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Research report

Glycine-induced CA1 excitotoxicity in the rat hippocampal slice

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Abstract

We evaluated the effects of glycine exposure upon CA1-evoked response in the rat hippocampal slice. Exposure to 10 mM glycine for 16 min produced rapid neuronal firing and increased orthodromic population spike (PS), followed by loss of CA1 neural transmission. Upon recovery, CA1 orthodromic and antidromic PS regained a mean of only $12 \pm 6\%$ and $8 \pm 5\%$, of initial amplitude. The electrophysiological pattern of glycine injury was similar to the excitotoxic damage produced by 8 min exposure to sodium glutamate (9 mM). L-Histidine, an inhibitor of glycine transport, exacerbated glycine-induced injury, just as dihydrokainic acid, a glutamate transport inhibitor, exacerbated glutamate-induced injury. The anticonvulsant felbamate (1.3 mM), as well as 100 μM zinc chloride, provided excellent protection from glycine-induced injury: 7-clorokynurenic acid appeared to be toxic. Blockers of the NMDA-associated ionic channel and methyl arginine prevented loss of neural transmission, but did not prevent accompanying hyper-excitability. Only 10 mM magnesium sulfate provided full protection against 9 mM glutamate exposure. Perfusion with low calcium ACSF protected against both glycine- and glutamate-induced injury. Thus, exposure to glycine resembled the excitotoxic effects of glutamate, but showed a different profile of protection. These results suggest that glycine elevations, as occur under physiologic and pathologic conditions, may modulate neuronal activity.

Keywords: Glycine; Glutamate; CA1

1. Introduction

The neuro-modulatory effects of the neutral amino acid, glycine, were first demonstrated in spinal neurones where glycine application was shown to produce neuronal hyperpolarization and inhibition [3,35]. These glycine inhibitory effects were later found to be blocked by strychnine [2], leading to the identification of strychnine-sensitive glycine receptors located predominantly in the hindbrain [39].

In addition to these glycine inhibitory receptors, excitatory effects of glycine have more recently been demonstrated in rostral brain regions. Glycine has been shown to facilitate neuronal excitation through binding at strychnine-insensitive glycine receptors [1] in the distribution of the NMDA macro-complex [11]. Within that receptor complex, glycine allosterically modulates activation of the NMDA-associated ionic channel [38], by promoting glutamate activation of the NMDA receptor [13,24].

The physiological significance of glycine binding at these sites, however, has been unclear. Although glycine appears to be an absolute requirement for activation of the NMDA-associated ionic channel [15], the NMDA-associated glycine binding site appears to be fully saturated at a glycine concentration of less than 1 μM [13]. If interstitial glycine concentrations, which have been found to be near 10 μM [8,7], were reflective of glycine concentration within the synaptic cleft, the NMDA-associated glycine site would most likely be occupied at all times. Under these conditions, glycine would produce a constant permissive effect for NMDA receptor activation, and no further activation would be expected from elevations in extracellular glycine concentrations occurring as the result of normal or pathological neuronal activity.

However, recent evidence suggests that glycine may act as a neurotransmitter in forebrain, as well as more caudal brain regions. Calcium-dependent glycine release has been demonstrated in rat cortical and hippocampal synaptosomes [17,23]. Additionally, a high-affinity sodium-dependent uptake system for glycine has also been identified in rat hippocampal slices [5],

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which may provide close regulation of glycine concentrations at the level of the synaptic cleft. If the NMDA-associated glycine site were to be left unoccupied, elevations in extracellular glycine concentration would then most likely modulate glutamate activation of the NMDA receptor producing changes in neuronal activity. To further investigate this question, we used the rat hippocampal slice preparation to examine the effect of glycine exposure upon the electrophysiological function of CA1 pyramidal cells.

2. Materials and methods

Fifty-two male Sprague–Dawley rats, 185–415 g were briefly anesthetized with halothane and decapitated. As previously described [30], the brain was removed and hippocampi were dissected in cold artificial cerebrospinal fluid (ACSF), which was composed of (in mM): NaCl, 126; KCl, 4.0; KH_2PO_4, 1.4; MgSO4, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; and glucose, 4.0; with a pH of 7.4 and saturated with 95% O_2/5% CO_2. Transverse slices, 475 μ thick, were cut using a McIlwain tissue chopper and placed into a recording well which was perfused at a rate of 2 ml/min, with upper slice surfaces submerged beneath approximately 2 mm ACSF, and temperature maintained at 34.0°C.

After 60 min, the orthodromic CA1-evoked response of each slice was elicited by stimulation of CA3 Schaffer collaterals, using a bipolar electrode with square wave pulses of 40 μs duration. Responses were recorded with a 4–5 MΩ tungsten electrode, placed within the CA1 pyramidal layer. Stimulating current and recording electrode depth were adjusted until a maximal CA1 PS amplitude was obtained. This generally occurred at a slice depth of approximately 200 μm. CA1 PS amplitude, CA1 field EPSP slope, and CA3 fiber volley response were analyzed [25]. CA1 antidromic responses were elicited by stimulation over the alveus. Only slices with orthodromic and antidromic CA1 PS of 3.0 mV amplitude or greater were utilized for further testing. PS amplitude was assessed as a sum of the negative and positive potential. No significant difference in mean initial CA1 PS amplitude were found by Wilcoxon rank sums between slices of different treatment groups. Orthodromic responses were monitored every 30 s through the course of each experiment. In prolonged recordings, orthodromic responses were assessed once per hour after the first hour of recovery for a total of 8 h monitoring. Antidromic responses were assessed at the beginning and end of each experiment.

Glycine, glutamate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid (AMPA), kainic acid and *N*-methyl-D-aspartate (NMDA) were dissolved in perfusion ACSF. In pharmacologic protection trials, glycine perfusion was continued for 16 min. Glutamate receptor agonists (glutamate, NMDA, AMPA and kainic acid) were perfused for 8 min. Following glycine and glutamate agonist exposure, original ACSF was perfused for a 60 min recovery period. Because recovery after AMPA exposure occurred more slowly, a 90 min recovery period was given following this exposure. Dihydrokainic acid and L-histidine were introduced simultaneously with glutamate and glycine. Other pharmacological agents and ionic modifications were introduced 30 min prior to glycine and glutamate agonist exposure, and continued for the first 15 min of recovery, for a total treatment time of 61 mis. With AMPA exposures, tested pharmacological agents were continued through the first 60 min of recovery.

