Ethanol Suppresses Fast Potentiation of Glycine Currents by Glutamate

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ABSTRACT

Excitatory (glutamate) and inhibitory (GABA A and glycine) receptor/channels coexist in many neurons. To assess effects of ethanol on the interaction of glutamate and glycine receptors, glycine-induced current (I Gly ) was recorded by a whole-cell patch-clamp technique from neurons freshly dissociated from the ventral tegmental area of rats. A conditioning prepulse of glutamate (1–3 s, 1 mM) significantly and reversibly potentiated responses to a pulse of glycine. This potentiation was increased when extracellular calcium was raised to 12 mM and reduced by including 10 mM of glycine. This potentiation was increased when extracellular calcium was raised to 12 mM and reduced by including 10 mM of glycine. When coapplied with glycine, ethanol (10 mM) potentiated I Gly in 35% of neurons from the ventral tegmental area. In contrast, when coapplied with glutamate and glycine, ethanol suppressed the glutamate-induced potentiation of I Gly in these neurons. This suppression was also observed when ethanol and glycine were coapplied after a glutamate prepulse. A similar effect was observed when ethanol alone did not potentiate I Gly . These findings suggest that glutamate-induced calcium influx modulates glycine receptors by a mechanism that can be blocked by ethanol.

Ethanol is the most abused substance in the United States. There is now compelling evidence that ethanol directly and/or indirectly affects many receptor/ion channels, including N-methyl-D-aspartate (NMDA), non-NMDA (AMPA), GABA A, glycine, 5-HT 3 , and nicotinic acetylcholine receptors (Narahashi et al., 2001). Modulation of these receptors by ethanol may be responsible for its behavioral effects. Because ethanol acts at many sites in the central nervous system, studies of the effects of ethanol on interactions between excitatory and inhibitory synaptic mechanisms are crucial.

The ventral tegmental area (VTA) contains the cells of origin of the mesolimbic system, which is important for the rewarding properties of drugs of abuse like ethanol (Gatto et al., 1994; Wise, 1996). There are two main types of neurons in the VTA: dopamine and nondopamine neurons (Lacey et al., 1989; Johnson and North, 1992). Both receive monosynaptic glutamatergic innervation from prefrontal cortex and have NMDA and non-NMDA receptors (Wang and French, 1993, 1995). According to Floresco et al. (2001), glutamatergic afferents from the hippocampus to the nucleus accumbens strongly excite VTA dopamine neurons.

We have already reported that glycine-activated current (I Gly ) can be recorded in most VTA neurons, and that ethanol (0.1–40 mM) potentiated I Gly in VTA neurons of 5- to 14-day-old rats and thus alters their excitability (Ye et al., 2001a). Bearing in mind that ethanol alters intracellular Ca 2+ (for review, see Little, 1991; Simasko et al., 1999; Mennerick and Zorumski, 2000), interactions between ethanol and glycine receptors may involve mechanisms linked to intracellular Ca 2+. Three recent studies have reported that glutamate-induced Ca 2+ entry greatly potentiated I Gly in spinal neurons or oocytes expressing glycine receptors (Xu et al., 1999, 2000; Fucile et al., 2000). However, the effects of glutamate on VTA glycine receptors have not been examined. In view of the important function of glycine receptors in the VTA and the pivotal role of the VTA in drug addiction, we initiated the current study on freshly dissociated VTA neurons to examine...
ine: 1) the enhancement of \( I_{\text{Gly}} \) by glutamate and 2) the effects of ethanol on this potentiating action of glutamate.

**Materials and Methods**

**Isolation of Neurons and Electrophysiological Recording.**

The care and use of animals and the experimental protocol of this study were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey (protocol number 00074). Sprague-Dawley rats (5- to 14-day-old) were decapitated as described earlier (Ye et al., 2001a). The brains were quickly excised, placed into ice-cold saline saturated with 95% O\(_2\) and 5% CO\(_2\), and then immersed in the standard external solution containing 1 mg of pronase/6 ml and saturated with O\(_2\) and incubated at 31°C for 20 min. After 20 min of additional incubation in 1 mg of thermolysin/6 ml, the VTA was identified by its striking purple color and lateral to the fasciculus retroflexus under a dissecting microscope. Micro-punches of the VTA were isolated and transferred to a 35-mm culture dish.

