Journal of Physiology (2002), **539.2**, *pp.* 495–502
DOI: 10.1013/jphysiol.2001.013508
© The Physiological Society 2002
www.jphysiol.org

Rapid Report

Tyrosine kinases enhance the function of glycine receptors in rat hippocampal neurons and human $\alpha_1\beta$ glycine receptors

Valerie B. Caraiscos [1], S. John Mihic [2], John F. MacDonald [1,3] and Beverley A. Orser [1,4,5,6]

Departments of [1] Physiology, [3] Pharmacology and [4] Anesthesia, University of Toronto, Canada, [2] Section of Neurobiology, University of Texas at Austin, USA, [5] Institute of Medical Science, University of Toronto, Canada and [6] Department of Anesthesia, Sunnybrook & Women's College Health Sciences Centre, 2075 Bayview Ave., Toronto, Ontario, Canada M4N 3M5

Glycine receptors (GlyRs) are transmitter-gated channels that mediate fast inhibitory neurotransmission in the spinal cord and brain. The GlyR β subunit contains a putative tyrosine phosphorylation site whose functional role has not been determined. To examine if protein tyrosine kinases (PTKs) regulate the function of GlyRs, we analysed whole-cell currents activated by applications of glycine to CA1 hippocampal neurons and spinal neurons. The role of a putative site for tyrosine phosphorylation at position 413 of the β subunit was examined using site-directed mutagenesis and expression of recombinant ($\alpha_1\beta^{Y413F}$) receptors in human embryonic kidney (HEK 293) cells. Lavendustin A, an inhibitor of PTKs, depressed glycine-evoked currents (I_{Gly}) in CA1 neurons and spinal neurons by 31 % and 40 %, respectively. In contrast, the intracellular application of the exogenous tyrosine kinase, cSrc, enhanced I_{Gly} in CA1 neurons by 56 %. cSrc also accelerated GlyR desensitization and increased the potency of glycine 2-fold (control EC_{50} = 143 μM; cSrc EC_{50} = 74 μM). Exogenous cSrc, applied intracellularly, upregulated heteromeric $\alpha_1\beta$ receptors but not homomeric α_1 receptors. Substitution mutation of the tyrosine to phenylalanine at position β-413 prevented this enhancement. Furthermore, a selective inhibitor of the Src family kinases, PP2, down-regulated wild-type $\alpha_1\beta$ but not $\alpha_1\beta^{Y413F}$ receptors. Together, these findings indicate that GlyR function is upregulated by PTKs and this modulation is dependent on the tyrosine-413 residue of the β subunit.

(Received 6 November 2001; accepted after revision 17 January 2002)
Corresponding author B. A. Orser: Department of Physiology, Room 3318, Medical Sciences Building, 1 King's College Circle, University of Toronto, Toronto, Ontario, Canada M5S 1A8. Email: beverley.orser@utoronto.ca

Reversible phosphorylation of protein targets by receptor and non-receptor PTKs was initially shown to influence signalling pathways involved in cell maturation and ontogeny. Recently, tyrosine phosphorylation was shown to rapidly regulate the function of ligand-gated channels involved in fast synaptic transmission. In particular, the Src family of endogenous PTKs upregulate inhibitory $GABA_A$ receptors (Moss *et al.* 1995; Wan *et al.* 1997) and excitatory NMDA receptor function (Ali & Salter, 2001). GlyRs are transmitter-gated anion channels with similar structural and biophysical properties as $GABA_A$ receptors; however, the functional consequence of GlyR modulation by tyrosine phosphorylation has not been previously examined.

Glycine is a major inhibitory transmitter in the spinal cord, brainstem and other parts of the brain. Activation of ionotropic GlyRs increases chloride conductance, hyperpolarizes the membrane and reduces neuronal excitability (for review see Legendre, 2001). Glycinergic inhibition regulates motor and sensory pathways involved in physiological processes such as locomotion, the coordination of spinal reflexes and nociception. Adult GlyRs are composed of α_{1-4} (48–50 kDa) and β (58 kDa) subunits that form hetero-oligomeric complexes (3α:2β). Only the α subunits form functional homomeric channels that contain binding sites for agonists and competitive antagonists (Grenningloh *et al.* 1990; Pribilla *et al.* 1992; Handford *et al.* 1996). The β subunit appears to serve a regulatory role by influencing receptor anchoring (Meyer *et al.* 1995), the allosteric modulation by pharmacological agents (Pribilla *et al.* 1992; Handford *et al.* 1996) and post-translational modifications including receptor phosphorylation (Grenningloh *et al.* 1990).

Cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC) and calcium-dependent calmodulin kinase II (CaMKII) have been shown to change the amplitude of I_{Gly}. Furthermore, PKA and PKC phosphorylate the α subunit (for review see Legendre, 2001). Although a

putative consensus site for tyrosine phosphorylation was identified on the large intracellular loop of the GlyR β subunit at position Y-413 (Grenningloh *et al.* 1990), the effects of PTKs on the function of native and recombinant receptors have not been reported. The objective of this study was to determine if inhibitors of PTKs or the exogenous cellular Src gene product, cSrc, influence I_{Gly} in hippocampal neurons and recombinant human GlyRs. Since GlyRs mediate the majority of fast inhibitory synaptic transmission in the spinal cord, GlyRs in spinal neurons were also examined. Here we show that PTKs upregulate GlyR function through a process involving β tyrosine-413.

METHODS

Various GlyR α_{1-4} subunit isoforms are expressed in distinct regional patterns in the CNS and are developmentally regulated. Since the isoform of the α subunit and spliced variants influence regulation by second messenger systems, two different experimental preparations were used to examine the effects of PTKs on GlyRs; these include CA1 neurons acutely isolated from postnatal rat hippocampus and cultured spinal neurons from fetal mice. Experiments were performed with the approval of the Animal Care Committee of the University of Toronto.