AMPA and 7-chlorokynurenic acid were purchased from Tocris Inc. Glycine and additional 7-chlorokynurenic acid were obtained from Research Biochemicals Inc. Glycine was analyzed by HPLC [12] and no contamination by other amino acids was detected. Felbamate was supplied by Carter Wallace Inc. All other chemicals were purchased from Sigma.

All treatment groups utilized *n* values of 4 or greater. PS injury

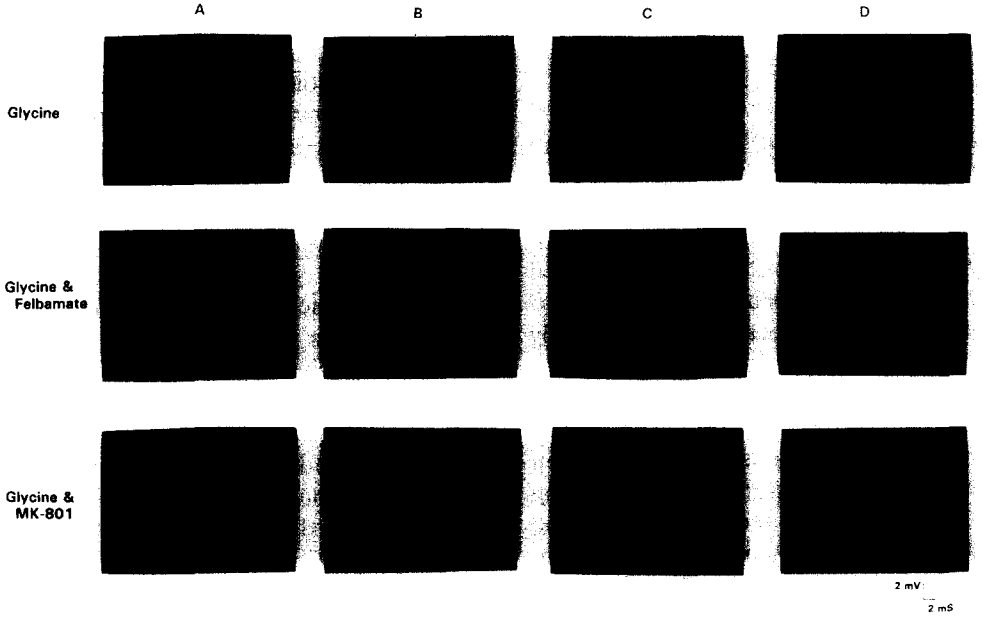

Fig. 1. Glycine exposure induces acute injury to CA1 neurons in rat hippocampal slices. Initial CA1 orthodromic evoked responses are shown in panel A. Tracings in panel B demonstrate effect of 30 min perfusion with ACSF alone (upper row), 1.3 mM felbamate (middle row) and 32 μM MK-801 (lower row). Panel C illustrates the effect of 8 min glycine exposure. The upper tracing of panel D illustrates the response after exposure to 10 mM glycine and 60 min recovery. The upper panel with glycine alone shows severe loss of PS amplitude. The middle panel shows good protection against this injury with felbamate, and the lower panel shows partial protection with potentiation with hyper-excitability and increased synaptic efficiency with MK-801.

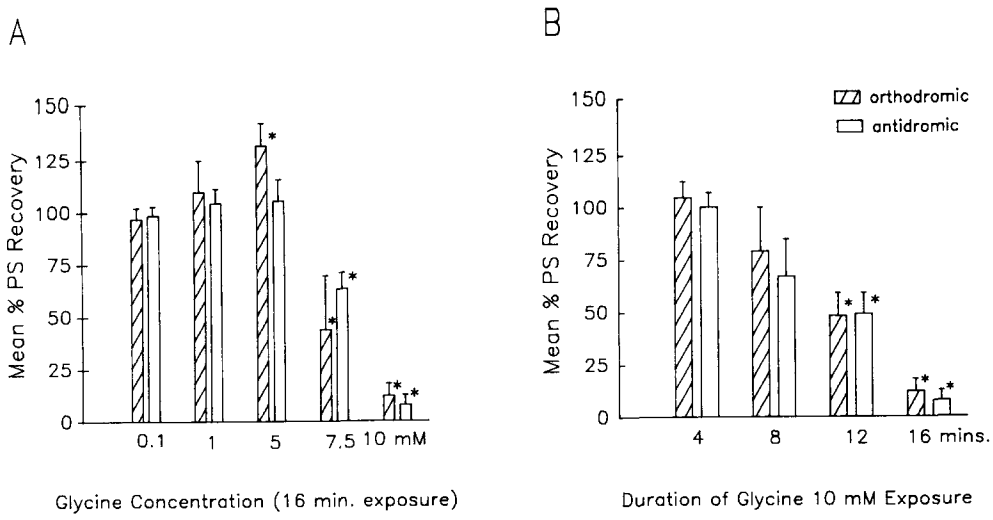

Fig. 2. Acute glycine-induced injury in CA1 is concentration- and time-dependent. Vertical bars indicate mean ± S.E.M. PS recovery after exposure to varying glycine concentrations (A) and durations (B). The EC_{50} for 8 min glycine exposure was 7.2 mM for loss of orthodromic PS, and 7.8 mM for loss of antidromic CA1 PS amplitude. The effective duration of 10 mM glycine exposure to produce 50% loss (ED_{50}) of evoked response amplitude was 11.8 for both orthodromic and antidromic PS. * $P < 0.05$.

was defined as loss of PS amplitude after recovery. Evoked potential percent recovery was calculated by dividing the final value by the initial value, multiplied by 100. In neurotoxic trials of glycine and glutamate agonists, evoked responses were compared to baseline values using Student's *t*-test. Other comparisons were made using the Wilcoxon rank-sum test.