**Mild**

**trituration through heat-polished pipettes of progressively smaller tip diameters dissociated single neurons.**

Within 20 min of trituration, isolated neurons attached to the bottom of the culture dish and were ready for electrophysiological experiments.

Under the light microscope, the cells acutely isolated from the VTA were divided into two groups: bipolar and multipolar. The majority was bipolar, with one to three dendritic processes emerging from each end of the fusiform soma (20–40 \( \mu \)m in length and 15–25 \( \mu \)m in diameter). The multipolar neurons were larger, with a diameter of 35 to 60 \( \mu \)m, and had four to five major dendrites. Most of the cells were sensitive to glycine. Glycine-induced current \( (I_{\text{Gly}}) \) was an enhancement of \( I_{\text{Gly}} \) by glutamate.

**The saline in which the brain was dissected contained 128 mM NaCl, 5 mM KCl, 1.2 mM Na\(_2\)PO\(_4\), 26 mM NaHCO\(_3\), 9 mM MgCl\(_2\), 0.3 mM CaCl\(_2\), and 2.5 mM glucose.**

The standard external solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with Tris base and the osmolarity to 320 mM with sucrose.

**Whole-cell current decays were fitted by a Chebyshev algorithm (pCLAMP).**

**Concentration-response data were analyzed with a nonlinear curve-fitting program (Sigma Plot; Jandel Scientific, San Rafael, CA).**

Data were statistically compared using Student's t test at a significance level of \( P < 0.05 \), unless otherwise indicated. For all experiments, average values are expressed as mean ± S.E.M., with the number of neurons indicated in brackets.

**Results**

**Glutamate Potentiates \( I_{\text{Gly}} \).**

In agreement with our previous observations, most neonatal VTA neurons (82%) were sensitive to glycine. Glycine-induced current \( (I_{\text{Gly}}) \) was antagonized by 0.1 \( \mu \)M strychnine (Ye et al., 1998). Glutamate (0.1 and 1 mM) elicited inward currents in all VTA neurons tested. At a holding potential of ~50 mV, larger peak currents were induced by 30 \( \mu \)M glycine \((-520 ± 68 \text{ pA, } n = 42)\) than by 1 mM glutamate \((-338 ± 44 \text{ pA, } n = 41)\). To examine the effect of glutamate on \( I_{\text{Gly}} \), a pulse of glycine (10–1000 \( \mu \)M) was preceded by a brief conditioning pulse of glutamate (1–3 s). For this purpose, 1 mM glutamate was routinely used, because of its very predictable action and its previous use in comparable experiments (Fucile et al., 2000). However, substantial potentiation of \( I_{\text{Gly}} \) could be obtained with 100 \( \mu \)M glutamate (see below).

The traces in Fig. 1A illustrate the effect of a glutamate prepulse (1 mM) on an immediately following \( I_{\text{Gly}} \), recorded from a neuron in standard external solution (2 mM Ca\(^{2+}\)).

**Involvement of Ca\(^{2+}\) in the Glutamate-Induced Potentiation of \( I_{\text{Gly}} \).**

According to recent reports, Ca\(^{2+}\) exerts...
a powerful and rapid modulation of glycine receptor/channels (Xu et al., 1999, 2000; Fucile et al., 2000). The following results suggest that Ca\(^{2+}\) also plays a role in $I_{\text{Gly}}$ potentiation in VTA neurons.