CA1 neurons were acutely isolated from hippocampal slices as previously described (Xiong *et al.* 1999). Briefly, Wistar rats (~14 days of age) were anaesthetized with halothane then killed by decapitation. Hippocampi were microdissected and cut into 400–500 μm slices then subjected to papain digestion (6.5 units ml^{-1}, Sigma, St Louis, MO, USA). Slices were rinsed in enzyme-free extracellular solution and electrophysiological recordings were undertaken in neurons ~15 min after isolation by mechanical trituration. Cultures of spinal neurons were prepared from Swiss white mice, using procedures previously described for hippocampal cultures (MacDonald *et al.* 1989) with additional 20 min incubation with trypsin-EDTA. Briefly, fetal pups (~14 days *in utero*) were removed from mice that were killed by cervical dislocation. Neurons were cultured at 35.6 °C in a 5 % CO_2–95 % air environment and recordings were made 15–21 days after plating, corresponding to postnatal days ~P9–P15. HEK 293 cells were transiently transfected with human GlyR α_1, $\alpha_1\beta$ (1:1) or $\alpha_1\beta^{Y413F}$ (1:1) cDNAs (Valenzuela *et al.* 1998) using the lipid method (Invitrogen, Carlsbad, CA, USA). The conservative single substitution of β tyrosine-413 to phenylalanine-413 (Y413F) was undertaken to examine the influence of this putative tyrosine phosphorylation site on GlyR function. According to Grenningloh *et al.* (1990), the predicted site for tyrosine phosphorylation was ELSNYDCYG. The Y413F point mutation was made using the protocol supplied in the Stratagene QuikChange Site-Directed Mutagenesis kit (La Jolla, CA, USA) and verified by double-stranded DNA sequencing. Recordings were undertaken 24–48 h after transfection of the cDNAs.

Whole-cell I_{Gly} were recorded at a holding potential of −60 mV at room temperature. Pipettes were prepared using a two-step puller (Narishige Scientific Instrument Lab, Tokyo, Japan, PP-83). A multi-barrelled perfusion system (solution exchange time of ~4 ms) was used to rapidly exchange the extracellular solutions. Glycine was applied at 2 min intervals to ensure sufficient time for GlyRs to recover from desensitization. Currents were filtered at 2 kHz, sampled at 500 μs per point, and analysed using pCLAMP6 software (Axon Instruments Inc., Foster City, CA, USA). Series resistance was monitored and cells with greater than a 20 % change in resistance were rejected.

The external solution contained (mM): 140 NaCl, 1.3 $CaCl_2$, 5.4 KCl, 2 $MgCl_2$, 25 Hepes, TTX (0.3 μM) and 33 glucose, at a pH of 7.4. Patch pipettes were filled with (mM): 63 CsCl, 70 CsF, 10 Hepes, 11 EGTA, 10 TEA-Cl, 2 $MgCl_2$, 1 $CaCl_2$ and 3.4 KATP, at a pH of 7.3. Identical results were obtained when CsCl replaced CsF for some experiments. Picrotoxin (50 mM) and strychnine (10 mM) were prepared in ethanol and distilled water, respectively, whereas the PTKs inhibitor, 5-amino-[(*N*-2,5-dihydroxybenzyl)-*N*′-2-hydroxybenzyl]salicylic acid (lavendustin A, 10 mM, Calbiochem, La Jolla, CA, USA) and its inactive analogue, 5-amino-(*N*,*N*′-bis-2-hydroxybenzyl)salicylic acid (lavendustin B) were dissolved in DMSO. In other experiments, active cSrc (30 U ml^{-1}, Upstate Biotechnology, Lake Placid, NY, USA) or cSrc that was inactivated by boiling at 100 °C for 1 h (inactive cSrc) were included in the pipette solution, while the selective inhibitor of the Src family of PTKs, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, 0.5 mM, Calbiochem) or its inactive analogue, 4-amino-7-phynelpyrazol[3,4-d]pyrimidine (PP3) were dissolved in DMSO.

Whole-cell currents evoked by glycine gradually declined or 'ran down' to a stable amplitude over 8–10 min. In CA1 neurons, this decline in amplitude was similar at 10 min whether currents were activated by saturating glycine (1 mM: 70 ± 7 % of the maximum current amplitude (I_{max}), $n = 6$) or sub-saturating concentrations of glycine (30 μM: 75 ± 5 % of I_{max}, $n = 4$). Similarly, in spinal neurons, glycine (45 μM)-evoked currents declined to 71 ± 7 % of I_{max} ($n = 4$) within 10 min. The amplitude of I_{Gly} was stable prior to the application of drugs that were intended to influence I_{Gly}. When inhibitors of PTKs or exogenous Src were applied intracellularly, currents were compared to controls at the same point in time. Concentration–response plots for glycine-activated currents were fitted to the equation: $I_{max}/I = 1+([Gly]/EC_{50})^n$ where I_{max} is maximal current, EC_{50} is the glycine concentration [Gly] that activates 50 % of the maximal response and n is the Hill coefficient. Results are expressed as means ± S.E.M. Statistical analysis was performed using Student's t test, Mann-Whitney test and two-way or one-way analysis of variance (ANOVA) with Dunnett's multiple comparison *post hoc* test, as appropriate (GraphPad Prism 3.02, GraphPad Software Inc.). P values < 0.05 were considered significant.

RESULTS

GlyRs are downregulated by inhibitors of PTKs and enhanced by exogenous cSrc

Initially, concentration–response plots for I_{Gly} in CA1 and spinal neurons were compared as the potency of glycine is influenced by GlyR subunit composition. The EC_{50} values for peak and steady-state currents in CA1 neurons were 134 ± 18 μM ($n = 6$) and 43 ± 8 μM ($n = 4$), respectively (Fig. 1*A* and *B*). In spinal neurons, the EC_{50} values were significantly less for peak (42 ± 6 μM, $n = 8$) and steady-state (18 ± 4 μM, $n = 6$) currents ($P < 0.05$, Fig. 1*C* and *D*). In subsequent experiments, concentrations of glycine that

approximated the EC_{50} for peak current were used to activate I_{Gly} in CA1 (140 μM) and spinal (45 μM) neurons, unless indicated otherwise.

