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A. ABSTRACT

MODULATION OF THE GLYCINE RECEPTOR BY TYROSINE PHOSPHORYLATION

Master of Science, 2000
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GlyRs mediate the majority of inhibitory synaptic neurotransmission in the spinal cord, however, their modulation by tyrosine phosphorylation has not been characterized. Native GlyRs are composed of α and β subunits. The GlyR β subunit contains a putative tyrosine phosphorylation site, Tyr-413, however, its function is unknown. Here we used electrophysiological techniques to characterize the effect of tyrosine phosphorylation and the role of the β subunit on GlyR function.

The tyrosine kinase inhibitor, lavendustin A depressed, while the exogenous tyrosine kinase, pp60<sup>C-src</sup> enhanced GlyR function and reduced the rundown of glycine-evoked currents. α<sub>1</sub> GlyRs were not influenced by pp60<sup>C-src</sup> whereas α<sub>1</sub>/β GlyRs were positively modulated by pp60<sup>C-src</sup>. Mutation of Tyr-413 of the GlyR β subunit abolished GlyR sensitivity to pp60<sup>C-src</sup>. These results demonstrate that tyrosine phosphorylation affects GlyR function in a positive fashion, and in a manner that is dependent on the presence of the GlyR β subunit Tyr-413 residue.
B. ACKNOWLEDGMENTS

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<th>Description</th>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole propionate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5-triphosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>concentration of agonist that produces 50% of the maximal response</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-O,O’-bis(2-aminoethyl)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>GABA_{AR}</td>
<td>a subtype of the γ-aminobutyric acid receptor</td>
</tr>
<tr>
<td>GLYR</td>
<td>glycine receptor</td>
</tr>
<tr>
<td>HEK 293</td>
<td>human embryonic kidney 293 cell line</td>
</tr>
<tr>
<td>HEPES</td>
<td>25 N-2-hydroxy-ethylpiperazine-N’-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>concentration of antagonist that produces 50% of the maximal inhibition</td>
</tr>
<tr>
<td>IPSC</td>
<td>inhibitory postsynaptic current</td>
</tr>
<tr>
<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential media</td>
</tr>
<tr>
<td>mIPSP</td>
<td>miniature inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>pA</td>
<td>picoamperes</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Ca^{2+}/phospholipid-dependent protein kinase C</td>
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PTK  protein tyrosine kinase
TEA  tetraethylammonium
TM  transmembrane domain
TTX  tetrodotoxin
1. INTRODUCTION

1.1. THE IMPORTANCE OF THE GLYCINE RECEPTOR IN HEALTH AND DISEASE

Glycine receptors (GlyRs) are the major ligand-gated ion channels involved in inhibitory synaptic neurotransmission in the spinal cord and activation of GlyRs causes membrane hyperpolarization and/or shunting of excitatory input. GlyRs are targets of general anaesthetics (Belelli et al., 1999) and have been implicated in various pathophysiological processes such as excitotoxicity and nociception (Chen et al., 1999; Maione et al., 2000). Excitotoxicity, a major mechanism that contributes to neural death is thought to be triggered by overactivation of postsynaptic receptors by the excitatory neurotransmitter, glutamate (Sattler & Tymianski, 2000). Studies indicate that blockers of GlyRs reduce neurotoxicity (Erdo, 1990; McNamara & Dingledine, 1990; Chen et al., 1999) by reducing Cl⁻ entry into cells (Chen et al., 1998), suggesting that activation of GlyRs is an important component of excitotoxic cell death. An increase in glycinerigic input may also modulate nociceptive processes. For example, allodynia, a painful sensation of otherwise innocuous stimuli (Sorkin & Puig, 1996), hyperalgesia (Zelhofer et al., 2000) and neuropathic pain (Huang et al., 1996) are associated with blockade of the GlyR. In addition, various reports indicate that GlyR function is enhanced by general anaesthetics (Belelli et al., 1999), suggesting a role for glycinerigic neurotransmission in the production of anaesthesia.

The physiological regulation and the pharmacological properties of GlyRs are of interest. The purpose of this thesis is to understand the regulation of the GlyR by second messengers/systems. In particular, the influence of tyrosine kinase activity
on the function of the GlyR was investigated. Such insights could suggest therapeutic strategies to attenuate the disastrous effects of excitotoxicity or modulate nociceptive processes.

1.2. PROTEIN PHOSPHORYLATION

Neurons utilize protein phosphorylation as a mechanism to regulate receptor function (Browning et al., 1985; Filippova et al., 1999). Such modulation can, in turn, regulate neuronal events such as synaptic transmission, learning, and memory (Salter, 1998).

Protein phosphorylation systems encompass three primary components: protein kinases, protein phosphatases, and substrate proteins. Protein kinases transfer the terminal phosphate from ATP to one or more serine, threonine or tyrosine residues of substrate proteins and hence, phosphorylate them (Browning et al., 1985; Hemmings, Jr. et al., 1989; Moss & Smart, 1996). The addition of a charged phosphate group to the protein presumably triggers a conformational change, which subsequently alters the functional properties of the protein. The added phosphate group can be removed by protein phosphatases (Browning et al., 1985; Hemmings, Jr. et al., 1989; Moss & Smart, 1996).

At any moment, the level of phosphorylation in the neuron is reflective of the combined activities of protein kinases and phosphatases. High expression levels of many protein kinases are observed in the central nervous system (CNS), suggesting that these proteins are important in regulating neuronal function (Hemmings, Jr. et al., 1989; Wagner et al., 1991).
Upon the binding of a neurotransmitter to a receptor, a receptor-associated protein kinase may be activated or the level of a second messenger may be altered, which, in turn, may regulate a specific protein kinase or phosphatase. This can lead to the alteration of the physiological properties of the target neuron (Hemmings, Jr. et al., 1989). Neurotransmitter binding to a receptor may also change the channel gating of receptor-associated ion channels, thus affecting the electrophysiological properties of the neuron. This signal transduction mechanism can be modulated by phosphorylation. As such, regulation by protein phosphorylation can be mediated either by direct phosphorylation of the ion channel or phosphorylation of a regulatory protein that is involved with the receptor complex (Swope et al., 1999).

A variety of ion channels in several neuronal preparations are regulated by protein phosphorylation, as described below. Ion channel phosphorylation is a primary mechanism involved in the regulation of receptor sensitivity to the agonist, desensitization, and gating as well as the clustering and subunit assembly of the receptors at the synapse (Moss & Smart, 1996; Swope et al., 1999). For example, phosphorylation affects the amplitude of currents of ligand-gated ion channels (Swope et al., 1999). The membrane current \( I \) of a certain population of ion channels, measured using voltage clamp techniques, can be described by the equation \( I = N \cdot p \cdot i \). In this equation, \( N \) is the number of functional channels in the membrane, \( p \) is the probability that an individual channel will be open, and \( i \) is the unitary current carried by a single open channel in the membrane. The activity of protein kinases and phosphatases most often alters \( N \) and \( p \) (Levitan, 1988), however \( i \) can also be affected (Eghbali et al., 1997; Benke et al., 1998). For
example, the channel conductance of hippocampal GABA$_A$Rs was increased by diazepam (Eghbali et al., 1997), and the induction of long term potentiation (LTP) in the CA1 region of the hippocampus was associated with an increase in the single-channel conductance of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptors (Benke et al., 1998). In addition, application of the protein tyrosine kinase (PTK), pp60$^{c-src}$ diminished the insulin-induced increase in Cl$^-$ channel conductance, while the protein tyrosine phosphatase (PTP), vanadate and insulin increased the single-channel Cl$^-$ conductance in the apical membrane of the distal nephron epithelium (Shintani & Marunaka, 1996). Hence, phosphorylation of ligand-gated ion channels can play a pivotal role in the efficiency of synaptic transmission.

1.3. PHOSPHORYLATION OF LIGAND-GATED ION CHANNELS BY PKA, PKC, PKG AND CaMKII

A variety of ligand-gated ion channels are known to be phosphorylated. The regulation of nicotinic acetylcholine receptors (nAChRs) by second messenger systems has been extensively characterized. At the neuromuscular junction, the nAChR is the ligand-gated ion channel that mediates rapid depolarization of the postsynaptic cell membrane upon binding of acetylcholine that is released from the motor neuron (Hall & Sanes, 1993). In the continued presence of acetylcholine, the nAChR desensitizes to a steady state. Phosphorylation of the nAChR by cAMP-dependent protein kinase (PKA) (Albuquerque et al., 1986; Middleton et al., 1986; Middleton et al., 1988) and Ca$^{2+}$/phospholipid-dependent protein kinase C (PKC)
(Eusebi et al., 1985) increases current desensitization. Phosphorylation by PKC also decreases the AchR's sensitivity to acetylcholine (Eusebi et al., 1985).

Ionotropic glutamate receptors are divided into N-methyl-D-aspartate (NMDA) and non-NMDA receptors. The latter group includes the AMPA and kainate receptors. Recent studies indicate that AMPA and kainate receptors are phosphorylated by PKA, PKC and the calcium/calmodulin-dependent protein kinase II (CaMKII). Phosphorylation by PKA enhances AMPA/kainate receptor-mediated currents (Greengard et al., 1991; Wang et al., 1991; Wang et al., 1993), through an increase in AMPA/kainate receptor channel opening frequency and open time (Greengard et al., 1991). Phosphorylation by PKC enhances responses evoked by AMPA and kainate at high agonist concentrations and it depresses currents evoked by kainate at lower concentrations (Wang et al., 1994), while CaMKII enhances kainate responses (McGlade-McCulloh et al., 1993). PKC and CaMKII are two protein kinases implicated in mechanisms underlying hippocampal LTP/LTD. However the mechanisms of action of these two kinases on the AMPA receptor have not yet been determined. The finding that previously 'silent' AMPA receptors may be activated following induction of hippocampal LTP (Liao et al., 1995; Issac et al., 1995) suggests that PKC and CaMKII may mediate the insertion and redistribution of AMPA receptors into the cell membrane.

The NMDA receptor is also phosphorylated by PKC, and phosphorylation is associated with potentiation of NMDA (N-methyl-D-aspartate) receptor-mediated currents (Chen & Huang, 1991). This potentiation may result from an increased probability of channel opening and/or a reduction in the block of NMDA receptors by
Mg\(^{2+}\) (Chen & Huang, 1992). Also, activation of PKC by phorbol esters or metabotropic receptor agonists resulted in potentiation of NMDA-evoked currents in hippocampal neurons (Aniksztejn et al., 1992; Lu et al., 1999). With regards to PKA phosphorylation, it has been suggested that PKA phosphorylates synaptic NMDA receptors in the resting state, which then become dephosphorylated during synaptic activity as the calcium-dependent serine/threonine phosphatase, calcineurin is activated upon NMDA receptor-mediated Ca\(^{2+}\) influx (Raman et al., 1996).

The GABA\(_{A}\)R, like the GlyR, is a major inhibitory receptor found in the CNS. Molecular cloning indicates that GABA\(_{A}\)Rs are members of a channel superfamily that includes GlyRs (Unwin, 1993). Activation of GABA\(_{A}\)Rs leads to an increase in cell membrane Cl\(^{-}\) permeability, which results in hyperpolarization of the neuronal membrane (Unwin, 1993).

PKA-mediated phosphorylation increases the rate of GABA\(_{A}\)R desensitization in chick cortical neurons (Tehrani et al., 1989). PKA also decreases GABA-gated Cl\(^{-}\) flux in cerebral cortical synaptoneurosomes (Leidenheimer et al., 1991) and depresses GABA\(_{A}\)R currents in spinal cord neurons and recombinant GABA\(_{A}\)Rs (Moss et al., 1992; Porter et al., 1990). In one study (Porter et al., 1990), this depression was the result of a reduction in the channel open frequency of GABA\(_{A}\) channels in spinal cord neurons. However, although these studies show that acute application of PKA produces a depression of GABA\(_{A}\)R-mediated current amplitude, chronic elevation of PKA activity can result in enhancement of GABA\(_{A}\)R-mediated currents (Angelotti et al., 1993). Receptor heterogeneity may underlie these differential effects of PKA on GABA-evoked currents. Various studies also indicate
that PKC phosphorylation affects GABA$_A$R function, where activation of PKC inhibits GABA-evoked currents (Sigel & Baur, 1988; Moran & Dascal, 1989; Leidenheimer et al., 1992). GABA$_A$Rs are also phosphorylated by cGMP-dependent protein kinase (PKG) and CaMKII; cGMP inhibits GABA-evoked currents from the tractus solitarius neurons of the rat (Glaum & Miller, 1993), while CaMKII potentiates GABA-evoked whole-cell currents in spinal cord neurons (Wang & Kelly, 1995).

1.4. PROTEIN TYROSINE PHOSPHORYLATION

Protein tyrosine phosphorylation was first recognized as a process regulating cellular function approximately 20 years ago (Eckhart et al., 1979). Since then it has become evident that tyrosine phosphorylation is crucial in cellular regulation through its involvement in various cellular events, including differentiation, growth and proliferation (Schlessinger, 1988; Schlessinger & Ullrich, 1992), as well as synaptogenesis (Catarsi & Drapeau, 1993), long-term potentiation (Terlau & Seifert, 1989; O'Dell et al., 1991; Lu et al., 1998), ischemia (Kindy, 1993), epilepsy (Jope et al., 1991; Stratton et al., 1991) and learning (Rosenblum et al., 1995).

The process of tyrosine phosphorylation involves the transfer of a phosphate group(s) from ATP to one or more tyrosine residues, which are invariably found in an intracellular location on the receptor (Browning et al., 1985; Hemmings, Jr. et al., 1989; Moss & Smart, 1996). Upon phosphorylation of a tyrosine residue, the charge and bulk size of a protein are changed. These changes in charge, steric hindrance and global conformation probably play an important role in producing the observed effects of tyrosine phosphorylation (Browning et al., 1985).
PTKs are classified as either receptor tyrosine kinases which contain a single transmembrane domain, or non-receptor tyrosine kinases which do not possess such a transmembrane domain. Upon ligand binding, receptor tyrosine kinases are activated, and this triggers their dimerization and the subsequent autophosphorylation of an intracellular kinase domain (Schlessinger & Ullrich, 1992).

Non-receptor tyrosine kinases, which are activated by intracellular cascades, mediate protein-protein interactions with a high degree of affinity and specificity (Superti-Furga & Courtneidge, 1995). The c-src family of PTKs, which is the prototype of the group of non-receptor PTKs, has nine known members (blk, c-fgr, fyn, hck, lck, lyn, c-src, c-yes and yrk), each encoding a cytoplasmic PTK believed to be involved in signal transduction (Kefalas et al., 1995). The c-src PTKs contain three Src homology (SH) domains: SH1, SH2 and SH3. The SH1 domain has PTK activity, while the SH2 and SH3 domains are involved in mediating protein-protein interactions by binding to phosphotyrosine-containing and proline-rich motifs, respectively. The PTK pp60<sup>c-src</sup> contains two tyrosine residues, Tyr-416 and Tyr-527 within the catalytic SH1 domain. Autophosphorylation of Tyr-416 and dephosphorylation of Tyr-527 by PTPs brings the enzyme to its active state. The PTK p50<sup>csk</sup> can phosphorylate Tyr-527 of pp60<sup>c-src</sup> and thus down-regulate its activity. It seems that phosphorylation of Tyr-527 causes a rearrangement of the molecule such that both the SH2 and SH3 domains are engaged intramolecularly and are therefore not accessible for interaction with other proteins. Dephosphorylation of Tyr-527 is sufficient to destabilize this conformation and de-repress the enzyme, although if pp60<sup>c-src</sup> is not phosphorylated at Tyr-416, the
enzymatic activity is low. It has been suggested that autophosphorylation may result in conformational changes that allow better access of other substrates to the catalytic site (Kefalas et al., 1995; Superti-Furga & Courtneidge, 1995).

The c-src gene is widely expressed, but attention has focused on its expression in neurons of the CNS. High-level expression is largely restricted to terminally differentiated, post-mitotic cells. It therefore seems unlikely that the c-src family members are primarily involved in signaling pathways that regulate cell division. Rather, they seem to be involved in signaling pathways that regulate the function of terminally differentiated cells (Kefalas et al., 1995; Superti-Furga & Courtneidge, 1995).

1.5. TYROSINE PHOSPHORYLATION OF LIGAND-GATED ION CHANNELS

Tyrosine phosphorylation of the nAChR at the neuromuscular junction increases the rate of desensitization of nAChR-mediated currents (Hopfield et al., 1988). Agrin-induced AchR clustering in muscle also appears to be mediated by tyrosine phosphorylation (Ferns et al., 1996).

NMDA receptor-mediated currents are depressed by the PTK inhibitors, genistein and lavendustin A (Wang & Salter, 1994), and enhanced by the PTK, pp60<sup>c-src</sup> (Wang & Salter, 1994; Xiong et al., 1999) and the PTP inhibitor, sodium orthovanadate (Wang & Salter, 1994). Pp60<sup>c-src</sup> increases NMDA receptor conductance but has no effect on the reversal potential of NMDA currents (Wang & Salter, 1994). Also, activation of pp60<sup>c-src</sup> increases the channel open probability and mean open time, while its inhibition significantly decreases those parameters.
These results suggest a functional role of tyrosine phosphorylation in NMDA receptor channel gating and synaptic transmission.

GABA$\alpha$R $\beta$ and $\gamma_2$ subunits are also targets of the PTKs pp60$^{v-src}$ and pp60$^{c-src}$ (Moss et al., 1995; Valenzuela et al., 1995). The sites of phosphorylation are located within the large cytoplasmic loops that connect the third and fourth putative transmembrane segments of the $\beta_1$ and $\gamma_2L$ subunits (Moss et al., 1995; Valenzuela et al., 1995). The tyrosine kinase inhibitors, genistein and tyrphostins, inhibited Cl$^-$ uptake and GABA-gated Cl$^-$ currents in Xenopus oocytes expressing GABA$\alpha$R subunits (Valenzuela et al., 1995), suggesting that PTKs regulate GABA$\alpha$R function. In addition, genistein depressed (Moss et al., 1995; Wan et al., 1997a) while pp60$^{c-src}$ (Moss et al., 1995; Wan et al., 1997a) and sodium vanadate (Moss et al., 1995) enhanced GABA$\alpha$R-mediated currents. Also, Moss et al. (1995) observed that modulation by tyrosine phosphorylation was observed in the presence of the wild type $\gamma_2L$ subunit but not when the mutant $\gamma_2L^{Y(365/367)F}$ subunit was present. Single-channel recordings of GABA-evoked currents suggest that tyrosine phosphorylation by pp60$^{c-src}$ increases the mean open time and probability of channel opening (Moss et al., 1995). These results indicate that neuronal GABA$\alpha$Rs can be modulated by tyrosine kinase activity, where PTKs enhance the function of GABA$\alpha$Rs.

