placed in the chamber to immerse the tissue block. The spinal segment was sectioned to yield several transverse slices of thickness 400 µm. Slices were preincubated in oxygenated incubation solution for 30 min at room temperature (22–25 °C) and then treated enzymatically in oxygenated incubation solution containing Pronase (1 mg/ml) for 20 min at 31 °C. This treatment was followed by exposure to thermolysin (1 mg/ml) for 15 min. After the enzyme treatment, slices were kept in enzyme-free incubation solution for 1 h. Then a portion of dorsal horn region was micropunched out and transferred into a culture dish filled with the standard external solution. Neurons were mechanically dissociated with fire-polished Pasteur pipettes under visual guidance under a phase contrast microscope (IX70, Olympus Optical Co., Ltd., Tokyo, Japan). Within 20 min, isolated neurons had attached to the bottom of the culture dish and were ready for electrical recording. The care and use of animals in these experiments followed guidelines and protocols approved by our institutional Animal Care and Use Committee.

Electrophysiology—Whole cell, voltage clamp recordings were made at room temperature (22–25 °C). Culture dishes (Corning 430165, Corning, Inc., Corning, NY) were used as recording chambers and were perfused at 0.5–2.0 ml/min with the standard external solution. 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), and had a resistance of 4–6 megohms. Membrane potentials were corrected for the liquid junction potential, which had a measured value of 3–4 mV. The series resistance (\( R_s \)) estimated from visual cancellation of the capacity transient, was 5–15 megohms. In most experiments, 80–90% series resistance compensation was applied. Artifacts caused by inadequate voltage clamp and space clamp were minimized by selecting for experiment neurons bearing no or short processes. Transmitter-evoked currents recorded from the cell soma normally did not exceed 5 nA. Unless otherwise noted, CsCl solution was used in the recording pipette. After taking these precautions, the largest recorded currents did not differ in time course from the smaller ones. To ensure cell dialysis, data for measurements were obtained at

FIG. 1. Glycine- and GABA-evoked currents in dissociated sacral dorsal commissural nucleus neurons. A, and B, sample recordings demonstrating inward currents induced by glycine and GABA at various concentrations. The bars above each trace indicate the drug application period. \( A_g \) and \( B_g \), concentration-response relationships for \( I_{Gly} \) and \( I_{GABA} \). \( I_{Gly} \) and \( I_{GABA} \) were normalized to the peak current amplitude induced by 1 mM Gly (\( A_g \)) and 1 mM GABA (\( B_g \)), respectively. Each point represents the mean ± S.E. of six to eight neurons. In this and subsequent figures, unless otherwise noted, the \( V_h \) was –50 mV.

FIG. 2. Cross-inhibition between \( I_{GABA} \) and \( I_{Gly} \). A, whole cell currents were induced by application of either 1 mM GABA or 1 mM Gly or by the simultaneous application of 1 mM GABA and 1 mM Gly. All three current traces were obtained from one neuron. The dashed trace represents the expected sumation of the \( I_{GABA} \) and \( I_{Gly} \). B, mean values of 36 experiments shown in A, C, summary of current inhibition data of different concentrations of GABA plus Gly. Ordinates represent \( I_{GABA} + I_{Gly}/I_{GABA} \) × 100%. Each bar, the concentrations of GABA and Gly were the same. The dashed line indicates \( I_{GABA} + I_{Gly}/I_{Gly} \). In this and subsequent figures, the number of experiments is shown in parentheses. **, \( p < 0.01 \).
least 3–5 min after the whole cell configuration was established. Solutions—The composition of incubation solution was (in mM) 124 NaCl, 24 NaHCO₃, 5 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgSO₄, and 10 glucose, and it was aerated with 95% O₂ and 5% CO₂ to a final pH of 7.4. The standard external solution contained (in mM) 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with Tris. The osmolarity of all bath solutions was adjusted to 310–320 mosm/liter with sucrose. The ionic composition of the internal solution medium was (in mM) 120 CsCl, 30 NaCl, 0.5 CaCl₂, 2H₂O, 1 MgCl₂, 6H₂O, 5 EGTA, 2 MgATP, and 10 HEPES with the pH adjusted to 7.2. Stocks of MgATP stored at −20 °C were dissolved in the intracellular solution shortly before use to a final concentration of 2 mM. Unless otherwise noted, the membrane potential was held at −50 mV in the voltage clamp studies. 