Next, the effects of lavendustin A, a membrane-permeable inhibitor of PTKs, and its inactive analogue, lavendustin B, on I_{Gly} in CA1 neurons were examined. Since genistein, another inhibitor of PTKs, directly blocked $GABA_A$ receptors when applied extracellularly (Dunne *et al.* 1998; Huang *et al.* 1999) but not intracellularly (Moss *et al.* 1995), lavendustin A (1 μM) or lavendustin B (1 μM) were added to the pipette solution. Lavendustin A caused a 21 ± 9 % ($P < 0.01$, $n = 7$, at 9 min) greater reduction in peak amplitude compared to lavendustin B, whereas lavendustin B had no effect compared to control (lavendustin B 84 ± 5 % of I_{max}, $n = 7$; control 82 ± 7 % of I_{max}, $n = 4$; Fig. 1*E* and *F*). Next, lavendustin A (10 μM) and lavendustin B (10 μM) were applied extracellularly. These compounds were shown not to directly block GlyRs in hypothalamic neurons (Huang & Dillon, 2000) or recombinant $GABA_A$ receptors (Huang *et al.* 1999). However, lavendustin B caused a rapid, reversible depression of I_{Gly} by 39 ± 3 % ($n = 24$) and this reduction was similar whether lavendustin B was pre-applied for 60 s or co-applied with glycine. Lavendustin A also caused a rapid inhibition of I_{Gly} by 33 ± 2 % ($n = 12$); however, this was followed by a gradual irreversible decline in current amplitude. We attributed the rapid, reversible inhibition by lavendustin A and lavendustin B to direct channel blockade whereas the slower irreversible reduction (observed only with lavendustin A) was due to inhibition of PTKs. We next examined whether lavendustin A reduced I_{Gly} in neurons pre-treated with lavendustin B and observed a 31 ± 11 % ($n = 4$) reduction in current amplitude ($P < 0.01$, Fig. 1G). In spinal neurons, lavendustin A reduced I_{Gly} by 40 ± 8 % ($P < 0.05$, $n = 4$, at 15 min) in cells pre-treated with lavendustin B. Moreover, I_{Gly} increased by 32 ± 11 % ($P < 0.01$, $n = 3$, over 8 min) following the washout of lavendustin B whereas I_{Gly} decreased by 8 ± 9 % ($n = 3$) following the washout of

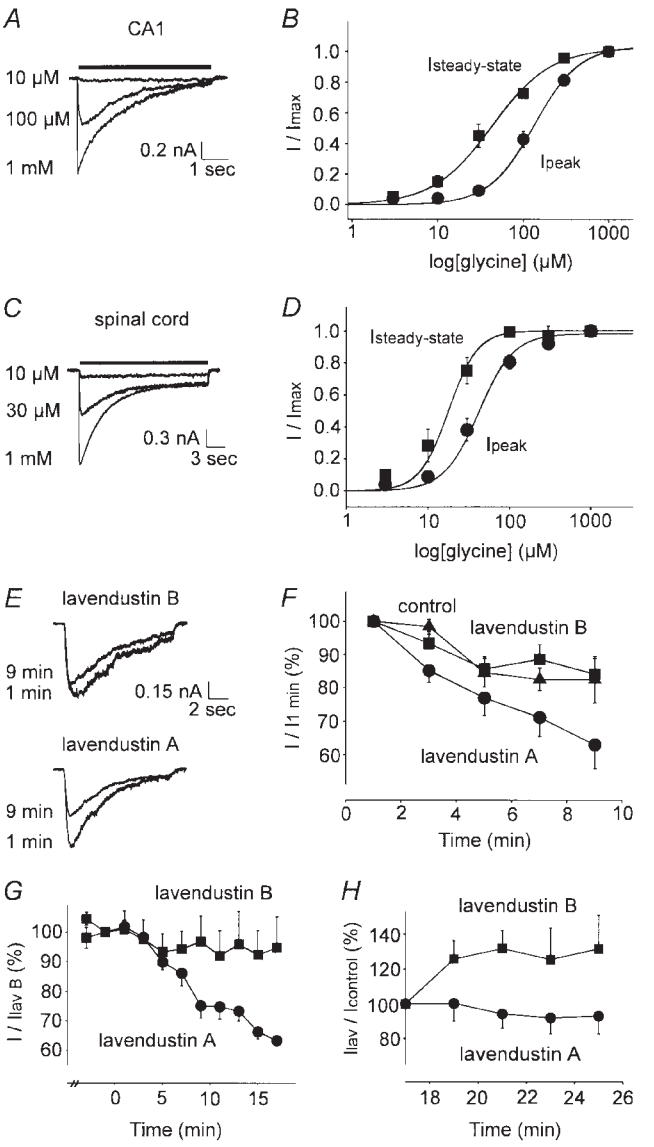

Figure 1. Concentration dependence of I_{Gly} in CA1 and spinal neurons. I_{Gly} is reduced by lavendustin A

A, superimposed traces of I_{Gly} recorded in CA1 neurons. *B*, dose–response relationships indicate EC_{50} values for peak (●) and steady-state currents (■) of 134 ± 18 μM ($n = 6$) and 43 ± 8 μM ($n = 4$) and Hill coefficients of 1.6 ± 0.1 and 1.3 ± 0.1, respectively. *C* and *D*, in spinal neurons, the EC_{50} and Hill coefficients for peak and steady-state currents were 42 ± 6 μM, 2.0 ± 0.4 ($n = 8$) and 18 ± 4 μM, 2.5 ± 0.5 ($n = 6$), respectively. *E* and *F*, peak I_{Gly} (140 μM) in CA1 neurons with control (▲, $n = 4$), lavendustin B (■, $n = 7$) or lavendustin A (●, $P < 0.01$, $n = 7$) in the pipette solution. *G* and *H*, peak I_{Gly} (140 μM) in CA1 neurons with lavendustin A applied extracellularly ($P < 0.01$, $n = 4$) following pre-treatment with lavendustin B, as compared to neurons continuously exposed to lavendustin B ($n = 5$). Recovery of I_{Gly} following washout of lavendustin A ($n = 3$) and lavendustin B ($P < 0.01$, $n = 3$) is shown. Currents were normalized to the peak amplitude measured immediately prior to drug washout.

lavendustin A (Fig. 1*H*), suggesting that the effects of lavendustin A were, in part, tyrosine kinase specific.