1.6. PHOSPHORYLATION OF THE GLYCINE RECEPTOR

1.6.1. PKA AND PKC PHOSPHORYLATION

Only a limited number of studies have been carried out in the domain of serine/threonine phosphorylation of the GlyR. These reports indicate that the GlyR is modulated by second-messenger signaling systems such as PKA and PKC.
PKA activation by forskolin and purified PKA lead to increased phosphorylation of the spinal cord GlyR α subunits (Vaello et al., 1994). Also, intracellular perfusion with cAMP reduces the fast-desensitizing component of glycine-evoked currents in acutely dissociated hypothalamic neurons while inhibition of PKA prevents the depression of the fast desensitizing glycine-activated current observed in the presence of cAMP (Agopyan et al., 1993). The observed effect is the result of a decrease in the open probability of the channels caused by PKA phosphorylation of the GlyR (Agopyan et al., 1993). In contrast, PKA increased glycine-induced currents in Xenopus oocytes, spinal cord neurons and neurons isolated from the ventral tegmental area (Song & Huang, 1990; Vaello et al., 1994; Ren et al., 1998). In spinal cord neurons, this potentiation was the result of increased probability of channel opening (Song & Huang, 1990).

PKC also phosphorylates the α1 subunit of the GlyR within the intracellular loop between the third and fourth transmembrane segments (Vaello et al., 1994). PKC activation by the phorbol ester, TPA, resulted in a time-dependent decrease in the glycine-elicited currents in Xenopus oocytes (Vaello et al., 1994), while the phorbol ester, PMA enhanced glycine-evoked currents in hippocampal neurons (Schonrock & Bormann, 1995).

These findings indicate that GlyRs are regulated by PKA- and PKC-dependent phosphorylation. However, both PKA and PKC have different effects on GlyR function, depending on the tissue preparation tested. These differential functional effects of PKA and PKC on GlyRs may be the result of receptor heterogeneity since the α subunit has 4 different isoforms, which form GlyR channels either alone or in
combination with the GlyR β subunit. Alternatively, the observed differences might be attributed to the activation of different signaling cascades in the various tissues utilized in each study, or differences in the mode of action of the various drugs used in these reports.

1.6.2. TYROSINE PHOSPHORYLATION

The GABA_A,R and the GlyR are similar in structure and function (Unwin, 1993). As previously explained, the GABA_A,R is phosphorylated by tyrosine kinases, which, in turn affect receptor function. By analogy, it is suspected that the GlyR might also be regulated by tyrosine phosphorylation.

Due to significant interest to determine the minimal requirements for substrate recognition by protein kinases, 'consensus sites' for phosphorylation within proteins have been identified. Consensus sites are usually of the form of a short linear sequence surrounding the site(s) of phosphorylation, identifying the minimum set of amino acids required for substrate recognition by the kinases (Moss & Smart, 1996).

There are, however, limitations to this approach. Many kinases show very broad substrate specificity, thus recognizing more than one consensus sequence with different affinities. In addition, secondary and tertiary structures can be important factors in substrate recognition. Hence, the existence of a consensus site for phosphorylation does not ensure phosphorylation of a protein nor is it an accurate indicator of which kinase(s) is responsible for phosphorylation. Consensus sites can only be used as a guide to possible phosphorylation sites, the presence of which should be experimentally determined (Moss & Smart, 1996).
Upon cloning of the GlyR β subunit, it was determined that a putative tyrosine phosphorylation site is present at position tyrosine-413 (Tyr-413), based on previously identified consensus sites for tyrosine kinases (Grenningloh et al., 1990a). However, no studies have been carried out to date as to the modulation of GlyR function by tyrosine phosphorylation.

1.7. GLYCINE RECEPTOR PROPERTIES

The GlyR belongs to the superfamily of ligand-gated ion channels which respond to a specific chemical neurotransmitter, such as acetylcholine, γ-aminobutyric acid (GABA), glutamate, serotonin or glycine (Bechade et al., 1994). The GlyR channel is formed from five integral glycoprotein subunits that co-assemble in a pentamer to form a functional Cl⁻ channel (Langosch et al., 1988). Binding of glycine to the GlyR results in the regulation of GlyR gating (opening and closing of the channel pore). The GlyR concentration response curve is sigmoidal with a Hill coefficient that is greater than 1, as will be explained later in more detail. The GlyR complex co-purifies with a peripheral membrane protein of 93 kDa, which has been named gephyrin (Figure 1) (Pfeiffer et al., 1982).
Figure 1

The GlyR complex

A model of the postsynaptic GlyR complex. The transmembrane channel of the receptor is composed of three copies of the α subunit and two copies of the β subunit. The co-purifying 93 kDa polypeptide, gephyrin which is localized at the cytoplasmic face of the postsynaptic membrane, binds to the β subunit and to the cytoskeleton and regulates GlyR clustering.
1.8. ROLE OF THE GLYCINE RECEPTOR IN INHIBITORY NEUROTRANSMISSION

Synaptic transmission in the mammalian CNS involves a balance between excitation and inhibition. Signal transmission between cells takes place at the synapses, which are sites where neurons make functional contact, through neurotransmitter release from presynaptic terminals. At glycineergic synapses, glycine is released into the synaptic cleft and binds to GlyRs on the postsynaptic side. Activation of the GlyR increases the Cl⁻ conductance and causes neuronal hyperpolarization (Curtis et al., 1967; Werman et al., 1967; Curtis et al., 1968a; Curtis et al., 1968b), thus creating a current shunt that opposes the depolarizing action of excitatory neurotransmitters on cellular membrane voltage (Figure 2).
Figure 2

A Glycinergic Synapse

Diagram of a glycinergic synapse, showing glycine synthesis, release into the synaptic cleft and actions on the postsynaptic neuron. Glycine is synthesized from serine by serine hydroxymethyltransferase (SHMT) and stored in the presynaptic neuron for release. Once released, glycine can bind to postsynaptic GlyRs and increase the Cl⁻ influx through the GlyR channel. Glycine is removed from the synaptic cleft by diffusion and by Na⁺/Cl⁻-dependent glycine transporters (GLYT1 and GLYT2).
Glycine was proposed to be the neurotransmitter involved in the initiation of spinal cord inhibitory postsynaptic potentials (IPSPs) approximately 35 years ago by detailed analysis of glycine distribution in various regions of the spinal cord (Weman et al., 1967; Curtis et al., 1968a; Curtis et al., 1968b). This was confirmed by autoradiographic studies, which localized \[^{3}\mathrm{H}\] glycine in synaptic regions of the spinal cord (Bechade et al., 1994; Rajendra et al., 1997; Vannier & Triller, 1997). To date, both inhibitory postsynaptic currents (IPSCs) and glycine-evoked responses have been observed in spinal cord neurons, pointing to an important role of GlyR inhibitory neurotransmission in the spinal cord.

Several reports indicate that the inhibitory GlyRs are present in the hippocampus (Ito & Cherubini, 1991; Shirasaki et al., 1991; Fatima-Shad & Barry, 1992; Fatima-Shad & Barry, 1995; Fatima-Shad & Barry, 1998). In hippocampal neurons, GlyRs produce an increase in the Cl\(^-\) permeability of the neurolemma and give rise to IPSCs (Ito & Cherubini, 1991; Fatima-Shad & Barry, 1998). In another report (Fatima-Shad & Barry, 1998), application of bicuculline, a GABA\(_A\)R antagonist, or strychnine, a GlyR antagonist, abolished the miniature spontaneous potentials (mIPSPs) observed in hippocampal cultures. Corelease of glycine and GABA has been reported in spinal interneurons, which release both glycine and GABA from individual synaptic vesicles to activate functionally distinct receptors in their postsynaptic target cells (Jonas et al., 1998). In addition, corelease of the inhibitory neurotransmitter, GABA with the fast excitatory neurotransmitter, ATP has been reported in spinal neurons, and adenosine, probably generated by extracellular metabolism of ATP, finely tuned GABAergic inhibitory postsynaptic currents in the
spinal cord (Jo & Schlichter, 1999). The glycine IPSCs observed in the hippocampus are thought to result from the spontaneous release of single quanta of glycine into the synaptic cleft. This evidence is in favour of a neurotransmitter role of glycine in the hippocampus, acting on strychnine-sensitive GlyRs.

1.9. GLYCINE RECEPTOR SUBUNITS

To date, five GlyR subunits have been identified and are classified according to the conservation of their amino acid sequence. There are two GlyR subunit types and various isoforms: \( \alpha_{1-4} \) and \( \beta \). In addition, some of the GlyR gene products undergo alternative splicing such as the rat \( \alpha_1 \) and \( \alpha_2 \) which can exist in the \( \alpha_1^{\text{ins}} \) (Malosio et al., 1991a), and \( \alpha_2A \) and \( \alpha_2B \), respectively (Kuhse et al., 1991). Another \( \alpha_2 \) subunit also exists, with a sequence identical to that of the \( \alpha_2A \)-subunit, except for one amino acid, suggesting that it is an allelic variant of the same gene rather than a product of a second \( \alpha_2 \) gene (Kuhse et al., 1990a). This allelic variant is referred to as \( \alpha_2^* \).

The \( \alpha \) and \( \beta \) GlyR subunits consist of approximately 420 (Grenningloh et al., 1990b; Kuhse et al., 1990a; Harvey et al., 2000) and 470 (Grenningloh et al., 1990a) amino acid residues, respectively. Each has extracellular N- and C-termini and four transmembrane domains designated TM1 - TM4, which are separated by short and long cytoplasmic loops (Bechade et al., 1994) (Figure 3).
Figure 3
The Topology of a GlyR Subunit

Generic GlyR protein subunit sequence and topological structure. The N- and C-termini of the polypeptide are suggested to be extracellular. There are four TM domains (TM1 – TM4). Between the third and fourth transmembrane domain there is a hydrophilic putative cytoplasmic region involved in intracellular regulatory mechanisms such as phosphorylation.
Extracellular Domain

COOH

TM1 TM2 TM3 TM4

Lipid Bilayer

H₂N

Intracellular Domain

FELS\text{N}({\text{Y-413}})\text{DCYGK}
on the $\beta$ subunit of the GlyR
The multiple $\alpha$ subunit isoforms that have been identified are: 1) the human, pig, and rat 48 kDa $\alpha_1$, and rat $\alpha_1^{\text{ins}}$ subunits, 2) the human, rat, and mouse 49 kDa $\alpha_2$, rat $\alpha_2A$ and $\alpha_2B$ and rat $\alpha_2^*$ subunits, 3) the 50 kDa $\alpha_3$ subunit in the rat, and 4) the mouse and chick $\alpha_4$ subunits (Harvey et al., 2000; Rajendra et al., 1997; Bechade & Triller, 1994; Vannier & Triller, 1997). The mature mouse and chick GlyR $\alpha_4$ subunits have predicted molecular weights of 49.7 and 49.5 kDa, respectively (Harvey et al., 2000). To date, no variants of the GlyR 58 kDa $\beta$ subunit have been identified (Grenningloh et al., 1990a).

The $\alpha_1$ transcript has been mainly found throughout the adult spinal cord, in the brainstem and in midbrain structures such as the colliculi, hypothalamus and the cerebellum (Malosio et al., 1991b). The $\alpha_2$ variants are mainly expressed in the neonatal spinal cord and the upper brain regions such as the cerebral cortex, the hippocampal formation and the thalamus (Malosio et al., 1991b). The $\alpha_3$ mRNA has been detected in the limbic system such as the infralimbic cortex and the hippocampal complex, in the cerebellum (Malosio et al., 1991b) and in the spinal cord (Kuhse et al., 1990b). $\alpha_4$ subunit transcripts have been found in low levels in the adult spinal cord and brain (Matzenbach et al., 1994; Harvey et al., 2000).

In contrast to the region-specific expression of the $\alpha$ subunits, the $\beta$ transcript has a widespread distribution in the brain, found in regions such as the brainstem, spinal cord, cerebral cortex, hippocampus and cerebellum, as well as in the thalamus, olfactory bulb and striatum (Malosio et al., 1991b).

The GlyR $\alpha$ subunits harbor the binding sites for glycinergic agonists and antagonists (Bechade & Triller, 1994; Rajendra et al., 1997; Vannier & Triller, 1997).
In addition, heterologous expression of recombinant GlyR \( \alpha_{1,4} \) subunit cDNAs in *Xenopus* oocytes and mammalian cell lines (Rajendra et al., 1997; Vannier & Triller, 1997; Harvey et al., 2000) has shown that the \( \alpha \) subunits are sufficient to generate homo-oligomeric channels which display pharmacological properties typical of native mammalian GlyRs. Whole-cell recordings confirmed the efficient formation of \( \alpha \)-subunit homo-oligomers, which generate glycine-gated channels that mediate large inward currents and reverse at the equilibrium potential of Cl\(^-\).

In a study by Bormann et al. (1993), no glycine-evoked currents were observed in cells expressing the \( \beta \) subunit alone in human embryonic kidney 293 (HEK 293) cells even when glycine concentrations up to 0.5 mM were used. In another study, very high glycine concentrations (EC\(_{50} = 24 \text{ mM} \)) elicited only small inward currents in *Xenopus* oocytes nuclei injected with GlyR \( \beta \) subunit cDNA (Grenningloh et al., 1990a). This evidence suggests that the GlyR \( \beta \) subunit carries only a very low affinity glycine site and/or does not assemble efficiently into functional channels (Bormann et al., 1993). Hence, the \( \beta \) subunit of the GlyR is not essential for ligand binding.

However, the GlyR \( \beta \) subunit modulates the channel properties of hetero-oligomeric GlyRs (Pribilla et al., 1992; Bormann et al., 1993; Handford et al., 1996). Its presence reduced the sensitivity of glycine-evoked currents to antagonism by picrotoxin (Pribilla et al., 1992; Handford et al., 1996). Thus the GlyR \( \beta \) subunit is an important determinant of blocker binding to the GlyR and also provides a means of assessing the functional co-expression of the GlyR \( \beta \) subunit with the GlyR \( \alpha \) subunit in recombinant expression systems. In addition, co-expression of the GlyR \( \alpha_1 \)
subunit with the β subunit in HEK 293 cells potentiated glycine-induced whole-cell currents by 5-fold (Bormann et al., 1993) and resulted in a 1.9-fold increase in the efficiency of receptor expression, as determined by $B_{\text{max}}$ values (Handford et al., 1996). These results suggest that the β subunit may facilitate and/or stabilize GlyR expression on the plasma membrane (Handford et al., 1996). Furthermore, the presence of the GlyR β subunit increased the EC$_{50}$ value of glycine-evoked currents (Handford et al., 1996). Homomeric $\alpha_1$ GlyRs displayed an EC$_{50}$ value of 18 μM while co-expression of the $\alpha_1$ and β subunits resulted in the formation of glycine-gated channels which had an EC$_{50}$ value of 74 μM.

The picrotoxinin binding site on the GlyR has been assigned to the TM2 segment of the GlyR $\alpha$ subunit (Pribilla et al., 1992). Since picrotoxinin is commonly regarded as a Cl$^-$ channel blocker, this suggests that the TM2 segment contributes to the wall of the GlyR pore, in analogy with the nAChR (Imoto et al., 1988; Imoto et al., 1991; Villarroel et al., 1992). Also, synthetic peptides corresponding to the TM2 segment of the $\alpha_1$ subunit of the GlyR elicit single-channel activity when incorporated into lipid bilayers (Langosch et al., 1991), further suggesting that the TM2 segments line the ion channel pore. The sensitivity of glycine-activated currents to inhibition by cyanotriphenylborate (CTB), a recently identified non-competitive blocker of the GlyR channel, is decreased in $\alpha_1$ GlyRs by mutating a residue in the TM2 region (Rundstrom et al., 1994). Also, mutations within the TM2 of the GlyR $\alpha_1$ and β subunits suggest that the TM2 segment determines the conductance differences observed between $\alpha$ and $\alpha/\beta$ GlyRs (Bormann et al., 1993), as will be explained
shortly. In summary, all these lines of evidence indicate that the TM2 segments line the GlyR channel pore.

1.10. DEVELOPMENTAL REGULATION OF GLYCINE RECEPTOR SUBUNIT EXPRESSION

In the developing rat spinal cord, during the embryonic period and just after birth up to postnatal day 3 (P3), the highest levels of GlyR α mRNA found are those of α2 while very low levels of α1 transcripts can be detected (Malosio et al., 1991a; Malosio et al., 1991b). A strong down-regulation of α2 subunits and up-regulation of α1 subunits occurs after P3, so that by P15 the α2 subunits are replaced by α1 subunits and adult levels of α1 subunit expression are reached (Benavides et al., 1981; Malosio et al., 1991b). A pattern comparable to that of GlyR α1 is observed for the GlyR α3 subunit, which accumulates late in development (Kuhse et al., 1990b; Malosio et al., 1991b).

In the spinal cord, fetal GlyRs are pentameric homomers composed of α2 subunits (Hoch et al., 1989; Grenningloh et al., 1990b; Bormann et al., 1993). In contrast, cross-linking studies indicate that adult GlyRs are pentameric heteromers composed of α1 and β subunits, in a likely ratio of three α1 to two β subunits (Langosch et al., 1988; Bormann et al., 1993; Kuhse et al., 1993). This developmental switch from the GlyR α2 to the GlyR α1 subunit is correlated with changes in the pharmacology of the GlyR complex. Neonatal GlyRs display a low affinity for strychnine, which is a potent antagonist of adult GlyRs (Vannier & Triller, 1997). Also, the switch from α2 to α1 reduces the channel open time of the GlyR channel from 180 ms to 24 ms, so that faster inhibitory postsynaptic currents are
observed in mature neurons (Takahashi et al., 1992). In addition, during embryonic development (E16-17), glycine preferentially activates a Cl\(^{-}\)-dependent depolarization of motoneurons in the rat spinal cord, at least until 1-2 days after birth but it is not known at what age glycine gives rise to Cl\(^{-}\)-dependent hyperpolarization (Barker & Ransom, 1978; Wu et al., 1992). The ionic mechanism or physiological significance of these glycine-induced depolarizations in embryonic motoneurons are not certain, although depolarizations are Cl\(^{-}\)-dependent and may result from an outward Cl\(^{-}\) current that is generated by modified Cl\(^{-}\) concentrations across developing membranes (Wu et al., 1992; Tapia et al., 1998).

In both fetal and adult rat hippocampi, \(\alpha_2\) and \(\beta\) are the main GlyR subunits expressed, but \(\alpha_3\) subunits are also present (Malosio et al., 1991b). Although glycine-activated currents or the presence of GlyR cDNAs have been reported in various regions of the brain such as the medulla oblongata, cerebral cortex, hypothalamus and brainstem, GlyR subunit composition in these areas has not been examined.