3. Results

3.1. Glycine-induced CA1 injury

Rat hippocampal slices exposed to glycine showed evidence of neuronal hyper-excitability and severe, rapidly evolving neuronal injury with irreversible loss of CA1 PS amplitude (Fig. 1). Perfusion of 10 mM glycine produced rapid neuronal firing and a transient increase in PS, which preceded PS loss. Upon recovery from glycine exposure for 16 min, CA1 orthodromic PS regained only a mean 12 ± 6% of initial amplitude (Fig. 2). CA1 antidromic PS was assessed to verify that loss of orthodromic PS was a consequence of neuronal cell body dysfunction, rather than synaptic depression. Unlike orthodromic CA1 PS, the generation of the antidromic potential is not dependent upon synaptic activation. With glycine exposure, antidromic PS was also lost, with only 8 ± 5% recovery of initial amplitude seen (Fig. 2).

To confirm that loss of CA1 activity after glycine exposure represented long-lasting neuronal injury, several slices were given prolonged monitoring over the

Table 1
CA1 orthodromic evoked responses during 10 mM glycine exposure

	Initial potential	% of initial potential after 30′ felbamate	% of initial potential after 16′ glycine	% of initial potential after 60′ recovery
Fiber volley				
Glycine (10 mM)	2.0 ± 0.4 mV	–	72 ± 12	101 ± 6
Glycine + felbamate (1.3 mM)	1.9 ± 0.5	96 ± 3	70 ± 10	96 ± 7
EPSP				
Glycine	0.7 ± 0.2 mV/mS	–	0	0
Glycine + felbamate	0.6 ± 0.1	78 ± 16	0	81 ± 12 *
Population spike				
Glycine	7.3 ± 0.9 mV	–	0	12 ± 6
Glycine + felbamate	6.1 ± 0.4	90 ± 4	0	97 ± 8 *

Values indicate mean response ± S.E.M. of CA3 fiber volley amplitude, CA1 EPSP slope and CA1 PS amplitude during 16 min exposure to 10 mM glycine and recovery. CA1 EPSP slope and orthodromic PS, which reflect activity of dendrites and neuronal cell bodies, show greatest vulnerability to glycine exposure, while fiber volley response, which reflects activity of CA3 axons, shows relative resistance to glycine. Treatment with felbamate at a concentration of 1.3 mM produces little effect when given alone for 30 min, and has little effect upon loss of EPSP and PS during glycine exposure, but provides good protection of both EPSP slope and orthodromic PS amplitude upon recovery. Initial potentials of slices treated with glycine, or glycine with felbamate, did not differ significantly.
* $P < 0.05$.

course of 8 h. In slices exposed to 10 mM glycine for 16 min, all recovery of evoked function occurred solely within the first hour of recovery. Beyond that time, only gradual deterioration of PS was seen, and after 4 h PS orthodromic response was lost altogether. Assessment of antidromic PS showed no response present after 8 h. This loss of PS activity appeared to be the result of glycine exposure rather than a consequence of slice deterioration, since slices exposed only to ACSF and similarly monitored for 8 h demonstrated little change in response, exhibiting a final mean orthodromic PS of 103 ± 2% of initial amplitude.

Exposure to glycine produced greatest effect upon post-synaptic elements. In addition to the loss of CA1 orthodromic PS, CA1 EPSP slope decreased to zero with glycine exposure (Table 1), and showed no recovery over the 8 h of subsequent recording. In comparison, axonal function as indicated by CA3 fiber volley response, showed relative resistance to glycine. Fiber volley amplitude was transiently lost during glycine perfusion but returned after this exposure to 101 ± 6% of initial amplitude at 1 h of recovery. After this time, fiber volley amplitude gradually decreased, with a mean 25 ± 10% of initial amplitude observed after 8 h of monitoring.

Consistent with reports of the production of long-term potentiation in CA1 with exposure to 10 mM glycine for 10 min [26], perfusion with 5 mM glycine for 16 min led to a significant increase in PS amplitude with recovery of CA1 orthodromic PS amplitude to 132 ± 11% (Fig. 2). This increase in response to CA1 orthodromic PS appeared to be the product of greater synaptic efficiency, since EPSP slope also showed similar increases. After 1 h of recovery, EPSP slope had increased to a mean 145 ± 33%, while no significant increase was observed in antidromic CA1 PS with 105 ± 10%, or fiber volley amplitude with 106 ± 6% recovery.

Glycine-induced CA1 injury showed several similarities with glutamate-induced injury in CA1. Both produced rapid loss of the orthodromic CA1 PS. This occurred in a mean 4.2 ± 0.8 min with exposure to 10 mM glycine, and in 2.8 ± 0.3 min with 9 mM sodium glutamate. In the case of glycine, this collapse of the PS was preceded by a small consistent increase in PS amplitude. Exposure to glycine and glutamate also produced rapid neuronal firing indicative of increased neuronal excitability. Although no spontaneous CA1 neuronal activity was detected at baseline, rapid single-unit firing began within 3 min of the onset of 10 mM glycine or 9 mM glutamate exposure. This neuronal firing was similar to that seen with exposure to the glutamate receptor sub-type agonists NMDA (20 μM), AMPA (25 μM) and kainic acid (65 μM), producing similar acute CA1 injury [32].

Glutamate exposure also produced a loss of neural transmission, with an electrophysiological pattern similar to that seen with glycine. Following 9 mM sodium glutamate exposure for 8 min and 1 h of recovery, CA1 orthodromic and antidromic PS amplitudes regained a mean of 24 ± 7% and 23 ± 6% of initial amplitude (Fig. 3). Near equal vulnerability to glutamate-induced injury was seen for dendritic and neuronal cell body function, with an EC_{50} for loss of CA1 orthodromic PS of 7.1 mM, and an EC_{50} for CA1 antidromic PS loss of 7.2 mM observed (Fig. 3). Similarly, the EC_{50} for glycine-induced PS loss was found to be 7.2 mM and 7.8 mM (Fig. 2). With both glycine and glutamate, loss of dendritic and neuronal cell body function developed concurrently, although injury from glycine exposure