To determine if members of the Src family of PTKs upregulate GlyR function, cSrc or inactive cSrc were applied intracellularly. Currents were first evoked by 1 mM glycine to mimic saturating concentrations at the synapse (Legendre, 1998). The maximum peak currents with cSrc or inactive cSrc in the pipette solution were 1.12 ± 0.48 nA ($n = 6$) and 1.37 ± 0.36 nA ($n = 6$), respectively. cSrc (30 U ml^{-1}) enhanced I_{Gly} by 25 ± 14 % at 13 min ($P < 0.05$, $n = 6$) while inactive cSrc had no effect ($n = 6$); I_{Gly} declined to 64 ± 7 % ($n = 6$) of the initial response in the presence of inactive cSrc, similar to control (62 ± 6 %, $n = 4$, Fig. 2*A–C*).

The effect of cSrc on I_{Gly} evoked by sub-saturating concentrations of glycine (30 μM) was examined in CA1 neurons. I_{Gly} increased by 56 ± 11 % with cSrc in the pipette solution ($P < 0.01$, $n = 4$, Fig. 2*D*, at 9 min) and the addition of lavendustin A (10 μM) to the pipette solution blocked the increase by cSrc ($n = 4$, Fig. 2*D*). The 2-fold greater enhancement (56 % *versus* 25 %, $P < 0.01$) of I_{Gly} evoked by a sub-saturating compared to saturating [Gly]

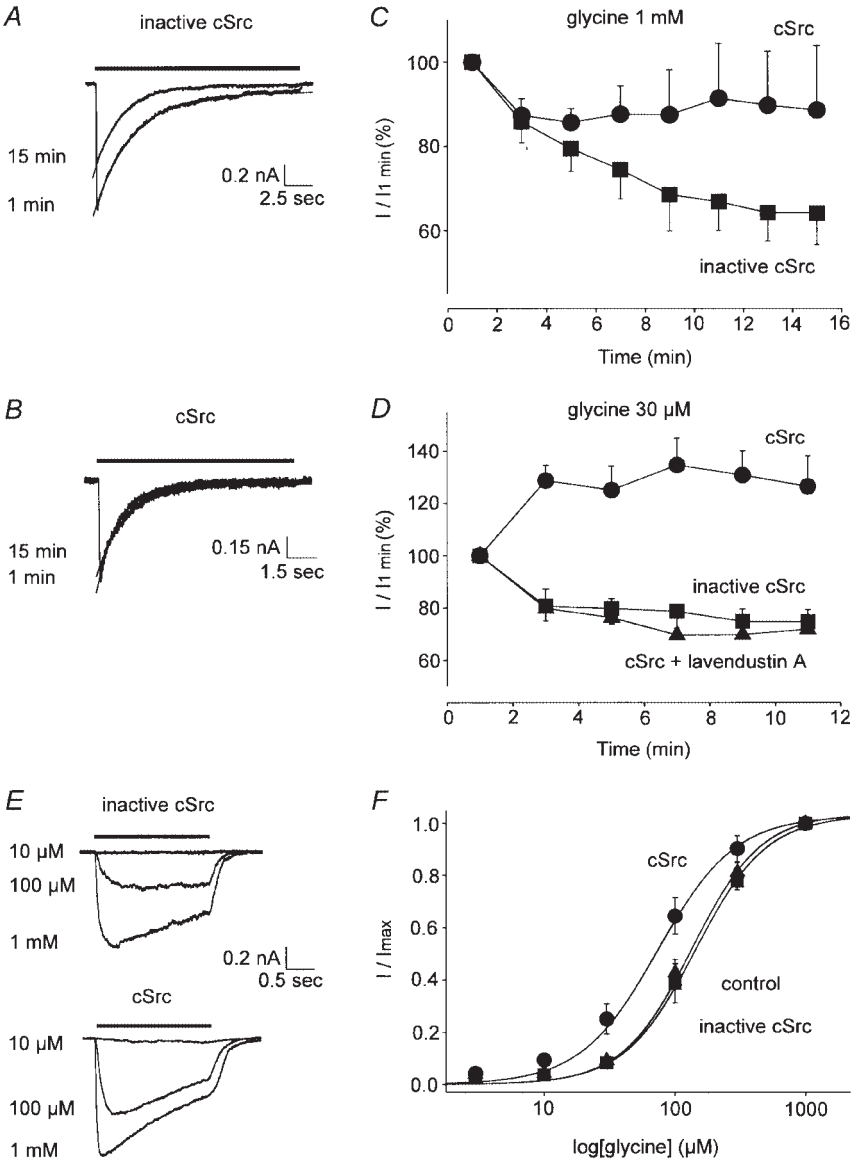

Figure 2. cSrc enhances I_{Gly} in CA1 neurons

A and *B*, superimposed traces of I_{Gly} activated by a *saturating* [Gly] at 1 and 15 min. *C*, I_{Gly} evoked by 1 mM glycine with cSrc (●) or inactive cSrc (■) applied intracellularly are shown ($P < 0.05$, each group $n = 6$). *D*, I_{Gly} activated by *sub-saturating* [Gly]= 30 μM with cSrc (●, $P < 0.01$), inactive cSrc (■) or cSrc plus lavendustin A (▲) in the pipette solution (each group $n = 4$). *E*, traces of I_{Gly} with inactive or active cSrc in the pipette solution. *F*, the [glycine]–response plots with cSrc (●, EC_{50}= 74 ± 14 μM, $P < 0.05$, $n = 5$), inactive cSrc (■) (EC_{50}= 143 ± 23 μM, $n = 3$) and control intracellular solution (▲, EC_{50} = 134 ± 18 μM, $n = 6$) are shown.