Drugs and Application System—Cyclosporin A (CSVPN), staurosporine, and Li₄ATP·S were obtained from Biomol. Okadaic acid (OA) and GF 109203X were from Tocris (Bristol, UK), and H89, KN93, and genistein were from Sigma. All other drugs were from Sigma. Phosphatase inhibitors and protein kinase inhibitors were added to the pipette solution. Agonists or antagonists of GlyR and GABAAR were diluted with extracellular solution to a final concentration and applied via the “Y-tube” method as described previously (22). The tip of the drug tube was positioned between 50 and 100 μm away from the patched neurons. This system allows a complete exchange of external solution surrounding a neuron within 20 ms. Throughout the experiment the bath was perfused continuously with the standard external solution. 

Data Acquisition and Analysis—Signals were filtered at 1 kHz, data were collected with an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA), which was connected to a Pentium III computer equipped with Digidata 1320A, Clampex, and Clampfit software (Axon Instruments). Data were analyzed with the pCLAMP software (Axon Instruments) and ORIGIN for Windows (Micrcal Software, Northampton, MA). Results are presented as the mean ± S.E. (n = number of cells), with statistical significance assessed by Student’s t test for two groups’ comparison or one-way analysis of variance test for multiple comparisons. A p value of < 0.05 or 0.01 was considered statistically significant. To evaluate the strychnine and bicuculline concentrations at half-maximal inhibition of I₆0, I₃₀GABA (IC₅₀), the mirror image of the Michaelis-Menten equation

\[ I(t) = I_0 \left[ 1 + \left( \frac{[A]}{IC_{50}} \right)^2 \right] \]  

(Eq. 2)

where I₉₀ and I₅₀ are the amplitudes of the fast and slow components, respectively; I₀ was the amplitude of the non-desensitized current; and τₙ and τₛ are respective time constants.

RESULTS

GABA- and Glycine-induced Responses—Cross-talk between GABAₐR and GlyR was analyzed on acutely dissociated sacral dorsal commissural nucleus neurons from P14 rats. At this age, neurons express the mature form of GABAₐR and GlyR (15, 23). Whole cell recording at the holding potential (Vₜₐₜ) of −50 mV showed that application of GABA or glycine evoked inward chloride currents in all cells tested. The reversal potentials of GABA- and glycine-induced currents (E₆0GABA and E₆0Gly) were −2.5 ± 1.1 (S.E., n = 6) and −1.3 ± 0.8 mV (n = 5), respectively, and were not significantly different (p > 0.1; unpaired t test). They were close to the theoretical Cl⁻ equilibrium potential (E_cl⁻) calculated from the Nernst equation (−1.3 mV), based on the external and internal Cl⁻ concentration used in the recording (see “Experimental Procedures”).

The saturating concentration and EC₅₀ for GABA or glycine were determined by analyzing concentration-response curves for responses to a 10-s application of agonists (Fig. 1). I₆0GABA and I₆0Gly were normalized to the peak amplitude of the currents evoked by the application of glycine and GABA at 1 μM, respectively. The normalized data were fitted using a single isotherm function of the form,

\[ \frac{I(t)}{I_{max}} = 1/(1 + (EC_{50}/[agonist])^{H}) \]  

