

D,L-*cis*-2,3-Pyrrolidine dicarboxylate alters [³H]-L-glutamate binding and induces convulsions in mice

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Abstract

This study investigated whether D,L-*cis*-2,3-Pyrrolidine dicarboxylate (D,L-*cis*-2,3-PDC), a new glutamate analogue, alters glutamate binding to cerebral plasma membranes and whether *N*-methyl-D-aspartate (NMDA) receptors are involved in the convulsant effect of this compound. D,L-*cis*-2,3-PDC reduced sodium-independent [³H]-L-glutamate binding to lysed membrane preparations from adult rat cortex and had no effect on sodium-dependent glutamate binding. Intracerebroventricular administration of D,L-*cis*-2,3-PDC (7.5–25 nmol/5 μl) induced generalized tonic–clonic convulsions in mice in a dose-dependent manner. The coadministration of MK-801 (7 nmol/2.5 μl), with D,L-*cis*-2,3-PDC (16.5 nmol/2.5 μl), fully protected the animals against D,L-*cis*-2,3-PDC-induced convulsions, while the coadministration of DNQX (10 nmol/2.5 μl) increased the latency to convulsions but did not alter the percentage of animals that had convulsions. These results suggest that D,L-*cis*-2,3-PDC-induced effects are mediated predominantly by NMDA receptors.

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1. Introduction

Glutamate and aspartate are the predominant excitatory amino acid (EAA) neurotransmitters in the mammalian brain (Kanai et al., 1993; Szatkowski and Attwell, 1994). The EAAs activate a family of ligand-gated ion channels, called ionotropic receptors [e.g., *N*-methyl-D-aspartate (NMDA), kainic acid (KA), and alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)], and a family of receptors coupled through GTP-binding proteins to a variety of intracellular signaling molecules, called “metabotropic” receptors (Conn and Patel, 1994; Hollman and Heinemann, 1994; Nakanishi, 1994; Nicolletti et al., 1996). Metabotropic receptors are activated by ligands such as trans-1-aminocyclopentane-1,3-dicarboxylate (1S,3R-ACPD), L-2-amino-4-phosphonobutyric acid (L-AP-4), ibotenate, and quisqualic acid (QA) (Hollman and Heinemann, 1994). EAA receptors

participate not only in fast excitatory transmission but also in more complex signaling processes, such as those required for synaptic plasticity and higher cognitive functions (Daw et al., 1993; Collingridge and Bliss, 1995; Cotman et al., 1995). In contrast to these normal signaling pathways, excessive activation of the ionotropic EAA receptors can trigger a cascade of events that eventually leads to neuronal death. This process, referred to as excitotoxicity, is thought to be an underlying pathological mechanism in a wide variety of neurological insults and degenerative disorders, such as ischemia, trauma, hypoglycemia, epilepsy, and Huntington’s and Parkinson’s diseases (Choi, 1990, 1994; Meldrum, 1993; Rothman and Olney, 1995).

L-Glutamate has an acyclic structure that has a free rotation at the space capable of assuming a wide range of conformations. Accumulating evidence suggests that glutamate may bind to each of the known EAA receptors and transporters in a distinct conformation. A cornerstone in the identification and characterization of the EAA receptors has been the utilization of conformationally constrained analogues of L-glutamate and L-aspartate. The application of the

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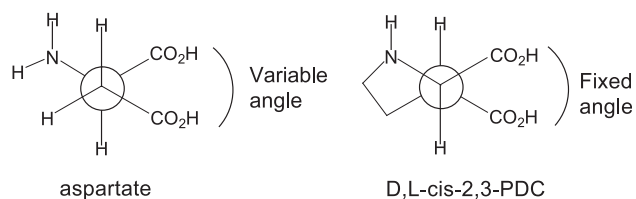


Fig. 1. Comparison of conformational mobility of aspartate to *cis*-2,3-PDC.

