Modulation of seizure susceptibility in the mouse by the strychnine-insensitive glycine recognition site of the NMDA receptor/ion channel complex

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1   In order to determine whether the strychnine-insensitive glycine modulatory site on the N-methyl-D-aspartate (NMDA) receptor/ion channel complex is fully activated in vivo, the ability of the selective glycine receptor agonist, D-serine, to modulate seizure susceptibility in the mouse has been examined.

2   D-Serine (10–200 μg per mouse, i.c.v.) dose-dependently increased the potency of NMDLA in inducing seizures in Swiss Webster mice by approximately 3 fold. L-Serine was without significant effect.

3   The potency of pentylenetetrazol in inducing seizures was also enhanced by D-, but not L-serine, although the magnitude of the shift (1.6 fold) was considerably less than for NMDLA.

4   Similar doses of D-serine were also able to block the anticonvulsant effect of the non-selective glycine receptor antagonist, kynurenic acid, against seizures induced by NMDLA, but were without effect on the anticonvulsant effect of the competitive NMDA receptor antagonist, 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP).

5   D-Serine completely antagonized the protective effect of the selective glycine receptor antagonist, 7-chlorokynurenic acid, against sound-induced seizures in DBA/2 mice, but was less effective in this model against the less selective antagonist, kynurenic acid.

6   The results indicate that in vivo, NMDA receptors are not maximally potentiated by endogenous glycine and suggest an important involvement of the glycine modulatory site on the NMDA receptor/ion channel complex in the pathophysiology of epilepsy.

Introduction

Electrophysiological studies indicate that glycine is able to potentiate the response evoked by N-methyl-D-aspartic acid (NMDA) in neurones cultured from rat cerebral cortex (Johnson & Ascher, 1987). Glycine is also able to enhance, by a strychnine-insensitive mechanism, the specific binding of radioligands interacting in a non-competitive manner with the ion channel of the NMDA receptor complex (Wong et al., 1987; Reynolds et al., 1987; Bonhaus et al., 1987). Taken with the nearly identical distribution of NMDA-sensitive, [3H]-glutamate binding sites and of strychnine-insensitive, [3H]-glycine binding sites (Monaghan & Cotman, 1985; Bristow et al., 1986; Hudson & Woodruff, 1988), these results suggest the presence of an allosteric glycine recognition site located in the NMDA receptor/ion channel complex (Bowery, 1987). As only low concentrations of glycine are required to occupy this site, and the free amino acid is present in appreciable amounts in body fluids (Ferraro & Hare, 1985), it might seem unlikely that glycine would have any significant influence on NMDA-mediated neurotransmission in vivo (Johnson & Ascher, 1987). However, in the present paper we provide evidence that this modulatory system is not normally fully activated since D-serine, an agonist at the glycine modulatory site (Wong et al., 1987; Reynolds et al., 1987; Kemp et al., 1988) is able to potentiate seizures induced in the mouse by NMDLA at doses similar to those antagonizing the anticonvulsant effects of the selective glycine receptor antagonist, 7-chlorokynurenic acid (Kemp et al., 1987; 1988).

Methods

Animals

Male Swiss Webster mice (20–30 g) and audiogenic-seizure-prone DBA/2 mice (21–23 days of age, 5–9 g) were obtained from Bantin and Kingman, Hull. Animals were housed in

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groups of five under a 12 h light/dark cycle (lights on at 07 h 00 min) with food and water available ad libitum.

Intracerebroventricular injections

For intracerebroventricular (i.c.v.) injections, mice were anaesthetized with ether and the skull exposed. Injections (5 μl) were made at lambda with a 3.5 mm long, 27 gauge needle attached to a 10 μl Hamilton syringe and the wound sealed with epoxy resin. Injections of dye by this procedure led to a uniform distribution of injectate throughout the ventricular system within 10 min.