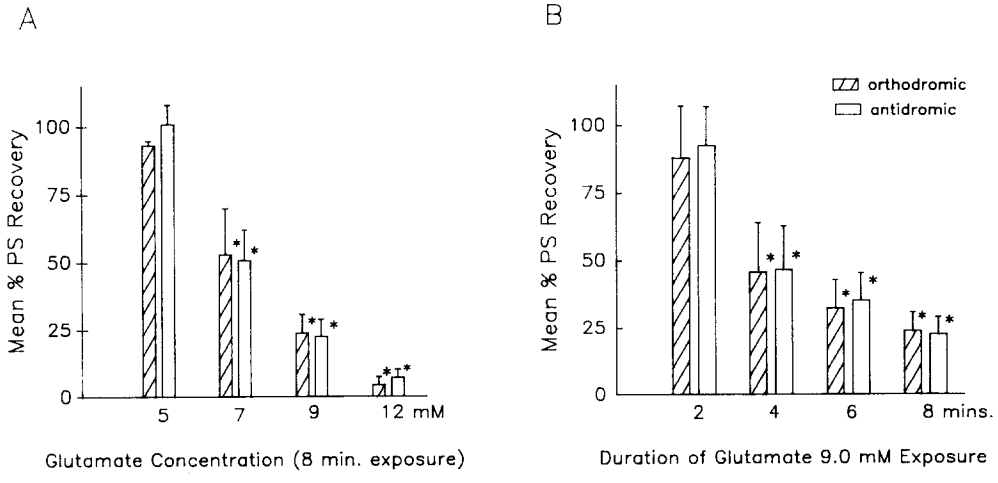

Fig. 3. Acute glutamate-induced injury in CA1 is concentration- and time-dependent. Vertical bars indicate mean ± S.E.M. PS recovery with varying sodium glutamate concentrations (A) and durations (B). The effective duration of 10 mM glycine exposure to produce 50% loss (ED_{50}) of EC_{50} for 8 min glutamate exposure was 7.1 and 7.2 mM for loss of orthodromic and antidromic CA1 PS amplitude. The ED_{50} of 9 mM sodium glutamate exposure was 3.6 and 3.8 min for loss of orthodromic and antidromic PS amplitude. * $P < 0.05$.

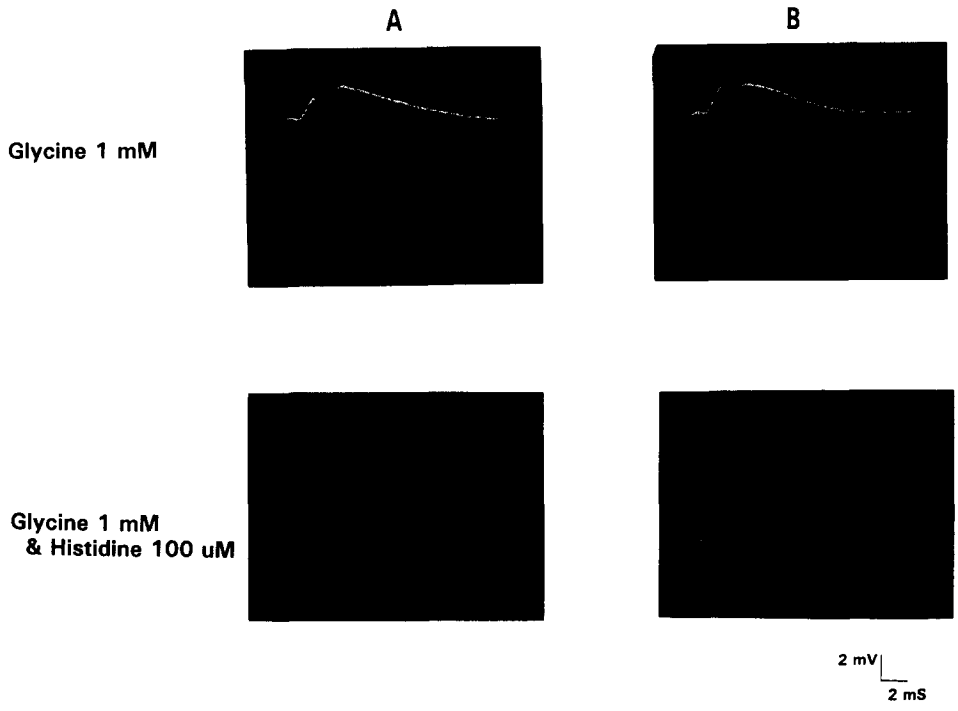

Fig. 4. The glycine uptake inhibitor, L-histidine, exacerbates glycine-induced CA1 injury. Initial CA1 orthodromic PS responses are shown in tracings of panel A. Tracings in panel B demonstrate effect of exposure for 8 min to 1 mM glycine followed by 60 min recovery with original ACSF (upper row), or 8 min exposure to 1 mM glycine with 100 μM L-histidine with recovery (lower row).

occurred with a longer overall time–course than glutamate-induced injury. The effective duration to produce 50% loss in CA1 PS amplitude upon recovery (ED_{50}) for 9 mM glutamate exposure was 3.6 min for orthodromic PS injury and 3.8 min for antidromic PS injury. This compared to an ED_{50} for 10 mM glycine exposure of 11.8 min for both orthodromic and antidromic PS injury. Thus, the evolution of neuronal injury occurred three-times faster with glutamate than with glycine exposure.

Similar to the effect of 10 mM glycine exposure, EPSP slope showed no recovery after 9 mM glutamate exposure, while CA3 fiber volley response showed relative resistance to this injury with a recovery of 74 ± 13% of initial amplitude.