(Eq. 3)

where I₉₀ is the maximum current amplitude, I₆0 is the normalized current amplitude, EC₅₀ is the agonist concentration producing 50% of the I₆0, and H is the Hill coefficient. This fit produced an EC₅₀ of 8.1 and 34 μM for GABA and glycine, respectively. The Hill coefficients for GABA and glycine were 1.18 and 1.27, respectively.
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Cross-inhibition between $I_{GABA}$ and $I_{Gly}$—The above experiments suggest that the saturating concentration for GABA or glycine for inducing neuronal responses was $\geq$1 mM. This suggests that responses evoked by the application of 1 mM GABA or 1 mM glycine should be additive if they are induced by the activation of independent receptors. We therefore compared peak amplitudes of currents induced by the application of either GABA or glycine (at 1 mM) and by the simultaneous application of both agonists. As shown in Fig. 2A, coapplication of GABA and glycine evoked currents with an amplitude that was significantly lower than the expected sum ($I_{EX}$) of currents evoked separately by GABA and glycine (Fig. 2B), suggesting a cross-inhibition between $I_{GABA}$ and $I_{Gly}$ (Fig. 2B; $n = 36; p < 0.01; t$ test). A quantitative analysis of this cross-inhibition and its dependence on concentrations of both agonists is shown in Fig. 2C. At 10 mM glycine and glycine, no significant cross-inhibition occurred (91.5 $\pm$ 5.3% of the $I_{EX}$; $n = 9; p > 0.05$). Significant cross-inhibition was observed at 30 mM GABA and glycine (55.1 $\pm$ 6.4% of the $I_{EX}$; $n = 11; p < 0.01$), and the effect appears to saturate beyond 30 mM concentrations of the two agonists.

Cross-inhibition Was Not Caused by Nonspecific Receptor Activation by GABA and Glycine—It has been suggested that at high concentrations GABA may activate GlyR (24, 25), resulting in an apparent cross-talk between GABA$_R$ and GlyR (20). We therefore used selective antagonists to determine the specificity of GABA and glycine in their receptor activation (Fig. 3). At 30 mM bicuculline, a selective GABA$_A$R antagonist, the $I_{GABA}$ induced by 1 mM GABA was totally abolished, whereas the $I_{Gly}$ induced by 1 mM glycine was unaffected (Fig. 3A and B). Conversely, 1 mM strychnine, a potent GlyR antagonist, completely abolished $I_{Gly}$ without affecting $I_{GABA}$. Dose-response curves for bicuculline and strychnine inhibition were obtained from currents evoked by glycine or GABA at 1 mM. The bicuculline inhibited $I_{GABA}$ in a concentration-dependent manner with an IC$_{50}$ of 0.68 mM (Fig. 3C). Strychnine also inhibited $I_{GABA}$, but only at concentrations higher than 1 mM (Fig. 3D), with IC$_{50}$ of 2.83 mM (Fig. 3D). The inhibitory effect of bicuculline on $I_{Gly}$ was also evident only at concentrations higher than 30 mM (Fig. 3C), a concentration that completely suppressed $I_{GABA}$. Strychnine was effective in suppressing $I_{Gly}$ at concentrations $\geq$0.01 mM (Fig. 3D). In contrast, the IC$_{50}$ values for the inhibition of $I_{Gly}$ were 0.04 and 74.2 uM for strychnine and bicuculline, respectively. Taken together, these results suggest that at a concentration of 1 mM, GABA and glycine are unlikely to cross-activate GlyR or GABA$_A$R, respectively, significantly.

Cross-inhibition between GABA$_A$R and GlyR Was Asymmetric—A different mode of drug application was used to explore further the interactions between $I_{Gly}$ and $I_{GABA}$ and to examine relative contributions of GABA$_A$R and GlyR to the cross-inhibition. As shown in Fig. 4A, GABA or glycine responses obtained in the presence of the other agonist were reduced in amplitude, with GABA-evoked responses more strongly affected by glycine than the reverse condition. The cross-inhibi-
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Fig. 6. Functional receptors are required for the current inhibition. A, whole cell currents induced by 1 mM GABA and sequential application of Gly and GABA at 1 mM in the absence or presence of strychnine (Str). B, whole cell currents induced by 1 mM Gly and sequential application of GABA and Gly at 1 mM in the absence or presence of bicuculline (Bic). In the presence of 1 μM strychnine or 30 μM bicuculline, the sequential application of GABA and Gly induced a current with amplitude and kinetics similar to the current induced by GABA or Gly alone. The recordings in A and B were from different neurons. C, statistical data showing the pooled results obtained on nine cells. Strychnine or bicuculline was pretreated for 30 s before simultaneous application with GABA or Gly. NS indicates no statistical significance. *, p < 0.05; **, p < 0.01.