conformational restricted analogues concept related to aspartate and glutamate is based on the construction of derivatives on a cyclic framework, which imposes more restricted angles on key structural and functional groups. The concept is best illustrated by the Fig. 1 where *cis*-2,3-PDC is compared to aspartate with regard to its flexibility (Watkins et al., 1990; Ortwine et al., 1992; Chamberlin and Bridges, 1993). By positioning the carboxylate groups at differing points around the ring, the carbon backbone of glutamate and aspartate can be embedded within a cyclic structure (Willis et al., 1997). These more rigid analogues can attain fewer of the required conformations and very often exhibit greater selectivity of binding than glutamate itself. These compounds become valuable not only in further refining the pharmacological requirements of the receptor classes but as probes of synaptic signaling and excitotoxic pathology (Willis et al., 1996). Previous studies have identified (2*S*,4*S*)-pyrrolidine dicarboxylate (*L*-trans-2,4-pyrrolidine dicarboxylate: *L*-trans-2,4-PDC) as a potent and selective inhibitor of the high-affinity, sodium-dependent glutamate transporter (Bridges et al., 1991). Positioning the distal COOH at the C3 position yielded *L*-*cis*-2,3-PDC (Humphrey et al., 1994) and *L*-trans-2,3-PDC. This latest proved to be a weaker uptake inhibitor but a potent NMDA agonist (Willis et al., 1996) whose excitotoxic potency and selectivity towards NMDA receptors are further increased by the introduction of a methyl group to the 5' position of the pyrrolidine ring (Willis et al., 1997). Nevertheless, the effects of *cis* pyrrolidine dicarboxylate derivatives on the glutamatergic system were not investigated to date. In the present study, we investigated whether *D,L*-*cis*-2,3-Pyrrolidine dicarboxylate (*D,L*-*cis*-2,3-PDC; Fig. 2), a new glutamate analogue and a *L*-trans-2,3-PDC diastereoisomer, causes convulsions and whether NMDA receptors are involved in the convulsant effect of this compound. In addition, due to the presently reported protective effect of MK-801 against *D,L*-*cis*-2,3-PDC induced-convulsions, we evaluated whether it alters glutamate binding to cerebral plasma membranes.

2. Material and methods

2.1. Reagents

All reagents were acquired from Sigma, MA, except [^3H]-*L*-glutamic acid, which was purchased from Amersham Pharmacia Biotech; MK-801, which was purchased from

RBI; and *D,L*-*cis*-2,3-PDC, which was synthesized by Carpes et al. (1997).

2.2. Animals

Adult male Wistar rats (230–250 g) and male albino mice (30–40 g), maintained in a 12:12-h dark/light cycle at controlled temperature ($22 \pm 1^\circ\text{C}$) with lab chow and tap water ad libitum, were used.

2.3. Membrane preparation

Membrane preparation was carried out as described by Emanuelli et al. (1998). Adult male Wistar rats were killed by decapitation; cerebral cortices were removed and homogenized in 20 volumes (ml/g of wet tissue) of 10 mM Tris–acetate buffer (pH 7.4) containing 320 mM sucrose, and 1 mM MgCl_2 using a hand-operated glass homogenizer. The homogenate was centrifuged at $1000 \times g$ for 15 min and the pellet was resuspended in 20 volumes (ml/g of wet tissue) of the same buffer and centrifuged again. The second pellet was discarded and the supernatant fractions were pooled and centrifuged at $27,000 \times g$ for 15 min. The resulting pellet was lysed in 20 volumes of 10 mM Tris–acetate buffer (pH 7.4) for 30 min and centrifuged at $27,000 \times g$ for 15 min. This pellet was washed three times in 20 volumes of 10 mM Tris–acetate buffer (pH 7.4) at $27,000 \times g$ for 15 min. The final pellet was resuspended in three volumes of 10 mM Tris–acetate buffer (pH 7.4). All steps were carried out at 4°C and the membranes were frozen at -20°C for no more than 1 month. On the day of binding assay, the membranes were rapidly thawed in a water bath (37°C), homogenized with 3 volumes of 10 mM Tris–acetate buffer (pH 7.4), and centrifuged at $27,000 \times g$ for 15 min. The resulting pellet was resuspended in three volumes (ml/ml of thawed membrane) of the same buffer, preincubated at 37°C for 30 min, and centrifuged at $27,000 \times g$ for 15 min. The pellet was resuspended in three volumes of 10 mM Tris–acetate buffer, washed four times in three volumes of the same buffer, and centrifuged at $27,000 \times g$ for 15 min. The final pellet was resuspended in the same buffer in order to yield a protein concentration of 1–2 mg/ml and was used for the binding assays.

2.4. [^3H]-*L*-glutamate binding

Sodium-dependent and -independent [^3H]-*L*-glutamate binding to cerebral plasma membranes was investigated

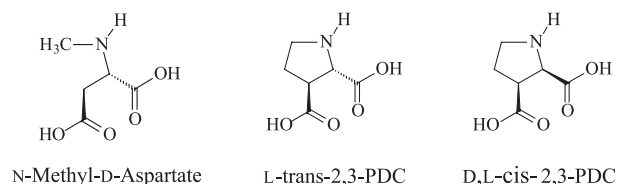


Fig. 2. Chemical structures of NMDA, *L*-trans-2,3-PDC, *D,L*-*cis*-2,3-PDC.