**Potentiation Was Greater when Extracellular Ca\(^{2+}\) Was Increased to 12 mM.** As shown in Fig. 2, when $[\text{Ca}^{2+}]_o$ was raised locally to 12 mM, $I_{\text{Gly}}$ increased by 65 ± 4% ($P < 0.01, n = 4$) and the magnitude of $I_{\text{Gly}}$ potentiation by glutamate by 55 ± 10% of control tests of glutamate in 2 mM $[\text{Ca}^{2+}]_o$ (Fig. 2B, $P < 0.01, n = 4$). This effect of $[\text{Ca}^{2+}]_o$ was

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**Fig. 1.** Glutamate prepulse enhances $I_{\text{Gly}}$ by a non-voltage-dependent mechanism. A, conventional whole-cell voltage-clamp recording with pipette containing 11 mM EGTA from a VTA neuron of a 13-day-old rat. $I_{\text{Gly}}$ was elicited by 30 μM glycine alone (a and c) and immediately after a conditioning prepulse of glutamate (Glu) (b; 1 mM, 2 s). Dotted lines d, e, and f indicate how current amplitude was measured: $I_{\text{Gly}}$ between baseline d and current peak e, peak $I_{\text{Gly}}$ between baseline d and f. B, similar recording from another cell shows rapid return of glutamate current to baseline after glutamate washout; in B-c, traces a and b are superimposed. This demonstrates the minimal overlap of glutamate tail current with the initial portion of $I_{\text{Gly}}$. Calibration: 10 s for traces a and b, 2.5 s for c. C, histogram shows variability of 1 mM glutamate-induced potentiation of $I_{\text{Gly}}$ (evoked by 30 μM glycine) in a population of 72 cells, all recorded with 11 mM EGTA-containing pipettes. D and E, glycine current-voltage relation was studied in a whole-cell recording with pairs of voltage ramps (from +40 to −80 mV), applied at a rate of 1 mV/10 ms. In each trace, the first, very small current ramp measured background/leakage current; the second measured the total current during 30 μM glycine application. The data plotted in F and G were obtained by subtracting the currents elicited by the first ramp from the currents elicited by the second ramp during glycine application, without (F) and with (G) the 1 mM glutamate prepulse. These current-voltage plots show that glutamate did not change the reversal potential of $I_{\text{Gly}}$. For this and the following figures, all the current traces were recorded at a holding potential of −50 mV. The boxes above or below each current trace indicate the duration of drug applications: blank and black boxes represent glycine and glutamate, respectively.

**Fig. 2.** Glutamate-induced potentiation of $I_{\text{Gly}}$ is enhanced by raising $[\text{Ca}^{2+}]_o$ but is unaffected by KN-62, a blocker of CAMKII. A, current traces from a neuron of an 8-day-old rat show $I_{\text{Gly}}$ elicited by 30 μM glycine before (a and c) and immediately after a brief conditioning prepulse of glutamate (Glu) (b; 1 mM, 2 s). B, similar recording from another cell shows rapid return of glutamate current to baseline after glutamate washout; in B-c, traces a and b are superimposed. This demonstrates the minimal overlap of glutamate tail current with the initial portion of $I_{\text{Gly}}$. Calibration: 10 s for traces a and b, 2.5 s for c. C, histogram shows variability of 1 mM glutamate-induced potentiation of $I_{\text{Gly}}$ (evoked by 30 μM glycine) in a population of 72 cells, all recorded with 11 mM EGTA-containing pipettes. D and E, glycine current-voltage relation was studied in a whole-cell recording with pairs of voltage ramps (from +40 to −80 mV), applied at a rate of 1 mV/10 ms. In each trace, the first, very small current ramp measured background/leakage current; the second measured the total current during 30 μM glycine application. The data plotted in F and G were obtained by subtracting the currents elicited by the first ramp from the currents elicited by the second ramp during glycine application, without (F) and with (G) the 1 mM glutamate prepulse. These current-voltage plots show that glutamate did not change the reversal potential of $I_{\text{Gly}}$. For this and the following figures, all the current traces were recorded at a holding potential of −50 mV. The boxes above or below each current trace indicate the duration of drug applications: blank and black boxes represent glycine and glutamate, respectively.
reducing the intracellular site of Ca^{2+} reflects the time required for BAPTA to equilibrate at the intracellular site of Ca^{2+} action. Note the unusually large effects of glutamate in traces A of Fig. 3, obtained with an electrode containing 4 mM EGTA; the potentiation persisted for more than 8 s (Fig. 3B). When I_{Gly} was recorded with such pipettes, even 100 μM glutamate induced a marked potentiation (146 ± 9%, n = 4; Fig. 3, C and D).