suggests that cSrc increases the potency of glycine. Consequently, [Gly]–response plots were constructed with active or inactive cSrc in the pipette solution. While inactive cSrc failed to influence the [Gly]–response plot, active cSrc shifted the curve to the left, consistent with an increase in glycine potency ($P < 0.05$, Fig. 2*E* and *F*). Since receptor phosphorylation may also influence desensitization, we examined the effect of cSrc on the time course of GlyR desensitization in CA1 neurons. I_{Gly} rapidly reached a peak then slowly decayed close to the baseline level during a 5 s application of 1 mM glycine (Fig. 1*A*). This decay was well described by a single exponential function (Fig. 2*A* and *B*). cSrc reduced the time constant of desensitization, τ, by 30 % (cSrc τ = 2.01 ± 0.29 s, inactive cSrc τ = 2.85 ± 0.35 s, $P < 0.05$, $n = 6$, at 15 min), suggesting that tyrosine phosphorylation enhances GlyR desensitization.

Tyrosine phosphorylation of recombinant GlyRs expressed in HEK 293 cells

To examine the specific role of α and β subunits, recombinant GlyR subunits were expressed in HEK 293 cells. First, [picrotoxin]–inhibition plots were examined to ensure the functional expression of heteromeric $\alpha_1\beta$ GlyRs as the β subunit reduced the sensitivity of heteromeric receptors to picrotoxin (Pribilla *et al.* 1992; Handford *et al.* 1996) and increased the amplitude of I_{Gly} (Bormann *et al.* 1993). The IC_{50} for picrotoxin inhibition of current activated by 100 μM glycine was lower in cells transfected with α_1 subunits compared to $\alpha_1\beta$ subunits (98 ± 41 μM, $n = 7$ *versus* 1060 ± 313 μM, $n = 6$, $P < 0.05$). Also, I_{max} was greater for $\alpha_1\beta$ heteromeric receptors (2.8 ± 0.6 nA, $n = 12$) compared to α_1 receptors (1.6 ± 0.7 nA, $n = 11$, $P < 0.05$), consistent with the functional expression of the β subunit.

Since a putative tyrosine phosphorylation site resides on the β subunit, we compared the sensitivity of homomeric α_1 and heteromeric $\alpha_1\beta$ receptors to exogenous cSrc. I_{Gly} (1 mM) in recombinant GlyRs gradually ran down over 8–10 min. cSrc (30 U ml^{-1}) reduced the run-down of I_{Gly} recorded from $\alpha_1\beta$ receptors by 15 ± 6 % ($P < 0.01$, $n = 12$, Fig. 3*A* and *B*) but not α_1 receptors ($n = 12$, Fig. 3*C* and *D*, at 13 min). The effects of cSrc on desensitization were examined by measuring the steady-state to peak current ratio (I_{ss}/I_p) and $\tau_{desensitization}$. For $\alpha_1\beta$ GlyRs, the I_{ss}/I_p ratio was reduced from 0.46 ± 0.09 to 0.32 ± 0.10 in the presence of active compared to inactive cSrc ($P < 0.01$, $n = 9$, at 1 min). In contrast, the I_{ss}/I_p ratio was similar with active and inactive cSrc for α_1 GlyRs (0.47 ± 0.08, $n = 10$ *versus* 0.53 ± 0.09, $n = 7$ at 1 min, respectively). These values did not change over time. Moreover, cSrc accelerated $\tau_{desensitization}$ of $\alpha_1\beta$ receptors (cSrc τ = 2.34 ± 0.49 s, $n = 10$; inactive cSrc τ = 6.38 ± 1.33 s, $n = 11$; $P < 0.05$, at 13 min)