Seizures induced by N-methyl-DL-aspartic acid and pentylenetetrazol

Mice were injected subcutaneously with N-methyl-DL-aspartic acid (NMDLA, 500 mg kg^{-1}) or pentylenetetrazol (PTZ, 120 mg kg^{-1}) and latency to the tonic extension of the forepaws noted. Animals not convulsing within 30 min of injection were considered protected and given a maximum latency score of 30 min. Putative antagonists of seizures were given i.c.v. 15 min before the convulsant and the ED$_{50}$ dose (dose protecting 50% of animals) was determined by probit analysis.

Audiogenic seizures

Tonic seizures were induced in male DBA/2 mice at 23 days of age by exposure to an electric bell (125 dB, 1.4 kHz) for 30 s. Protection was defined as the absence of a tonic seizure during the exposure to sound. Vehicle-treated animals convulsed within 10 s of sound onset.

Drugs

Kynurenic acid, N-methyl-DL-aspartic acid, pentylenetetrazol and D- and L-serine were obtained from Sigma (Poole, Dorset). 3-((+)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) was purchased from Tocris Neuramin, Buckhurst Hill, 7-chlorokynurenic acid was synthesized by the Department of Medicinal Chemistry, Merck Sharp & Dohme

Research Laboratories, Terlings Park, Harlow, Essex. All compounds were dissolved in 0.9% NaCl with the aid of HCl or NaOH and the pH adjusted to 6.5–7.5 with acid or base as appropriate.

Results

Effect of D- and L-serine on seizures induced by NMDLA and PTZ

The administration of NMDLA to mice induces wild running, barrell rolling and, at high doses ($>200\,mg\,kg^{-1}$, s.c.) tonic seizures and death. NMDLA (50–$500\,mg\,kg^{-1}$) dose-dependently increased the incidence of tonic seizures and decreased the latency to convulse (Figure 1a). When D-serine (50–$200\,\mu g$ per mouse) was injected i.c.v., no seizure activity was seen over the following 45 min, but when given 15 min before NMDLA, the dose-response relationship to NMDLA was shifted to the left. After a $100\,\mu g$ dose of D-serine, the ED_{50} for the induction of convulsions by NMDLA decreased by more than 3 fold from $245\,mg\,kg^{-1}$ to $80\,mg\,kg^{-1}$ (Figure 1a). Increasing the dose of D-serine to $200\,\mu g$ per mouse had no greater effect than $100\,\mu g$ D-serine (results not shown). In contrast, L-serine ($100\,\mu g$ per mouse) was without significant effect on either the latency to convulse or the number of animals convulsing in response to NMDLA (Figure 1a).

Pentylenetetrazol (30–$120\,mg\,kg^{-1}$) also dose-dependently induced tonic seizures in the mouse and the dose-response relationship was again shifted to the left by administration of D-, but not L-serine (Figure 1b). However, the magnitude of the shift was considerably less than that seen when NMDLA was the convulsant, the ED_{50} dose of PTZ decreasing from $93.7\,mg\,kg^{-1}$ to $60.4\,mg\,kg^{-1}$ following administration of $200\,\mu g$ of D-serine.

Effects of D-serine on the blockade of NMDLA-induced seizures by kynurenic acid and CPP

In addition to blockade of non-NMDA excitatory amino acid receptors, kynurenic acid can act as an antagonist of the potentiating effects of glycine on NMDA-mediated neurotransmission (Kemp *et al.*, 1987; Watson *et al.*, 1988). When given i.c.v., 15 min beforehand, kynurenic acid dose-