Cross-inhibition was also observed during sequential application of 1 mM GABA and 1 mM glycine or vice versa. As shown in Fig. 4B, when glycine was applied before GABA, the amplitude of $I_{GABA}$ became $37.4 \pm 2.5\%$ ($n = 12$; $p < 0.01$) of the control values observed in the absence of glycine. Similarly, $I_{Gly}$ was reduced to $77.9 \pm 4.0\%$ ($n = 15$; $p < 0.05$) of the control value after a prepulse of 1 mM GABA. Thus the suppression of $I_{GABA}$ by GlyR activation was significantly larger than inhibition of $I_{Gly}$ by GABA<sub>A</sub>R activation ($p < 0.01$). Fig. 4C summarizes the percent reduction in the mean amplitude of $I_{GABA}$ and $I_{Gly}$ when different prepulse concentrations of glycine and GABA were used, respectively. Glycine became effective in cross-inhibition at concentrations ≥ 30 μM, whereas GABA was effective at 100 μM. This asymmetric cross-inhibition between GABA<sub>A</sub>R and GlyR did not depend on chloride fluxes or changes in $E_{Cl}$. As shown in Fig. 5A, cross-inhibition was observed at both $V_h = -50$ mV and $+50$ mV ($V_h = 10$). This asymmetry between GABA<sub>A</sub>R and GlyR did not depend on chloride fluxes or changes in $E_{Cl}$. The suppression of $I_{GABA}$ by GlyR activation was significantly larger than inhibition of $I_{Gly}$ by GABA<sub>A</sub>R activation ($p < 0.01$). Fig. 4C summarizes the percent reduction in the mean amplitude of $I_{GABA}$ and $I_{Gly}$ when different prepulse concentrations of glycine and GABA were used, respectively.

Response evoked by successive applications of glycine and GABA at 1 mM were estimated by voltage ramp application during the steady-state phase of the evoked currents (Fig. 5A). As shown in Fig. 5C, successive glycine- and GABA-evoked responses had similar reversal potentials. During the glycine prepulse $E_{Cl} = -4.15 \pm 0.4$ mV, whereas it was $-3.98 \pm 0.58$ mV during successive GABA application ($n = 8$).

Receptor Channel Activation Is Required for Cross-inhibition—The data shown in Fig. 4 suggest that cross-inhibition depends on the prepulse agonist concentration. However, it remained unclear whether specific receptor channel opening and thus changes in receptor conformation triggered by the prepulse application are required for asymmetric cross-inhibition. To address this issue, we have used specific competitive antagonists for GABA<sub>A</sub>R and GlyR, bicuculline and strychnine, respectively. Sequential applications of glycine-GABA at 1 mM (Fig. 6A), or GABA-glycine at 1 mM (Fig. 6B) were performed in the presence of 1 μM strychnine and 30 μM bicuculline, respectively, at a concentration that selectively inhibits GlyR or GABA<sub>A</sub>R completely (Fig. 3, C and D). As shown in Fig. 6, A and B, during sequential applications of transmitters, application of the antagonist known to compete with transmitter binding of the preactivated receptor prevented cross-inhibition. Fig. 6C summarizes the results from all experiments ($n = 9$) by comparing the amplitudes of control currents (in the absence of prepulses and antagonists) and currents observed after prepulse application, in the absence or presence of either strychnine (for $I_{GABA}$) or bicuculline ($I_{Gly}$). We found that in the presence of bicuculline, $I_{Gly}$ observed after the GABA prepulse had the same amplitude as the control $I_{Gly}$ (2.151 ± 0.243 versus 2.213 ± 0.259 with or without bicuculline, $p > 0.1$). Similarly, in the presence of strychnine, $I_{GABA}$ observed after the glycine prepulse had the same amplitude as the control $I_{GABA}$ (1.615 ± 0.226 versus 1.638 ± 0.204 with or without strychnine, $p > 0.1$). These data indicate that cross-inhibition between GlyR and GABA<sub>A</sub>R cannot be explained solely by ligand binding and suggest that activation of receptor channel is required.