1.11. GLYCINE RECEPTOR AGONISTS

The most potent agonist of the GlyR is the amino acid glycine. In various cultured and acutely isolated in vitro preparations, the glycine concentration response curves have yielded values that produce 50% of the maximal response (EC\(_{50}\)), that range from \(~ 20\) to \(120\) \(\mu\)M (Rajendra et al., 1997; Tapia et al., 1997; Yoon et al., 1998; Xu et al., 1999; Harvey et al., 2000). These values are similar to those obtained with recombinant GlyRs expressed in HEK 293 cells (Rajendra et al., 1997). However, in Xenopus oocytes, the EC\(_{50}\) values of recombinant GlyRs
range from ~ 0.1 to 0.3 mM upon 10 – 25 sec applications of glycine, thereby displaying a lower sensitivity to glycine (Grenningloh et al., 1990b; Schmieden et al., 1992; Laube et al., 1995; Griffon et al., 1999; Harvey et al., 2000).

Various studies have shown that the Hill coefficients for glycine activation in preparations containing $\alpha_1$, $\alpha_2$, $\alpha_4$-homomeric and $\alpha_1/\beta$-heteromeric GlyRs range from 1.6 – 5.7 (Rajendra et al., 1997; Yoon et al., 1998; Harvey et al., 2000). Hence, at least two glycine molecules bind to the GlyR in a cooperative manner to open the associated Cl⁻ channel.

The amino acids, β-alanine and taurine are also GlyR agonists, although not as potent as glycine. The EC₅₀ values for β-alanine and taurine are ~ 0.3 and 0.4 mM respectively, in the medulla oblongata and hippocampus (Krishtal et al., 1988). In recombinant expression systems, the EC₅₀ values for β-alanine for $\alpha_1$- and $\alpha_2$-homomeric GlyRs are ~0.7 and 3 mM, respectively (Schmieden et al., 1992), while for taurine, the respective EC₅₀ values are ~ 0.2 – 3 mM (Grenningloh et al., 1990b; Schmieden et al., 1992; Rajendra et al., 1995) and 4 - 6 mM (Grenningloh et al., 1990b; Schmieden et al., 1992). These differences suggest that the binding sites of the ligands may be different in $\alpha_1$ and $\alpha_2$ GlyRs or there might exist distinct agonist subsites within the ligand-binding sites of $\alpha_1$ and $\alpha_2$ GlyR subunits (Schmieden et al., 1992).

For $\alpha_1$ GlyRs expressed in Xenopus oocytes, β-alanine is a full agonist compared to glycine while taurine acts as a partial agonist, eliciting amplitudes corresponding to ~28% of the glycine response (Schmieden et al., 1992). In contrast, for $\alpha_2$ GlyRs, both β-alanine and taurine act as partial agonists, giving rise to currents that are only
29

~ 20% and 5% of the response activated by glycine (Schmieden et al., 1992). Hence, the relative current amplitudes induced by β-alanine and taurine are much smaller with neonatal α2 receptors than with adult-type α1 GlyRs (Schmieden et al., 1992). Since taurine concentrations are elevated in the CNS of newborn rodents (Cutler & Dudzinski, 1974), this difference in agonist efficacy of α1 and α2 GlyR subunits may reflect an adaptation to alterations in the endogenous levels of the putative modulators of glycinergic transmission, taurine and β-alanine during development. The Hill coefficients for β-alanine and taurine are similar to those of glycine (Lewis et al., 1991; Schmieden et al., 1992; Rajendra et al., 1995).

α2*-containing GlyRs expressed in Xenopus oocytes display much lower agonist sensitivity to glycine, β-alanine and taurine. The EC50 value for glycine was 12 mM, while β-alanine and taurine acted as partial agonists with Imax values of ~ 55 and 50%, respectively and EC50 values of 7 and 18 mM, respectively (Kuhse et al., 1990a). The Hill coefficients ranged from 1.5 – 1.8 for all three agonists (Kuhse et al., 1990a). Given that the α2* subunit is suspected to be present only in early development, the comparatively low agonist affinity of the rat α2* GlyR subunit may reflect adaptation to high cerebrospinal amino acid levels (Cutler & Dudzinski, 1974; Kuhse et al., 1990a). Alternatively, it is possible that co-assembly of the GlyR α2* with other subunits may alter agonist binding, in vivo, and increase heteromeric receptor affinity for an agonist (Kuhse et al., 1990a).

Expression of α4-homomeric GlyRs in Xenopus oocytes yielded EC50 values of 0.12, 0.5 and 1.12 mM for glycine, β-alanine and taurine, respectively (Harvey et al., 2000). These values are similar to those obtained for the GlyR α1 subunit
(Schmieden et al., 1992) but not to those obtained for the GlyR $\alpha_2$ and $\alpha_3$ subunits (Kuhse et al., 1990b; Schmieden et al., 1992). The Hill coefficients were $\geq 1.5$ for all three agonists (Harvey et al., 2000). The regional distribution and characteristics of the various GlyR subunits are summarized in Table 1.
<table>
<thead>
<tr>
<th>SUBUNIT(S)</th>
<th>LOCATION</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>HILL</th>
<th>SINGLE CHANNELS</th>
<th>EXPRESSION SYSTEM</th>
<th>REFERENCE(S)</th>
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<tr>
<td>Hippocampus &amp; Medulla Oblongata</td>
<td>100</td>
<td>1.4</td>
<td></td>
<td></td>
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<td>61</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
<td>Fatima-Shad &amp; Barry, 1992</td>
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<tr>
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<td>63</td>
<td>2.0</td>
<td>19 and 24 pS</td>
<td>1.4 pA</td>
<td></td>
<td>Fatima-Shad &amp; Barry, 1995</td>
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<tr>
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<td>47 and 100 pS</td>
<td></td>
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<td>Yoon et al., 1998</td>
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<td></td>
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<td>29</td>
<td>2.9</td>
<td>53 pS [gly] = 6 µM</td>
<td>t&lt;sub&gt;r&lt;/sub&gt; = 10 ms</td>
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<td>42</td>
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<td></td>
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<td>30 and 43 pS</td>
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<td>Smith et al., 1989</td>
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<td></td>
<td>32, 47 and 94 pS</td>
<td>t&lt;sub&gt;1&lt;/sub&gt; = 14.8 ms</td>
<td>t&lt;sub&gt;2&lt;/sub&gt; = 48.1 ms</td>
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<td>Spinal Cord (P16)</td>
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<td>27 and 45 pS</td>
<td>t&lt;sub&gt;1&lt;/sub&gt; = 4.73 ms</td>
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<td>Ren et al., 1998</td>
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<td>1.6</td>
<td>HEK 293 cells</td>
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<td>2.4</td>
<td>Xenopus oocytes</td>
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<td>1.8</td>
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<td>95</td>
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<td>$\alpha_2$</td>
<td>30</td>
<td>5.7</td>
<td>86 pS</td>
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*Values in parentheses represent the channel density (pmol/mg protein).
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<td>54</td>
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<td>48 pS</td>
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<td>Xenopus oocytes</td>
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Table 1.

Properties of Native and Recombinant GlyRs

Pharmacological and single channel properties of native and recombinant GlyRs are shown.
1.12. BIOPHYSICAL PROPERTIES OF THE GLYCINE RECEPTOR

1.12.1. CONDUCTANCES

The electrical conductance (g) of an ion channel is a measure of the ease of current flow across the membrane. The native GlyR has multiple conductance states as indicated by single-channel studies. Channel currents with two predominant amplitudes have been recorded in spinal cord neurons, suggesting a dominant or main-conductance state of approximately 42 pS and a sub-conductance state of about 27 pS (Bormann et al., 1987; Smith et al., 1989; Takahashi et al., 1992). For both conductance states, three exponential components were required to fit the open time frequency distribution histograms at all glycine concentrations tested, suggesting that both the main and sub-conductance states of the channel open into at least three open states (Twyman & Macdonald, 1991). For the main-conductance state the time constants were ~ 1.09, 4.06 and 9.79 ms, while for the sub-conductance state the time constants were ~ 0.55, 2.64 and 8.57 ms (Twyman & Macdonald, 1991). Closed time frequency distributions were fitted with multiple exponential components, suggesting that the channel has several closed states (Twyman & Macdonald, 1991). The two shortest time constants were ~ 0.16 and 1.26 ms, while the longer time constant varied with concentration (Twyman & Macdonald, 1991).

GlyR α1, α2 and α3 homomeric channels expressed in HEK 293 cells have single-channel conductances of 86, 111 and 105 pS, respectively, while smaller and less frequent conductances have been observed for each subunit type (Bormann et al., 1993; Harvey et al., 2000). In contrast, α1/β, α2/β and α3/β GlyRs have main-state conductances of 44, 54 and 48 pS respectively, with each α/β combination exhibiting
several smaller conductance states (Bormann et al., 1993). The latter values are similar to those found in spinal cord neurons, suggesting that native GlyRs are predominantly α/β hetero-oligomers. Also, as the main-state conductances of α/β heteromers are much lower than those of the corresponding α homo-oligomers, it appears that selection of the main-state conductance depends on the presence of the β subunit and that the β subunit is important in determining ion flux through the GlyR channel. Site-directed mutagenesis revealed that residues within and C-terminal to the TM2 segment determine the difference in the conductance pattern displayed by α1 and α1/β GlyR channels (Bormann et al., 1993). The main conductance of the α4 subunit is 90 pS when expressed in HEK 293 cells (Harvey et al., 2000), a value similar to that of the 86 pS main conductance of the α1 subunit (Bormann et al., 1993). However, smaller and less frequent conductances are also observed (Harvey et al., 2000).

In the hippocampus, single-channel studies show that there exist two types of channels distinguished by a difference in conductances. The two groups have single channel conductances of approximately 47 pS and 100 pS (Yoon et al., 1998). Conductances of 24 and 18 pS have also been reported in hippocampal neurons (Fatima-Shad & Barry, 1995). These results suggest that both α-homomeric and α/β heteromeric GlyRs are present in the hippocampal region.

Detailed studies of GlyR ion permeation mechanisms using single-channel recording techniques in embryonic spinal cord neurons (Bormann et al., 1987) and in cultures of postnatal hippocampal neurons (Fatima-Shad & Barry, 1992; Fatima-Shad & Barry, 1993) found the relative permeability sequence to be: SCN− > NO3− > I− > Br− > Cl− > F−, whereas the relative conductances were Cl− > Br− > NO3− > I− > SCN− > F−. The
permeabilities are proportional to the ionic hydration energies, indicating that hydrogen forces, rather than ion-site interactions within the channel, form the major barrier to ion channel entry. However, the approximate inverse relationship between the permeation and conductance sequences is evidence for an anion-selectivity mechanism within the channel. Based on the relationship between anion size and permeation ability, GlyR channels have an effective pore diameter of ~ 5.2 – 5.6 Å when tested in various tissue preparations, such as embryonic spinal cord neurons, postnatal hippocampal neurons and recombinant GlyRs expressed in HEK 293 cells (Bormann et al., 1987; Fatima-Shad & Barry, 1993; Rundstrom et al., 1994).

1.12.2. VOLTAGE

There is a distinct heterogeneity in the voltage dependence of glycine-activated responses. Various studies report the absence of or strong inward or outward rectification of glycine-evoked currents. A linear current-voltage relationship is observed for glycine-activated currents in the hippocampus, spinal cord, medulla oblongata and sacral dorsal commissural nucleus as well as brainstem, ventromedial hypothalamus and ventral tegmental area, and $\alpha_1$ and $\alpha_1/\beta$ recombinant GlyRs (Rajendra et al., 1997; Ren et al., 1998; Singer et al., 1998). Inward-rectifying current-voltage relationships have been observed in the spinal cord and hypothalamus (Rajendra et al., 1997; Levi et al., 1999), while outward rectification was observed in the hippocampus and spinal cord (Rajendra et al., 1997).

1.13. DESENSITIZATION
Ligand-gated channels open in response to the binding of neurotransmitter but can also close or 'desensitize' while the agonist is still bound (Jones & Westbrook, 1996) (Figure 4). Desensitization has been traditionally viewed as a negative-feedback mechanism to prevent the undesirable consequences of excess receptor activation such as excitotoxicity (Jones & Westbrook, 1996).

The desensitization properties of ligand-gated ion channels are still not clearly understood. Various studies of the glycine receptor have reported that desensitization follows a biphasic time course, with higher glycine concentrations causing an increase in the rate of current decay (Rajendra et al., 1997). Reports indicate a fast $\tau$ of 0.7 – 0.9 sec and a slow desensitizing component ($\tau_{\text{slow}} = 2.7 – 4.4$ sec) in response to applications of 100 $\mu$M glycine in acutely isolated hypothalamic neurons (Akaike & Kaneda, 1989; Agopyan et al., 1993). Also, the decay constants increased with depolarization and decreased with hyperpolarization (Faber & Korn, 1987; Akaike & Kaneda, 1989), indicating that the rate of desensitization is considerably voltage dependent. The voltage kinetics of the GlyR responses may serve to enhance the magnitude and duration of inhibitory responses appreciably in the event of increased excitation. GlyRs fully recover from desensitization within 60 sec (Akaike & Kaneda, 1989).
Figure 4

Desensitization and Deactivation of Glycine-Evoked Currents

A glycine-evoked response recorded from α₁/β GlyRs expressed in HEK 293 cells is shown. The bar on top of the current represents the duration of drug application. Desensitization refers to the process whereby receptors are inactivated upon prolonged or repeated exposure to an agonist. The extent of receptor desensitization can be estimated from the steady state to peak current ratio (Iₘₜ/Iₚ). After agonist application, the glycine-evoked current returns to a baseline current. This process is termed deactivation. The factors contributing to deactivation are 1) dissociation of the agonist from the receptor binding site(s), 2) receptor recovery from desensitization, and 3) wash-out of the agonist.
Extent of receptor desensitization is calculated from:
\[
\frac{\text{steady state current} \ (I_{ss})}{\text{peak current} \ (I_p)}
\]
To explain the complex gating properties of the GlyR, the previous kinetic model has been proposed (Legendre, 1998). The model presented above possesses two sequential agonist binding steps (A2 + C to A + AC to A2C), and a doubly-ligated closed state, A2C providing access to another closed state, A2C*. These two closed states provide access to two doubly-ligated independent open states, O1 and O2. The rates of channel opening and closing are $\beta_1$, $\beta_2$ and $\alpha_1$, $\alpha_2$, respectively. The rates of association and dissociation of agonist molecules are $2k_{on}$, $k_{on}$ and $k_{off}$, $2k_{off}$, respectively. The parameters d and r represent the switching rates between A2C and A2C*. A more rudimentary model previously suggested by Akaike & Kaneda (1989) is in agreement with the model presented above (Legendre, 1998).

The model possesses two sequential binding steps (yielding A + AC and A2C) as determined by analysis of the onset of outside-out glycine-evoked currents. Two open states (O1 and O2) are incorporated, because two mean open times were detected during single-channel recordings. For preparations where desensitization has been
observed, this model should be modified to include entry into, and recovery from a nonconducting desensitized state. In such a model, desensitization could proceed from both the closed and open states of the GlyR, as proposed for the GABA<sub>A</sub>R (Bai et al., 1999). However, no such model has been proposed to date.

1.15. PHARMACOLOGICAL PROPERTIES OF THE GLYCINE RECEPTOR

Strychnine acts as a highly selective, extremely potent, competitive antagonist of glycine, β-alanine and taurine in various preparations (Bechade et al., 1994; Rajendra et al., 1997; Vannier & Triller, 1997). In cells transfected with only the human GlyR β subunit cDNA, no specific binding of [<sup>3</sup>H] strychnine was detected (Handford et al., 1996). In contrast, specific glycine-displaceable [<sup>3</sup>H] strychnine binding was observed in cells transfected with either α or α/β cDNAs and co-expression of α and β subunits caused no significant change in the binding affinities for strychnine or glycine when compared to homomeric α GlyRs (Handford et al., 1996). Hence, the binding site for strychnine is localized on the α subunit of the GlyR complex.

The concentrations of strychnine that produce 50% of the maximal inhibition (IC<sub>50</sub>) range from ~20 – 35 nM in the spinal cord (Pfeiffer & Betz, 1981), hypothalamus (Abe et al., 1994) and hippocampus (Shirasaki et al., 1991). For α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>,α<sub>1</sub>/β, α<sub>2</sub>/β and α<sub>3</sub>/β GlyRs transfected in HEK 293 cells, the IC<sub>50</sub> values of strychnine ranged from 110-200 nM (Pribilla et al., 1992) and was as low as 20 nM for the α<sub>1</sub> subunit in another report (Sontheimer et al., 1989). The IC<sub>50</sub> values of strychnine for α<sub>1</sub> and α<sub>2</sub> GlyRs expressed in Xenopus oocytes were 37 and 50 nM, respectively for glycine-activated responses, 30 and 145 nM, respectively, for β-alanine-activated responses, and 47 and 1200,
respectively, for taurine-activated responses (Schmieden et al., 1992). The neonatal GlyR isoform, α2 has low strychnine binding affinity (Benavides et al., 1981; Becker et al., 1988). α2*, the allelic variant of α2, also generates Cl− channels of low strychnine sensitivity (no inhibition of glycine currents was observed at strychnine concentrations below 1 μM) upon expression in Xenopus oocytes (Kuhse et al., 1990a). Strychnine (100 nM) inhibited the α3 GlyR-mediated glycine response by > 80 % in Xenopus oocytes (Kuhse et al., 1990b). For α4-homomeric GlyRs expressed in Xenopus oocytes, the IC50 of strychnine was 30 nM (Harvey et al., 2000). Hence, IC50 values vary with experimental preparation, subunit expression and agonist used in each study.

Picrotoxin, a plant alkaloid that contains an equimolar mixture of picrotin and picrotoxinin is generally accepted as a non-competitive antagonist of GABAARs (Pribilla et al., 1992), although the precise mechanism of its blocking action is unclear. An open channel blocking mechanism has been proposed for GABAARs, based on the following findings: 1) both the onset of and the recovery from picrotoxinin inhibition are accelerated by GABA, i.e. a clear use dependency of the block is seen; and 2) in sensory neurons, picrotoxinin also inhibits GABA responses when applied intracellularly (Pribilla et al., 1992). Newland & Cull-Candy (1992) have suggested a mechanism whereby picrotoxin preferentially binds to an agonist bound form of the GABAAR and stabilizes an agonist-bound shut state, suggesting that picrotoxin enhances the occurrence of a desensitized state or an allosterically blocked state.

For glycine-induced currents, reports as to picrotoxin’s binding action are controversial. In adult spinal cord, no inhibition is seen, but picrotoxinin blocks glycine-evoked responses of brainstem neurons, hypothalamic neurons and motoneurons
(Pribilla et al., 1992). In the hippocampus, where two types of glycine channels have been distinguished by a difference in conductances, picrotoxinin had differential effects on the two types of channels. Single-channel studies have shown that at low concentrations, picrotoxinin (20 μM) selectively blocked large conductance channels (100 pS), while at high picrotoxinin concentrations (0.5 – 1 mM), the small conductance (47 pS) glycine channels were inhibited by a ‘flickering’ blockade (Yoon et al., 1998).