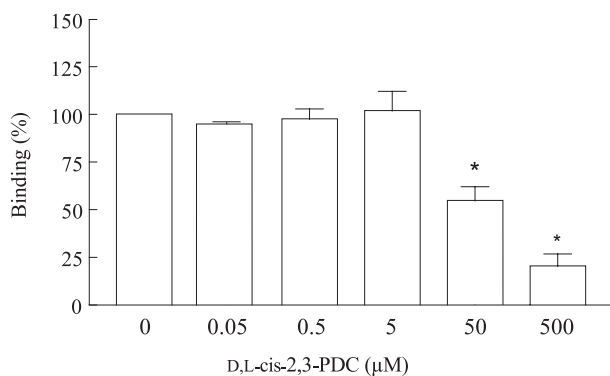


Fig. 3. Effect of D,L-cis-2,3-PDC on [^3H]-L-glutamate binding in rat brain plasma membranes. Results are presented as means (S.E.M.) of three experiments and are expressed as activity percentage of control. * Significantly different from control ($P < .05$ —SNK test).

according to Rao and Murthy (1993). Briefly, membranes were incubated in a 0.5-ml reaction mixture containing 50 mM Tris-acetate buffer (pH 7.4), 40 nM [^3H]-L-glutamate, and 0, 0.05, 0.5, 5, 50, and 500 μM D,L-cis-2,3-PDC (dissolved in water, pH adjusted to 7.4 with KOH). Sodium-dependent binding was carried out in the same incubation medium described above, except that it contained 150 mM sodium acetate. Incubation was carried out at 30 °C for 30 min and the reaction was stopped by filtration using GF/B glass microfiber filters. Dried filters were transferred to eppendorf tubes containing scintillation liquid, and the radioactivity was determined with a Packard scintillation spectrometer at 40–45% efficiency. Specific binding was calculated as the difference between total binding and nonspecific binding, which was measured in the presence of a 10,000-fold excess (4 mM) of the unlabeled L-glutamate. All determinations were made in triplicate. Protein concentration was measured using bovine serum albumin as standard (Bradford, 1976). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple range test, when appropriate. Standard errors were less than 10%.

2.5. Behavioral effects of D,L-cis-2,3-PDC on mice

Freehand intracerebroventricular injections into the lateral ventricles of the conscious mice were made using a 29G needle attached to a 10- μl Hamilton syringe (3 mm of the needle tip exposed) according to Clark et al. (1988). The site of injection was an imaginary line drawn through the anterior lobe of the ears and from an imaginary midsagittal line, and the whole injection procedure was completed within 5–10 s in order to minimize discomfort and pain. Immediately after behavioral evaluation, the animals were decapitated and had the site of the intracerebroventricular injection confirmed by needle track verification with a PZO MST 131 stereomicroscope. Only data from animals with the needle track aiming the lateral ventricle were considered. In those experiments designed to evaluate the convulsant

action of D,L-cis-2,3-PDC, the animals were injected (intra-cerebroventricular) with 5 μl of D,L-cis-2,3-PDC (2.5, 7.5, and 25 nmol) or 0.85% NaCl. Immediately thereafter, the animals were individually placed in a round open field (35 cm of internal diameter) and observed for 10 min for the appearance of tonic–clonic convulsions. The latency for the first convulsive episode (a full motor seizure with loss of postural control, usually reported as a Class 5 motor seizure according to the Racine scale) and the percent of animals that presented convulsions or death were recorded (Racine, 1972). For statistical purposes, the animals that did not present tonic–clonic convulsions up to 600 s were attributed a score of 600. The involvement of NMDA or AMPA and KA receptors in the D,L-cis-2,3-PDC-induced convulsions was assessed by coinjecting the animals (intracerebroventricular) with 7.0 nmol MK-801 or 10 nmol DNQX and 16.5 nmol D,L-cis-2,3-PDC or 0.85% NaCl alone and 0.85% NaCl with the drugs above, in 2.5 μl plus 2.5 μl volumes in the same syringe, separated by an air bubble (0.1 μl). The animals were immediately transferred to the open field and observed for 10 min for the signs of convulsions, as described above. The D,L-cis-2,3-PDC dose was chosen on the basis of its effectiveness to cause convulsions in 100% of the animals without death (the dose–effect curve and pilot experiments).