Kinetics of Cross-inhibition—Inhibition of $I_{GABA}$ by preactivation of GlyR was accompanied by significant changes in the time course of the desensitizing $I_{GABA}$ component. This was not the case for $I_{Gly}$ when GABA<sub>A</sub>R was preactivated. To examine potential changes in the desensitization of $I_{Gly}$ or $I_{GABA}$ during cross-inhibition, time constants of the desensitizing phase of the corresponding current were measured. The desensitization time course of $I_{Gly}$ evoked by 10-s applications of 1 mM glycine with or without GABA prepulses could be fitted by the sum of two exponential curves with time constants $\tau_1$ and $\tau_2$. As shown in Fig. 7, preactivation of GlyR accelerated the desensitization of GABA<sub>A</sub>R (Fig. 7, A and B). In contrast, preactivation of GABA<sub>A</sub>R did not change GlyR desensitization kinetics significantly (Fig. 7C).

We have also examined whether cross-inhibition between GlyR and GABA<sub>A</sub>R was reversible and whether it requires simultaneous activation of the two amino acid receptors. As shown in Fig. 8A, we found that increasing the time interval between sequential applications of glycine and GABA at 1 mM (or vice versa) resulted in a progressively reduced cross-inhibition. The effect of time interval between successive applications of agonist on cross-inhibition was analyzed on 12 and 14 cells for $I_{GABA}$ and $I_{Gly}$ inhibition, respectively. Half-inhibition of $I_{GABA}$ was obtained at interval times close to 41 s (Fig. 8B). Cross-inhibition cannot be evoked for $I_{GABA}$ for interpulse intervals ≥ 55 s. The time interval for $I_{Gly}$ inhibition evoked by
GABA receptors at which no cross-talk occurred was shortened (≥22.5%). Half-inhibition of \( I_{\text{Gly}} \) occurred at time intervals close to 14 s (Fig. 8B).

Inhibition of \( I_{\text{GABA}} \) by GlyR Activation Depends on Phosphatase 2B—It is known that phosphatase 2B (calcineurin) activity can inhibit \( I_{\text{GABA}} \) activation and \( I_{\text{Gly}} \) desensitization kinetics (29, 30). We therefore asked whether phosphatase activity might be involved in the cross-inhibition between \( I_{\text{GABA}} \) and GlyR. We first examined the effect of the phosphatase 2B inhibitor CSPN on the asymmetric cross-inhibition elicited by sequential application of 1 mM glycine and GABA at 1-s intervals. When loaded into neurons via the patch electrode, 500 nM CSPN prevented \( I_{\text{GABA}} \) inhibition by preapplication of glycine or \( I_{\text{Gly}} \) activation (Fig. 9, A2, A3, and A). OA became nonspecific at concentration higher than 1 μM and mimics the effect of CSPN. Similar to that found for CSPN, 5 μM OA had no significant effect on \( I_{\text{Gly}} \) inhibition evoked by GABA preapplications. Because phosphatase 2B activation may depend on intracellular \( \text{Ca}^{2+} \) concentration, we have examined the effect of buffering intracellular \( \text{Ca}^{2+} \) with BAPTA. When the neurons were loaded with 15 mM BAPTA via the recording pipette, \( I_{\text{GABA}} \) was decreased to 39.7 ± 4.5% of its control value, whereas \( I_{\text{Gly}} \) was reduced to 81.2 ± 4.5% of the control following the glycine or GABA prepulse, respectively (Fig. 9, A4 and B). These values did not differ significantly from the cross-inhibition obtained in control conditions (\( p > 0.1; \) unpaired \( t \) test). Thus the results indicate that changes in intracellular \( \text{Ca}^{2+} \) were not directly involved in the cross-inhibition. Inhibition of \( I_{\text{GABA}} \), but not of \( I_{\text{Gly}} \), depends on phosphatase 2B activity, whereas phosphatase 1 and 2A did not appear to be involved.