Application of uptake inhibitors exacerbated neu-

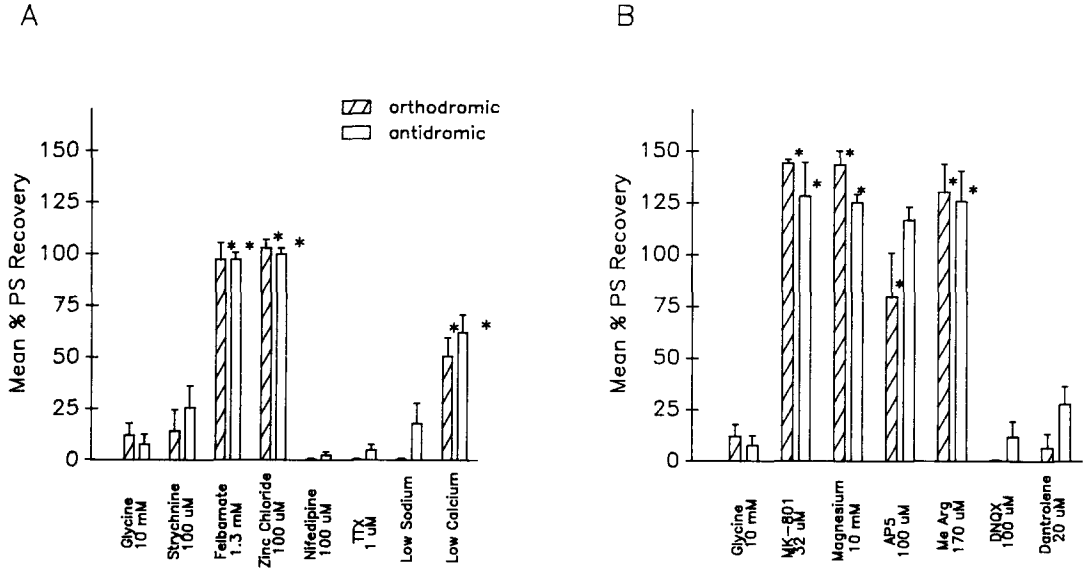

Fig. 5. Protection from glycine-induced CA1 injury with felbamate, NMDA antagonists and low calcium ACSF. Vertical bars indicate mean ± S.E.M. orthodromic and antidromic recovery after exposure to 10 mM glycine for 16 min. * $P < 0.05$.

ronal injury induced by both glycine and glutamate. Exposure to 5 mM glutamate alone for 8 min produced little evidence of injury, with CA1 PS orthodromic and antidromic PS recovering to a mean 93 ± 2% and 101 ± 7%. However, the co-application of 100 μM dihydrokainic acid, a glutamate transport inhibitor [14], significantly increased neuronal injury, and yielded orthodromic and antidromic PS recoveries of only 28 ± 16% and 19 ± 16%, of initial amplitude. This injury did not appear to be due to any toxicity from dihydrokainic acid, since perfusion of this compound alone produced essentially no change, with orthodromic and antidromic PS recovering to 100 ± 0% and 102 ± 4%.

Glycine-induced injury showed a similar pattern of exacerbation with co-application of L-histidine, a glycine uptake inhibitor [5] (Fig. 4). Exposure to 1 mM glycine for 16 min was non-lethal and produced orthodromic and antidromic recoveries of 110 ± 15% and 104 ± 7%. However, the addition of 100 μM L-histidine, during 1 mM glycine exposure, reduced orthodromic and antidromic PS recovery to only 6 ± 3% and 7 ± 4%. This reduction in PS amplitude did not appear to be the consequence of any toxic effect of L-histidine, since perfusion of an even greater L-histidine concentration of 10 mM for 16 min produced very little effect, with orthodromic and antidromic PS recovering to 108 ± 10% and 102 ± 4% of initial amplitude.

The injury produced by glycine in CA1 did not appear to be mediated by strychnine-sensitive mechanisms. When 100 μM strychnine was given with 10 mM glycine, no significant protection was found, and orthodromic and antidromic PS recovered to only 14 ± 10% and 26 ± 11% (Fig. 5). Strychnine 100 μM given alone had little effect with orthodromic and antidromic PS recovering to 115 ± 1% and 93 ± 2%, of initial amplitude.

Felbamate, however, provided excellent protection against glycine-induced hyper-excitability and neuronal injury (Fig. 1). This anticonvulsant displaces 5,7-dichlorokynurenic acid [18], a highly specific ligand of the NMDA-associated glycine binding site. With 1.3 mM felbamate treatment during 10 mM glycine exposure, CA1 orthodromic and antidromic PS recovered to 97 ± 8% and 97 ± 3% (Fig. 5). This protection was concentration-dependent, with a felbamate EC_{50} of 1.04 and 1.06 mM found for orthodromic and antidromic PS protection against 10 mM glycine exposure (Fig. 6). Felbamate also preserved orthodromic PS configuration, and prevented secondary population spike generation (Fig. 1). Felbamate, however, did not prevent PS disappearance occurring during glycine application, nor did it prevent rapid single-unit firing during glycine exposure. This recovery, despite the persistence of rapid neuronal firing, suggests that felbamate protection is not mediated through an anticonvulsant effect.

Felbamate protection was specific for glycine-induced injury and did not extend to glutamate-induced injury. Orthodromic and antidromic CA1 PS recovered to only 11 ± 7% and 22 ± 5% with 1.3 mM felbamate treatment during 9 mM sodium glutamate exposure for 8 min. Additionally, no significant felbamate protection was seen against the acute injury produced by glutamate receptor subtype agonists. Felbamate 1.3 mM given during exposure to 20 μM NMDA, 25 μM AMPA or 65 μM kainic acid for 8 min produced orthodromic PS recoveries of only 28 ± 19% 3 ± 2%, and 3 ± 3%, with antidromic recoveries of 23 ± 11%, 18 ± 5%, and 15 ± 4%. These results are comparable to previous findings where similar treatment with NMDA, AMPA and kainic acid alone for 8 min produced CA1 PS recoveries of less than 25% [32]. To help exclude subtle felbamate protection against NMDA injury, slices were also treated with a higher felbamate concentration of 1.8 mM and given a shorter duration exposure to 20 NMDA for 6 min. Here again no felbamate protection was seen and orthodromic PS amplitude recovered to only 10 ± 7%.

Similar to felbamate, zinc chloride also afforded excellent protection from injury induced by exposure to 10 mM glycine for 16 min (Fig. 3). With 100 μM zinc chloride, orthodromic and antidromic CA1 PS recovered to 103 ± 4% and 100 ± 3%. As with felbamate, zinc chloride preserved PS waveform configuration and did not prevent rapid single unit firing. Also similar to felbamate, zinc did not protect against 20 μM NMDA exposure, yielding orthodromic and antidromic PS recovery of 0% and 10 ± 5%.