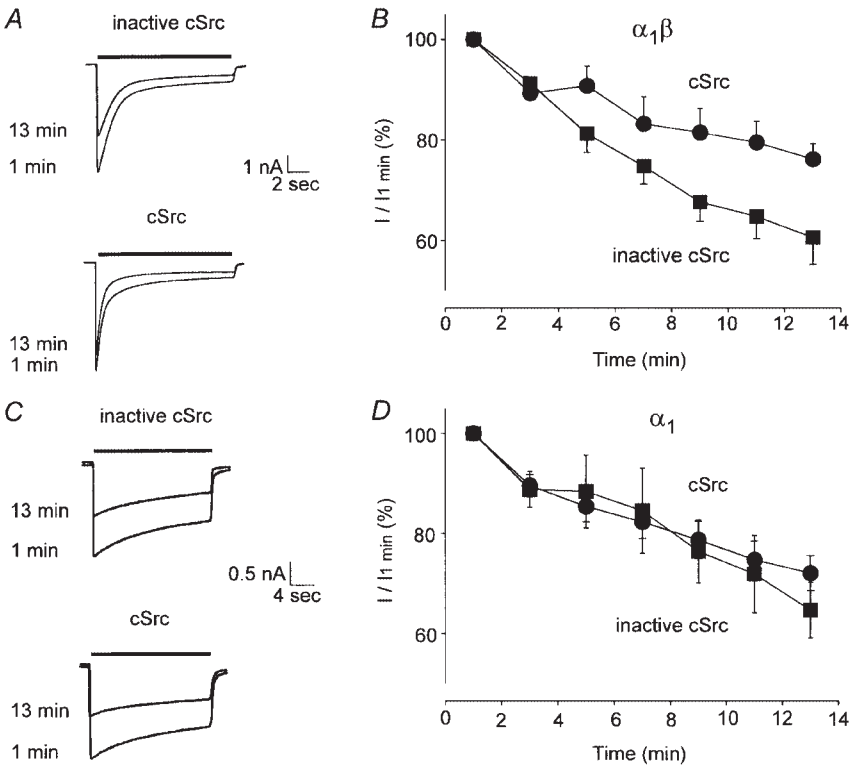

Figure 3. cSrc reduces the rundown of $\alpha_1\beta$ GlyR-mediated currents

A and *B*, peak I_{Gly} (1 mM) from $\alpha_1\beta$ GlyRs with cSrc (●) and inactive cSrc (■) applied intracellularly ($P < 0.01$, each group $n = 12$). *C* and *D*, I_{Gly} (1 mM) for α_1 GlyRs with cSrc ($n = 12$) or inactive cSrc ($n = 11$) applied intracellularly.

but not α_1 receptors (cSrc $\tau = 4.42 \pm 0.98$ s, $n = 12$; inactive cSrc $\tau = 4.61 \pm 1.20$ s, $n = 11$; at 13 min), indicating that tyrosine kinases enhance desensitization of $\alpha_1\beta$ but not α_1 GlyRs.

The specific role of β-Y413 in mediating the sensitivity to PTKs was next examined (Fig. 4*A*). The maximal amplitude of current recorded from $\alpha_1\beta^{Y413F}$ receptors (3.08 ± 0.67 nA, $n = 9$) was not different from wild-type $\alpha_1\beta$ receptors (3.02 ± 0.74 nA, $n = 6$). Also, consistent with the functional expression of the mutant β^{Y413F} subunit, I_{Gly} from $\alpha_1\beta^{Y413F}$ receptors was less sensitive to inhibition by picrotoxin (1 mM) compared to homomeric α_1 receptors as measured by percentage inhibition of control ($\alpha_1\beta$ 46 ± 9 %, $n = 5$; $\alpha_1\beta^{Y413F}$ 47 ± 10 %, $n = 8$ *versus* α_1 79 ± 9 %, $n = 5$, $P < 0.05$).

The run-down of I_{Gly} in $\alpha_1\beta$ and $\alpha_1\beta^{Y413F}$ GlyRs, with active or inactive cSrc in the pipette solution, was examined (Fig. 4*B*). For $\alpha_1\beta$ GlyRs, the current ran down to 61 ± 5 and 39 ± 4 % of the control values measured at 1 min with active and inactive cSrc in the pipette solution, respectively