There was less potentiation when recording with electrodes containing 10 to 30 mM BAPTA. In these recordings, the potentiation induced by glutamate decreased with time: 4 min after the start of whole-cell recording, I_{Gly} was potentiated to 153 ± 8% of control, but by 16 min, to only 126 ± 4% of control (paired t test, P = 0.029, n = 4; cf. traces in Fig. 3E and histograms in Fig. 3F). Presumably, this reflects the time required for BAPTA to equilibrate at the intracellular site of Ca^{2+} action.

Although the glutamate-induced potentiation of I_{Gly} thus seems to depend on an increase in intracellular free Ca^{2+}, it was not affected by pretreating cells for 8 min with 5 mM KN-62, a selective calcium/calmodulin-dependent protein kinase II inhibitor: the large potentiations observed in the same five cells before and after applying KN-62 were to 372 ± 18 and 387 ± 21% of control, respectively (P = 0.67; Fig. 2, C and D).

Glutamate-Induced Potentiation Is Sensitive to Glycine Concentration. Glutamate could augment I_{Gly} either by increasing the number or conductance of functional glycine receptor channels or by modifying their sensitivity to glycine. To distinguish between these possibilities, we examined the effect of glutamate on I_{Gly} induced by 10 to 1000 μM glycine. The traces in Fig. 4 show I_{Gly} evoked by 30, 100, and 300 μM glycine, in the absence (Fig. 4A) and presence (Fig. 4B) of prepulses of glutamate (1 mM). Glutamate strongly potentiated I_{Gly} induced by submaximal concentrations of glycine (30 and 100 μM; Fig. 4, a and b); but it had a weaker effect on I_{Gly} induced by supramaximal concentrations of glycine (≥300 μM; Fig. 4C). On average, 1 mM glutamate potentiated peak I_{Gly} elicited by 30, 100, 300, and 1000 μM glycine to 180 ± 21, 152 ± 22, 118 ± 22, and 121 ± 20%, respectively (n = 4, Fig. 4D).

Concentration-response data obtained in the absence and presence of conditioning pulses of glutamate (1 mM) are illustrated in Fig. 4C. The EC_{50} and Hill coefficient were 87 μM and 1.96 in the absence of glutamate, and 47 μM and 1.83 after glutamate conditioning pulses. Thus glutamate reduced the EC_{50} by nearly 50%. Similar observations on three other cells gave a mean reduction to 64 ± 3% (n = 4). The conditioning pulses of glutamate thus increased the apparent affinity of the glycine receptor for its agonist. In addition, maximal I_{Gly} was also larger after glutamate (121 ± 8%, n = 4), as found by Xu et al. (1999).

Glutamate Alters the Kinetics of I_{Gly}. Changes in either agonist affinity or channel opening efficacy can alter the EC_{50} values of agonists (Colquhoun, 1998). Indeed, data from human embryonic kidney-AMPA cells transfected with α1H demonstrate different kinetics of I_{Gly} before and after a glutamate prepulse (Fucile et al., 2000). Therefore, we examined I_{Gly} channel activation, deactivation, and desensitization, before and after glutamate conditioning pulses. To allow accurate measurement within the limits of the fast perfusion system (time constant of ~10 ms), we applied glycine at a concentration of 30 μM (Fig. 5A). As previously observed (Ye et al., 2001a), both the onset and the decay of I_{Gly} could be fitted by a single exponential function (Fig. 5, C and D). The activation time constant (τ_{on}) was significantly shortened by a glutamate prepulse, from 340 ± 14 ms to 133 ± 22 ms (paired t test, P < 0.01, n = 8). In contrast, glutamate prolonged the deactivation time constant (τ_{off}), from 261 ± 19 ms to 350 ± 31 ms (Fig. 5B; paired t test, P < 0.01, n = 8). The slower decay indicates that glutamate increases the affinity of glycine for its receptor (Fucile et al., 2000).