Although excellent protection against glycine-induced injury was seen with felbamate and zinc chloride, comparable protection was not seen with 7-chlo-

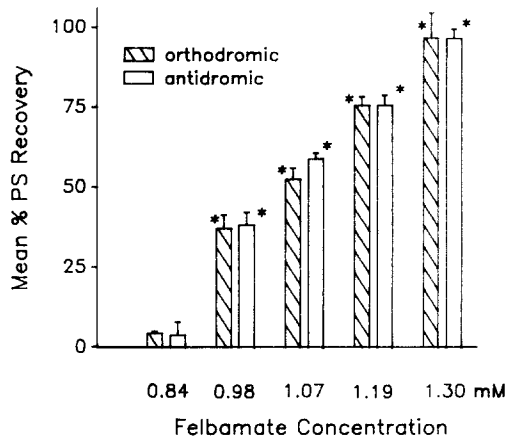

Fig. 6. Felbamate protection against glycine-induced CA1 injury is concentration-dependent. Vertical bars indicate mean ± S.E.M. orthodromic and antidromic recovery after exposure to 10 mM glycine for 16 min with varying concentrations of felbamate. The EC_{50} for recovery of orthodromic and antidromic CA1 PS amplitude against 10 mM glycine exposure was 1.04 mM and 1.06 mM felbamate. * $P < 0.05$.

rokynurenic acid. This compound which serves as an antagonist for the NMDA-associated glycine binding site [9], appeared to be toxic in this preparation. Exposure to 50 μM 7-chlorokynurenic acid alone for 61 min yielded CA1 orthodromic and antidromic PS recoveries of only 57 ± 16% and 40 ± 11%. This toxicity may have been the cause of the relatively poor response to 10 mM glycine with 7-chlorokynurenic acid, where 5, 10, 25, and 35 μM 7-chlorokynurenic acid produced only modest evidence of protection, with 0%, 34 ± 22%, 38 ± 32% and 40 ± 29% recoveries of orthodromic PS amplitude seen. Antidromic recovery was equally poor for 5, 10, 25 and 35 μM 7-chlorokynurenic acid, yielding recovery of 9 ± 3%, 39 ± 13%, 56 ± 35%, and 49 ± 33%, respectively. Despite reports of 7-chlorokynurenic acid protection against NMDA in dissociated neuronal cultures [19,22] and with intrastriatal injection [6,29], 10 μM 7-chlorokynurenic acid produced no significant protection against 20 μM NMDA exposure for 8 min, with CA1 orthodromic and antidromic PS amplitude recovering to only 27 ± 22% and 30 ± 21% of initial amplitude. No difference in response was seen with 7-chlorokynurenic acid from different sources.

Cycloleucine, a weaker antagonist of the NMDA-associated glycine site [34], was also evaluated for glycine protection. As opposed to 7-chlorokynurenic acid, little evidence of toxicity was observed with this compound. Given alone at a concentration of 500 μM for 61 min, cycloleucine produced orthodromic and antidromic CA1 PS recoveries of 110 ± 4% and 108 ± 2%. Cycloleucine, however, failed to protect against 10 mM glycine exposure, with 50, 100, 500 and 1,500 μM cycloleucine producing orthodromic PS recoveries of only 21 ± 5%, 24 ± 10%, 38 ± 25% and 44 ± 17%, and respective antidromic recoveries of 3 ± 3%, 13 ± 8%, 0% and 7 ± 4%.

The NMDA antagonist, MK-801, which blocks the NMDA-associated ionic channel, provided partial protection against glycine-induced neuronal injury (Fig. 5). After recovery from 10 mM glycine treatment with 32 μM MK-801, evidence of neuronal hyper-excitability was seen with the orthodromic PS increasing to 128 ± 16%. Orthodromic CA1 PS amplitude showed an even greater increase to 144 ± 2% of initial amplitude, in contrast to the full protection against NMDA-induced injury of similar degree produced by this same MK-801 concentration [32]. Magnesium (10 mM) provided nearly identical glycine protection to that of MK-801 with CA1 orthodromic and antidromic PS recovering to 143 ± 7% and 125 ± 4%. Likewise, methylarginine at a concentration of 170 μM, which blocks nitric oxide production as may be generated by NMDA receptor activation, produced orthodromic and antidromic recoveries of 130 ± 13% and 126 ± 15% after exposure to 10 mM glycine injury. All NMDA antagonists tested, including competitive antagonists and channel blockers, as well as methylarginine, abolished rapid unit firing during glycine exposure. The NMDA competitive antagonist, DL-2-amino-5-phosphonopentanoate (AP5; 50 μM), which also allosterically inhibits glycine binding at the NMDA-associated glycine site [21], provided partial protection against glycine exposure, yielding orthodromic and antidromic recoveries of 81 ± 15% and 83 ± 15%. DL-2-amino-7-phosphonoheptanoate (AP7; 50 μM), which inhibits this glycine binding to a much lesser extent, afforded no protection against glycine exposure, and produced orthodromic and antidromic recoveries of 21 ± 10% and 20 ± 9%. In contrast to this differential antagonism against glycine, both AP5 and AP7 at 50 μM provided good protection against exposure against 20 μM NMDA for 8 min. This NMDA exposure produces severe neuronal injury in this model with CA1 orthodromic and antidromic PS, recovering to only 8 ± 5% and 2 ± 2% [32]. With 50 μM AP5 treatment during NMDA exposure, PS recoveries improved to 111 ± 10% and 104 ± 8%. Similarly, treatment with (50 μM) AP7 improved PS recoveries to 98 ± 1% and 99 ± 6%.

Mild protection against glycine injury was also seen with the perfusion of ACSF without added calcium. This intervention increased orthodromic and antidromic recovery after glycine exposure to 51 ± 9% and 62 ± 9%. No protection was provided by 100 μM 6,7-dinitroquinoxalone-2,3-dione (DNQX) which acts as a non-NMDA antagonist, 20 μM dantrolene which helps block calcium-mediated intracellular calcium release, 100 μM nifedipine which blocks L-type voltage-dependent calcium channels, 1 μM tetrodotoxin which blocks voltage-dependent sodium channels, or low sodium solutions, containing 26 mM sodium.