3. Results

3.1. Binding

Fig. 3 shows the effect of D,L-cis-2,3-PDC on sodium-independent [^3H]-L-glutamate binding to cerebral plasma membranes. D,L-cis-2,3-PDC reduced sodium-independent [^3H]-L-glutamate binding by 50% in membrane preparations from adult rat cortex [$F(5,12) = 28.1$, $P < .00001$ ANOVA, considering D,L-cis-2,3-PDC concentrations (0–500 μM) as a within-subject factor]. Partitioning of the sum of squares into trend components revealed a significant linear trend [$F(1,12) = 112.2$, $P < .001$], indicating that sodium-independent [^3H]-L-glutamate binding decreased

Table 1
D,L-cis-2,3-PDC (intracerebroventricular) induces convulsive behavior in mice

Treatment	Onset latency in seconds (interquartile range)	Convulsions n_{cv}/n_t (%)	Mortality n_d/n_t (%)
0.85% NaCl	600 (600–600)	0/7 (0%)	0/7 (0%)
D,L-cis-2,3-PDC			
2.5 nmol	600 (600–600)	0/8 (0%)	0/8 (0%)
7.5 nmol	68.0 (20.0–264.0) [#]	5/6 (83.3%)*	0/6 (0%)
25 nmol	10.5 (3.7–15.2) [#]	9/9 (100%)*	3/9 (33.4%)*

$n = 6$ –9 animals in each group; n_{cv} —number of animals which had convulsions; n_d —number of animals that died; n_t —total number of animals.

* $P < .05$ compared to 0.85% NaCl (Fisher's test).

[#] $P < .0001$ compared to 0.85% NaCl (Kruskal–Wallis test).

linearly with increasing D,L -*cis*-2,3-PDC concentrations. Interestingly, D,L -*cis*-2,3-PDC had no effect on sodium-dependent [3 H]-L-glutamate binding (data not shown).

3.2. Behavioral evaluation

Intracerebroventricular administration of 2.5 nmol of D,L -*cis*-2,3-PDC did not cause convulsions, while 7.5 nmol of D,L -*cis*-2,3-PDC induced generalized tonic–clonic convulsions, which lasted a few minutes. D,L -*cis*-2,3-PDC (25 nmol) induced long-lasting generalized tonic–clonic convulsions immediately after its injection. Table 1 shows the effect of the injection of increasing amounts of D,L -*cis*-2,3-PDC (0, 2.5 or 7.5, and 25 nmol icv) on convulsive behavior. Statistical analysis ($H=23.05$, $df=3$, $P<.0001$; Kruskal–Wallis H test) revealed that increasing amounts of D,L -*cis*-2,3-PDC decreased the latency to convulsion and increased the percentage of animals that presented convulsions and death ($P<.05$, Fisher test). The most of animals that received 25 nmol D,L -*cis*-2,3-PDC (66.6%) remained alive after a 24-h period.

The involvement of NMDA receptors on the convulsant effect of D,L -*cis*-2,3-PDC was assessed by coadministering MK-801 (7 nmol/ 2.5 μ l), a noncompetitive NMDA receptor antagonist, with D,L -*cis*-2,3-PDC (16.5 nmol/ 2.5 μ l). The coadministration of MK-801 protected the animals against D,L -*cis*-2,3-PDC-induced convulsions, measured by the frequency of convulsions ($P<.05$, Fisher test—Table 2) and by the latency to the first convulsive episode ($H=19.55$, $df=3$, $P<.0001$; Kruskal–Wallis H test).

The involvement of AMPA and KA receptors on the convulsant effect of D,L -*cis*-2,3-PDC was assessed by coadministering DNQX (10 nmol/2.5 μ l), a competitive AMPA and KA receptor antagonist, with D,L -*cis*-2,3-PDC (16.5 nmol/2.5 μ l). The coadministration of DNQX afforded a slight protection against D,L -*cis*-2,3-PDC-induced convulsions since it increased the latency to convulsion ($H=23.72$, $df=3$, $P<.0001$; Kruskal–Wallis H test—Table 2).

