3.1 OBJETIVOS ESPECÍFICOS:

a) Investigar o efeito do D,L-*cis*-2,3-PDC sobre a ligação do [^{3}H]-L-glutamato em receptores de membranas plasmáticas de córtex de ratos.

b) Investigar o potencial convulsivante do D,L-*cis*-2,3-PDC em camundongos.

c) Investigar o possível mecanismo de ação do D,L-*cis*-2,3-PDC em camundongos usando antagonistas específicos de receptores glutamatérgicos.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Pharmacology, Biochemistry and Behavior 76 (2003) 295–299
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

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

Valéria Dornelles Gindri Sinhorin[a], Marcos José Souza Carpes[b], Cheila Roehrs[a], Melissa Freire Zimmer[a], Patricia Dutra Sauzem[a], Maribel Antonello Rubin[a], Carlos Roque Duarte Correia[b], Carlos Fernando Mello[a,*]

[a]*Centro de Ciências Naturais e Exatas, Departamento de Química, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS Brazil*
[b]*Instituto de Química, Universidade Estadual de Campinas, 13083-970 Campinas, SP Brazil*

Received 11 March 2003; received in revised form 22 July 2003; accepted 5 August 2003

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 [^{3}H]-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.

Keywords: Binding; Convulsion; Glutamate; MK-801; DNQX; Intracerebroventricular administration

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-amino-cyclopentane-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

* Corresponding author. Tel./fax: +55-55-220-8031.
E-mail address: cf.mello@smail.ufsm.br (C.F. Mello).

0091-3057/$ – see front matter
doi:10.1016/j.pbb.2003.08.012

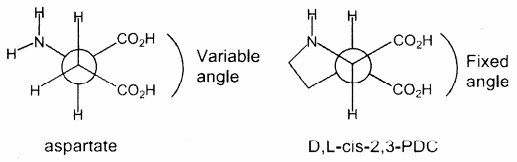

Fig. 1. Comparison of conformational mobility of aspartate to *cis*-2,3-PDC.

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 (2S,4S)-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 [^{3}H]-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 ± 1 °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 × *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 × *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 × *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 × *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 × *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 × *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 × *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. [^{3}H]-L-glutamate binding

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

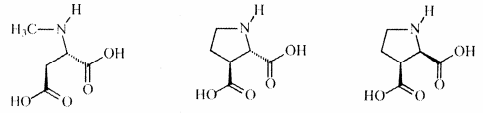

Fig. 2. Chemical structures of NMDA, L-trans-2,3-PDC, D,L-*cis*-2,3-PDC.

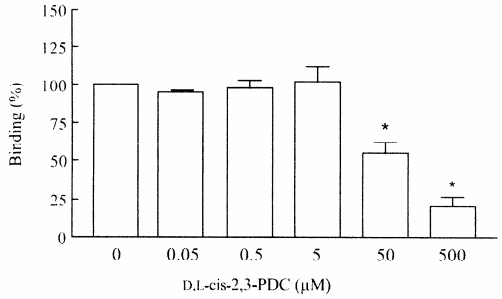

Fig. 3. Effect of D,L-*cis*-2,3-PDC on [^{3}H]-L-glutamate binding in rat brain plasma membranes. Results are presented as means (S.E.M.) of three experiments and are expressed as activity percentage of control. * Significantly different from control ($P<.05$—SNK test).

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 [^{3}H]-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 (intracerebroventricular) 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 [^{3}H]-L-glutamate binding to cerebral plasma membranes. D,L-*cis*-2,3-PDC reduced sodium-independent [^{3}H]-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 [^{3}H]-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 coadministrating MK-801 (7 nmol/ 2.5 μl), a noncompetitive NMDA receptor antagonist, with D,L-*cis*-2,3-PDC (16.5 nmol/ 2.5 μl). 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 coadministrating DNQX (10 nmol/2.5 μl), a competitive AMPA and KA receptor antagonist, with D,L-*cis*-2,3-PDC (16.5 nmol/2.5 μl). 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-*cis*-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-*cis*-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-*cis*-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.

Acknowledgements

This study was supported by CNPq (351127/97-6), CAPES, FAPESP, and FAPERGS.

References

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

Bridges RJ, Stanley MS, Anderson MW, Cotman CW, Chamberlin AR. Conformationally defined neurotransmitter analogues. Selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate diastereoisomer. J Med Chem 1991;34:717–25.