Figure 2 The anticonvulsant activity of compounds against (a) N-methyl-DL-aspartic acid (NMDLA)-induced seizures in Swiss Webster mice and (b) sound-induced seizures in DBA/2 mice. In (a) 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (O) or kynurenic acid (●) were given i.c.v. 15 min before NMDLA ($500\,mg\,kg^{-1}$, s.c.) and the latency to the tonic extension of the fore-paws noted. Animals not convulsing within 30 min of injection of NMDLA were considered protected. Values shown are the mean latencies (s.e.mean shown by vertical bars) of at least 8 animals per dose. The ED_{50} dose (dose protecting 50% of the animals tested) for CPP and kynurenic acid was $0.08\,\mu g$ per mouse and $25\,\mu g$ per mouse respectively. In (b), CPP (O), kynurenic acid (●) or 7-chlorokynurenic acid (□) were given i.c.v. 15 min before exposure to an electric bell. Protection was defined as the absence of a tonic seizure during 30 s of sound exposure. Results are shown as the percentage of animals protected ($n = 8$ or more per group). The ED_{50} doses for CPP, kynurenic acid and 7-chlorokynurenic acid were 0.008, 3.75 and $2.32\,\mu g$ per mouse respectively.

dependently increased the latency to convulse to a supra-maximal dose of NMDLA ($500\,mg\,kg^{-1}$, s.c.; Figure 2a). In order to investigate the involvement of the glycine/NMDA receptor in the anticonvulsant effect of kynurenic acid, D-serine (30–$100\,\mu g$ per mouse) was administered i.c.v. concomitantly with a submaximal anticonvulsant dose of kynurenic acid ($25\,\mu g$ per mouse): the increase in the latency to convulse induced by kynurenic acid was antagonized dose-dependently by D-serine (Figure 3a). The competitive NMDA receptor antagonist, CPP (3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, Davies *et al.*, 1986) also dose-dependently antagonized NMDLA-induced seizures over the dose range of 0.015–$0.15\,\mu g$ per mouse, i.c.v. (Figure 2a). However, D-serine (3–$300\,\mu g$ per mouse) failed to antagonize the protective effect of a submaximal dose of CPP ($0.075\,\mu g$ per mouse, Figure 3b).
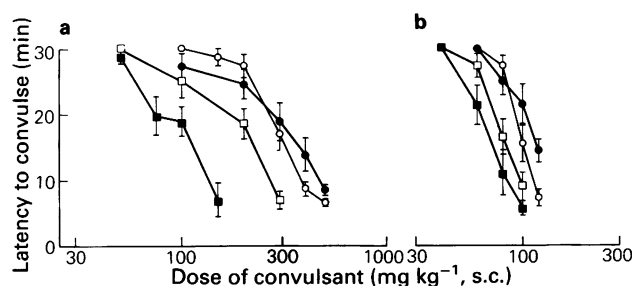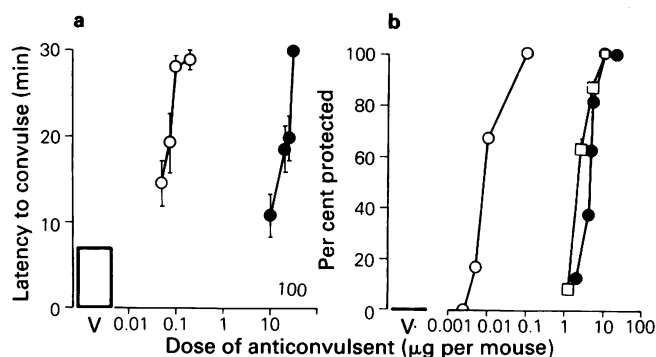

