Strychnine sensitive glycine receptors depress hyperexcitability in rat dentate gyrus

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ABSTRACT

Previously we have shown that strychnine-sensitive glycine-gated chloride channels (GlyRs) are functionally expressed by CA1 pyramidal cells and GABAergic interneurons in mature rat hippocampal slices. We now report that glycine application to dentate granule cells and hilar interneurons recorded in acute slices from adolescent rats elicits a strychnine-sensitive current similar to glycine-mediated currents recorded in area CA1, indicating that GlyRs are also present on neurons in the dentate gyrus. This finding suggests that GlyRs have a widespread distribution in the hippocampal region. The physiological role of GlyRs in forebrain is unclear, but it is possible that these receptors mediate neuronal inhibition, like GABA_A receptors, and thus could be a novel target for antiepileptic therapy. Therefore, we tested the hypothesis that activation of inhibitory GlyRs could suppress neuronal hyperexcitability in dentate, a brain region vulnerable to epileptic activity. In whole-cell current clamp recordings of granule cells, we observed a membrane potential hyperpolarization followed by cessation of the action potential firing pattern in hyperexcitable slices induced by elevated extracellular K^+ or by blocking GABA_A receptors with bicuculline. The GlyR antagonist, strychnine, prevented the anti-epileptic effect of glycine. These results demonstrate that glycine acting at GlyRs elicits neuronal inhibition in dentate. Further, our findings suggest the possibility that these receptors could be a therapeutic target for the treatment of epilepsy.
INTRODUCTION

The dentate gyrus (DG) is a brain region highly susceptible to seizure activity (Parent and Lowenstein 2002; Scharfman 2000). Several lines of evidence suggest that loss of GABA-mediated inhibition in DG plays a role in the pathophysiology and pathology of epilepsy (Coulter 2000; Dalby and Mody 2001; Dudek et al. 2002; Sloviter 1996). Thus, the availability of additional or alternate inhibitory mechanisms would be useful since effective neuronal inhibition is required to maintain the normal excitatory balance for proper function of DG as well as other regions throughout the forebrain. Glycine-gated chloride channels (GlyRs) could subserve this role. In fact, accumulating evidence shows that strychnine-sensitive GlyRs are functionally expressed in many regions of developing and mature brain (Ye 2000; Sergeeva and Haas 2001) including hippocampus (Mori et al. 2002; Sergeeva and Haas 2001; Ye 2000; Ye et al. 1999; Yoon et al. 1998) where we have previously characterized the pharmacological properties of GlyRs expressed by CA1 pyramidal cells and GABAergic interneurons recorded in hippocampal slices (Chattipakorn and McMahon 2002). Interestingly, previous work in vivo has demonstrated that exogenous glycine application can depress seizure activity in an animal model of epilepsy (Cherubini et al. 1981) and can potentiate the antiepileptic effects of GABA_\text{A} receptor (GABA_\text{A}R) agonists (Seiler and Sarhan 1984a; Seiler and Sarhan 1984b). Therefore, activation of GlyRs either alone or in combination with GABA_\text{A}Rs may provide an alternative therapeutic approach for the better treatment of epilepsy.

An inhibitory role of glycine acting at GlyRs in DG has not been examined previously despite the possibility that these receptors may provide an important inhibitory mechanism in this highly excitable brain region. Therefore, in the present study, we used patch clamp electrophysiology to provide evidence that both granule cells and hilar interneurons express
functional GlyRs. Furthermore, we investigated whether GlyR activation can inhibit bursting activity elicited in DG slices. Our findings indicate that GlyRs are expressed by neurons in DG and their activation provides an inhibitory mechanism that has been overlooked previously.