Glutamate Accelerates Glycine Receptor Desensitization. The potentiation of I_{Gly} by glutamate could result from a slower rate of receptor desensitization. To test for this possibility, we compared I_{Gly} desensitization in the absence and presence of glutamate prepulses. As shown in Fig. 5E, the current activated by a long pulse of glycine (30 μM) decayed more rapidly when applied after a brief pulse of glutamate. The ratio of the decay time constants (τ_{off}/τ_{control}) in Fig. 5E was 0.56. For six neurons (Fig. 5F), 1 mM glutamate significantly shortened the time constant of desensitization from 6.9 ± 1.4 to 4.7 ± 0.8 s (paired t test, P < 0.05). Because glutamate enhanced the peak more than the steady-state I_{Gly}, the ratio of steady-state to peak current amplitude declined from 0.92 ± 0.02 to 0.71 ± 0.04 (paired t test, P < 0.01, n = 17).

Ethanol Potentiates I_{Gly} But Inhibits Glutamate Current. In agreement with previous findings (Ye et al., 2001a), 0.1 to 100 mM ethanol enhanced I_{Gly} in 35% of VTA neurons from 5- to 14-day-old rats. This effect is illustrated in Fig. 6A, where I_{Gly} evoked by 30 μM glycine was potentiated by 0.1, 1, and 10 mM ethanol (Fig. 6, A-b–A-d). After washout of ethanol, I_{Gly} recovered to control amplitude (Fig. 6, A-e). For a series of neurons, 0.1, 1, 10, and 100 mM ethanol enhanced peak I_{Gly} to 116 ± 5% (n = 3), 135 ± 4% (n = 34), 127 ± 3% (n = 34), and 117 ± 6% (n = 4) of control, respectively. When a brief pulse of 10 mM ethanol was coapplied during a longer pulse of glycine, there was an immediate and rapidly reversible increase in I_{Gly} (Fig. 6B). In contrast to this potentiation of I_{Gly}, 10 mM ethanol depressed glutamate-evoked current to 74 ± 3% (n = 10) of control (Fig. 6C), in agreement with previous reports (Narahashi et al., 2001).

Ethanol Suppresses the Glutamate-Induced Potentiation of I_{Gly}. Records a and b of Fig. 6D illustrate the usual potentiation of I_{Gly} by a conditioning prepulse of glutamate. When 10 mM ethanol was coapplied with glutamate and glycine (Fig. 6D-d), the potentiation of I_{Gly} by glutamate was significantly reduced: from 188 ± 72% of control in the absence of ethanol to 146 ± 26% in its presence (Fig. 6E; P < 0.05, n = 4).

In the experiments illustrated in Fig. 6D, ethanol was present when glutamate was applied. Therefore, the suppression of glutamate-induced potentiation of I_{Gly} could result from ethanol-induced reduction of Ca^{2+} entry via glutamate receptors. To assess this possibility, ethanol was coapplied with glycine immediately after the end of the glutamate prepulse (Fig. 7A). Although either glutamate or ethanol alone increased I_{Gly} (Fig. 7, A-b and A-c), there was no fur-
ther enhancement of $I_{Gly}$ by ethanol (Fig. 7A-d). The data from 13 cells studied with this protocol are summarized in Fig. 7B: there was a similar potentiation of $I_{Gly}$ by condition-
ing pulses of glutamate (as in Fig. 7A-b; $146 \pm 11\%$), ethanol alone (Fig. 7A-c; $145 \pm 11\%$), and ethanol applied after the glutamate conditioning pulse (Fig. 7A-d; $148 \pm 16\%$). These findings suggest different mechanisms of potentiation by eth-
anol and by glutamate. As shown in Fig. 7A-b, glutamate enhanced mainly peak $I_{Gly}$ and its rate of decay, whereas
ethanol potentiated both peak and steady-state $I_{Gly}$ (Fig. 7A-d). When ethanol was coapplied with glycine after a con-
ditioning pulse of glutamate, both peak and steady-state $I_{Gly}$ were enhanced, as during ethanol treatment alone (Fig. 7A-
d).