Carpes MJS, Miranda PCML, Correia CRD. Stereoselective synthesis of conformationally restricted analogues of aspartic and glutamic acids from endocyclic enecarbamates. Tetrahedron Lett 1997;38:1869–72.

Chamberlin AR, Bridges RJ. Conformationally constrained amino acids as probes of glutamate receptors and transporters. In: Kozikowski AP, editor. Drug design for neuroscience. New York: Raven; 1993. p. 231–59.

Choi DW. The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death. Annu Rev Neurosci 1990;13:171–82.

Choi DW. Glutamate receptors and the induction of excitotoxic neuronal death. Prog Brain Res 1994;100:47–51.

Clark WG, Vivonia CA, Baxter CF. Accurate freehand injection into a lateral brain ventricle of the conscious mouse. J Appl Physiol 1988;25:319–21.

Collingridge GL, Bliss TVP. Memories of NMDA receptors and LTP. Trends Neurosci 1995;18:54–6.

Conn PJ, Patel J. The metabotropic glutamate receptors. Toyota (NJ): Humana; 1994. p. 1–277.

Cotman CW, Kahle JS, Miller SE, Ulas J, Bridges RJ. Excitatory amino acid neurotransmission. In: Bloom FE, Kupfer DJ, editors. Psychopharmacology: the fourth generation of progress. New York: Raven Press; 1995. p. 75–85.

Daw NW, Stein PS, Fox K. The role of NMDA receptors in information processing. Annu Rev Neurosci 1993;16:207–22.

Debonnel G, Beauchesne L, Montigny C. Domoic acid, the alleged "mussel toxin," might produce its neurotoxic effect through kainate receptor activation: an electrophysiological study in rat dorsal hippocampus. Can J Physiol Pharm 1989;67:29–33.

Emanuelli T, Antunes VF, Souza DOG. Characterization of L-[^{3}H]glutamate binding to fresh and frozen crude plasma membranes isolated from cerebral cortex of adult rats. Biochem Mol Biol Int 1998;44:1265–72.

Hollman M, Heinemann S. Cloned glutamate receptors. Annu Rev Neurosci 1994;17:31–108.

Humphrey JM, Bridges RJ, Chamberlin AR. 2,3-pyrrolidine dicarboxylates as neurotransmitter conformer mimics: enantioselective synthesis via chelation-controlled enolate alkylation. J Org Chem 1994;59:2467–72.

Ishida M, Shinozaki H. Acromelic acid is a much more potent excitant than kainic acid or domoic acid in the rat spinal cord. Brain Res 1988;474:386–9.

Kanai Y, Smith CP, Hediger MA. The elusive transporters with a high affinity for glutamate. Trends Neurosci 1993;16:365–70.

Kudo Y, Akita M, Ishida M, Shinozaki H. A significant increase in intracellular Ca^{2+} concentrations induced by (2S,3R,4S)-2-(carboxycyclopropyl)glycine, a new potent NMDA agonist, in cultured rat hippocampal neurons. Brain Res 1991;567:342.

Lanthorn TH, Hood WF, Watson GB, Compton RP, Rader RK, Gaoni Y, et al. *Cis*-2,4-Methanoglutamate is a potent and selective *N*-methyl-D-aspartate receptor agonist. Eur J Pharmacol 1990;182:397–404.

Madsen U, Bang-Andersen B, Brehm L, Christensen IT, Ebert B, Kristoffersen ITS, et al. Synthesis and pharmacology of highly selective carboxy and phosphono isoxazole amino acid AMPA receptor antagonists. J Med Chem 1996;39:1682–91.

Meldrum BS. Excitotoxicity and selective neuronal loss in epilepsy. Brain Pathol 1993;3:405–12.

Nakanishi S. Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. Neuron 1994;13:1031–7.

Nicolletti F, Bruno V, Capani A, Casabona G, Knöpfel T. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? Trends Neurosci 1996;19:267–71.

Ortwine DF, Malone TC, Bigge CF, Drummond JT, Humblet C, Johnson G, et al. Generation of *N*-methyl-D-aspartate agonist and competitive antagonist pharmacophore models. Design and synthesis of phosphonoalkyl-substituted tetrahydroisoquinolines as novel antagonists. J Med Chem 1992;35:1345–70.

Racine RJ. Modification of seizure activity by electrical stimulation: II. Motor seizure. Electroenceph Clin Neurophysiol 1972;32:281–94.