METHODS

Electrophysiological recordings

Coronal hippocampal slices (400 $\mu$M) were prepared from 3-4 week old Sprague-Dawley rats using a high sucrose, low calcium aCSF solution containing (in mM): NaCl 85; KCl 2.5; CaCl$_2$ 0.5; Mg$_2$SO$_4$ 4; NaH$_2$PO$_4$ 1.25; NaHCO$_3$ 25; glucose 25; kynurenic acid 2, ascorbate 0.5 and saturated with 95% O$_2$/5% CO$_2$ (pH 7.4). Slices were maintained in a submersion chamber at room temperature in the high sucrose solution for 30 min before switching to a standard aCSF solution containing (in mM): NaCl 119, KCl 2.5, CaCl$_2$ 2.5, MgSO$_4$ 1.3, NaH$_2$PO$_4$ 1, NaHCO$_3$ 26, glucose 10, kynurenic acid 2, and saturated with 95% O$_2$-5% CO$_2$ (pH 7.4). For experiments, slices were placed in a submersion recording chamber and continually perfused with aCSF containing 2 mM kynurenic acid (voltage-clamp experiments only) at 2-3 ml/min and warmed to 28–30°C. Whole-cell voltage and current clamp recordings of visually identified granule cells and hilar interneurons were obtained using IR-DIC microscopy and standard recording techniques as previously described (Chattipakorn and McMahon 2002). The interneurons recorded in this study were limited to those cells located at the border between the granule cell layer and the hilus (Ribak and Seress 1983). Patch electrodes had resistances between 4-6 M$\Omega$ and for voltage-clamp recordings were filled with (in mM): CsCl 100; EGTA 0.6; MgCl$_2$ 5; ATP-Na$_2$ 2; GTP-Na 3; HEPES 40; and biocytin 0.4%, pH 7.2, 260-270 mOsm. In some recordings, 5 mM QX 314 was added to the internal solution to block voltage-dependent Na$^+$ channels. Series resistance was continuously monitored throughout the experiments and
recordings were terminated when there was a greater than 20% change. For current-clamp recordings of granule cells, 100 mM K+ gluconate was substituted for CsCl. These granule cells had an average input resistance of 373 ± 30 MΩ and resting membrane potential of 69 ± 1 mV (n=21). Recordings were terminated if there was a greater than 20% change in these parameters and if action potential amplitude was not overshooting. All slices were fixed in 4% paraformaldehyde following experiments and processed for biocytin staining of recorded neurons to confirm cell identity as a granule cell or hilar interneuron. Filled cells were visualized at the light level and images were reconstructed using camera lucida. Representative cells are shown in Fig 1B. Our visual identification of recorded cells using IR-DIC optics consistently matched the identity obtained with the biocytin fill.

**Drug Delivery**

All chemicals used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO). Agonists and antagonists were prepared as stock solutions and diluted to appropriate concentrations in the recording solution. To test for the functional expression of GlyRs by granule cells and hilar interneurons, glycine (300 µM) was applied by pressure ejection (3-6s) using a picospritzer with the drug containing pipet positioned 50-100 µm from the recorded cell. The method of glycine application for obtaining dose-response measurements was performed using a drug pipet connected to a valve system which permits switching between drug solutions (4-5 ml/min; complete bath exchange within 30 s) as previously (Chattipakorn and McMahon 2002). Dose-response data were normalized to the amplitude measured at 1 mM glycine and were fit to the Hill equation \( I = I_{max}/1 + EC_{50}/[\text{agonist}]^n \) using Origin 5.0 software (Chattipakorn and McMahon 2002). To test whether GlyR activation could depress neuronal hyperexcitability,
glycine (300 µM) was applied to granule cells recorded in whole-cell current clamp while perfusing the slices with high extracellular K⁺ (8.5mM) or 10 µM bicuculline to block GABA<sub>A</sub> receptor mediated inhibition.

**RESULTS**

**Glycine currents recorded in rat dentate gyrus**

To demonstrate that dentate neurons have functional GlyRs, we obtained whole-cell recordings of visually identified granule cells and hilar cells in slices prepared from 3-4 week old animals. Short pulses (3s) of glycine (300 µM) pressure-applied to both granule cells (n=11/11) and hilar cells (n=15/15) elicited inward currents (I<sub>gly</sub>; E<sub>Cl</sub> = 0 mV) that rapidly decayed to baseline following cessation of glycine application (Fig. 1A). Bath application of the GABA<sub>A</sub> antagonist, bicuculline (10µM; n=10), had no effect on I<sub>gly</sub>. In contrast, bath application of strychnine (1µM; n=10) reversibly depressed or abolished I<sub>gly</sub>, reaching maximal block within 8-10 minutes. A partial recovery was obtained following washout of the antagonist (20-30 minutes). The mean I<sub>gly</sub> elicited by 300 µM glycine (near EC<sub>50</sub> concentration) recorded from granule cells (500-1500 pA, 916±162 pA) and hilar cells (350-3000 pA, 1050±345 pA) were not significantly different (P=0.73). To confirm cell identity, recorded cells were filled with biocytin for post-hoc analysis. Fig. 1B shows camera lucida reconstructions of a typically recorded granule cell and hilar interneuron that responded to glycine with a strychnine-sensitive inward current.