It is unlikely that maximal activation of glycine receptors explains these observations because the outcome was the

Fig. 3. When recording with pipettes containing 4 instead of 11 mM EGTA, potentiation by glutamate was enhanced, could be elicited with 100 μM glutamate, but was reduced when pipettes also contained 10 mM BAPTA. A, current traces obtained with pipette containing 4 mM EGTA from a VTA neuron of a 10-day-old rat. B, time course of change of peak $I_{Gly}$ after glutamate prepulse from neuron illustrated in A. Similar data were obtained from another five cells. C, current traces obtained from a VTA neuron of a 15-day-old rat produced by 30 μM glycine alone (a and c) and immediately after a conditioning prepulse of 100 μM glutamate (b). D, histogram shows mean potentiation of $I_{Gly}$ (evoked by 30 μM glycine) by 100 μM glutamate ($n = 4$). E, current traces obtained from a VTA neuron of an 11-day-old rat with 10 mM BAPTA-containing pipette illustrate progressive reduction of glutamate-induced potentiation after 4 min of whole-cell recording (traces a and b) and after 16 min (traces c and d). F, mean data from four cells show significantly reduced potentiation by 16 min. All data in this figure were obtained with 4 mM EGTA pipette solution.

Fig. 4. Glutamate prepulse reduces EC$_{50}$ and increases maximal $I_{Gly}$. A and B, current traces (obtained from a neuron of an 11-day-old rat) induced by increasing glycine concentrations without (A) and with (B) preceding pulse of 1 mM glutamate. C, concentration-response curves for glycine alone (open circles) or glycine after glutamate prepulse (filled circles), from the same neuron as in A and B. Continuous lines are fits of the Hill equation: $I = I_{max} \cdot (C/EC_{50} + 1)^n$, where $I$ is $I_{Gly}, I_{max}$ is maximal $I_{Gly}, C$ is glycine concentration, $EC_{50}$ is glycine concentration for half-
maximal response, and $n$ is the Hill coefficient. D, mean potentiation of $I_{Gly}$ (four neurons) by 1 mM glutamate, at various concentrations of glycine. Before pooling data, amplitudes of peak $I_{Gly}$ were normalized to response elicited by $30 \mu M$ glycine alone.

ther enhancement of $I_{Gly}$ by ethanol (Fig. 7A-d). The data from 13 cells studied with this protocol are summarized in Fig. 7B: there was a similar potentiation of $I_{Gly}$ by condition-
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ethanol potentiated both peak and steady-state $I_{Gly}$ (Fig. 7A-d). When ethanol was coapplied with glycine after a con-
ditioning pulse of glutamate, both peak and steady-state $I_{Gly}$ were enhanced, as during ethanol treatment alone (Fig. 7A-
d).

It is unlikely that maximal activation of glycine receptors explains these observations because the outcome was the
same when ethanol was applied at a lower concentration (1 mM) (data not shown). Moreover, ethanol suppressed the potentiation by glutamate even in cells that showed no enhancement of \( I_{\text{Gly}} \) by ethanol alone. This is illustrated in Fig. 7C, where 10 mM ethanol did not alter \( I_{\text{Gly}} \) (Fig. 7C-c) but suppressed its enhancement by glutamate (cf. Fig. 7A-b and A-d). The results obtained from five cells are summarized in Fig. 7D, in which \( I_{\text{Gly}} \) was not altered by ethanol alone (\( I_{\text{Gly}} \) was 103 ± 1% of control), although after glutamate, \( I_{\text{Gly}} \) was enhanced to 124 ± 4%. When glycine + ethanol were applied after the glutamate prepulse, \( I_{\text{Gly}} \) was 105 ± 5% of control, although peak glutamate currents were the same for the first and second pulse (274 ± 91 and 271 ± 97 pA; \( P > 0.1, n = 5 \)). Thus, even when ethanol did not change the response to glycine, it still prevented the interaction between glutamate and glycine.