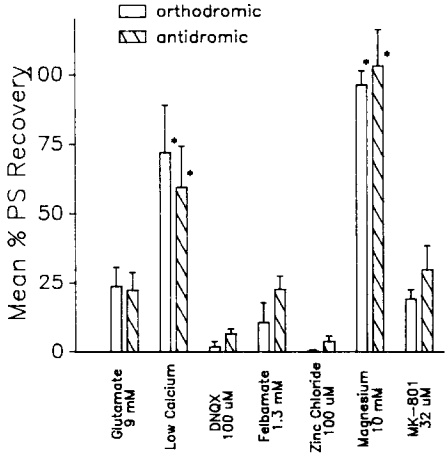

Fig. 7. Protection from acute glutamate-induced injury in CA1 with 10 mM magnesium sulfate and low calcium ACSF. Vertical bars indicate mean ± S.E.M. orthodromic and antidromic recovery after exposure to 9 mM sodium glutamate for 8 min, or to glutamate with additional pharmacological interventions. * $P < 0.05$.

Exposure to 9 mM sodium glutamate for 8 min also produced similar severe acute injury, with orthodromic and antidromic CA1 PS recovering to only $7 \pm 1\%$ and $7 \pm 2\%$ of initial amplitude. Treatment with 10 mM magnesium sulfate provided robust protection from glutamate-induced injury, recoveries of $96 \pm 5\%$ and $103 \pm 13\%$ (Fig. 7). As with glycine injury, perfusion of ACSF without added calcium afforded mild protection with orthodromic and antidromic recovery of $72 \pm 17\%$ and $62 \pm 15\%$. No protection from glutamate-induced injury was provided by 1.3 mM felbamate, 32 μM MK-801 or 100 μM DNQX.

4. Discussion

The results of these experiments demonstrate that elevations in extracellular glycine concentration can produce CA1 neuronal hyper-excitability and irreversible loss of neural transmission, consistent with neuronal excitotoxicity. Hyper-excitability induced by glycine exposure was evidenced by the rapid single-unit firing and transient increase in CA1 orthodromic PS amplitude which preceded loss of neural transmission. Additionally, increased synaptic activity indicative of long-term potentiation was found with non-lethal glycine exposures. Likewise a pattern consistent with excitotoxicity was demonstrated in the preferential loss of EPSP and orthodromic PS activity with glycine exposure. These post-synaptic responses reflect the function of dendrites and neuronal cell bodies and were also lost in excitotoxic injury produced by glutamate application. In contrast, the fiber volley, an indicator of pre-synaptic function, showed relative resistance to glycine injury. Fiber volley response demonstrated resistance to glutamate exposure as well.

The glycine concentrations needed to produce loss of neural transmission were similar to the concentrations of glutamate needed to produce comparable injury in this preparation. The glycine EC_{50} for irreversible loss of CA1 orthodromic PS was 7.1 mM, which was near to the EC_{50} of 7.2 mM for glutamate induced PS loss. This glutamate concentration is also consistent with those needed to produce glutamate-mediated effects in other brain slice preparations where, for example, 10 mM glutamate is needed to induce robust cGMP production in cerebellar slices [27]. These relatively high concentrations most likely reflect the action of amino acid uptake systems which must be overcome before effects of exogenously applied glycine or glutamate can be observed. The likely action of uptake systems was demonstrated in the exacerbation of glycine-induced injury produced by histidine, a glycine uptake inhibitor. This exacerbation of glycine injury with histidine, similar to the worsening of glutamate injury with dihydrokainic acid, suggests that glycine uptake systems play an important role in the regulation of local glycine concentrations at the synaptic level, and in the prevention of glycine-induced neuronal injury.

Although similarity was seen in the electrophysiological pattern of injury with both glycine and glutamate, the profile of pharmacological protection for each showed distinctive differences. Only magnesium antagonized the action of glutamate, while glycine injury was prevented by felbamate and zinc. This magnesium block against glutamate effect could conceivably have occurred either through the prevention of neurotransmitter release, or the blockade of the NMDA-associated ionic channel. However, since MK-801 which blocks the NMDA-associated channel, provided no protection against glutamate exposure, magnesium antagonism of glutamate effects most likely occurred through the prevention of glutamate-induced neurotransmitter release.

The differing profiles of protection seen with glycine and glutamate also suggest that glycine injury is not mediated through non-specific mechanisms, such as depolarization-induced glutamate release. If glycine injury were mediated predominantly by non-specific release of glutamate, antagonists of glutamate would be expected to protect against glycine injury. However, magnesium, which fully protected against glutamate-induced injury, offered only partial protection against glycine and did not prevent neuronal hyper-excitability. This lack of full antagonism by magnesium against glycine would indicate, therefore, that glycine injury is not mediated through non-specific glutamate release.

The results of magnesium treatment during glycine exposure were nearly identical to the results of MK-801 treatment during glycine exposure. This similarity suggests that glycine-induced injury is mediated in major part through opening of the NMDA-associated ionic channel. The finding of similar protection with methyl arginine, a nitric oxide inhibitor which prevents NMDA excitotoxic injury [4], is also consistent with a mediation of glycine injury through NMDA mechanisms.

Other findings would indicate that this opening of NMDA channels is likely due to activation of the NMDA-associated glycine receptor. Felbamate and zinc both protected from this injury, and both of these agents are active at NMDA-associated glycine site. Felbamate displaces 5,7-dichlorokynurenic acid [18] a highly specific ligand of the NMDA associated glycine site. In addition, zinc has been shown to antagonize activation of the NMDA-associated glycine site through noncompetitive inhibition [37]. The finding of significant protection from glycine injury with AP5, but not AP7, also suggests that this injury occurred through glycine activation of the NMDA-associated glycine receptor. Although all NMDA competitive antagonists inhibit NMDA and glutamate binding at the NMDA

recognition site, NMDA competitive antagonists having a five carbon chain, such as AP5, also inhibit glycine binding at the NMDA-associated glycine site [21]. Competitive NMDA antagonists having seven carbon chains, in contrast, inhibit this glycine binding to a much lesser extent. This differential inhibition of glycine binding is consistent with the findings in our present study, where both AP5 and AP7 gave nearly full protection against NMDA, but only AP5 provided significant protection against glycine-induced injury.