To further characterize the sensitivity of GlyRs to glycine, we generated dose-response curves from both cell types (Fig. 2). Normalized dose-response relationships demonstrate that GlyRs expressed by granule cells (EC<sub>50</sub> = 360 µM and Hill coefficient n=1.2) and hilar cells (EC<sub>50</sub> = 370 µM and Hill coefficient n=1.6) respond similarly to glycine (Fig. 2B). Additionally,
we observed that $I_{\text{gly}}$ recorded from both cell types only partially desensitize during the 1 minute exposure to glycine (concentrations up to 1 mM, Fig. 2A) and reach a steady-state level. This is an important issue since the CSF glycine concentration can be elevated for prolonged periods following seizures and hypoxic episodes (Andine et al. 1991; Castillo et al. 1996; Sherwin 1999). Our data suggest therefore that under these pathological conditions, GlyR-mediated inhibition will remain functional, although at a reduced level.

**GlyR activation inhibits neuronal hyperexcitability.**

To test the antiepileptic potential of GlyR activation, glycine was applied to hyperexcitable dentate slices. Hyperexcitability was induced either by increasing extracellular $[K^+]_o$ (8.5 mM), which elicits spontaneous discharges in granule cells resembling epileptic spikes (Chamberlin et al. 1990) ($n=6$) or by blocking GABA$_A$ mediated inhibition with 10 $\mu$M bicuculline ($n=6$). GABA$_A$R blockade alone does not elicit spontaneous discharges, therefore we applied depolarizing current (usually 5-6 mV) to bring cells to action potential threshold. In the majority of granule cell recordings ($n=8/12$) bath application of 300 $\mu$M glycine hyperpolarized the membrane potential (8 ± 2 mV in 8.5 mM $[K^+]_o$ and 9 ± 2 mV in bicuculline) and completely interrupted the hyperexcitable firing pattern (Fig. 3). In the remaining 4 cells (2 recorded in 8.5 mM K+ and 2 in bicuculline) action potential firing was considerably depressed (26±6% of control; 120±6 spikes/min in control vs 33±6 spikes/min in glycine) (data not shown). The decrementing action potential amplitude observed in some of our experiments has been previously reported by others (Pan and Stringer 1996; Schweitzer et al. 1992) and is not related to deterioration of cell health, since no changes were observed in membrane input resistance or membrane potential. Thus, this effect is likely a result of decreased ionic driving force resulting from the prolonged high rate of action potential firing that is occurring over tens of minutes to an
hour. Co-application of strychnine (1µM) and glycine prevented the antiepileptic effect of glycine without changing the firing rate (n=4). These data suggest that the anti-bursting activity of glycine was mediated through the activation of GlyRs. Furthermore, the ability of GlyR activation to depress action potential generation in the presence of GABA<sub>A</sub>R blockade demonstrates that GlyRs can provide effective inhibition when GABAergic inhibition is compromised.

**DISCUSSION**

This study represents the first report of strychnine-sensitive glycine currents recorded from granule cells and hilar interneurons, indicating the expression of glycine-gated chloride channels (GlyRs) in the dentate gyrus. In addition, our data show clearly that glycine, via GlyR activation, depresses granule cell bursting activity under hyperexcitable conditions, even when GABA<sub>A</sub>R inhibition is compromised. These findings imply that GlyR-mediated inhibition is an alternative or additional inhibitory mechanism to the familiar GABA<sub>A</sub>R-mediated mechanism and that GlyR activation is capable of controlling the activity of excitatory circuits in DG. Furthermore, these data suggest the strong possibility that GlyRs could be an important target for antiepileptic therapy. Surprisingly, these receptors have not received strong consideration previously despite the fact that a few studies have shown glycine to be antiepileptic in some animal models of epilepsy (Cherubini et al. 1981; Seiler and Sarhan 1984a) and to potentiate the antiepileptic effect of GABA<sub>A</sub>R agonists (Seiler and Sarhan 1984a). Therefore, we propose that activation of GlyRs alone or in combination with GABA<sub>A</sub> receptors may provide an alternative therapeutic approach for the better treatment of epilepsy.