Table 2
Effect of MK-801 (7 nmol) or DNQX (10 nmol) on D,L -*cis*-2,3-PDC-induced convulsions in mice

Treatment	Onset latency (interquartile range)	Convulsions n_{cv}/n_t (%)
0.85% NaCl + 0.85% NaCl	600 (600–600)	0/6 (0%)
0.85% NaCl + D,L - <i>cis</i> -2,3-PDC (16.5 nmol)	14.0 (11.0–16.0)*	6/6 (100%)*
MK-801 + 0.85% NaCl	600 (600–600)	0/5 (0%)
MK-801 + D,L - <i>cis</i> -2,3-PDC (16.5 nmol)	600 (600–600)	0/5 (0%)
DNQX + 0.85% NaCl	600 (600–600)	0/7 (0%)
DNQX + D,L - <i>cis</i> -2,3-PDC (16.5 nmol)	26.0 (16.0–38.0)*	7/7 (100%)*

$n=5$ –7 animals in each group; n_{cv} —number of animals which had convulsions; n_t —total number of animals. D,L -*cis*-2,3-PDC (16.5 nmol) did not cause death.

* $P<.0001$ compared to NaCl–NaCl (Kruskal–Wallis test).

We also assessed the locomotor behavior of the animals. Statistical analysis of open-field data (one-way ANOVA) revealed that coadministration of MK-801 increased immobility scores [$F(3,17)=5.63$, $P<.05$] and had no effect on number of crossing [$F(3,17)=2.41$, $P>.05$] or rearing responses [$F(3,17)=2.38$, $P>.05$].

4. Discussion

The action of L-glutamate at the various EAA receptors plays a central role in both neuronal communication and CNS pathology (Cotman et al., 1995). Given their role, it is not surprising that considerable attention has focused on EAA receptor pharmacology and on the development of selective agonists and antagonists of these receptors. It is remarkable that recent progress has expanded the library of EAA analogues beyond those originally used to delineate the basic receptor classes (e.g., NMDA, KA, and AMPA). These newer agonists have been valuable in characterizing receptor channel properties, elucidating intracellular processes triggered by receptor activation, and associating specific pathological cascades with individual types of receptors (Ishida and Shinozaki, 1988; Debonnel et al., 1989; Shinozaki et al., 1989; Lanthorn et al., 1990; Kudo et al., 1991; Schoepp et al., 1991, 1994; Madsen et al., 1996).

In the present study, we demonstrated that D,L -*cis*-2,3-PDC, a glutamate analogue, inhibited only sodium-independent [3 H]-L-glutamate binding in brain plasma membranes, indicating an interaction with glutamate receptors. These results suggest that this compound, differently from its diastereoisomer L-*trans*-2,3-PDC (Willis et al., 1996) and L-*trans*-2,4-PDC (Bridges et al., 1991), does not interact with glutamate uptake binding sites. One should be aware, however, that such a lack of effect of D,L -*cis*-2,3-PDC on sodium-dependent binding does not imply a lack of effect of this compound on amino acid uptake or release since binding studies do not directly address functional activity but do identify specific sites of action. Nevertheless, the fact that a *cis* pyrrolidine dicarboxylate derivative does not alter sodium-dependent [3 H]-L-glutamate binding while *trans* isomers alter it suggests that the *cis* configuration of the carboxyl groups in the pyrrolidine ring affords some selectivity towards nontransport glutamate binding sites. It remains to be determined whether D,L -*cis*-2,3-PDC selectively binds to ionotropic or metabotropic receptors, but pharmacological evidence supports the involvement of NMDA receptors in the convulsant effects of D,L -*cis*-2,3-PDC, as discussed below.

It is remarkable that the intracerebral injection of D,L -*cis*-2,3-PDC caused generalized tonic–clonic convulsions in all mice in a dose-dependent manner (see Table 1). Moreover, these convulsions were completely prevented by the coadministration of the NMDA receptor antagonist MK-801, but not by DNQX, a competitive AMPA and KA receptor

antagonist, which caused a only a partial protection against D,L -*cis*-2,3-PDC-induced convulsions. These results indicate that D,L -*cis*-2,3-PDC cause convulsions by activating NMDA receptors, and that the participation non-NMDA ionotropic receptors in the convulsant action of D,L -*cis*-2,3-PDC is of minor relevance.

In conclusion, in this study, we report that the glutamate analogue D,L -*cis*-2,3-PDC interacts with nontransport glutamate binding sites and causes convulsions in mice, which seem to be due to the activation of NMDA receptors. Further studies are still necessary to determine whether this novel neurotoxin affects other glutamate-related functions as well as its value as a pharmacological tool.

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