Fig. 5. Glutamate prepulse reduces deactivation rate and accelerates desensitization of \( I_{\text{Gly}} \). A, whole-cell currents activated by 30 \( \mu \)M glycine before (a) and after (b) glutamate prepulse (from a VTA neuron of an 11-day-old rat). B, histograms show that glutamate prepulse (1 mM, 2 s) decreased the activation time constant (\( \tau_{\text{on}} \)) and increased the deactivation time constant (\( \tau_{\text{off}} \)) of \( I_{\text{Gly}} \); data are from eight neurons. C and D are from the same neuron as in A. Both activation (C) and deactivation (D) could be fitted by a single exponential (continuous curves). E, current traces from another neuron of an 11-day-old rat. \( I_{\text{Gly}} \) was elicited by 30 \( \mu \)M glycine before (a) and immediately after a conditioning prepulse of 1 mM glutamate (b). Superimposed traces in c illustrate decay of \( I_{\text{Gly}} \) elicited by long application of 30 \( \mu \)M glycine, with and without glutamate prepulse. F, histograms indicate average values of time constants of decay (\( \tau_{\text{d}} \)) for currents activated by 30 \( \mu \)M glycine; values obtained with and without 1 mM glutamate prepulse differed significantly (\( P < 0.01, n = 6 \)).

Fig. 6. Ethanol enhances \( I_{\text{Gly}} \), depresses \( I_{\text{Glu}} \), and suppresses glutamate-induced potentiation of \( I_{\text{Gly}} \). A, traces from a VTA neuron of a 7-day-old rat show \( I_{\text{Gly}} \) elicited by 30 \( \mu \)M glycine alone (a and e, open horizontal bars) or together with 0.1 (b), 1 (c), and 10 mM ethanol (d, hatched bars). B, brief pulse of 10 mM ethanol potentiated \( I_{\text{Gly}} \) induced by 30 \( \mu \)M glycine (from a VTA neuron of an 8-day-old rat). C, brief pulse of 10 mM ethanol inhibited \( I_{\text{Gly}} \) induced by 1 mM glutamate (from the same neuron as in B). D, in a VTA neuron of a 12-day-old rat, \( I_{\text{Gly}} \) was elicited by 30 \( \mu \)M glycine alone (a), immediately after 1 mM glutamate prepulse (b), together with 10 mM ethanol (c), and immediately after coapplication of 1 mM glutamate and 10 mM ethanol (d). E, mean values of peak \( I_{\text{Gly}} \) activated by 30 \( \mu \)M glycine obtained in the three recording conditions as in D-a, D-b, -c, and -d. Before plotting, all values were normalized to the value of peak \( I_{\text{Gly}} \) obtained in the control condition (D-a).
Our previous research revealed glycine receptors in the majority of freshly dissociated VTA neurons (Ye et al., 1998), all of which also respond to glutamate (Wang and French, 1993; Wu and Johnson, 1996; Ye et al., 2001b). The present results show that in such VTA neurons, glutamate consistently enhances $I_{\text{gly}}$, as previously observed in spinal neurons by Xu et al. (1999, 2000), and spinal and transfected cells by Fucile et al. (2000). Our principal new finding is that ethanol suppresses the glutamate-induced potentiation of $I_{\text{gly}}$ in VTA neurons. In keeping with the previous authors, our results point to the involvement of Ca$^{2+}$, perhaps Ca$^{2+}$ influx, in this phenomenon, although probably not calcium/calmodulin-dependent protein kinase II.

Comparison with Previous Reports of Interactions between Glutamate and Glycine. The previous studies of glutamate-induced fast potentiation of $I_{\text{gly}}$ (Xu et al., 1999, 2000; Fucile et al., 2000) attributed this effect to a rise in intracellular free Ca$^{2+}$. Although outwardly similar, these reports differed in some important respects. The results of the perforated-patch recordings from freshly dissociated spinal neurons (Xu et al., 1999, 2000) led to the conclusion that the increase in $I_{\text{gly}}$ is not caused by a change in the affinity of glycine for its receptor and that the potentiation is mediated by activation of CAMKII. In contrast, conventional whole-cell recordings from transfected cells (Fucile et al., 2000) suggested potentiation was due to a higher affinity of glycine receptors and not mediated by known protein kinases. In our experiments, an increase in glycine receptor affinity was indicated by the slower deactivation of $I_{\text{gly}}$ and the consistent reduction in $EC_{50}$. However, the glycine concentration-response plots also showed a clear increase in the maximal $I_{\text{gly}}$. In VTA neurons, the potentiation thus appeared to be mediated by both mechanisms.