The effect of picrotoxinin at 20 μM on the glycine concentration response curve suggested that picrotoxinin is a competitive inhibitor because the amount of block decreased with agonist concentration with no decrease in the maximal currents observed. At 500 μM, the picrotoxinin effect was almost totally non-competitive since the maximum glycine response was significantly attenuated at such high concentrations (Yoon et al., 1998). Hence, the picrotoxinin effect seems to be competitive for the large conductance channels (probably α homomers) while its effect on the small conductance channels (probably α/β heteromers) is non-competitive.

Various reports show that picrotoxin efficiently antagonizes activation of recombinant homo-oligomeric α subunit GlyRs, being 17- to 200-fold more potent for homomeric α₁-subunit GlyRs than for heteromeric α₁/β GlyRs (Pribilla et al., 1992; Handford et al., 1996). Site-directed mutagenesis identified residues 278-295 within the GlyR β subunit TM2 as major determinants of picrotoxin resistance (Pribilla et al., 1992). These results suggest that the contradictory information regarding picrotoxinin inhibition of GlyR channels may reflect developmental and/or regional differences in the occurrence of homomeric and heteromeric GlyRs. Interestingly, this difference in picrotoxin sensitivity
between α and α/β glycine channels is the most effective means of pharmacologically
discriminating between homomeric α and heteromeric α/β GlyRs, to date.

The Cl⁻ channel of the recombinant α₁ homo-oligomeric GlyR is efficiently blocked by
CTB with an IC₅₀ of 1.3 μM in the presence of 50 μM glycine (Rundstrom et al., 1994).
The antagonistic effect of CTB is non-competitive, use-dependent, and more
pronounced at positive membrane potentials, suggesting open-channel block
(Rundstrom et al., 1994). In contrast, the GlyR α₂ homo-oligomers are resistant to CTB
with an IC₅₀ of >>20 μM (Rundstrom et al., 1994). Thus CTB may prove useful as a tool
to elucidate the subunit structure of native neuronal GlyRs. This is of importance since
the expression of different α GlyR subunits is developmentally and spatially regulated in
the mammalian CNS (Becker et al., 1988; Malosio et al., 1991b).

1.16. MODULATORS

Glycine responses were enhanced by Zn²⁺ concentrations of 20 nM to 10 μM, while
higher concentrations caused a reversal of the potentiation, followed by a progressive
inhibition of the glycine response when > 20 μM Zn²⁺ was applied to spinal cord neurons
(Bloomenthal et al., 1994; Laube et al., 1995). Specifically, as much as a 3-fold
increase and an 80% decrease in the amplitude of glycine-evoked responses was
observed upon extracellular application of Zn²⁺ (Bloomenthal et al., 1994; Laube et al.,
1995). Neither the enhancement nor the inhibition by this metal ion was associated with
any changes in the reversal potential of the glycine-induced currents (Bloomenthal et
al., 1994). The biphasic modulation by Zn²⁺ appeared essentially identical in native
spinal cord neurons and recombinant α/β GlyRs (Bloomenthal et al., 1994). Analysis of
chimeric constructs of the GlyR α₁ and β subunits revealed that the positive and
negative modulatory effects of Zn\(^{2+}\) are mediated by different regions of the \(\alpha_1\) subunit (Laube et al., 1995). Low concentrations of Zn\(^{2+}\), which potentiated GlyR responses, and high concentrations of Zn\(^{2+}\), which inhibited glycine receptor responses, reduced and increased the \(EC_{50}\) values for glycine, respectively, indicating the existence of distinct high- and low-affinity binding sites for Zn\(^{2+}\) on the GlyR \(\alpha_1\) subunit (Laube et al., 1995). At both potentiating and inhibitory concentrations of Zn\(^{2+}\), the slopes of the dose-response curves were not different from that obtained in the absence of the metal ion, suggesting that the number of agonist molecules required for channel gating was identical in both the presence and absence of Zn\(^{2+}\) (Laube et al., 1995). These results suggest that Zn\(^{2+}\) may act as both an allosteric activator and inhibitor of glycine binding and/or channel gating (Bloomenthal et al., 1994; Laube et al., 1995). In view of the low concentrations of Zn\(^{2+}\) required for potentiation of glycine currents, it is possible that vesicular release of Zn\(^{2+}\) from nerve terminals may be implicated in the regulation of inhibitory pathways (Laube et al., 1995).

1.17. TIME-DEPENDENT DECREASE IN RESPONSIVENESS TO THE AGONIST

During whole-cell voltage clamp, due to the large diameter (1 \(\mu\)M) of the patch electrode, a low resistance pathway into the cell’s interior is established (~ 2-10 M\(\Omega\)). However, this large diameter of the pipette tip also allows for the rapid diffusion of small molecules from the cell interior to the relatively large volume of the pipette solution and vice versa. Due to this exchange, some co-factors or second messengers might be lost from the interior of the cell, a process that is sometimes referred to as ‘dialysis’. Thus, if channels are regulated intracellularly, their activity may decrease as various cytoplasmic factors are lost from the cell’s interior to the solution of the electrode.
Consequently, the repeated application of neurotransmitters such as glycine may cause currents to exhibit a time-dependent decrease in responsiveness, termed 'wash-out' or 'rundown'. Low intracellular [ATP] and/or high intracellular [Ca\textsuperscript{2+}] are involved in the rundown of several voltage- and ligand-gated ion channels (Behrends et al., 1988; Belles et al., 1988; Gyenes et al., 1988; Ohno-Shosaku et al., 1989; Chen et al., 1990; Rosenmund & Westbrook, 1993; Wang et al., 1993; Huang & Dillon, 1998).

For NMDA receptors, an increase in intracellular Ca\textsuperscript{2+} leads to channel rundown during whole-cell recording in hippocampal neurons by reducing the open probability of the NMDA channel (Rosenmund & Westbrook, 1993). The rate and reversibility of rundown is use-dependent since no rundown is observed with infrequent applications of NMDA (Rosenmund & Westbrook, 1993).

For GABA-activated Cl\textsuperscript{-} currents, rundown was prevented and induced in the presence of tyrosine phosphatase inhibitors and tyrosine kinase inhibitors, respectively, in cerebellar granule cells and recombinant GABA\textsubscript{A}Rs (Amico et al., 1998; Huang & Dillon, 1998). The rundown was the result of a reduction in the open probability of GABA\textsubscript{A} channels in cerebellar granule cells (Amico et al., 1998). These results suggest that tyrosine phosphorylation is important in maintaining the functional state of the GABA\textsubscript{A}R by preventing rundown. In addition, inhibition of the Ca\textsuperscript{2+}/calmodulin-dependent phosphatase, calcineurin also prevented rundown of the response to GABA (Huang & Dillon, 1998). Hence, protein phosphorylation of a site that is dephosphorylated by calcineurin, maintains the function of GABA\textsubscript{A}Rs (Huang & Dillon, 1998). Also, rundown of the GABA-evoked response was reduced when ATP was present, suggesting that an ATP-dependent process, possibly phosphorylation, is
involved in the maintenance of GABA\textsubscript{A}R function in spinal cord neurons (Gyenes et al., 1988). Hence, a number of processes causing cellular-, membrane- or receptor-dependent changes can contribute to the phenomenon of rundown.

1.18. Gephyrin

The synaptic localization of neurotransmitter-gated receptors and their aggregation to high receptor densities are prerequisites for efficient neurotransmission. For ligand-gated receptors, receptor-associated proteins are thought to play an important role in the mechanism of anchoring and modulating the function of receptors at postsynaptic sites through their interaction with structural elements beneath the plasma membrane, such as components of postsynaptic densities (Froehner, 1991; Kirsch et al., 1996; Kins et al., 2000).

Affinity chromatography has revealed that the GlyR co-purifies with a peripheral membrane protein of 93 kDa that has been named gephyrin (Bechade et al., 1994; Rajendra et al., 1997; Vannier & Triller, 1997). Immunoelectron microscopy has shown that gephyrin decorates the cytoplasmic face of the glycinergic postsynaptic membranes (Triller et al., 1985). Gephyrin is widely expressed throughout the CNS in the spinal cord, cortex and cerebellum as determined by immunohistochemical methods (Kirsch & Betz, 1993) and in situ hybridization (Kirsch et al., 1993a). Northern blot analysis has revealed the existence of at least five different gephyrin mRNAs, which are generated by alternative splicing (Prior et al., 1992), suggesting the possible existence of tissue-specific gephyrin isoforms. Gephyrin binds to the \(\beta\) subunit of the GlyR through the cytoplasmic loop between TM3 and TM4 of the \(\beta\) subunit (Meyer et al., 1995) and it also binds to polymerized tubulin with high affinity (Kirsch et al., 1991). In addition, the
localization of gephyrin coincides with that of the GlyR β subunit transcripts (Malosio et al., 1991b; Kirsch et al., 1993a). Thus, it has been proposed that gephyrin acts as a bridge between the GlyR and the cytoskeleton, and is thought to anchor the GlyR at postsynaptic sites via binding to subsynaptic tubulin (Kirsch & Betz, 1995; Meyer et al., 1995).

Gephyrin also regulates the degree of clustering and localization of GlyRs at the postsynaptic membrane. Treatment of spinal cord neurons with gephyrin antisense oligonucleotides prevents the formation of GlyR clusters in the plasma membrane, while oligonucleotide withdrawal results in the formation of GlyR clusters (Kirsch et al., 1993b). Also, gephyrin aggregates are detected earlier than GlyR clusters, indicating that the assembly of gephyrin-rich domains in the plasma membrane may precede the formation of glycinergic membrane specializations (Kirsch et al., 1993b). In another report (Kirsch & Betz, 1995), depolymerization of microtubules reduced the number of GlyR clusters per cell as well as the density of the remaining clusters. Depolymerization of microfilaments generated smaller clusters of increased gephyrin density as compared to those observed under normal conditions, suggesting that interaction of gephyrin with microtubules or microfilaments may serve opposite functions. Recent evidence suggests a model in which GlyR activation that results in Ca\(^{2+}\) influx is required for the clustering of gephyrin and GlyRs at developing postsynaptic sites (Kirsch & Betz, 1998).

Co-expression of α2 GlyR subunits with gephyrin in HEK 293 cells reduced the sensitivity of the homomeric GlyR to strychnine inhibition, with concentration response curves revealing IC\(_{50}\) values for strychnine of 140 nM for α2 and 1.8 μM for co-transfected cells (Takagi et al., 1992). In addition, co-expression with gephyrin
produced an increase in glycine affinity, whereby the concentration of glycine required to elicit a half-maximal response decreased from ~ 80 to 20 μM (Takagi et al., 1992). Thus, gephyrin alters the agonist affinity of α2 GlyRs, presumably by binding to and inducing the clustering of the receptors, which are otherwise diffusely distributed in the plasma membrane (Takagi et al., 1992). Hence, the dense packing of GlyRs in the postsynaptic membrane may have functional consequences on the neurotransmitter-induced response. This modulation of the α2 subunit properties by gephyrin was not affected by the presence of the β subunit and was not observed with α3 GlyRs (Takagi et al., 1992), suggesting that different isoforms of gephyrin generated by alternative splicing might be differentially associated with the various GlyR isoforms.

Upon incubation of extracts from spinal cord membranes containing the GlyR α and β subunits as well as gephyrin with [γ-32P]ATP, radioactivity was incorporated in the 93 kDa band, indicating that a kinase activity endogenous to purified GlyR preparations phosphorylates gephyrin (Langosch et al., 1992). Phosphoamino acid analysis of phosphorylated gephyrin revealed that phosphorylation occurred mainly on serine and to a lesser extent, on threonine residues (Langosch et al., 1992), indicating that the endogenous protein kinase belongs to the serine/threonine class of kinases, such as PKA or PKC. Hence, phosphorylation of gephyrin might control its binding to tubulin and thus affect the proposed function of gephyrin as a linker between the GlyR and the cytoskeleton.

A novel gephyrin-binding protein, collybistin II was recently cloned. Collybistin II induced the formation of submembrane gephyrin clusters and recruited heteromeric GlyRs to the plasma membrane (Kins et al., 2000). Thus, collybistin II is speculated to
regulate the degree of GlyR clustering at the plasma membrane through its involvement in gephyrin clustering. In summary, previous studies suggest that GlyR function can be regulated by several mechanisms including 1) modulation by kinases such as PKA (Song & Huang, 1990; Agopyan et al., 1993; Vaello et al., 1994; Ren et al., 1998) and PKC (Ruiz-Gomez et al., 1991; Vaello et al., 1994; Schonrock & Bormann, 1995) and 2) modulation by collybistin (Kins et al., 2000) or the serine/threonine kinase that phosphorylates gephyrin (Langosch et al., 1992).

1.19. RECOMBINANT RECEPTORS

Heterologous systems have been widely used to investigate the modulation of ligand-gated ion channels by protein kinases. The human embryonic kidney cell line (HEK 293) is commonly used for the expression of cloned channels, in part because this cell line expresses few endogenous channels. Hence, use of this cell line eliminates possible interactions among receptors/channels that may exist in neuronal preparations.

In terms of the GlyR, no response to glycine was detected in non-transfected HEK 293 cells (Sontheimer et al., 1989). Also, no endogenous $\alpha_1$ subunit was detected by using an antibody that recognizes the $\alpha_1$ GlyR subunit (Sontheimer et al., 1989). However, glycine currents were observed in HEK 293 cells transfected with the GlyR $\alpha_1$ subunit (Sontheimer et al., 1989; Bormann et al., 1993; Handford et al., 1996). These homomeric-$\alpha_1$ GlyR responses were sensitive to the antagonistic alkaloids strychnine (Sontheimer et al., 1989) and picrotoxin (Handford et al., 1996). Hence, the functional GlyR channels detected in the transfected cells possess agonist and antagonist binding properties similar to those characterizing native GlyRs. It is interesting to note that
although no gephyrin band was detected in a cytosolic extract of untransfected HEK 293 cells (Meyer et al., 1995), endogenous gephyrin was detected by David-Watine et al. (1999) in HEK 293 cells.

Recombinant receptors expressed in HEK 293 cells have been previously used to discover the target receptor subunits of PTKs (Moss et al., 1995). The most effective approach to determine the targets of PTKs is to express homomeric and heteromeric channels in HEK 293 cells, and then examine the functional differences of these channels using electrophysiological methods. Site-directed mutagenesis is an additional tool that can be used to delete various putative phosphorylation sites on the receptor under investigation, and identify the amino acid residues involved in the process of receptor phosphorylation by PTKs such as pp60<sup>c-src</sup> (Moss et al., 1995). However, caution must be used in interpreting data from such studies because substitution of even a single amino acid might cause profound changes in the three dimensional conformation of the receptor subunit, leading to altered channel function that is not the result of phosphorylation-induced changes.

1.20. SITE-DIRECTED MUTAGENESIS

Recombinant DNA technology enables us to engineer a known change in a gene and then observe the phenotypic result. One of the most interesting and most powerful of these techniques involves the insertion of an altered nucleotide(s) into a gene. Thus, it is possible to arrange matters so that the gene codes for a protein with one or more unusual amino acids at specific points in its primary sequence. This technique was originally engineered by Smith (1985) and is called 'site-directed mutagenesis'.
In order to achieve this result, the base sequence of the amplified DNA molecule must first be determined. It is then possible to synthesize an oligonucleotide of approximately 10-15 bases that is complementary to the region of interest, but with one or a few mismatches. The plasmid is then denatured and the primers containing the desired mutation are annealed. Using the nonstrand-displacing action of *Pfu Turbo* DNA polymerase under the correct hybridization conditions, complementary base pairing takes place, and the primers are incorporated and extended. The methylated, nonmutated parental DNA template is digested with the endonuclease DpnI, and the dsDNA is transformed into *E.coli*, where it replicates and gives rise to mutant DNA. The DNA is usually sequenced in order to make sure that it contains the desired mutation. The mutant is then amplified by cloning and introduced into an expression vector in order to examine the function of the slightly altered protein.
2. OBJECTIVES AND WORKING HYPOTHESIS

2.1. SECTION 1

Previous studies suggest that the function of the GlyR is influenced by PKA- and PKC-induced phosphorylation. However, the effect of tyrosine phosphorylation on GlyR function has not yet been examined. Here, we test the hypothesis that tyrosine phosphorylation modulates the function of GlyRs. We will determine the effects of various modulators of tyrosine phosphorylation on GlyR function.

In Section 1, we will address the following questions:

1. Does inhibition of tyrosine phosphorylation by lavendustin A decrease the function of the GlyR in CA1 hippocampal and spinal cord neurons?
2. Does tyrosine phosphorylation by pp60^c-src enhance glycine-evoked responses in CA1 hippocampal neurons?

2.2. SECTION 2

The β subunit of the GlyR contains a putative tyrosine phosphorylation site at Tyr-413. However, the function of this site is not well defined. Our hypothesis is that the β subunit of the GlyR, and specifically Tyr-413, is required for the modulation of GlyR function by tyrosine phosphorylation.

In Section 2, we will address the following questions:

1. Is the function of α1-homomeric GlyRs affected by tyrosine phosphorylation by pp60^c-src?
2. Is the function of α1/β-heteromeric GlyRs affected by tyrosine phosphorylation by pp60^c-src?
3. Does the β subunit affect the kinetics of desensitization of glycine-evoked currents?

4. Does mutation of Tyr-413 of the GlyR β subunit affect the function of α1/β-heteromeric GlyRs?
3. METHODS

3.1. PREPARATION OF ACUTELY ISOLATED CA1 HIPPOCAMPAL NEURONS

Acutely isolated hippocampal neurons were prepared using previously described procedures (Wang & MacDonald, 1995). Briefly, postnatal Wistar rats (10-21 day old) were anaesthetized with halothane and then sacrificed by decapitation, using a guillotine. The entire brain was rapidly removed and placed in cold extracellular solution (composition described below). Tetrodotoxin (TTX, 0.3 μM) was added to the extracellular solution in order to block the voltage-activated sodium channels in the brain and thus reduce the damage caused by excitotoxicity upon the insult imposed by the dissection. Both hippocampi were then microdissected and manually cut into 400-500 μm thick slices using a razor blade. The slices were incubated in oxygen-bubbled extracellular solution (TTX included) at room temperature (20-25 °C) and subjected to papain (derived from papaya latex, Sigma Chemical Co., St. Louis, MO) digestion (5 mg/ml) for 30 min. Following papain digestion, slices were rinsed and kept in enzyme-free extracellular solution (0.3 μM TTX included) until used. To obtain CA1 acutely isolated neurons, a single slice was transferred into a plastic 35 mm tissue culture dish containing the incubation solution described above, the CA1 hippocampal region was isolated and CA1 neurons were obtained using mechanical trituration. Electrophysiological recordings using the acutely isolated neurons began approximately 15 minutes after this mechanical trituration so as to allow neurons to settle at the bottom of the dish. Only neurons which retained their pyramidal shape were used for recording.
3.2. SPINAL CORD CELL CULTURES

Cultures of spinal cord neurons (12-16 days postnatally) were prepared from Swiss white mice as previously described (MacDonald et al., 1989). Briefly, fetal pups (15 days in utero) were removed from mice sacrificed by cervical dislocation. The spinal cord was dissected from each fetus and was subjected to trypsin digestion for 30 min. It was then dissociated by mechanical trituration using two Pasteur pipettes (tip diameter of 150 - 200 μm) and plated at a density of $1 \times 10^6$ cells/ml on 35-mm culture dishes. The culture dishes had been coated with collagen from calf skin to aid neurons in adhering to the bottom of the dish (Sigma Chemical Co., St. Louis, MO). For the first 4 - 7 days in vitro, cells were maintained in Minimal Essential Media (MEM) (Life Technologies, Grand Island, NY) supplemented with glucose (final concentration 33.6 mM), NaHCO₃ (final concentration 31.56 mM), 10% horse serum, 10% fetal bovine serum, and 1% insulin (Life Technologies, Grand Island, NY). The neurons were cultured at 37 °C in a 5% CO₂ / 95% air environment. Once the background cells had grown to confluence (4 to 7 days), 0.1 mL of FUDR (fluorodeoxy uridine) mixture (4 mg 5-fluorodeoxyuridine and 10 mg uridine in 20 mL MEM) were added to each dish to arrest mitotic division of glial cells. The supplemented media was changed every 3 - 4 days. After 7 days in culture, the media was changed to a new media containing MEM supplemented with 10% horse serum, 1% insulin, 31.56 mM NaHCO₃ and glucose. There was a heterogeneous population in culture as there were neurons from all the regions of the spinal cord.