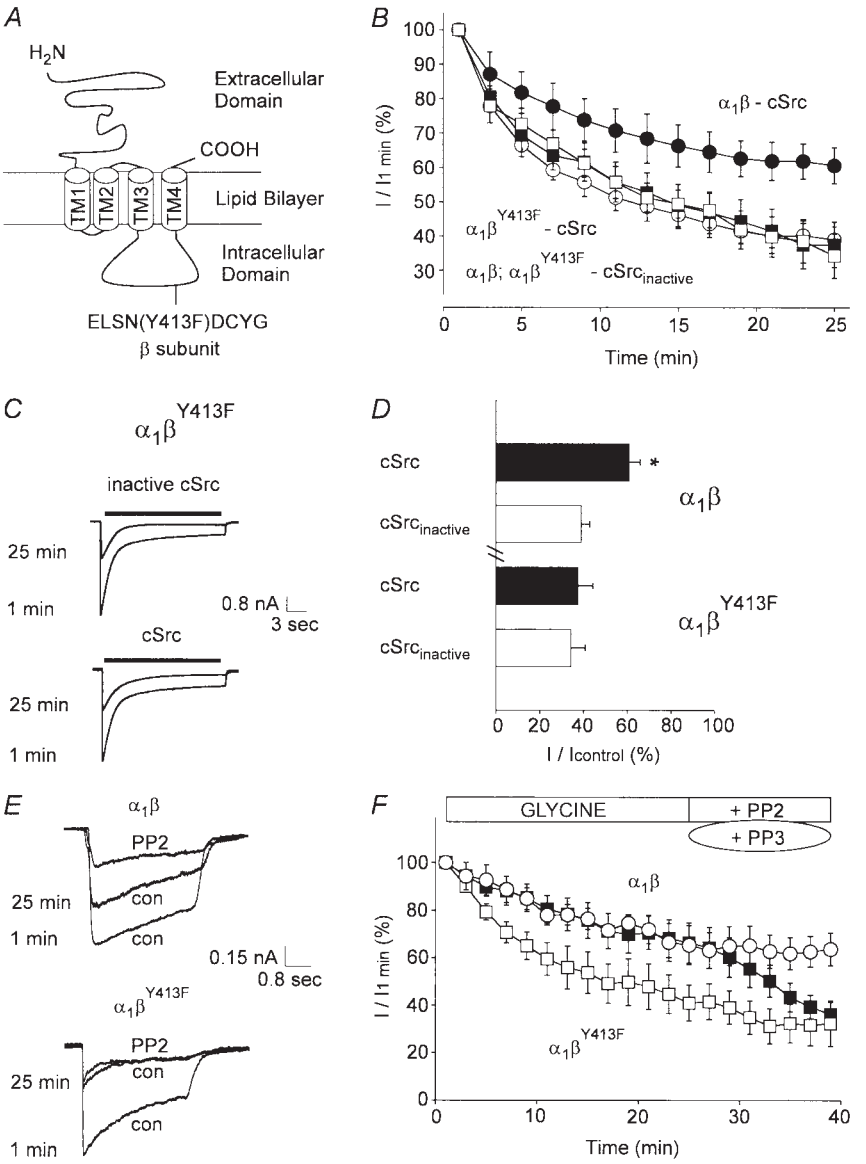

Figure 4. Point mutation of β^{Y413} abolishes GlyR sensitivity to cSrc

A, the location of tyrosine-413 of the GlyR β subunit. *B*, the time course of I_{Gly} from $\alpha_1\beta$ receptors with active (●, $n = 6$) or inactive cSrc (○, $n = 6$) applied intracellularly. Also, I_{Gly} from $\alpha_1\beta^{Y413F}$ receptors with active (■, $n = 10$) or inactive cSrc (□, $n = 9$) applied intracellularly are shown. *C*, I_{Gly} from $\alpha_1\beta^{Y413F}$ receptors. *D*, mean value of I_{Gly} (1 mM) from $\alpha_1\beta$ and $\alpha_1\beta^{Y413F}$ GlyRs. Currents were measured at 25 min and were normalized to the response measured 1 min following patch breakthrough (n values are listed above). *E*, traces of I_{Gly} at 1 and 25 min after breakthrough in control solution, followed by PP2 applied extracellularly to $\alpha_1\beta$ and $\alpha_1\beta^{Y413F}$ GlyRs. *F*, PP2 (■, $P < 0.05$, $n = 6$) but not PP3 (○, $n = 4$) inhibited $\alpha_1\beta$ GlyRs whereas PP2 did not inhibit $\alpha_1\beta^{Y413F}$ GlyRs (□, $n = 6$).

($P < 0.01$, $n = 6$, Fig. 4*D*, at 25 min). However, for $\alpha_1\beta^{Y413F}$ GlyRs, the current ran down to 37 ± 7 % ($n = 10$) and 34 ± 6 % ($n = 9$) in the presence of active and inactive cSrc, respectively (Fig. 4*C* and *D*, at 25 min). Hence, cSrc reduced the run-down of $\alpha_1\beta$ GlyR-mediated currents by 22 ± 7 % ($P < 0.01$) but did not affect $\alpha_1\beta^{Y413F}$ GlyRs. Next, the effect of a selective inhibitor of the Src family of PTKs, PP2, was examined in GlyRs containing the β or β^{Y413F} subunit. PP2 (0.5 μM) depressed $\alpha_1\beta$-mediated currents ($P < 0.05$, $n = 6$, at 13 min) but had no effect on $\alpha_1\beta^{Y413F}$-mediated currents ($n = 6$). A control analogue for PP2, PP3, failed to inhibit $\alpha_1\beta$ currents ($n = 4$, Fig. 4*E* and *F*), consistent with the hypothesis that phosphorylation of β^{Y413} enhances GlyR function.

DISCUSSION

This study provides evidence that PTKs play a role in maintaining and upregulating the function of GlyRs in neurons as well as recombinant heteromeric GlyRs. Lavendustin A, an inhibitor of PTKs, reduced I_{Gly} while intracellularly applied cSrc increased I_{Gly}. The enhancement by cSrc required the β subunit and point substitution of tyrosine-413 abolished both the enhancement by cSrc and the inhibition by PP2. Furthermore, cSrc increased the potency of glycine and accelerated GlyR desensitization.

The simplest explanation for our findings is that tyrosine phosphorylation of the β subunit at position 413 increases GlyR-mediated conductance by at least two possible mechanisms: (1) enhanced glycine potency and (2) alterations in intrinsic receptor properties, including channel conductance or gating. However, our results do not exclude interactions of PTKs with other intermediary cytosolic proteins that modulate GlyR function. The β subunit regulates interactions with the anchoring protein, gephyrin (Meyer *et al.* 1995) or scaffolding proteins that can be modified by phosphorylation. Also, GlyR activity is modulated by divalent cations, including Ca^{2+} (Xu *et al.* 1999, 2000) and Zn^{2+} (for review see Legendre, 2001). Src specifically interacts with the intracellular domains of the $GABA_A$ receptor β and $\gamma 2$ subunits (Brandon *et al.* 2001) and potentiates the function of recombinant NMDA receptors by reducing voltage-independent inhibition by Zn^{2+} (Zheng *et al.* 1998). Furthermore, PTKs may be involved in cross-modulation of GlyR function with other intracellular kinases, including PKA and PKC (for review see Moss & Smart, 1996). PKA increased glycine potency in neurons from the ventral tegmental area (Ren *et al.* 1998), although neither PKA- nor PKC-dependent mechanisms influenced the EC_{50} of glycine in trigeminal neurons (Song & Huang, 1990; Gu & Huang, 1998). Biochemical experiments to examine whether the GlyR β subunit is tyrosine phosphorylated will address protein phosphorylation directly although such studies have been hampered by unsuccessful attempts to develop antibodies selective for the GlyR β subunit (correspondence with C. F. Valenzuela, University of New Mexico, Health Science Center).

Potentiation of GlyRs by tyrosine phosphorylation may have therapeutic implications. For example, GlyRs have been implicated in the regulation of nociceptive input to the spinal cord. Inhibition of GlyRs by intrathecal administration of strychnine yields touch-evoked allodynia (Yaksh, 1989) and contributes to hyperalgesia (Beyer *et al.* 1988) and neuropathic pain (Simpson *et al.* 1996). Thus, the manipulation of GlyR function by second messenger regulation may represent a novel therapeutic strategy, particularly for patients with narcotic-resistant pain states.

REFERENCES

Ali, D. W. & Salter, M. W. (2001). NMDA receptor regulation by Src kinase signaling in excitatory synaptic transmission and plasticity. *Current Opinion in Neurobiology* **11**, 336–342.

Beyer, C., Banas, C., Gomora, P. & Komisaruk, B. R. (1988). Prevention of the convulsant and hyperalgesic action of strychnine by intrathecal glycine and related amino acids. *Pharmacology, Biochemistry, and Behavior* **29**, 73–78.

Bormann, J., Rundstrom, N., Betz, H. & Langosch, D. (1993). Residues within transmembrane segment M2 determine chloride conductance of glycine receptor homo- and hetero-oligomers. *EMBO Journal* **12**, 3729–3737.