The level of substrate phosphorylation depends on the action of kinases and phosphatases in the neuronal cytoplasm. Prevention of cross-inhibition by phosphatase 2B inhibition may result from a shift in balance in favor of phosphorylation of the substrate by protein kinases. We first tested this hypothesis by loading the neuron with ATPγS, which facilitates protein phosphorylation by donating a thioisophosphate group in a kinase-mediated reaction that resists hydrolysis by phosphatases (32). As shown in Fig. 10, loading the cells with 150 μM ATPγS (with \( \text{Li}^+ \)) prevented \( I_{\text{GABA}} \) inhibition induced by preapplication of GlyR. The effect of ATPγS on \( I_{\text{GABA}} \) inhibition increased with
time after the onset of whole cell configuration and completely abolished the inhibition within 15 min. The effect is unlikely to result from the loading of Li\(^+\) because loading of LiCl (up to 20 mM) did not affect the cross-inhibition (data not shown; \(n = 4\)). In contrast to the effect on \(I_{\text{GABA}}\) cross-inhibition, \(I_{\text{Gly}}\) inhibition evoked by GABA prepulse application was not affected by loading of ATP\(_\gamma\)S (Fig. 10).

To confirm further the role of protein phosphorylation, we analyzed the effect of 5 mM staurosporine, a nonspecific protein kinase inhibitor, on the cross-inhibition between GABA\(_A\)R and GlyR (Fig. 11). If changes in GABA\(_A\)R activation properties resulted from changes in the balance between phosphorylation and dephosphorylation processes, protein kinase inhibition should render cross-inhibition irreversible. The protocol used to determine time interval dependence of cross-inhibition in the presence of staurosporine was identical to that described in Fig. 8. In control conditions, recovery from cross-inhibition occurred in less than 50 s (Fig. 8). However, staurosporine evoked a rundown of \(I_{\text{GABA}}\) when applied alone. This was not the case for \(I_{\text{Gly}}\). A 50% rundown of \(I_{\text{GABA}}\) was found after 40–50 min. To overcome this problem, the effects of staurosporine on cross-inhibition were tested when \(I_{\text{GABA}}\) amplitude had declined to 50%. Change in GABA response amplitude evoked by glycine prepulse in the presence of staurosporine was compared with change in GABA response amplitude with time in the presence of staurosporine but without prepulse application of glycine. As shown in Fig. 11B, \(I_{\text{GABA}}\) was only slightly decreased 100 s after attaining 50% rundown. Staurosporine cannot block the inhibition of \(I_{\text{GABA}}\) or \(I_{\text{Gly}}\) evoked by preactivation of GlyR or GABA\(_A\)R, respectively. However, in these conditions we observed no recovery from \(I_{\text{GABA}}\) inhibition up to 2 min after evoking cross-inhibition. In contrast, staurosporine had no effect on the recovery of \(I_{\text{Gly}}\) (Fig. 11B). These results indicate that kinase activity is needed for GABA\(_A\)R but not for GlyR to recover from cross-inhibition. Interestingly, specific antagonists of protein kinase A (H89, 10 mM, \(n = 6\) cells), protein kinase C (GF 109203X, 3 mM, \(n = 6\) cells), tyrosine kinase (genistein, 5 mM, \(n = 10\) cells) or calmodulin-dependent protein kinase II (KN93, 10 mM, \(n = 10\) cells) had no effect on cross-inhibition and its recovery. However, applying a mixture of all four kinase inhibitors mimicked the staurosporine effect on the recovery from cross-inhibition.

**DISCUSSION**

In this study, we have demonstrated that activation of GABA\(_A\)R and GlyR in acutely dissociated rat spinal dorsal horn neurons can induce specific asymmetric cross-inhibition of currents induced by GABA and glycine. Our results reveal a novel form of interaction between signal events mediated by two distinct anionic channels and show that specific involvement of intracellular phosphorylation pathways triggered by GlyR activation underlies the asymmetry in the cross-inhibition between the GABA\(_A\)R and GlyR.