3.3. HUMAN EMBRYONIC KIDNEY 293 (HEK 293) CELL TRANSFECTIONS
HEK-293 cells (American Type Culture Collection, CRL, Rockville, MD, USA) were grown and maintained in culture media, as previously described (Valenzuela et al., 1998; Wick et al., 1999). HEK 293 cells were maintained in MEM media supplemented with 10% fetal bovine serum and MEM with Earle’s salts and L-glutamine (Gibco BRL), and were kept at 37 °C in a humidified atmosphere containing 5% CO₂. Culture dishes were treated with a 35 µg/ml fibronectin (Boehringer Mannheim GmbH, Germany) solution 45 minutes before plating and cells were plated to a density of ~10⁶/35 mm plastic culture dish. These cells were transiently transfected with the wild-type human α₁ or α₁ plus β (1:1) cDNAs, using the lipid method (Invitrogen). Briefly, HEK 293 cells were incubated with the PerFect Lipid, pFx-2 (Invitrogen, Carlsbad, CA) and α or α plus β subunits in OPTI-MEM Reduced Serum medium (Gibco BRL) for four hours at 37 °C with 5% CO₂. Following the four-hour incubation period, cells were transferred to the complete medium described above and the plates were returned to the 37 °C, 5% CO₂ incubator. Each of the cDNAs was subcloned in a modified version of the expression vector pBK-CMV (called NB-200). pBK-CMV was modified by removal of the lac promoter and the lacZ ATG (Wick et al., 1999), in order to increase CMV promoter activity for transient transfections. In all transfections, the amount of cDNA was kept constant (2 µg / 35 mm petri dish). Recordings were made from monolayer cultures of HEK 293 cells within 24-48 hours after transfection.

**Site-Directed Mutagenesis:**

To determine the effects of abolishing the putative tyrosine phosphorylation site on the β subunit, a substitution mutation of tyrosine-413 to phenylalanine-413 was constructed for the β subunit of the human GlyR (Y413F). The single letter code for
amino acids is used to describe mutations, with the letter preceding the position number referring to the amino acid in the wild-type and the letter following the number referring to the amino acid replacing the wild-type amino acid. Each of the cDNAs was subcloned in a modified version of pBK-CMV (called NB-200), as explained above. HEK 293 cells were transiently transfected with the GlyR wild type α₁ and β mutant (Y413F), using the lipid method previously described. The Y413F point mutation was made using the protocol supplied in the Stratagene QuikChange Site-Directed Mutagenesis kit (La Jolla, CA, USA). The mutation was verified by double-stranded DNA sequencing. In all transfections, the amount of cDNA was kept constant (2 µg / 35 mm petri dish). Recordings were made from monolayer cultures of HEK 293 cells within 24-48 hours after transfection.

3.4. RECORDING PIPETTES

Whole cell patch-pipettes were constructed from thin-walled borosilicate glass capillary tubing (World Precision Instruments, Sarasota, Florida). Electrodes were pulled using a two-stage vertical puller (Narishige Scientific Instruments Laboratory, Tokyo, Japan, model PP-83) in order to produce pipettes of approximately uniform tip diameter and shape. Patch pipettes had a final diameter of approximately 1 µm. The resistance of the electrodes once filled with recording solution was about 3 - 5 MΩ.

Pipettes were filled with pipette solution (see below) and were mounted and secured in a teflon holder which contained a suction port through which negative pressure was applied using a syringe in order to achieve seal formation. This teflon holder was electrically coupled with the solution in the pipette through a silver/silver chloride-coated silver wire (Ag/AgCl). The Ag/AgCl behaved as a reversible electrode, converting the
ionic Cl⁻ current in the pipette solution into electron current in the wire. The holder was connected to the headstage of the amplifier and a ground silver electrode was placed in the bath solution. The junction potential was nullified before seal formation was achieved.

3.5. PIPETTE SOLUTIONS

For recordings with spinal cord neurons, patch pipettes were filled with a solution containing (in mM) 140 CsCl, 10 HEPES, 11 EGTA, 10 tetraethylammonium chloride (TEA-Cl), 2 MgCl₂, 1 CaCl₂, and 4 MgATP (pH 7.3, 290-300 mOsm). For recordings with hippocampal neurons and HEK 293 cells, the following changes were made: the pipette solution contained (in mM) 63 CsCl, 70 CsF and 3.4 KATP. This was done because previous investigators in our lab have observed that acutely isolated neurons die shortly after whole-cell configuration is achieved when only CsCl and no CsF is present in the pipette solution. Also, KATP was used because MgATP was insoluble in the intracellular solution containing both CsCl and CsF. The rest of the chemical concentrations were as described for the pipette solution prepared for recordings with spinal cord neurons. For experiments that involved the intracellular application of lavendustin A, lavendustin B or active and heat-inactivated pp60⁵⁺⁻src, these drugs were included in the pipette solution.

3.6. EXTRACELLULAR SOLUTION

The extracellular fluid (ECF) bathing the spinal cord and hippocampal neurons and HEK 293 cells contained (in mM) 140 NaCl, 1.3 CaCl₂, 5.4 KCl, 2 MgCl₂, 25 HEPES and 33 glucose, with a pH of 7.4. Tetrodotoxin (TTX) (0.3 μM) was included in the ECF
bathing the cultured spinal cord neurons. This was necessary because neurons in cultures usually form synapses and action potentials are observed in the recordings. Inclusion of TTX prevented the firing of any action potentials.

3.7. DRUGS AND OTHER CHEMICALS

3.7.1. GLYCINE

A stock solution of 100 mM glycine was prepared in distilled water. Stocks of glycine were prepared every month and stored at 4 °C. Aliquots of this stock solution were diluted to the desired concentration in the appropriate extracellular solution just prior to use. Glycine was applied at 2-minute intervals in order to ensure complete recovery of GlyRs from desensitization (Akaike & Kaneda, 1989).

3.7.2. LAVENDUSTIN B and LAVENDUSTIN A

The chemical names of lavendustin B and lavendustin A are 5-Amino-(N,N'-bis-2-hydroxybenzyl)salicylic acid and 5-Amino-[(N-2,5-dihydroxybenzyl)-N'-2-hydroxybenzyl]salicylic acid, respectively. Lavendustin A and lavendustin B were purchased from Calbiochem (La Jolla, California), and stock solutions of 10 mM were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C.

Lavendustin A has been shown to inhibit the epidermal growth factor (EGF) receptor-associated tyrosine kinase, but it does not inhibit PKA or PKC (Onoda et al., 1989). Also, O'Dell et al. (1991) has reported that lavendustin A is at least 200-fold less effective at inhibiting PKA, PKC and CaMKII (IC₅₀ > 100 μM) than pp60⁵⁺⁻src (IC₅₀ = 0.5 μM). Lavendustin A has been used as a protein tyrosine kinase inhibitor in various studies involving ligand-gated ion channels (Huang et al., 1999; Lu et al., 1999).
Although, other tyrosine kinase inhibitors and their assumed inactive analogues such as genistein and daidzein, and tyrphostin A25 and A1 have been reported to have direct effects on ion channels (Dunne et al., 1998), the effects of lavendustin A on GABA\(_A\)R function have been reported to be due exclusively to inhibition of PTKs (Huang et al., 1999).

Lavendustin B has been accepted as the negative control of lavendustin A since it fails to exert the effects of lavendustin A on vascular endothelial growth factor (VEGF) (Hu & Fan, 1995) and is inactive as to inhibition of NMDA currents compared to lavendustin A (Wang & Salter, 1994).

3.7.3. PP60\(^{\text{C-SRC}}\)

The biologically active native enzyme, pp60\(^{\text{C-src}}\) was purchased from Upstate Biotechnology (Lake Placid, New York). Pp60\(^{\text{C-src}}\) was expressed in Sf9 insect cells by recombinant baculovirus containing the human c-src gene. A unit of pp60\(^{\text{C-src}}\) is defined as 1 picomole of phosphate transferred to the src substrate peptide per minute. A sample of the enzyme was run on SDS-PAGE gel and stained by Coomassi or silver stain. A high purity would indicate little or no other bands other than that of the purified enzyme. According to the certificate of analysis (Upstate Biotechnology), pp60\(^{\text{C-src}}\) was essentially free of other protein kinase contamination. A Src kinase assay revealed Src kinase activity (Upstate Biotechnology).

The enzyme solution was aliquoted and stored in a \(-70\) °C freezer to avoid degradation of activity. Individual aliquots were thawed just prior to use and diluted to the desired concentration in the appropriate intracellular solution. As a negative control for experiments involving the use of the active form of pp60\(^{\text{C-src}}\), aliquots of pp60\(^{\text{C-src}}\)
were heated at 100 °C for one hour in order to inactivate the enzyme as was done in previous studies (Wang & Salter, 1994; Xiong et al., 1999).

3.7.4. PICROTOXIN

A picrotoxin stock solution of 50 mM was prepared in pure anhydrous ethanol. Stocks of picrotoxin were stored at 4 °C. Aliquots of this stock solution were diluted to the desired concentration in the appropriate extracellular solution just prior to use.

3.7.5. STRYCHNINE

A strychnine stock solution of 10 mM was prepared in distilled water and was stored at 4 °C. Aliquots of this stock solution were diluted to the desired concentration in the appropriate extracellular solution just prior to use.

3.8. WHOLE-CELL VOLTAGE-CLAMP RECORDINGS

The voltage-clamp technique has been successfully used by investigators for the last 40 years to study ion channels. The whole-cell voltage-clamp technique provides a means for controlling the membrane potential and allows for the direct measurement of ion flow across the cell membrane at a uniform membrane potential. Hence, it can provide information about receptor activity. Whole-cell recording measures a macroscopic current, which is the sum of many single channel openings and closings throughout the entire cell membrane in response to a stimulus such as the application of an agonist or a voltage step. In the voltage-clamp system, when a stimulus triggers a change in ionic permeability, a negative feedback amplifier with a high frequency response is used to compare the incoming signal received from the recording electrode to a fixed "command potential". The potential difference between the input signal and
the command potential is amplified and transmitted back to the recording electrode. As a result, the feedback amplifier is used to rapidly readjust the current continuously in order to oppose any change away from the holding potential. A single electrode voltage clamp such as the one used to perform our experiments uses a single electrode to both measure and inject current to maintain the membrane potential constant via a feedback circuit (Axopatch 200 or the Axopatch 1D amplifier, Axon Instruments Inc., Foster City, CA).

To record from a neuron or a cell, the recording pipette was filled with the appropriate pipette solution and secured into the pipette holder, which was in turn inserted into the headstage of the amplifier. The electrode was lowered into the ECF solution of the culture dish and positioned close to the cell, at a distance of approximately 700 μm in front of the control barrel. This was done using a coarse manipulator first and then a fine hydraulic micromanipulator. The junction potential between the bath and pipette solutions was nullified by adjusting the input offset control of the amplifier. A +20 mV voltage step was used to measure the electrode resistance, which was monitored on an oscilloscope. This voltage step was also used to monitor seal formation. Once the electrode touched the cell, the size of the signal from the test pulse was decreased, indicating that the seal resistance between the tip of the electrode and the cell membrane had increased. Then, gentle suction was applied and a high resistance seal was established. It is important that a high resistance seal is achieved because the higher the seal resistance, the less the current that can leak across the seal and the less the noise in the recordings due to this leakage current. Hence, seal resistance has a crucial bearing on the quality of current recording. Following the
formation of a high resistance seal, the neuron / cell was voltage-clamped at -60 mV, and the membrane patch under the pipette tip was disrupted by applying negative pressure and a brief current pulse. The electrode thus provided a low resistance pathway into the cell interior. Once a cell was patched, responses were recorded and analyzed on a computer using pClamp6 software (Axon Instruments Inc., Foster City, CA). The acutely isolated hippocampal neurons were lifted into the solution stream after whole-cell configuration was achieved in order for all the GlyRs, including those on the bottom surface of the patched neuron, to come in contact with the applied solution. A +10 mV voltage step was used to measure the series or access resistance during recording. Where appropriate, currents were allowed to stabilize for approximately 10 – 15 minutes before proceeding with the intended experiment. All experiments were carried out at room temperature (20-25 °C).

3.9. DRUG AND AGONIST PERFUSION SYSTEM

The glycine agonist and drugs used during our experiments were applied to the neurons and HEK 293 cells using a multi-barreled perfusion system (Figure 5). Three square-glass capillary tubes (Longreach Scientific Resources, Orr’s Island, Maine) were horizontally aligned, glued together by cyanoacyramide (Krazy Glue), and placed in a holder. The switching of the barrels was executed by a stepping motor, which was controlled either by an Atari 520ST or by pClamp6 on a personal computer. By laterally displacing the perfusion barrels, the solution bathing the cell at any particular time could be controlled. The barrels were connected to solution reservoirs through silastic tubing. Adjustment of the height of the reservoirs above the bath determined the flow rate of the solution (1 ml / min). Glycine-evoked currents were recorded every 2 min so as to allow
the neuron / cell to recover from desensitization after agonist application. Bath perfusion of the agonist or drug lasted from 5-20 sec at a time, thus obtaining the peak or both the peak and steady state glycine-evoked currents.
Figure 5

Multi-Barrel Perfusion System

Three square glass capillary tubes (400 x 400 μM) were aligned and glued together as shown. The barrels were connected by silastic tubing to separate solution reservoirs. Perfusion solutions were exchanged by laterally displacing the barrels a distance of one barrel diameter. Solutions perfused acutely isolated rat hippocampal neurons, murine spinal cord neurons or HEK 293 cells. Currents were recorded using the whole-cell voltage clamp technique.
3.10. WHOLE-CELL CURRENT ANALYSIS

The whole-cell patch-clamp data were analyzed using the ClampFit program of pClamp (Axon Instruments Inc., Foster City, CA).

In analyzing concentration response relationships, current amplitudes were normalized to the response recorded for the saturating agonist concentration and were plotted against the log of the agonist concentration. Responses were fitted using a standard logistic equation of the form: \( I = I_{\text{max}} / [1 + (d / EC_{50})^{n_H}] \) where \( I \) is the current amplitude and \( d \) is the agonist concentration. The \( EC_{50} \) is the agonist concentration that produced currents of 50% the amplitude of the maximal response and \( n_H \) is the estimated Hill coefficient, which is a measure of agonist cooperativity in binding to the receptor. Both the \( EC_{50} \) and \( n_H \) were determined from the logistic equation given above.

The extent of receptor desensitization was found by calculating the \( I_{ss}/I_p \) ratio, where \( I_{ss} \) refers to steady state current and \( I_p \) refers to peak current.

3.11. STATISTICAL ANALYSIS

All data are presented as mean ± S.E.M. (SigmaPlot, versions 4.0 and 5.0). Two-way analysis of variance (ANOVA) (GraphPad Prism, version 2.00) or Student's paired or unpaired t-tests (SigmaPlot, versions 4.0 and 5.0) were employed when appropriate. Briefly, the paired t-test was selected when sets of pairs were compared, while the unpaired t-test was selected when the values to be compared when not paired. Two-way ANOVA was used to determine differences between group means when two populations were compared. The level of accepted significance was \( p < 0.05 \), a value that has traditionally been accepted as the threshold value for determining statistical difference. A \( p \) value of 0.05 indicates that random sampling from identical populations
would lead to a difference larger than the one observed in only 5% of experiments and smaller than the one observed in 95% of experiments. With this p value, the degree of confidence is 95%, meaning that one can be 95% sure that the confidence interval includes the true difference and that the difference observed is a true difference.
4. RESULTS

4.1. SECTION 1

The GlyR is found in various regions of the brain, where it is involved in synaptic inhibitory neurotransmission. Although its structure has been well characterized, studies investigating the phosphorylation of the GlyR by various kinases are limited. The purpose of these experiments was to determine if GlyRs found in CA1 hippocampal and spinal cord neurons are modulated by tyrosine phosphorylation.

4.1.1. CONCENTRATION-RESPONSE RELATIONS FOR GLYCINE-EVOKED PEAK AND STEADY STATE CURRENTS IN CA1 HIPPOCAMPAL NEURONS

To characterize glycine-evoked currents in CA1 hippocampal neurons, we examined the concentration-response relations for peak and steady state currents (Figure 6). The threshold concentration for glycine that activated currents was approximately 3 μM whereas maximal currents were observed upon application of 1 mM glycine. Glycine currents were normalized to the response evoked by a saturating concentration of glycine (1 mM) and were plotted against the log of the glycine concentration. The EC\textsubscript{50} value for peak currents was 138 ± 20 μM and the Hill coefficient was 1.6 ± 0.1 (n = 6, Figure 6B). For the steady state currents, the EC\textsubscript{50} value was 43 ± 8 μM and the Hill coefficient was 1.3 ± 0.1 (n = 4, Figure 6B). The maximal peak current evoked in each cell by 1 mM glycine ranged from ~ 0.5 to ~ 2.5 nA (n = 6). These glycine-evoked currents were not affected by bicuculline (10 μM), a GABA\textsubscript{A}R antagonist, suggesting that they were not mediated by the GABA\textsubscript{A}R. However, currents were depressed by strychnine (1 μM), a competitive antagonist of the GlyR, suggesting that the recorded currents were mediated by GlyRs.
Figure 6

Concentration-Response Relations for Peak and Steady State Glycine-Evoked Currents in CA1 Hippocampal Neurons

A. Responses evoked by 10 μM, 100 μM, and 1 mM glycine in hippocampal neurons are shown. Responses peaked and then desensitized to a steady state. The peak and steady state current amplitudes increased with applications of increasing concentrations of glycine, until maximal current amplitude was reached at the saturating glycine concentration of 1 mM.