Is the Potentiating Action of Glutamate in VTA Neurons Mediated by Intracellular Ca$^{2+}$? Our findings that glutamate was more effective when [Ca$^{2+}$]$_{\text{i}}$ was raised to 12 mM and less effective when a stronger Ca$^{2+}$ buffer, BAPTA or 11 mM EGTA (as compared with 4 mM EGTA) was present in the electrodes are consistent with some involvement of Ca$^{2+}$. However, the relatively modest effects produced by these manipulations, especially when compared with those seen in the experiments of Xu et al. (1999, 2000) and Fucile et al. (2000), seem more in keeping with a Ca$^{2+}$-sensitive than a Ca$^{2+}$-dependent process. Admittedly, by buffering slow changes in [Ca$^{2+}$]$_{\text{i}}$, the routine presence of EGTA in the electrodes would tend to reduce Ca$^{2+}$-mediated mechanisms. This may, at least in part, account for the relatively small and transient effects of glutamate observed in the current study as compared with the potentiation observed in previous studies, where weaker or no buffers were used. Since even 30 mM BAPTA failed to abolish the action of glutamate, the potentiation of glycine receptors may occur at a site that is not entirely intracellular, or at least not easily accessible to intracellular chelators. Judging by the lack of effect of KN-62, CAMKII is probably not the agent of Ca$^{2+}$-mediated modulation. Whether phosphorylation or dephosphorylation plays a significant role should be clarified by further tests of selective blockers.

Mechanisms of Ethanol's Actions. Because ethanol alone inhibits glutamate-induced currents, it could exert its...
effect by a direct depression of glutamate receptor/channels and consequently a smaller rise in [Ca\(^{2+}\)]\(_i\), (Gruol et al., 1997). This is unlikely because ethanol suppressed glutamate-induced potentiation when ethanol was applied after the end of the glutamate prepulse. Therefore, ethanol probably exerts its suppressant action at a site closer to the glycine receptors; for example, where Ca\(^{2+}\)-sensitive phosphorylation occurs.

In VTA neurons, both glutamate prepulses and ethanol potentiated glycine responses. These effects showed several similarities. First, the potentiation of glycine receptors by either ethanol (Ye et al., 2001a) or glutamate decreases with higher glycine concentrations. Second, both agents lower the EC\(_{50}\) of glycine. Third, they both accelerate glycine receptor desensitization. Thus, some occlusion between glutamate- and ethanol-induced potentiations is highly probable. However, because ethanol was effective in neurons that showed no potentiation of \(I_{Gly}\) by ethanol alone (Fig. 7 D), another mechanism is also likely involved.

Consequences of Ethanol’s Inhibition of Glutamate-Induced Potentiation of Glycine Responses and Other Cellular Activities. Because excitatory and inhibitory receptors coexist in many neurons, it is essential for us to understand how they interact. Modulation of glycine receptors by agents such as glutamate and ethanol is important because changes in the efficacy of glycineric transmission have pathophysiological implications in nociception and motor behavior (Breitinger and Becker, 1998; Simpson and Huang, 1998). Moreover, Ca\(^{2+}\)-dependent clustering of glycine receptors during synaptogenesis has been demonstrated (Kirsch and Betz, 1998). Being present in most VTA neurons, glycine receptors are likely to play an important role in modulating the excitability of VTA dopaminergic and nondopaminergic neurons and, consequently, the release of dopamine and other agents in the brain.

In conclusion, glutamate induced a fast potentiation of \(I_{Gly}\) in VTA neurons of 5- to 14-day-old rats. This was enhanced when extracellular Ca\(^{2+}\) was raised from 2 to 12 mM and diminished by intraneuronal chelators, indicating the possible involvement of intracellular Ca\(^{2+}\). Glutamate increased both the apparent affinity of the glycine receptor for its agonist and the maximal response. Ethanol suppressed these effects of glutamate, even when ethanol was applied after a glutamate prepulse. Therefore, ethanol may act on a Ca\(^{2+}\)-sensitive kinase or other pathways that regulate the function of glycine receptor channels.