Figure 1 Effect of D- and L-serine on (a) N-methyl-DL-aspartic acid (NMDLA) and (b) pentylenetetrazol (PTZ)-induced tonic seizures. D- or L-serine (50–$200\,\mu g$ per mouse) were injected i.c.v. in Swiss Webster mice. Fifteen min later NMDLA (50–$500\,mg\,kg^{-1}$) or PTZ (60–$120\,mg\,kg^{-1}$) were injected s.c. and the latency to the tonic extension of the forepaws noted. Animals not showing a tonic seizure within 30 min were given a maximum latency score of 30 min. Values shown are the mean latency scores (s.e.mean shown by vertical bars) of at least 8 animals per group. (a): (O) Vehicle; (□) D-serine $50\,\mu g$; (■) D-serine $100\,\mu g$; (●) L-serine $100\,\mu g$. (b) (O) Vehicle; (□) D-serine $100\,\mu g$; (■) D-serine $200\,\mu g$; (●) L-serine $100\,\mu g$. The ED_{50} dose (dose inducing seizures in 50% of the animals tested) was calculated by probit analysis, animals not convulsing within 30 min of injection of the convulsant being considered protected. The ED_{50} dose of NMDLA was decreased from $245\,mg\,kg^{-1}$ to $80\,mg\,kg^{-1}$ by pretreatment with $100\,\mu g$ D-serine ($P < 0.05$, F test) whilst L-serine was without significant effect. For PTZ, D-serine significantly reduced the ED_{50} dose from $93.7\,mg\,kg^{-1}$ to $60.4\,mg\,kg^{-1}$ ($P < 0.05$, F test) and again L-serine was without significant effect.
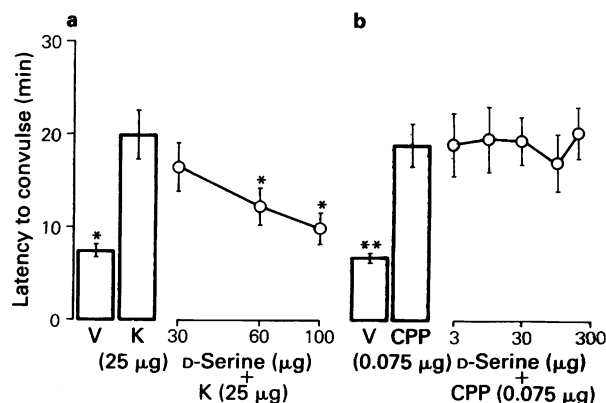

Figure 3 The antagonism of the protective effects of (a) kynurenic acid (K) and (b) 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) against N-methyl-DL-aspartic acid (NMDLA)-induced seizures. D-Serine was co-administered i.c.v. with either kynurenic acid ($25\,\mu g$ per mouse) or CPP ($0.075\,\mu g$ per mouse) 15 min before the injection of NMDLA ($500\,mg\,kg^{-1}$, s.c.) and the latency to convulse determined. Values shown are the mean latencies (s.e.mean shown by vertical bars) of at least 8 animals per group. *Significantly different from animals given only kynurenic acid, $P < 0.05$ (Student Newman Keuls test). **Significantly different from animals given only CPP, $P < 0.05$.
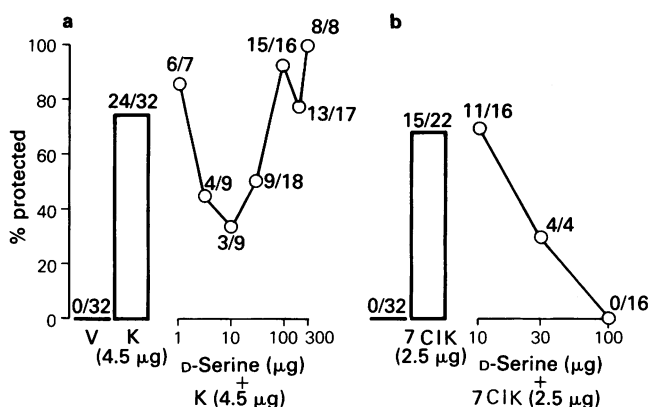
Figure 4 The antagonism of the protective effects of (a) kynurenic acid (K) and (b) 7-chlorokynurenic acid (7 ClK) against audiogenic seizures. D-Serine was co-administered i.c.v. with kynurenic acid (4.5 μg per mouse) or 7-chlorokynurenic acid (2.5 μg per mouse) 15 min before exposure to sound (for further details see legend to Figure 2). The number of animals not convulsing was noted. Results are shown as the percentage of animals protected. The numbers at each point on the graph represent the number of mice protected/number tested.