B. The concentration response curves for the peak (circles) and steady state (squares) glycine-evoked currents in hippocampal neurons are shown. The EC₅₀ value for peak currents was $138 \pm 20$ μM and the Hill coefficient was $1.6 \pm 0.1$ (n = 6). For the steady state currents, the EC₅₀ value was $43 \pm 8$ μM and the Hill coefficient was $1.3 \pm 0.1$ (n = 4).
Concentration-Response Relations of Glycine-Evoked Currents in CA1 Hippocampal Neurons

A

10 µM glycine

100 µM glycine

1 mM glycine

B

Concentration-Response Relations of Glycine-Evoked Currents in CA1 Hippocampal Neurons

$\frac{I}{I_{\text{max}}}$ vs. log [glycine] (µM)

- Peak
- Steady state
4.1.2. CONCENTRATION-RESPONSE RELATIONS FOR GLYCINE-EVOKED PEAK AND STEADY STATE CURRENTS IN SPINAL CORD NEURONS

To characterize glycine-evoked currents in spinal cord neurons, we examined the concentration-response relations for peak and steady state currents (Figure 7). Similar to results obtained from hippocampal neurons, the threshold concentration for glycine that activated currents was approximately 3 μM whereas maximal currents were observed upon application of 1 mM glycine. Glycine-evoked currents were normalized to the saturating glycine concentration of 1 mM and were plotted against the log of the glycine concentration applied in the bath. The EC₅₀ value for peak currents was 42 ± 6 μM and the Hill coefficient was 2.0 ± 0.4 (n = 8, Figure 7B). For the steady state, the EC₅₀ value was 18 ± 4 μM and the Hill coefficient was 2.5 ± 0.5 (n = 6, Figure 7B). The maximal current evoked in each cell upon glycine application of 1 mM ranged from ~1.7 to 6.6 nA (n = 8).

The concentration that evoked half the maximal response was higher for peak glycine-evoked currents in CA1 hippocampal neurons (138 ± 20 μM, n = 6) compared to spinal cord neurons (42 ± 6 μM, n = 8) (p < 0.05, unpaired t-test). Similarly, the EC₅₀ value for glycine was higher for steady state glycine-activated currents in CA1 hippocampal neurons (43 ± 8 μM, n = 4) compared to spinal cord neurons (18 ± 4 μM, n = 6) (p < 0.05, unpaired t-test). No difference was observed between the Hill coefficients of peak and steady state glycine-evoked currents in hippocampal and spinal cord neurons (p > 0.05, unpaired t-test).
Figure 7

Concentration-Response Relations for Peak and Steady State Glycine-Evoked Currents in Spinal Cord Neurons

A. Responses evoked by 10 μM, 30 μM, and 1 mM glycine in spinal cord neurons are shown. Responses peaked and then desensitized to a steady state. The peak and steady state current amplitudes increased with applications of increasing concentrations of glycine, until maximal current amplitude was reached at the saturating glycine concentration of 1 mM.

B. The concentration response curves for the peak (circles) and steady state (squares) glycine-evoked currents in spinal cord neurons are shown. The EC$_{50}$ value for peak currents was 42 ± 6 μM and the Hill coefficient was 2.0 ± 0.4 (n = 8). For the steady state, the EC$_{50}$ value was 18 ± 4 μM and the Hill coefficient was 2.5 ± 0.5 (n = 6).
Concentration-Response Relations for Peak and Steady State Glycine-Evoked Currents in Spinal Cord Neurons
4.1.3. LAVENDUSTIN A REDUCED THE FUNCTION OF THE GLYCINE RECEPTOR IN CA1 HIPPOCAMPAL AND SPINAL CORD NEURONS

To test the effect of tyrosine phosphorylation inhibition on GlyRs, the tyrosine kinase inhibitor, lavendustin A and its inactive analogue, lavendustin B were used. Lavendustin A and B were selected as these compounds are reported to not directly block GABA<sub>a</sub>Rs (Huang et al., 1999) or GlyRs (Huang & Dillon, 2000). A glycine concentration that was approximately equal to the EC<sub>50</sub> concentration for peak glycine-activated currents in hippocampal neurons (140 μM) was used. Contrary to the report by Huang & Dillon (2000), lavendustin B (10 μM) rapidly reduced peak glycine-evoked currents by 51 ± 4 % (p < 0.01, paired t-test, n = 9) within the first minute of application with no further reduction observed with time. This effect of lavendustin B was rapidly reversed upon wash-out with a lavendustin B-free solution. Therefore, we investigated the additional inhibitory actions of lavendustin A compared to lavendustin B. Lavendustin B was first applied and a stable current was recorded. Lavendustin A (10 μM) was extracellularly-applied next, which depressed peak glycine-activated currents by 31 ± 8 % (n = 4) compared to lavendustin B (10 μM) (n = 5) (p < 0.01, 2-way ANOVA, Figure 8C, at 17 minutes following application). Glycine-activated currents did not recover from the depression observed in the presence of lavendustin A even after 10 minutes of wash-out. These results suggest that inhibition of tyrosine phosphorylation reduces the function of GlyRs in the CA1 region of the hippocampus, whereas lavendustin B has a direct blocking action.

We next determined that the modulation of glycine-evoked currents by tyrosine phosphorylation observed in hippocampal neurons is also observed in spinal cord neurons. A glycine concentration near the EC<sub>50</sub> value for peak currents (45 μM) was
used to evoke currents. Lavendustin B (10 μM) was applied in the bath until stable currents were recorded and then lavendustin A (10 μM) was applied. When lavendustin A was applied, both peak and steady state glycine-evoked currents were reduced by 40 ± 8 % (p < 0.05, paired t-test, n = 4, Figure 8C inset, 15 minutes following application) and 41 ± 6 % (p < 0.05, paired t-test, n = 3, Figure 8C inset, 15 minutes following application), respectively compared to lavendustin B (10 μM). Glycine-activated currents did not recover from the depression by lavendustin A even after 10 minutes of wash-out. These results suggest that inhibition of tyrosine phosphorylation reduces the function of GlyRs in spinal cord neurons.
Figure 8

Extracellular Application of Lavendustin A Depressed Peak Glycine-Evoked Currents in CA1 Hippocampal and Spinal Cord Neurons

A, B. Responses evoked by glycine (140 μM) in the presence of lavendustin B (10 μM) and lavendustin A (10 μM), 1 and 17 minutes after establishment of whole-cell configuration, are shown.

C. The graph illustrates the effect of lavendustin A and lavendustin B on peak glycine-evoked currents in hippocampal neurons. The inhibition observed immediately after application of lavendustin A was no different than that observed following application of lavendustin B. However, GlyR-mediated responses were depressed in the presence of lavendustin A (n = 4) in a time-dependent fashion, an effect that was not observed with lavendustin B (n = 5) (p < 0.01, 2-way ANOVA).

*Inset (right):* The bar graph illustrates the effect of lavendustin A on peak glycine-evoked currents in spinal cord neurons. Bath application of lavendustin A depressed both peak (p < 0.05, paired t-test, n = 4, 15 minutes following application) and steady state (p < 0.05, paired t-test, n = 3, 15 minutes following application) glycine-evoked currents, as indicated by an asterisk.
Extracellular Application of Lavendustin A Depresses Peak Glycine-Evoked Currents in Hippocampal Neurons

Extracellular Application of Lavendustin A Depresses Glycine-Evoked Currents in Spinal Cord Neurons
Genistein, a tyrosine kinase inhibitor has a direct blocking effect on the GABA<sub>A</sub>R when applied to the extracellular solution but not at the intracellular domain of the receptor (Moss et al., 1995; Dunne et al., 1998; Huang et al., 1999). In an attempt to avoid the direct inhibitory effects of lavendustin B and lavendustin A on GlyRs, additional experiments were conducted with lavendustin A or lavendustin B included in the pipette solution. To make certain that lavendustin B had no direct effects on the GlyR when included in the pipette solution, currents recorded in control intracellular solution (n = 4) and with lavendustin B (1 μM) in the pipette (n = 7) were compared. These current amplitudes were not different upon achievement of whole-cell configuration (p > 0.05, unpaired t-test) and did not become different from each other with time (p > 0.05, 2-way ANOVA, Figure 9D), suggesting that the direct blocking effects of lavendustin B were avoided when it was included in the pipette solution.

In contrast, intracellular application of lavendustin A (1 μM) reduced peak glycine-induced currents by 21 ± 9% compared to lavendustin B (1 μM) (p < 0.01, 2-way ANOVA, n = 7, Figure 9E). These results further support the hypothesis that tyrosine phosphorylation inhibition decreases GlyR function in CA1 hippocampal neurons.
Figure 9

Intracellular Application of Lavendustin A Depressed Glycine-Evoked Currents in CA1 Hippocampal Neurons

A, B, C. Responses evoked by glycine (140 μM) using control intracellular solution, in the presence of lavendustin B, and in the presence of lavendustin A in the intracellular solution, 1 and 9 minutes after establishment of whole-cell configuration, are shown.

D. The graph illustrates peak glycine-evoked currents in the absence (triangles, n = 4) and presence (squares, n = 7) of lavendustin B. Currents are normalized to the current recorded 1 minute after achievement of whole-cell configuration. Intracellularly-applied lavendustin B had no effect on the amplitude of peak glycine-evoked currents (p > 0.05, 2-way ANOVA).

E. The graph illustrates the effect of intracellular lavendustin A (circles) and lavendustin B (squares) on peak glycine-evoked currents. Currents are normalized to control glycine-evoked currents. Lavendustin A depressed the amplitude of peak glycine-evoked currents compared to lavendustin B (p < 0.01, 2-way ANOVA, n = 7).
A control

B lavendustin B

C lavendustin A

D Intracellular Application of Lavendustin B Does Not Depress Peak Glycine-Evoked Currents in Hippocampal Neurons

E Intracellular Application of Lavendustin A Depresses Peak Glycine-Evoked Currents in Hippocampal Neurons
4.1.4. THE EXOGENOUS PROTEIN TYROSINE KINASE, PP60c-src ENHANCED THE FUNCTION OF GLYCINE RECEPTORS IN CA1 HIPPOCAMPAL NEURONS

To examine the effect of an exogenous PTK, pp60c-src was added to the pipette solution of CA1 hippocampal neurons. A saturating glycine concentration (1 mM) was used, as determined by the concentration-response relation for hippocampal neurons (Figure 6B). Changes in GlyR affinity by pp60c-src would be evident at low but not saturating glycine concentrations. Hence, a saturating glycine concentration was applied to minimize any possible changes in GlyR-mediated currents due to alterations in affinity by pp60c-src. Heat-inactivated pp60c-src and no pp60c-src were used as control experiments.

Heat-inactivated pp60c-src (n = 6) did not affect the amplitude of peak glycine-evoked currents compared to control currents (n=4) (p > 0.05, 2-way ANOVA, Figure 10D). In contrast, in the presence of pp60c-src (30 U/ml), peak glycine-activated currents were larger by 25.5 ± 14.4 % compared to currents generated in the presence of heat-inactivated pp60c-src (30 U/ml) (p < 0.05, 2-way ANOVA, n = 6, Figure 10E, measured at 13 minutes). These results suggest that tyrosine phosphorylation reduces rundown of glycine-evoked currents in CA1 hippocampal neurons.

As rundown of GABAAR-mediated currents is reduced at lower GABA concentrations (Huang & Dillon, 1998), we also tested the effect of pp60c-src on glycine-evoked currents using a low concentration of glycine (30 µM). However, contrary to this previous report on GABAARs, rundown was not different with 30 µM glycine (n = 4) versus 1 mM glycine in the presence of heat-inactivated pp60c-src (n = 6) (p > 0.05, 2-way ANOVA). Hence, rundown of glycine-evoked currents was not concentration-dependent. Pp60c-src (30 U/ml) enhanced the amplitude of peak glycine-evoked currents by 56 ± 11 % upon 9
minutes of application compared to heat-inactivated pp60\textsuperscript{c-src} (30 U/ml) \((p < 0.01, 2\text{-way ANOVA, } n = 4, \text{Figure 11D})\). This increase in current amplitude by pp60\textsuperscript{c-src} was not observed when lavendustin A (10 \(\mu\text{M}\)) was co-applied with pp60\textsuperscript{c-src} in the pipette solution (30 U/ml) \((p > 0.05, 2\text{-way ANOVA, } n = 4, \text{Figure 11D})\), further suggesting that tyrosine phosphorylation by pp60\textsuperscript{c-src} modulates GlyR function in CA1 hippocampal neurons in a positive fashion.

Also, the increase in current amplitude observed at the low glycine concentration (30 \(\mu\text{M, } n = 4\)) in the presence of pp60\textsuperscript{c-src} was greater than that observed with the saturating glycine concentration (1 mM, \(n = 6\)) \((p < 0.01, 2\text{-way ANOVA})\), compared to heat-inactivated pp60\textsuperscript{c-src}.
Figure 10

Pp60\textsuperscript{C-src} Caused a Reduction in the Rundown of Peak Glycine-Evoked Currents (1 mM Glycine) in CA1 Hippocampal Neurons

A, B, C. Control responses evoked in hippocampal neurons by 1 mM glycine using control intracellular solution, heat-inactivated pp60\textsuperscript{C-src} (30 U/ml), and pp60\textsuperscript{C-src} (30 U/ml) in the intracellular solution, 1 and 15 minutes after establishment of whole-cell configuration, are shown.

D. The graph illustrates the effect of control solution (triangles) and heat-inactivated pp60\textsuperscript{C-src} (squares) on peak glycine-evoked currents. Currents are normalized to the current recorded 1 minute after achievement of whole-cell configuration. Heat inactivated pp60\textsuperscript{C-src} (n=6) had the same effect on the amplitude of peak glycine-evoked currents as the control solution (n = 4) (p > 0.05, 2-way ANOVA).

E. The graph illustrates the effect of pp60\textsuperscript{C-src} (circles) and heat-inactivated pp60\textsuperscript{C-src} (squares) on peak glycine-evoked currents upon inclusion of active and heat-inactivated pp60\textsuperscript{C-src} in the pipette solution. Currents are normalized to control glycine-evoked currents. Pp60\textsuperscript{C-src} reduced the amount of rundown of glycine-evoked currents compared to currents generated in the presence of heat-inactivated pp60\textsuperscript{C-src} (p < 0.05, 2-way ANOVA, n = 6).
Intracellular Application of Heat-inactivated pp60\textsuperscript{c-src} Does Not Affect Peak Glycine-Evoked Currents in Hippocampal Neurons

\[ \frac{I}{I_{\text{1min}}} \% \]

\begin{align*}
\text{Time (min):} & 0 & 2 & 4 & 6 & 8 & 10 & 12 & 14 & 16 \\
\text{Normalized to Control:} & 100 & 80 & 60 & 40 & 20 & 0 & 0 & 0 & 0
\end{align*}
Figure 11

Pp60<sup>c-src</sup> Enhanced Peak Glycine-Evoked Currents (30 μM Glycine) in CA1 Hippocampal Neurons

A, B, C. Responses evoked in hippocampal neurons by 30 μM glycine in the presence of heat-inactivated pp60<sup>c-src</sup> (30 U/ml), pp60<sup>c-src</sup> (30 U/ml), and pp60<sup>c-src</sup> (30 U/ml) plus lavendustin A (10 μM) in the intracellular solution, 1 and 11 minutes after establishment of whole-cell configuration, are shown.

D. The graph illustrates the effect of pp60<sup>c-src</sup> on peak glycine-evoked currents. Currents are normalized to the current recorded 1 minute after achievement of whole-cell configuration. Pp60<sup>c-src</sup> (circles) enhanced the amplitude of glycine-evoked currents compared to currents obtained in the presence of heat-inactivated pp60<sup>c-src</sup> (squares) (p < 0.01, 2-way ANOVA, n = 4). When both pp60<sup>c-src</sup> (30 U/ml) and lavendustin A (10 μM) were included in the pipette solution (triangles), the peak glycine-evoked currents were not different than those generated in the presence of heat-inactivated pp60<sup>c-src</sup> (p > 0.05, 2-way ANOVA, n = 4).
D

Intracellular Application of pp60^c-src Potentiates Peak Glycine-Evoked Currents in Hippocampal Neurons

\[
\frac{I}{I_{1\text{ min}}} (\%) \quad 0 \quad 2 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12
\]

- pp60^c-src
- heat-inactivated pp60^c-src
- pp60^c-src plus lavendustin A (10 μM)
4.2. SECTION 2

Previous studies (Grenningloh et al., 1990b) indicate that a putative tyrosine phosphorylation site is present on the β subunit of the GlyR, specifically Tyr-413. However, the function of Tyr-413 has not been investigated. The purpose of these experiments was to determine if the α and/or β subunits of the GlyR are involved in the modulation of GlyR function by tyrosine phosphorylation as well as determine the role of Tyr-413 in GlyR tyrosine phosphorylation.

4.2.1. THE GLYCINE RECEPTOR β SUBUNIT DECREASED THE AFFINITY OF THE GLYCINE RECEPTOR FOR PICROTOXIN

In order to examine the effect of tyrosine phosphorylation on the GlyR α₁ and β subunits, we transfected HEK 293 cells with GlyR α₁ or α₁ and β cDNAs. To ensure co-expression of the β subunit, we compared the sensitivity of recombinant α₁ and α₁/β GlyRs to picrotoxin inhibition. Previous reports suggest that the presence of the β subunit decreases the sensitivity of the GlyR to inhibition by picrotoxin. Pribilla et al. (1992) reported that the IC₅₀ value of picrotoxin for GlyR α₁ subunits was 9 μM whereas in the presence of both the α₁ and β subunits of the GlyR, the IC₅₀ value of picrotoxin was > 1 mM. Similar results were obtained by Handford et al. (1996) where the IC₅₀ value of picrotoxin for GlyR α₁- and α₁/β-mediated currents were 25 μM and 420 μM, respectively.

Concentration-response curves for inhibition by picrotoxin for currents recorded from HEK 293 cells transfected with GlyR α₁ and α₁ and β cDNAs were constructed. To mimic the experiments of Pribilla et al. (1992), 100 μM glycine was used. As illustrated in Figure 12C, the presence of the GlyR β subunit shifted the picrotoxin concentration-
response curve to the right, increasing the $\text{lC}_{50}$ from $98 \pm 41 \, \mu\text{M} \ (n = 7)$ to $1060 \pm 313 \, \mu\text{M} \ (n = 6) \ (p < 0.05, \text{unpaired t-test})$. These results indicated that the GlyR $\beta$ subunit was expressed, and confirmed previous reports that the GlyR $\beta$ subunit decreases the sensitivity of the GlyR to picrotoxin inhibition.