Brandon, N. J., Delmas, P., Hill, J., Smart, T. G. & Moss, S. J. (2001). Constitutive tyrosine phosphorylation of the $GABA_A$ receptor $\gamma 2$ subunit in rat brain. *Neuropharmacology* **41**, 745–752.

Dunne, E. L., Moss, S. J. & Smart, T. G. (1998). Inhibition of $GABA_A$ receptor function by tyrosine kinase inhibitors and their inactive analogues. *Molecular and Cellular Neuroscience* **12**, 300–310.

Grenningloh, G., Pribilla, I., Prior, P., Multhaup, G., Beyreuther, K., Taleb, O. & Betz, H. (1990). Cloning and expression of the 58 kD beta subunit of the inhibitory glycine receptor. *Neuron* **4**, 963–970.

Gu, Y. & Huang, L. Y. M. (1998). Cross-modulation of glycine-activated Cl- channels by protein kinase C and cAMP-dependent protein kinase in the rat. *Journal of Physiology* **506**, 331–339.

Handford, C. A., Lynch, J. W., Baker, E., Webb, G. C., Ford, J. H., Sutherland, G. R. & Schofield, P. R. (1996). The human glycine receptor beta subunit: primary structure, functional characterization and chromosomal localization of the human and murine genes. *Molecular Brain Research* **35**, 211–219.

Huang, R. & Dillon, G. H. (2000). Direct inhibition of glycine receptors by genistein, a tyrosine kinase inhibitor. *Neuropharmacology* **39**, 2195–2204.

Huang, R. Q., Fang, M. J. & Dillon, G. H. (1999). The tyrosine kinase inhibitor genistein directly inhibits $GABA_A$ receptors. *Molecular Brain Research* **67**, 177–183.

Legendre, P. (1998). A reluctant gating mode of glycine receptor channels determines the time course of inhibitory miniature synaptic events in zebrafish hindbrain neurons. *Journal of Neuroscience* **18**, 2856–2870.

Legendre, P. (2001). The glycinergic inhibitory synapse. *Cellular and Molecular Life Sciences* **58**, 760–793.

MacDonald, J. F., Mody, I. & Salter, M. W. (1989). Regulation of *N*-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurones in culture. *Journal of Physiology* **414**, 17–34.

Meyer, G., Kirsch, J., Betz, H. & Langosch, D. (1995). Identification of a gephyrin binding motif on the glycine receptor beta subunit. *Neuron* **15**, 563–572.

Moss, S. J., Gorrie, G. H., Amato, A. & Smart, T. G. (1995). Modulation of $GABA_A$ receptors by tyrosine phosphorylation. *Nature* **377**, 344–348.

Moss, S. J. & Smart, T. G. (1996). Modulation of amino acid-gated ion channels by protein phosphorylation. *International Review of Neurobiology* **39**, 1–52.

Pribilla, I., Takagi, T., Langosch, D., Bormann, J. & Betz, H. (1992). The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. *EMBO Journal* **11**, 4305–4311.

Ren, J., Ye, J. H. & McArdle, J. J. (1998). cAMP-dependent protein kinase modulation of glycine-activated chloride current in neurons freshly isolated from rat ventral tegmental area. *Brain Research* **811**, 71–78.

Simpson, R. K. Jr, Gondo, M., Robertson, C. S. & Goodman, J. C. (1996). Reduction in the mechanonociceptive response by intrathecal administration of glycine and related compounds. *Neurochemical Research* **21**, 1221–1226.

Song, Y. & Huang, L. Y. M. (1990). Modulation of glycine receptor chloride channels by cAMP-dependent protein kinase in spinal trigeminal neurons. *Nature* **348**, 242–245.

Valenzuela, C. F., Cardoso, R. A., Wick, M. J., Weiner, J. L., Dunwiddie, T. V. & Harris, R. A. (1998). Effects of ethanol on recombinant glycine receptors expressed in mammalian cell lines. *Alcoholism, Clinical and Experimental Research* **22**, 1132–1136.

Wan, Q., Man, H. Y., Braunton, J., Wang, W., Salter, M. W., Becker, L. & Wang, Y. T. (1997). Modulation of $GABA_A$ receptor function by tyrosine phosphorylation of beta subunits. *Journal of Neuroscience* **17**, 5062–5069.

Xiong, Z., Pelkey, K. A., Lu, W. Y., Lu, Y. M., Roder, J. C., MacDonald, J. F. & Salter, M. W. (1999). Src potentiation of NMDA receptors in hippocampal and spinal neurons is not mediated by reducing zinc inhibition. *Journal of Neuroscience* **19**, RC37.

Xu, T. L., Dong, X. P. & Wang D. S. (2000). N-methyl-D-aspartate enhancement of the glycine response in the rat sacral dorsal commissural neurons. *European Journal of Neuroscience* **12**, 1647–1653.

Xu, T. L., Li, J. S., Jin, Y. H. & Akaike, N. (1999). Modulation of the glycine response by Ca^{2+}-permeable AMPA receptors in rat spinal neurones. *Journal of Physiology* **514**, 701–711.

Yaksh, T. L. (1989). Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. *Pain* **37**, 111–123.

Zheng, F., Gingrich, M. B., Traynelis, S. F. & Conn, P. J. (1998). Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. *Nature Neuroscience* **1**, 185–191.

Acknowledgements

We thank Dr C. Fernando Valenzuela (Department of Neuroscience, University of New Mexico Health Sciences Centre, Albuquerque, New Mexico, USA) for providing the cDNAs, Dr Michael W. Salter (Programmes in Brain and Behaviour & Cell Biology, Hospital for Sick Children) for reviewing the manuscript, and Lidia Brandes and Elzbieta Czerwinska for technical assistance. Grants from CIHR to B.A.O. and J.F.M. and the International Anesthesia Research Society to B.A.O. supported these studies.