Cross-inhibition Depends on Intracellular GABA\(_A\)R and GlyR Signaling—Although negative cross-talk between GABA\(_A\)R and GlyR has been suspected previously (20, 21, 33), the underlying mechanisms remain controversial. Trombley et
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activation of GABA<sub>α</sub> and glycine receptor channels is required for the cross-inhibition.

What downstream signaling events associated with the receptor channel activation are involved in the cross-inhibition? It is unlikely that alterations in the Cl<sup>-</sup> gradient resulting from channel activation mediate the cross-inhibition, as proposed previously by Grassi (33). We found that the inhibition remained when I<sub>Gly</sub> and I<sub>GABA</sub> were changed from inward to outward, indicating that the movement of Cl<sup>-</sup> is not critical for the inhibition. Furthermore, measurements of agonist-induced Cl<sup>-</sup> currents with the application of a voltage ramp also showed no change in the reversal potential for the Cl<sup>-</sup> current (34). These data indicate that cross-inhibition is a receptor-mediated event unrelated to the Cl<sup>-</sup> flux across the membrane.

**Asymmetry in Cross-inhibition**—When GABA and glycine were coapplied, the Cl<sup>-</sup> current was smaller than the sum of the two individual currents evoked by the application of each agonist. The occlusion level depended on the concentration of the two agonists, and it was nearly maximal when the concentration was 30 μM. Thus, the intensity of the cross-inhibition between GABA<sub>α</sub>R and GlyR might be determined by the number of activated receptors, as proposed previously for the interactions between P2X and GABA<sub>α</sub>R (7).

To determine the respective contribution of the two agonists to the cross-inhibition, we examined the responses evoked by sequential application of these two compounds. Preapplication of glycine more strongly inhibited I<sub>GABA</sub> than preapplication of GABA did to I<sub>Gly</sub>. The level of current inhibition depended on the concentration of the prepulse agonist, in a manner consistent with that found when GABA and glycine were coapplied. Furthermore, the effect of prepulse glycine on I<sub>GABA</sub> is accompanied by a decrease in the desensitization time constants of the current response. This was not the case for the effect of GABA prepulse on I<sub>Gly</sub>. Because the effect of desensitization was observed during successive applications of the two agonists, the changes in GABA<sub>α</sub>R desensitization kinetics by GlyR activation remain even when GlyR is fully deactivated. This effect on the nonactivated GABA<sub>α</sub>R appears to persist for more than 45 s after the end of the application of glycine. This effect cannot be accounted for simply by cross-desensitization between these two receptors (35) because only GABA<sub>α</sub>R desensitization properties were modified. These results are also in sharp contrast to the previous reports on the inhibitory cross-talk between ionotropic receptors. No changes in receptor desensitization properties were observed during cross-inhibition between GABA<sub>α</sub>R and P2X (7) or between P2X and nicotinic acetylcholine receptors (2).

**Cross-inhibition of GABA<sub>α</sub>R by Glycine Depends on Dephosphorylation**—Our results indicate that inhibition of I<sub>GABA</sub> by preapplied glycine involves phosphatase 2B activity, whereas its recovery requires protein kinase activity. Our findings suggest the following model of cross-inhibition: Glycine preapplication results in dephosphorylation by phosphatase 2B and inhibition of I<sub>GABA</sub>, whereas recovery from inhibition depends on rephosphorylation of GABA<sub>α</sub>R or of associated proteins. Activation of phosphatase 2B requires the activation of GlyR, but rephosphorylation of GABA<sub>α</sub>R or of an associated protein does not require the activation of either GlyR or GABA<sub>α</sub>R. Our observations also indicate that in the absence of glycine prepulse, the balance between basal phosphorylation/dephosphorylation results in stable kinetic properties of the GABA<sub>α</sub>R during repetitive applications of GABA.

There are three potential mechanisms by which GlyR activation may evoke dephosphorylation by phosphatase 2B. First, activated GlyR may directly activate phosphatase 2B. To our knowledge, there is no existing evidence in support of this
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