A previous report by Bormann et al. (1993) indicates that co-transfection of the GlyR $\beta$ subunit in HEK 293 cells enhances glycine-evoked currents. In our experiments, peak $\alpha_1$ GlyR-mediated currents had an average current amplitude of $-1.7 \pm 0.4 \, \text{nA}$ while $\alpha_1/\beta$ GlyRs had an average current amplitude of $-3.3 \pm 0.4 \, \text{nA} \ (p < 0.05, \text{unpaired t-test})$. This two-fold enhancement of glycine-evoked currents adds further support of efficient co-transfection of the GlyR $\alpha_1$ and $\beta$ subunits in our studies.
**Figure 12**

**The GlyR \( \beta \) Subunit Reduced the Sensitivity of Peak Glycine-Evoked Currents to Picrotoxin Inhibition**

A, B. Responses evoked by 100 \( \mu \text{M} \) glycine in HEK 293 cells expressing the GlyR \( \alpha_1 \), and \( \alpha_1 \) and \( \beta \) subunits, in the absence and presence of 100 \( \mu \text{M} \) picrotoxin are shown. The glycine-evoked peak current decreased with applications of increasing concentrations of picrotoxin.

C. The concentration response curves for peak glycine-evoked currents for \( \alpha_1 \) homomeric GlyRs (squares) and \( \alpha_1/\beta \) heteromeric GlyRs (circles) in the presence of increasing concentrations of picrotoxin are shown. The \( \text{IC}_{50} \) value of 98 ± 41 \( \mu \text{M} \) (\( n = 7 \)) for peak glycine-evoked currents mediated by \( \alpha_1 \) homomeric GlyRs was smaller than the \( \text{IC}_{50} \) value of 1060 ± 313 \( \mu \text{M} \) (\( n = 6 \)) for peak glycine-evoked currents mediated by \( \alpha_1/\beta \) heteromeric GlyRs (\( p < 0.05 \), unpaired t-test).
The GlyR β Subunit Reduces the Sensitivity of Peak Glycine-Evoked Currents to Picrotoxin Inhibition
4.2.2. THE EXOGENOUS TYROSINE KINASE, PP60\(^c\)-src DID NOT AFFECT THE FUNCTION OF THE \(\alpha_1\) HOMOMERIC GLYCINE RECEPTORS

The effect of the exogenous PTK, pp60\(^c\)-src on glycine-evoked currents was examined in HEK 293 cells expressing the \(\alpha_1\) subunit of the GlyR. The presence of pp60\(^c\)-src (30 U/ml) \((n = 12)\) in the pipette solution did not affect peak glycine-activated currents compared to currents recorded in the presence of heat-inactivated pp60\(^c\)-src (30 U/ml) \((n = 11)\) in the pipette solution \((p > 0.05,\) 2-way ANOVA, Figure 13C). These results suggest that the \(\alpha_1\) subunit of the GlyR is not involved in the modulation of GlyR function by tyrosine phosphorylation by pp60\(^c\)-src.
Figure 13

Pp60c-src Did Not Affect the Amplitude of Glycine-Evoked Currents of α1 GlyRs

A, B. Responses evoked by 1 mM glycine in HEK 293 cells expressing α1 homomeric GlyRs, in the presence of heat-inactivated pp60c-src (30 U/ml) and pp60c-src (30 U/ml) in the intracellular solution. 1 and 13 minutes after establishment of whole-cell configuration, are shown.

C. The graph illustrates the effect of pp60c-src (circles) and heat-inactivated pp60c-src (squares) on peak glycine-evoked currents mediated by α1 GlyRs. Currents are normalized to the response recorded 1 minute after achievement of whole-cell configuration. Pp60c-src had no effect on the amplitude of peak glycine-evoked currents (n = 12) compared to currents recorded in the presence of heat-inactivated pp60c-src (n = 11) (p > 0.05, 2-way ANOVA).
Intracellular Application of pp60^{c-src} Does Not Affect the Peak Amplitude of Glycine-Evoked Currents of α_{1} GlyRs
4.2.3. THE EXOGENOUS TYROSINE KINASE, pp60<sup>C-src</sup> REDUCED THE RUNDOWN OF CURRENTS MEDIATED BY α<sub>1</sub>/β HETEROMERIC GLYCINE RECEPTORS

To examine the effect of pp60<sup>C-src</sup> on α<sub>1</sub>/β GlyR-mediated currents, glycine-evoked currents were recorded in the presence of active and heat-inactivated pp60<sup>C-src</sup>. Intracellular pp60<sup>C-src</sup> (30 U/ml) caused a 15 ± 6 % reduction in the rundown of peak glycine-evoked currents compared to heat-inactivated pp60<sup>C-src</sup> (30 U/ml) (p < 0.01, 2-way ANOVA, n = 12, Figure 14C, measured at 13 minutes). In light of our finding for the peak glycine-evoked currents for the α<sub>1</sub> subunit (Figure 13C), these results indicate that the GlyR β subunit is involved and is a necessary component for the modulation of GlyR function by tyrosine phosphorylation by pp60<sup>C-src</sup>.
Figure 14

Pp60c-src Caused a Reduction in the Rundown of Glycine-Evoked Currents of α/β GlyRs

A, B. Responses evoked by 1 mM glycine in HEK 293 cells expressing α/β heteromeric GlyRs, with heat-inactivated pp60c-src (30 U/ml) and pp60c-src (30 U/ml) in the pipette solution, 1 and 13 minutes after establishment of whole-cell configuration, are shown.

C. The graph illustrates the effect of pp60c-src (circles) and heat-inactivated pp60c-src (squares) on peak glycine-evoked currents mediated by α/β GlyRs. Currents are normalized to the response recorded 1 minute after achievement of whole-cell configuration. Pp60c-src reduced the amount of rundown of peak glycine-evoked currents compared to heat-inactivated pp60c-src (p < 0.01, 2-way ANOVA, n = 12).
Intracellular Application of pp60<sup>c-src</sup> Enhances the Amplitude of Peak Glycine-Evoked Currents of α<sub>1</sub>/β GlyRs
4.2.4. THE PROTEIN TYROSINE KINASE, PP60\textsuperscript{c-src} INCREASED THE EXTENT OF DESENSITIZATION OF GLYCINE-EVOKED CURRENTS IN THE PRESENCE OF THE GLYCINE RECEPTOR $\beta$ SUBUNIT

The role of either the $\alpha$ or $\beta$ GlyR subunits as well as the role of tyrosine phosphorylation on GlyR desensitization has not been examined to date. Here we investigated 1) the role of the $\beta$ subunit on desensitization and 2) the role of tyrosine phosphorylation on GlyR desensitization.

There was no difference in the $I_{ss} / I_p$ ratios for GlyR $\alpha_1$- ($I_{ss} / I_p = 0.53 \pm 0.09$, $n=7$) versus $\alpha_1/\beta$-mediated currents ($I_{ss} / I_p = 0.46 \pm 0.09$, $n=9$) in the presence of heat-inactivated pp60\textsuperscript{c-src} ($p > 0.05$, Mann-Whitney test, Figure 15A, measured at 1 minute). Hence, the presence of the $\beta$ subunit does not influence the extent of desensitization of the GlyR.

The role of the $\beta$ subunit on GlyR desensitization in the presence of pp60\textsuperscript{c-src} was then investigated. The $I_{ss} / I_p$ ratios for currents mediated by $\alpha_1$ and $\alpha_1/\beta$ GlyRs were plotted versus time. The $I_{ss} / I_p$ ratio for $\alpha_1$ GlyRs was not affected by the presence of pp60\textsuperscript{c-src} ($I_{ss} / I_p = 0.47 \pm 0.08$, $n = 10$) as compared to the $I_{ss} / I_p$ ratio obtained when heat-inactivated pp60\textsuperscript{c-src} ($I_{ss} / I_p = 0.53 \pm 0.09$, $n = 7$) was included in the intracellular solution ($p > 0.05$, Mann-Whitney test, Figure 15B, measured at 1 minute). However, for $\alpha_1/\beta$ GlyRs, the $I_{ss} / I_p$ ratio was less when pp60\textsuperscript{c-src} was included in the pipette solution ($I_{ss} / I_p = 0.32 \pm 0.10$) as compared to heat-inactivated pp60\textsuperscript{c-src} ($I_{ss} / I_p = 0.46 \pm 0.09$) ($p < 0.01$, Mann-Whitney test, $n = 9$, Figure 15C, measured at 1 minute). The reduction in the $I_{ss} / I_p$ ratio took place within the first minute of pp60\textsuperscript{c-src} application and remained stable over the recording period (Figure 15C). These results suggest that
tyrosine phosphorylation of the β subunit by pp60⁵⁶src increases the extent of desensitization of glycine-evoked currents.
Figure 15

Tyrosine Phosphorylation of the GlyR β Subunit by pp60c-src Increased the Extent of Desensitization of Glycine-Evoked Currents

A. The $I_{ss}/I_p$ ratios of glycine-evoked currents in the presence of heat-inactivated pp60c-src (30 U/ml) for $\alpha_1$ (triangles, $n = 7$) and $\alpha_1/\beta$ (diamonds, $n = 9$) GlyRs, plotted against time are shown. The presence of the β subunit did not affect the $I_{ss}/I_p$ ratio ($p > 0.05$, Mann-Whitney test, measured at 1 minute).

B. The $I_{ss}/I_p$ ratios of glycine-evoked currents mediated by $\alpha_1$ GlyRs in the presence of active (30 U/ml) (circles) and heat-inactivated (30 U/ml) (squares) pp60c-src plotted against time are shown. Pp60c-src did not change the $I_{ss}/I_p$ value ($n=10$) compared to heat-inactivated pp60c-src ($n = 7$) ($p > 0.05$, Mann-Whitney test, measured at 1 minute).

C. The $I_{ss}/I_p$ ratios of glycine-evoked currents mediated by $\alpha_1/\beta$ GlyRs in the presence of active (30 U/ml) (circles) and heat-inactivated (30 U/ml) (squares) pp60c-src plotted against time are shown. Pp60c-src reduced the $I_{ss}/I_p$ value compared to heat-inactivated pp60c-src ($p < 0.01$, Mann-Whitney test, $n = 9$).
A. The Presence of the GlyR β Subunit Does Not Affect the Extent of Desensitization of Glycine-Evoked Currents

![Graph showing the effect of GlyR β subunit on desensitization of glycine-evoked currents.]

B. Intracellular Application of pp60c-src Does Not Affect the Extent of Desensitization of Glycine-Evoked Currents of α1 GlyRs

![Graph showing the effect of pp60c-src application on desensitization of glycine-evoked currents.]

C. Intracellular Application of pp60c-src Increases the Extent of Desensitization of Glycine-Evoked Currents of α1/β GlyRs

![Graph showing the effect of pp60c-src heat-inactivation on desensitization of glycine-evoked currents.]

Legend:
- ▲ α1 GlyRs
- ♦ α1/β GlyRs
- ■ pp60c-src
- □ heat-inactivated pp60c-src
4.2.5. MUTATION OF TYR-413 OF THE GLYCINE RECEPTOR β SUBUNIT
ABOLISHED MODULATION OF THE GLYCINE RECEPTOR BY TYROSINE
PHOSPHORYLATION

In order to examine the specific role of the putative tyrosine phosphorylation site (Tyr-413) of the GlyR β subunit, the Y413F mutant was created. The effect of pp60<sup>c-src</sup> was investigated at a saturating glycine concentration of 1 mM (Handford et al., 1996). In agreement with previous results in HEK 293 cells (Figure 14C), pp60<sup>c-src</sup> reduced the amount of rundown of peak glycine-evoked currents recorded from cells expressing wild type α<sub>1</sub>/β GlyRs compared to heat-inactivated pp60<sup>c-src</sup> (p < 0.01, unpaired t-test, n=6, Figure 16F, measured at 25 minutes). However, peak glycine-evoked currents mediated by α<sub>1</sub>/β<sup>Y413F</sup> GlyRs were reduced by the same amount in the presence of pp60<sup>c-src</sup> (n = 10) and heat-inactivated pp60<sup>c-src</sup> (n = 9) (p > 0.05, unpaired t-test, Figure 16G, measured at 25 minutes). Moreover, this reduction was similar to that observed with wild type α<sub>1</sub>/β GlyRs in the presence of heat-inactivated pp60<sup>c-src</sup> (p > 0.05, unpaired t-test, measured at 25 minutes). Hence, mutation of the Tyr-413 residue of the GlyR β subunit abolished the reduction in the rundown of glycine-evoked currents induced by pp60<sup>c-src</sup> on wild type α<sub>1</sub>/β GlyRs.

The effect of the mutation Y413F of the GlyR β subunit on current amplitude was also investigated. There was no difference in current amplitudes 1 minute after whole-cell configuration was achieved between wild type α<sub>1</sub>/β (n = 6) and α<sub>1</sub>/β<sup>Y413F</sup> (n = 9) GlyRs in the presence of heat-inactivated pp60<sup>c-src</sup> (p > 0.05, unpaired t-test, Figure 16E). Hence, the Y413F β subunit mutation does not affect the initial amplitude of peak-glycine evoked currents.
Figure 16

Tyrosine-413 of the GlyR β Subunit is the Amino Acid Residue that is Tyrosine Phosphorylated by pp60c-src

A, B. Responses evoked by 1 mM glycine in HEK 293 cells expressing wild type α1/β GlyRs, in the presence of heat-inactivated pp60c-src (30 U/ml) and pp60c-src (30 U/ml) in the pipette solution, 1 and 25 minutes after achievement of whole-cell configuration, are shown.

C, D. Responses evoked by 1 mM glycine in HEK 293 cells expressing α1/β GlyRs, with the β subunit containing the mutation Y413F. Traces obtained in the presence of heat-inactivated pp60c-src (30 U/ml) and pp60c-src (30 U/ml) in the intracellular solution, 1 and 25 minutes after establishment of whole-cell configuration, are shown.

E. Average initial amplitudes of GlyR α1/β- and α1/βY413F-mediated currents are shown. The bar graph illustrates that mutation of Tyr-413 of the GlyR β subunit (n = 9) did not affect the amplitude of initial glycine-evoked currents compared to wild type α1/β-mediated currents (n = 6) in the presence of heat-inactivated pp60c-src (30 U/ml) (p > 0.05, unpaired t-test).

F. The effect of pp60c-src on GlyR α1/β-mediated currents is shown. Pp60c-src caused a reduction in the rundown of glycine-evoked currents mediated by wild type α1/β GlyRs compared to heat-inactivated pp60c-src (p < 0.01, unpaired t-test, n = 6).

G. The bar graph illustrates the effect of the GlyR β subunit mutation, Y413F on the modulation of GlyR function by pp60c-src. Glycine-evoked currents mediated by
\(\alpha_1/\beta^{Y413F}\) GlyRs, generated in the presence of active \((n = 10)\) and heat-inactivated \((n = 9)\) pp60\(^{c-src}\) showed an equal amount of rundown \((p > 0.05,\) unpaired t-test\) and were reduced to a similar extent as those mediated by the wild type \(\alpha_1/\beta\) GlyRs in the presence of heat-inactivated pp60\(^{c-src}\) \((p > 0.05,\) unpaired t-test\).
A. Wild Type

heat-inactivated pp60c-src

1 min

25 min

0.5 nA
3 sec

B. Wild Type

pp60c-src

1 min

25 min

0.5 nA
3 sec

C. β Y413F

heat-inactivated pp60c-src

1 min

25 min

0.8 nA
3 sec

D. β Y413F

pp60c-src

1 min

25 min

0.8 nA
3 sec

E. The Mutation βY413F Does Not Affect the Initial Amplitude of α/β-Mediated Glycine-Evoked Currents

F. Pp60c-src Causes a Reduction in the Rundown of α/β-Mediated Glycine-Evoked Currents

G. Pp60c-src Does Not Affect the Rundown of α/β-Y413F-Mediated Glycine-Evoked Currents

Current Amplitude (pA)

I_{25 \text{ min}} / I_{1 \text{ min}} (pA)
5. DISCUSSION

5.1. SECTION 1

Inhibition of tyrosine phosphorylation by lavendustin A depressed glycine-evoked currents in both hippocampal and spinal cord neurons, while the tyrosine kinase, pp60-src enhanced GlyR function and reduced the rundown of glycine-evoked currents. These results are consistent with tyrosine phosphorylation modulation of GlyR function in a positive fashion.

5.1.1. LAVENDUSTIN A DEPRESSION OF GLYCINE-EVOKED CURRENTS

Extracellular application of lavendustin A significantly depressed the amplitude of glycine-evoked currents in both hippocampal and spinal cord neurons. We interpret these results to indicate that inhibition of tyrosine phosphorylation decreases the function of GlyRs.

This is a novel finding since there are no reports as to the effects of tyrosine phosphorylation on GlyR function to date. Studies on the GABA_A R, which is similar to the GlyR in structure and function, have reported that the tyrosine kinase inhibitors, genistein and the tyrphostins B-42 and B-44 inhibited muscimol-stimulated Cl^- uptake (Valenzuela et al., 1995). Genistein and lavendustin A also reduced the amplitude of GABA_A R- (Moss et al., 1995; Wan et al., 1997a; Huang & Dillon, 1998) and NMDA receptor-mediated currents (Wang & Salter, 1994). These studies, which indicate that tyrosine phosphorylation inhibition decreases the function of ligand-gated ion channels are consistent with our results as to modulation of the GlyR by tyrosine phosphorylation.

Previous reports indicate that tyrosine kinase inhibitors and their inactive controls such as genistein and its inactive analogue, daidzein, as well as tyrphostin A25 and the
inactive analogue, tyrphostin A1 can directly interact with GABAₐRs, independently of any effects on tyrosine kinases (Dunne et al., 1998; Huang et al., 1999). Furthermore, a recent report also indicates that genistein and daidzein directly block GlyRs in hypothalamic neurons (Huang & Dillon, 2000). Consistent with these reports, in our studies we observed that lavendustin B, the inactive analogue of lavendustin A decreased glycine-evoked currents. However, lavendustin B caused an immediate and rapidly reversible depression of glycine-activated currents while a gradual decrease in current amplitude was observed upon the replacement of lavendustin B with lavendustin A. The immediate decrease observed with lavendustin B is consistent with direct effects of the drug on the GlyR. On the other hand, the additional gradual decrease in glycine-evoked currents that was observed in the presence of lavendustin A suggests that a phosphorylation process is a more likely cause of the reduction in glycine-evoked current amplitude. This observation is supported by the results of Huang et al. (1999) and Huang & Dillon (2000), which indicate that the effects of lavendustin A on GABAₐR- and GlyR-activated currents are due exclusively to inhibition of PTKs.

In order to avoid the direct effects of the lavendustins on the GlyR, we directly applied lavendustin B and lavendustin A into the internal milieu of neurons. This intracellular application method was previously used by Moss et al. (1995) who observed that intracellularly-applied genistein had no direct effects on GABAₐRs since it decreased the amplitude of wild type GABAₐR-mediated currents but had no effect on GABAₐRs incorporating the mutant γ2L Y(365/367)F subunit. In our experiments, we observed no difference in the amplitude of glycine-evoked currents in the absence or presence of lavendustin B, suggesting that intracellularly-applied lavendustin B has no
direct effect on GlyR-mediated currents. However, we observed that lavendustin A depressed glycine-evoked currents compared to lavendustin B in a time-dependent fashion. We suggest that the gradual inhibition observed in the presence of lavendustin A is due to its action on tyrosine kinases.