Our finding that both principal cells and interneurons in DG express GlyRs, consistent with our studies in the CA1 region (Chattipakorn and McMahon 2002), indicates that these
receptors are likely of fundamental importance in the control of neuronal excitability in this region. Because both excitatory and inhibitory neurons respond to glycine, the effect of GlyR activation on dentate circuitry will entirely depend upon when then the receptors are activated and on what cell type. For example, if these receptors are selectively activated on granule cells, the output of the dentate will be decreased. However, selective activation of GlyRs on interneurons could cause disinhibition of the granule cells, thereby increasing the output of the dentate. The source of glycine and other GlyR agonists (taurine) (Mori et al., 2002) in forebrain, including dentate, is not presently known. However, synaptosomes prepared from hippocampus release glycine via both Ca^{2+}-dependent and independent mechanisms (Engblom et al. 1996), suggesting both vesicular and transporter-mediated release. Knowledge of the specific source of GlyR agonists and under what conditions these agonists are released, will shed light on the role of these receptors in controlling dentate circuitry.

Since our study was performed in acutely prepared slices from 3-4 week old rats, our data demonstrate that GlyR expression by dentate neurons is not transient and limited to early development but persists through mature developmental stages. The glycine induced inhibition of action potential firing occurs in the absence of N-methyl-D-aspartate receptor (NMDAR) blockade suggesting that increases in the CSF glycine concentration, particularly when excitability is high, can elicit neuronal inhibition, thus overcoming any potential increase in glycine-mediated NMDAR-induced excitability. The physiological role of GlyRs in the hippocampal formation is currently unknown and whether these receptors mediate fast synaptic inhibition as they do in spinal cord and brainstem has yet to be documented. In cerebellum, GlyRs are involved in synaptic inhibition following the vesicular release of glycine, while in cortex GlyRs appear to be activated by non-synaptically released taurine (Flint et al. 1998)
suggesting an extrasynaptic location of GlyRs. To increase our understanding of these understudied receptors in hippocampus, future investigations are needed to determine the synaptic versus extrasynaptic location of GlyRs and the conditions under which these receptors are activated.
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FIGURE LEGENDS

FIG. 1: GlyRs are expressed by granule cells and hilar interneurons in rat dentate gyrus slices. A, $I_{\text{gly}}$ induced by a 3s pressure application of 300µM glycine (arrow) is completely unaffected by 10µM bicuculline but reversibly blocked with 1µM strychnine. Cells were held at -70 mV and recorded with a CsCl pipette solution. B, Camera lucida reconstructions of a typically recorded granule cell and hilar interneuron that express strychnine-sensitive GlyRs.

FIG. 2: Glycine dose-response relationship. A, top: representative traces of $I_{\text{gly}}$ recorded from a granule cell and a hilar interneuron at increasing glycine concentrations. Glycine was applied via a drug pipette (see Methods) for duration of 1 min and at increasing concentrations as noted. Glycine was reapplied at 1 minute intervals. B, bottom: Plot compares glycine dose-response curves generated from granule cell (n=6) and hilar interneuron (n=6) recordings. Currents were normalized to the peak amplitude of the response at 1 mM glycine. Each point is the mean ± standard error. Dose–response data were fit with the Hill equation (see Methods). Cells were held at -70 mV and recorded with a CsCl pipette solution.

FIG. 3: Bath application of 300 µM glycine can inhibit or disrupt bursting behavior recorded from granule cells induced by (A) 8.5 mM extracellular $[K^+]_o$ (n=6) or (B) 10 µM bicuculline (n=6). Average membrane potentials recorded in 8.5 mM extracellular $[K^+]_o$ were 43 ± 1 mV and in 10 µM bicuculline were 52 ± 4 mV compared to 69 ± 1 mV in control conditions. Depolarizing current injection was required to bring cells to action potential threshold when recorded in bicuculline. Strychnine (1 µM) reverses the antiepileptic effects of glycine.
application. Each trace was obtained from a different cell. Cells were recorded with a K\(^+\) gluconate pipet solution and were held at their resting membrane potential.


A  granule cell
  glycine
  control  bic  wash
  control  stry  wash

B  granule cell
  molecular layer
  granule cell layer
  hilus

hilar cell
  glycine
  control  bic  wash
  control  stry  wash

1s  250 pA

100 μM
A granule cell

B  

granule cells  
EC$_{50}$ = 0.37 mM, n=1.55

hilar cells

EC$_{50}$ = 0.36 mM, n=1.27

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granule cells

hilar cells
A 8.5 mM [K⁺],

B 10 µM bicuculline