Effects of D-serine on the blockade of audiogenic seizures by kynurenic acid, 7-chlorokynurenic acid and diazepam

Kynurenic acid was more than 6 fold as potent as an antagonist of sound-induced seizures in DBA/2 mice than of seizures induced by NMDLA (ED_{50} = 3.8 and 25 μg per mouse respectively, Figure 2a,b). The anticonvulsant effect of a submaximal dose of kynurenic acid (4.5 μg per mouse) was antagonized only partially by D-serine (Figure 4a) and at doses somewhat lower (1–10 μg per mouse) than those required to block the action of kynurenic acid against NMDLA (10–100 μg per mouse). Furthermore, the effect of D-serine was biphasic, 100 μg D-serine giving less antagonism than a 10 μg dose. The possibility that D-serine might even potentiate the anticonvulsant effect of kynurenic acid was examined by co-administration of D-serine (100 μg per mouse) with a lower dose of kynurenic acid (3 μg per mouse): the proportion of mice convulsing was not altered (number of animals convulsing/number tested: controls = 0/8; kynurenic acid, 3 μg = 1/7; kynurenic acid, 3 μg + D-serine, 100 μg = 1/8).

Unlike kynurenic acid, its 7-substituted chloro analogue has appreciably higher affinity for the strychnine-insensitive glycine recognition site than for NMDA, quisqualate or kainate receptors and potently antagonizes the actions of glycine at the NMDA receptor ((Kemp et al., 1988). 7-Chlorokynurenic acid at doses up to 10 μg per mouse was without effect on seizures induced by NMDLA (doses in excess of this induced marked sedation, ataxia and respiratory depression), but dose-dependently blocked sound-induced seizures in DBA/2 mice with an approximate ED_{50} of 2 μg per mouse. As for the anticonvulsant effect of kynurenic acid against NMDLA-induced seizures, concomitant administration of D-serine (10–100 μg per mouse) antagonized the protective effect of a submaximal dose of 7-chlorokynurenic acid (2.5 μg per mouse, Figure 4b). In contrast, D-serine (10–300 μg per mouse) was unable to antagonize an equivalent submaximal protective effect of the benzodiazepine receptor agonist, diazepam (1 mg kg^{-1}, i.p., results not shown).

Discussion

Neuronal responses to the excitatory amino acid, N-methyl-D-aspartate, can be potentiated by glycine acting at a strychnine-insensitive binding site on the NMDA receptor/ion channel complex (Johnson & Ascher, 1987; Wong et al., 1987; Bonhaus et al., 1987; Reynolds et al., 1987; Bowery, 1987). In patch clamp experiments with cultured mouse neurones,

glycine potentiates responses to NMDA at high perfusion rates, when leakage from the cells contributes little to the concentration of glycine in the external milieu: concentrations greater than 1 μM are saturating (Johnson & Ascher, 1987). Given the high concentrations of glycine in body fluids (i.e. > 1 μM in CSF, Ferraro & Hare, 1985) it has been argued that the glycine modulatory site on the NMDA receptor may be maximally activated in vivo and thus of little relevance to the phasic control of NMDA-mediated neurotransmission (Johnson & Ascher, 1987). However, the concentration of glycine in the synaptic cleft in vivo may not always exceed 1 μM. Indeed, when the perfusion rate of rat neocortical slices is kept at a level permitting the ebb and flow of neurotransmitters and neuromodulators to be controlled by natural release and degradation processes, exogenously applied glycine enhanced responses to NMDA (Thomson et al., 1989). Furthermore, there is evidence that the intraventricular injection of glycine can potentiate the rat cerebellar cyclic GMP response to co-administered NMDA (Danysz et al., 1989).

Seizures induced in the mouse by NMDLA are thought to reflect the activation of central NMDA receptors (Leander et al., 1988; Price et al., 1988) and thus present a model with which to probe the functional relevance of the glycine modulatory site on the NMDA receptor in vivo. Because of its lack of selectivity between strychnine-sensitive and insensitive receptors, glycine itself is not a suitable tool with which to work. However, this problem can be overcome by use of the glycine receptor agonist D-serine (Bristow et al., 1986; Wong et al., 1987; Kemp et al., 1988) that is devoid of significant affinity for strychnine-sensitive glycine receptors (A. Foster, personal communication). When given directly into the cerebral ventricles, D-serine was not itself convulsant, but clearly potentiated the convulsant action of NMDLA. In contrast, L-serine, which in vitro is about 100 fold less potent than the D-enantiomer, was without significant effect. Thus, serine stereoselectively potentiated the convulsant activity of NMDLA, consistent with the glycine modulatory site on the NMDA receptor being unsaturated with respect to substrate in vivo.