In summary, these findings show that tyrosine phosphorylation might be an important mechanism involved in the enhancement of GlyR function in both CA1 hippocampal and spinal cord neurons.

5.1.2. PP60\textsuperscript{C-src} ENHANCEMENT OF GLYCINE-EVOKED CURRENTS

The PTK, pp60\textsuperscript{C-src} enhanced GlyR function and reduced rundown of glycine-evoked currents in hippocampal neurons in the presence of a low (30 \textmu M) and a saturating (1 mM) glycine concentration, respectively, while heat-inactivated pp60\textsuperscript{C-src} did not affect glycine-induced currents. The enhancing effect of pp60\textsuperscript{C-src} was blocked by lavendustin A. These results suggest that tyrosine phosphorylation affects GlyR function in a positive fashion.

The effect of pp60\textsuperscript{C-src} on GlyR function has not been previously investigated. Various studies on GABA\textsubscript{A}Rs have reported that intracellular application of pp60\textsuperscript{C-src} caused a progressive increase in current amplitude (Moss et al., 1995; Wan et al., 1997a), a potentiation that was prevented by pretreatment of the neurons with genistein (Wan et al., 1997a). Intracellular pp60\textsuperscript{C-src} / phosphatase inhibitor, sodium vanadate treatment increased both the mean open time and the probability of GABA\textsubscript{A}R channel opening (Moss et al., 1995). A recent report indicates that co-activation of TrkB receptor by BDNF and Src by mGluR1 enhances the amplitude of GABAergic miniature IPSCs (mIPSCs) in cerebellar Purkinje cells (Boxall, 2000). Several studies on the
NMDA receptor have also reported that intracellularly-applied pp60c-src potentiated NMDA currents and increased channel open probability (Wang & Salter, 1994; Yu & Salter, 1999). The antibody, anti-Src1, which selectively blocks the function of the tyrosine kinase Src, decreased the open probability and mean open time of NMDA channels (Yu et al., 1997). These data are in agreement with our results and suggest that PTKs are important in regulating the function of ligand-gated receptors.

Previous studies indicate that receptor function can be altered by phosphorylation through changes in conductance and/or open channel probability (Wang & Salter, 1994; Moss et al., 1995; Yu & Salter, 1999; Brandon et al., 2000), as well as changes in receptor localization (Strong et al., 1987; Ehlers et al., 1995; Chapell et al., 1998). Hence, pp60c-src may enhance GlyR function by changing single channel properties or by recruiting functional GlyRs to the plasma membrane.

The effects of pp60c-src on GlyR function might be brought about by 1) direct phosphorylation of the GlyR subunits at a tyrosine residue(s) or 2) an indirect effect of tyrosine kinases through phosphorylation of a peripheral protein such as gephyrin or collybistin II. Section 2 will attempt to answer this question in light of the results obtained in this study.

The observed effect of tyrosine phosphorylation on GlyR function might oppose or enhance the effects of serine/threonine-specific phosphorylation. PKA- and PKC reduce or enhance GlyR function, depending on the tissue under investigation (Song & Huang, 1990; Agopyan et al., 1993; Inomata et al., 1993; Vello et al., 1994; Schonrock & Bormann, 1995; Ren et al., 1998). Hence, tyrosine phosphorylation might either have
a synergistic or antagonistic effect on the actions of PKA and PKC in regards to GlyR function, their combined effects reflecting the function of the GlyR at any given time.

Interestingly, pp60c-src affected glycine-evoked responses to a greater extent at the low (30 μM: 56 ± 11 % larger than heat-inactivated pp60c-src, measured at 9 minutes) compared to the saturating (1 mM: 25.5 ± 14.4 % larger than heat-inactivated pp60c-src, measured at 13 minutes) glycine concentration (p < 0.01, 2-way ANOVA). Two possible explanations can account for these results. The first is that pp60c-src increases receptor affinity and hence, the enhancement is greatest at low concentrations of agonist. Alternatively, the pp60c-src-mediated increase measured with a saturating concentration of glycine was underestimated due to concurrent receptor desensitization. We favour the latter explanation for several reasons. Results from HEK 293 cells transfected with α1/β GlyRs indicate a decrease in the I_{ss} / I_{p} ratio in pp60c-src-treated cells using a saturating glycine concentration (1 mM) (I_{ss} / I_{p} pp60c-src: 0.32 ± 0.10, I_{ss}/I_{p} heat-inactivated pp60c-src: 0.46 ± 0.09, p < 0.01, Mann Whitney test, n = 9, measured at 1 minute). Furthermore, previous findings in the lab (unpublished observations) indicate that 1) pp60c-src increases the extent of desensitization of GABA-evoked currents, 2) pp60c-src does not change the affinity of GABAARs for GABA, and 3) other modulators of glycine that possibly act through tyrosine phosphorylation to increase glycine-evoked currents did not influence the affinity of GlyRs for glycine.

### 5.1.3. Differential Affinities of Glycine Receptors in Hippocampal and Spinal Cord Neurons

In CA1 hippocampal neurons, the EC₅₀ values for peak and steady state glycine-evoked currents were 138 ± 20 μM and 43 ± 8 μM, respectively, while in spinal cord
neurons, the EC_{50} values were 42 ± 6 and 18 ± 4 µM, respectively. The values for the Hill coefficients for peak and steady state currents in the hippocampus were 1.6 ± 0.1 and 1.3 ± 0.1, respectively, while in the spinal cord, the respective values were 2 ± 0.4 and 2.5 ± 0.5.

In previous reports, the EC_{50} values and Hill coefficients in hippocampal neurons have been shown to range between 61 - 100 µM and 1.4 - 2.0 respectively (Krishtal et al., 1988; Fatima-Shad & Barry, 1992; Fatima-Shad & Barry, 1995; Yoon et al., 1998). The difference between these EC_{50} values and our reported EC_{50} value is most likely the result of the usage of neurons from the whole hippocampus and/or cultured hippocampal neurons in the studies mentioned above and not acutely isolated CA1 hippocampal neurons, as was done in our studies. The values we obtained for the Hill coefficients are similar to those reported in the aforementioned studies, suggesting cooperativity of glycine binding for receptors present in neurons from different regions of the hippocampus. With respect to spinal cord neurons, previous reports indicate EC_{50} values and a Hill coefficient of ~ 40 µM (Laube et al., 1995; Xu et al., 1996; Tapia et al., 1997; Xu et al., 1999) and 2.6 (Laube et al., 1995), respectively. These data are similar to our reported EC_{50} and Hill coefficient values.

The affinity of the GlyR for glycine was higher in spinal cord neurons than in hippocampal neurons, as indicated by the lower EC_{50} values for peak (p < 0.05, unpaired t-test) and steady state (p < 0.05, unpaired t-test) glycine-evoked currents in spinal cord neurons. This difference in affinity probably reflects the different subunit composition of GlyRs in the spinal cord and hippocampus. Adult spinal cord GlyRs are composed primarily of α_1/β subunits (Langosch et al., 1988; Bormann et al., 1993;
Kuhse et al., 1993), while hippocampal GlyRs are composed primarily of $\alpha_2/\beta$ subunits (Malosio et al., 1991b). Previous studies on recombinant $\alpha_1/\beta$ GlyRs expressed in HEK 293 cells have reported $EC_{50}$ values of 54 – 74 $\mu$M (Pribilla et al., 1992; Bormann et al., 1993; Handford et al., 1996), while those of $\alpha_2/\beta$ GlyRs ranged from 80 – 95 $\mu$M (Pribilla et al., 1992; Takagi et al., 1992; Rundstrom et al., 1994). Our values for the spinal cord and hippocampal GlyRs are similar to those obtained for $\alpha_1/\beta$ and $\alpha_2/\beta$ recombinant GlyRs. Any differences are probably the result of possible other subunit combinations in the spinal cord where the $\alpha_3$ (Kuhse et al., 1990b) and $\alpha_4$ (Matzenbach et al., 1994; Harvey et al., 2000) subunits are also present, and in the hippocampus where the presence of the $\alpha_3$ subunit has been reported (Malosio et al., 1991b). Hence, our results are in agreement with previous reports and indicate that $\alpha_1/\beta$ GlyRs have a greater affinity for glycine compared to $\alpha_2/\beta$ GlyRs.

The $EC_{50}$ value for the steady state glycine-evoked currents was lower than the $EC_{50}$ value for the peak glycine-evoked currents both in hippocampal and spinal cord neurons. This difference in the $EC_{50}$ values indicates that the potency of glycine is greater when steady state is reached as compared to the peak phase of the current. This phenomenon has also been observed with AMPA receptors (Raman & Trussell, 1992; Shen et al., 1999). In order to explain it, investigators have proposed the existence of many different states for receptors. At any given state, the receptor has different binding and/or gating properties, thereby influencing the affinity of the receptor for the agonist or the ability of the agonist to gate the receptor (Jones & Westbrook, 1996).
5.2. SECTION 2

By using recombinant GlyR subunits expressed in HEK 293 cells, we were able to observe the modulation of GlyRs by the tyrosine kinase, pp60C-src in the presence and absence of the GlyR β subunit. In addition, the role of a specific amino acid residue, Tyr-413 of the GlyR β subunit, on GlyR modulation by tyrosine phosphorylation was investigated.

5.2.1. REDUCTION IN THE RUNDOWN OF GLYCINE-EVOKED CURRENTS BY PP60C-SRC IN THE PRESENCE OF THE GLYCINE RECEPTOR β SUBUNIT

Homomeric GlyRs composed of α1 subunits were not affected by the PTK, pp60C-src. However, upon co-expression of the GlyR α1 subunits with the wild type GlyR β subunit, pp60C-src caused a reduction in the rundown of glycine-evoked currents compared to currents obtained in the presence of heat-inactivated pp60C-src. This reduction in the amount of current rundown was not observed when the GlyR β mutant Y413F was present instead. These results suggest that Tyr-413 of the GlyR β subunit is essential for the phosphorylation of the GlyR and hence, modulation of GlyR function by the tyrosine kinase, pp60C-src.

Upon cloning of the GlyR β subunit, Grenningloh et al. (1990a) identified Tyr-413 of the GlyR β subunit as a putative tyrosine phosphorylation site, but its actual phosphorylation by a PTK has never been investigated.

Various studies have demonstrated that the regulation of ligand-gated ion channels by phosphorylation depends on subunit composition. A study on the NMDA receptor has reported that the increase of NMDA receptor-mediated currents by src is dependent upon the presence of the NR2A subunit, which becomes phosphorylated and thus
affects the current flux through NMDA receptor channels (Kohr & Seeburg, 1996). A recent report has shown that PKA-dependent phosphorylation enhanced the amplitude of mIPSCs in the presence of the $\text{GABA}_A\beta_3$ subunit but not in the presence of the $\beta_2$ subunit (Nusser et al., 1999). An electrophysiological study by Moss et al. (1995) has reported that site-specific mutagenesis of two putative tyrosine phosphorylation sites within the $\gamma_2\text{L} \text{GABA}_A\text{R}$ subunit eliminated GABA-evoked current enhancement by pp60$^{\text{c-src}}$ / sodium vanadate treatment. Another study has reported that the $\beta_2/\beta_3$ subunit(s) of the $\text{GABA}_A\text{R}$ are tyrosine phosphorylated in situ and that pp60$^{\text{c-src}}$ caused a progressive increase in $\text{GABA}_A\text{R}$-mediated current amplitude (Wan et al., 1997a). These results indicate that modulation of ligand-gated ion channels by phosphorylation depends upon the subunit composition of the receptor, and suggest that control of receptor subunit composition might be an important method utilized by neurons to regulate receptor function.

Our data show for the first time that the GlyR is subject to subunit-dependent modulation by tyrosine phosphorylation, which occurs on Tyr-413 of the GlyR $\beta$ subunit. The reduction in current rundown observed in the presence of the GlyR $\beta$ subunit could possibly be the result of an increase in the open probability, open time and/or number of expressed postsynaptic GlyRs on the plasma membrane (Wang & Salter, 1994; Ehlers et al., 1995; Moss et al., 1995; Yu & Salter, 1999; Brandon et al., 2000).

The loss of pp60$^{\text{c-src}}$-mediated reduction of rundown of glycine-evoked currents upon mutation of Tyr-413 of the GlyR $\beta$ subunit is consistent with the direct phosphorylation of the GlyR complex by pp60$^{\text{c-src}}$. However, it is possible that pp60$^{\text{c-src}}$ acts on other
kinases, which in turn affect the function of the GlyR through phosphorylation of Tyr-413 of the β subunit.

5.2.2. TYROSINE PHOSPHORYLATION OF THE β SUBUNIT INFLUENCES THE DESENSITIZATION OF THE GLYCINE RECEPTOR

In the presence of the α1 subunit alone, pp60c-src did not affect the $I_{se}/I_p$ ratio of glycine-evoked currents, however, pp60c-src reduced the $I_{se}/I_p$ ratio of α1/β GlyRs. These results suggest that tyrosine phosphorylation of the GlyR β subunit increases the extent of desensitization of the GlyR complex.

Previous studies on ligand-gated ion channels indicate that phosphorylation affects the desensitization state of other ionotropic receptors. PKA-mediated (Albuquerque et al., 1986; Middleton et al., 1986; Middleton et al., 1988; Hoffman et al., 1994) and tyrosine (Hopfield et al., 1988) phosphorylation increased the desensitization of nAChR-mediated currents. Interestingly, the PKA-mediated increase in current desensitization was the result of the phosphorylation of the nAChR γ and δ subunits (Huganir et al., 1986). A recent study has reported that in the presence of the GABAAR β2 subunit, PKA-dependent phosphorylation prolonged the decay time course of mIPSCs, while in the presence of the GABAAR β3 subunit, PKA-dependent phosphorylation did not change the time course of GABA mIPSCs (Nusser et al., 1999). In another report, PKA- (Greengard et al., 1991) and PKC-mediated phosphorylation increased the time constant of decay of mIPSCs mediated by AMPA/kainate receptors (Wang et al., 1994). These studies suggest that phosphorylation affects the desensitization properties of ligand-gated ion channels, with specific subunits being the targets of phosphorylation. Our data are in agreement with these observations and show for the first time that
tyrosine phosphorylation of the GlyR β subunit increases the extent of desensitization of glycine-evoked currents.

5.2.3. GLYCINE RECEPTOR SENSITIVITY TO PICROTOXIN INHIBITION AND ENHANCEMENT OF GLYCINE RECEPTOR CURRENT AMPLITUDE IN THE PRESENCE OF THE GLYCINE RECEPTOR β SUBUNIT

How can we be certain that the β subunit assembled with the α subunit in our co-transfection studies? A previous electrophysiological study has reported the picrotoxin IC_{50} values for recombinant α_1 and α_1/β GlyRs expressed in HEK 293 cells to be 9 and >1000 μM, respectively (Pribilla et al., 1992). Another electrophysiological study that used HEK 293 cells has reported IC_{50} values of 25 and 420 μM for recombinant α_1 and α_1/β GlyRs, respectively (Handford et al., 1996). In our studies, the IC_{50} value for picrotoxin inhibition of α_1/β GlyRs was increased to 1060 ± 313 μM compared to the IC_{50} value of 98 ± 41 μM obtained in the presence of the GlyR α_1 subunit only (p < 0.05, unpaired t-test). Our results are in agreement with previous data, which indicate that the presence of the GlyR β subunit decreases the sensitivity of the GlyR to picrotoxin inhibition. The 11-fold increase that we observed in the IC_{50} of α_1/β GlyRs compared to α_1 GlyRs was taken as an indicator of the successful transfection of the β subunit in our experiments and its efficient co-assembly with the GlyR α_1 subunit.

In addition, an electrophysiological study by Bormann et al. (1993) has reported that co-expression of the GlyR α_1 subunit with the GlyR β subunit in HEK 293 cells resulted in a 5-fold enhancement of glycine-evoked currents. In our studies, co-transfection of the α_1 GlyR subunit with the GlyR β subunit resulted in a 2-fold enhancement of glycine-
activated currents ($p < 0.05$, unpaired t-test). These data add further support of efficient co-transfection of the GlyR $\alpha_1$ and $\beta$ subunits in our studies.
5.3. FUTURE DIRECTIONS

By using the cytosolic tyrosine kinase, pp60<sup>C-src</sup> we have provided the first evidence for tyrosine phosphorylation of GlyRs at a specific site - at Tyr-413 of the GlyR β subunit. To follow this study, experiments should be carried out with a phosphotyrosine antibody to test if the GlyR wild type β subunit is tyrosine phosphorylated as opposed to β<sup>Y413F</sup> or the α<sub>1</sub> subunits expressed in HEK 293 cells. These experiments should be extended to hippocampal and spinal cord GlyRs, which were modulated by tyrosine phosphorylation in this study. The results of these studies should provide evidence as to the localization of tyrosine phosphorylation sites on GlyR subunits.

Also, mIPSCs should be recorded in spinal cord neurons in the presence of pp60<sup>C-src</sup> and heat-inactivated pp60<sup>C-src</sup> in order to investigate modulation of glycinergic synaptic activity by tyrosine phosphorylation.

Preliminary data in our lab indicate that GlyR-mediated currents are enhanced by insulin, a physiological ligand that binds to insulin receptors. Insulin receptors are receptor tyrosine kinases that are thought to exert their effects on cellular function through tyrosine phosphorylation. Since we have found that a cytoplasmic PTK regulate GlyR function in a subunit-dependent manner, it would be interesting to test whether the insulin effect is subunit-dependent as well. These studies should shed light into the site of action of insulin on the GlyR as well as the role of the β subunit and in specific, β subunit Tyr-413 on GlyR modulation by insulin.

The anaesthetic, propofol increases both α<sub>1</sub> and α<sub>1</sub>/β GlyR function (Pistis et al., 1997; Daniels & Roberts, 1998; Belelli et al., 1999). However, the effect of insulin on
the propofol-induced enhancement has never been investigated. These experiments should further clarify the mechanisms by which propofol enhances GlyR function.
6. CONCLUSIONS

The tyrosine kinase inhibitor, lavendustin A depressed glycine-evoked currents. On the contrary, the protein tyrosine kinase pp60c-src enhanced GlyR function or caused a reduction in the rundown of currents activated by glycine. The enhancing effect was abolished by lavendustin A. Pp60c-src did not affect glycine-evoked currents mediated by α1 GlyRs. However, pp60c-src reduced the rundown of α1/β GlyR-mediated currents. The effect was abolished when Tyr-413 on the GlyR β subunit was mutated to Phe-413. These observations suggest that the GlyR is modulated by tyrosine phosphorylation through the Tyr-413 residue of the GlyR β subunit.
7. REFERENCES


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