Although these data suggest that glycine injury was mediated in large part through activation of the NMDA-associated glycine site, the injury produced by glycine did not appear to occur exclusively through NMDA mechanisms. Blockers of the NMDA channel rendered only partial antagonism against glycine effects, and they did not prevent glycine-induced hyperexcitability. This lack of full protection from glycine effects with NMDA channel blockers did not appear to be due to use of suboptimal concentrations of MK-801 or magnesium, since these NMDA channel blockers given at these concentrations provide full protection against NMDA injury of similar magnitude [32]. Additionally, the lack of full protection from glycine with NMDA channel antagonists did not appear to be caused by use of an overwhelming glycine concentration, since exposure to 10 mM glycine induced only an approximate 90% loss of PS amplitude, and this injury was fully protected by felbamate and zinc. The failure of NMDA antagonists with sites of action beyond the NMDA-associated glycine site to fully protect against glycine, while such protection was seen with felbamate and zinc, would therefore suggest that glycine effects may not be generated entirely through opening of the NMDA channel.

Although felbamate and zinc clearly antagonized the effects of glycine, they did not protect against the action of NMDA. NMDA protection by felbamate might have been expected if felbamate inhibits activation of the NMDA-associated glycine site, and if activation of that site is a prerequisite for NMDA receptor activation. However, two classes of NMDA receptors have been described with differential responses to glycine [20], and it is possible that NMDA toxicity in this preparation not blocked by felbamate may occur through the action of NMDA receptors which are not regulated by glycine. Zinc might also have been expected to protect against NMDA, since zinc has been reported to protect cultured neurons from NMDA exposure [16]. However, in the present experiments zinc did not protect against NMDA exposure. This lack of protection with zinc against NMDA-induced injury may reflect differences in receptor expression in these two preparations. 7-Chlorokynurenic acid, an antagonist of the NMDA-associated glycine site, also did not to protect from glycine injury. However, the lack of protection in this case appeared to be due to toxic effects of from 7-chlorokynurenic acid.

In contrast to the many differences seen in the protection profiles of glycine and glutamate, one common feature in that protection was found. Perfusion with low calcium solution provided partial protection against both glycine and glutamate injury. These results suggest that injury from both glycine and glutamate is mediated by calcium influx. The point of entry for this influx did not appear to be through voltage-dependent calcium channels since neither glycine nor glutamate injury was prevented by nifedipine, a blocker of L-type voltage-dependent calcium channels found predominantly in post-synaptic regions. In the case of glutamate, it is likely that this calcium entry occurred through the opening of both NMDA and non-NMDA receptor-associated channels, since the ionic channels gated by these receptors are permeable to calcium, and the acute injury produced by both NMDA and non-NMDA agonists is reduced with low calcium solutions in this preparation [32]. With injury from glycine, this calcium influx probably occurred, in part, through the NMDA receptor-associated channel since blockers of this channel also afforded partial protection against glycine injury.

The possibility that NMDA channels did not exclusively mediate glycine injury was also suggested by the pattern of single unit firing occurring during glycine exposure. All NMDA antagonists tested, including both competitive NMDA antagonists and NMDA channel blockers, completely abolished the rapid neuronal firing which accompanied PS collapse induced by glycine. Therefore, it is likely that this firing occurred as a consequence of opening of the NMDA channel. Glycine-induced injury manifested by irreversible PS loss, however, still occurred in several cases despite the abolition of this firing. This dichotomy was best illustrated by the effects of AP7. Treatment with AP7 treatment totally prevented rapid neuronal firing induced by glycine, but provided no substantial protection against glycine-induced PS loss. Conversely, protection from glycine-induced PS loss did not predict the prevention of this single-unit firing. Felbamate and zinc both protected from PS loss, but did not prevent rapid neuronal firing. This differential pharmacological antagonism of rapid neuronal firing and PS loss would further indicate that glycine injury is mediated in part through mechanisms other than NMDA channel activation. They additionally suggest that felbamate protection does not occur through an anti-convulsant action.

The concentrations of felbamate needed to prevent glycine injury in CA1 neurons showed similarity to felbamate concentrations needed to protect against hypoxic injury to CA1 neurons [30]. Felbamate demonstrated an EC_{50} of about 1,050 μM against glycine-in-

duced PS loss which was near the felbamate EC_{50} of 830 μM seen for hypoxic protection. These concentrations are also similar to EC_{50} of 374 μM for felbamate inhibition of 5,7-dichlorokynurenic acid binding [18]. These findings suggest that felbamate protection for both hypoxic and glycine-induced injury occurs through an interaction at the NMDA-associated glycine site. These felbamate concentrations also compare to those needed for anti-convulsant effects, where a felbamate effective dose (ED_{50}) of 238 mg/kg or 1000 μmol/kg) is demonstrated for the prevention of pentylenetetrazol-induced seizures [28].

Although the question of actual glycine concentration within the synaptic cleft remains to be resolved, several studies point to the importance of glycine both in synaptic plasticity, and in the evolution of neuronal injury. The glycine antagonists, 7-chlorokynurenic acid and cycloleucine, both block long-term potentiation in rat hippocampal slices [10], and this block is reversed by 100 μM glycine. Exposure to 10 mM glycine for 10 min has additionally been shown to produce long-term potentiation in hippocampal slices [26], similar to the results seen in our experiments. Extracellular glycine concentrations also show a marked increase during ischemia, and, unlike glutamate, these elevations continue for some time after the re-establishment of blood flow [7,8]. Further evidence of the importance of glycine in the development of neuronal injury from stroke is seen in the protection afforded by ligands of the NMDA-associated glycine site. Felbamate, which displaces 5,7-dichlorokynurenic acid [18], provides neuronal protection against neuronal injury from both hypoxia [30] and ischemia [33]. Additionally, felbamate hypoxic protection is reversed by small amounts of glycine [31]. Ischemic protection has also been reported with another ligand of the NMDA-associated glycine binding site, HA-966 [36]. These findings suggest that glycine plays an active role both in the normal electrophysiological function and the development of excitotoxic neuronal injury.

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