Since NMDA receptor antagonists also block seizures induced by pentylenetetrazol (Croucher et al., 1982; Hayes & Balster, 1985) it was of interest to determine whether D-serine was also able to potentiate the convulsant action of PTZ. D-, but not L-serine also dose-dependently increased the potency of PTZ in inducing tonic seizures over the same dose range that was effective in the NMDLA seizure model. The magnitude of the effect was, nevertheless, considerably smaller than that seen with NMDLA, possibly reflecting the pharmacological specificity of the D-serine/NMDA receptor interaction. On the other hand, the relative insensitivity of PTZ-induced seizures could also be explained by a differential penetration of D-serine to disparate epileptogenic foci.

Modulation of NMDA receptor activation by the D-serine-sensitive glycine receptor was also illustrated by the ability of D-serine to antagonize the anticonvulsant action of the glycine receptor antagonists, kynurenic acid and 7-chlorokynurenic acid. The blockade of NMDLA-induced seizures by kynurenic acid was reversed by D-serine at doses similar to those potentiating the actions of NMDLA alone. The specificity of this effect is shown by the inability of D-serine to antagonize a similar anticonvulsant effect of the competitive NMDA receptor antagonist, CPP. This result is entirely consistent with electrophysiological studies in which both D-serine and glycine were unable to reverse the competitive antagonist action of CPP and D-2-aminophosphonovaleric acid against NMDA in the rat cortical slice (Kemp et al., 1988; Fletcher & Lodge, 1988).

Kynurenic acid was also an effective anticonvulsant in the audiogenic seizure prone DBA/2 mouse. However, D-serine was less efficacious (although somewhat more potent) in blocking the protective effect of kynurenic acid in DBA/2 mice than in the NMDLA seizure model. Furthermore, the antagonism of kynurenic acid by D-serine in DBA/2 mice was clearly

biphasic. Interestingly, glycine is reported to reverse only partially the kynurenate-induced block of responses to NMDA in rat cortical wedges (Fletcher et al., 1988). These paradoxical results could be explained by the lack of selectivity of kynurenic acid for the glycine modulatory site: in addition to antagonism of the potentiating effects of glycine at the NMDA receptor, kynurenate also antagonizes the actions of glutamate at quisqualate and kainate receptors (Kemp et al., 1987). Blockade of these 'glycine-insensitive' receptors (Johnson & Ascher, 1987) is also likely to have anticonvulsant consequences (Turski et al., 1987). Indeed, when the selective glycine receptor antagonist, 7-chlorokynurenic acid, was used as the anticonvulsant in the DBA/2 mouse, D-serine was able to block totally its protective effect, but was without effect on a similar degree of protection afforded by diazepam.

Thus, the selective potentiation of the convulsant action of NMDLA by D-serine suggests that NMDA receptors are not maximally potentiated by endogenous glycine. A similar con-

clusion was also recently reached by Larson & Beitz (1988) who showed that the intrathecal injection of glycine in animals pretreated with a subconvulsant dose of strychnine (to block strychnine-sensitive glycine receptors) enhanced the convulsant action of co-administered NMDA. Consequently, it is probable that variations in the availability of glycine occurring in the vicinity of the receptor in vivo are sufficient to influence significantly the tone of NMDA-mediated neurotransmission. Given the high concentrations of glycine in epileptogenic foci (Van Gelder et al., 1972) and the anticonvulsant properties of the selective glycine receptor antagonist, 7-chlorokynurenic acid, these results strongly suggest an important involvement of the glycine/NMDA receptor/ion channel complex in the pathophysiology of epilepsy.

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