Rao R, Murthy RK. Characteristics of [^{3}H] glutamate binding sites in rat cerebellum. Biochem Mol Biol Int 1993;30:861–6.

Rothman SM, Olney JW. Excitotoxicity and the NMDA receptor-still lethal after eight years. Trends Neurosci 1995;18:57–8.

Schoepp DD, Smith CL, Lodge D, Millar JD, Leander JD, Sacaan AI, et al. D,L-(tetrazol-5-yl)glycine: a novel and highly potent NMDA receptor agonist. Eur J Pharmacol 1991;203:237–43.

Schoepp DD, Lunn WHW, Salhoff CR, McDonald JW. The NMDA receptor agonist D,L-(tetrazol-5-yl)glycine is a highly potent excitotoxin. Eur J Pharmacol 1994;270:67–72.

Shinozaki H, Ishida M, Shimamoto K, Ohfune Y. A conformationally restricted analogue of L-glutamate, the (2S,3R,4S) isomer of L-α-(carboxycyclopropyl)glycine, activates the NMDA-type receptor more markedly than NMDA in the isolated rat spinal cord. Brain Res 1989;480:355–9.

Szatkowski M, Attwell D. Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. Trends Neurosci 1994;17:359–65.

Watkins JC, Krogsgaard-Larsen P, Honore T. Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. Trends Pharmacol Sci 1990;11:25–33.

Willis CL, Humphrey JM, Koch HP, Blakely T, Ralston L, Baker CA, et al. L-Trans-2,3-pyrrolidine dicarboxylate: characterization of a novel excitotoxin. Neuropharmacology 1996;35:531–9.

Willis CL, Dauenhauer DL, Humphrey JM, Chamberlin AR, Buller AL, Monaghan DT, et al. Methylation of the NMDA receptor agonist L-trans-2,3-pyrrolidine-dicarboxylate: enhanced excitotoxic potency and selectivity. Toxicol Appl Pharmacol 1997;144:45–55.

3.2 OBJETIVOS ESPECÍFICOS:

a) Investigar os efeitos comportamentais da injeção intracerebroventricular de sucinato em camundongos.

b) Investigar o efeito da injeção intracerebroventricular de sucinato sobre a produção de Substâncias que reagem ao Ácido Tiobarbitúrico (TBARS) em cérebros de camundongos *ex vivo*.

c) Investigar o efeito da injeção intracerebroventricular de sucinato sobre a carbonilação de proteínas em cérebros de camundongos *ex vivo*.

d) Investigar se o MK-801, um bloqueador do canal NMDA, altera a carbonilação de proteínas em cérebros de camundongos *ex vivo.*

DTD 5

ARTICLE IN PRESS

BRES-34412; No. of pages: 6; 4C:

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Brain Research xx (2005) xxx – xxx

BRAIN RESEARCH

www.elsevier.com/locate/brainres

Research report

Succinate causes oxidative damage through *N*-methyl-D-aspartate-mediated mechanisms

V.D.G. Sinhorin[a], C. Roehrs[c], J.S.M. Pasin[a], N.A.V. Bellé[a], M.A. Rubin[b], C.F. Mello[a,*]

[a]*Department of Physiology, Federal University of Santa Maria, 97105-900, Santa Maria RS, Brazil*
[b]*Department of Chemistry, Federal University of Santa Maria, 97105-900, Santa Maria RS, Brazil*
[c]*Department of Health Sciences, Integrated Regional University of Alto Uruguai and Missões, 98400-00, Frederico Westphalen RS, Brazil*

Accepted 23 May 2005

Abstract

In this study we investigated whether succinate, the accumulating substrate in succinate dehydrogenase (SDH) deficiencies and SDH inhibitor intoxication, causes lipoperoxidation and protein carbonylation, and if NMDA receptors are involved in the succinate-induced oxidative damage. Adult male mice (30–40 g) received an intracerebroventricular injection of succinic acid (0.7, 1.0 and 1.7 μmol/5 μl) or 0.9% NaCl (5 μl) and had their exploratory behavior assessed in an open field for 10 min. Succinate (0.7 and 1.0 μmol/5 μl) decreased locomotor activity behavior and increased thiobarbituric acid reactive substances (TBARS) and protein carbonylation in the forebrain. Conversely, 1.7 μmol of succinate did not alter locomotor activity or oxidative damage parameters. The involvement of NMDA receptors in the succinate-induced increase of total protein carbonylation content and exploratory behavior inhibition was assessed by co-administrating MK-801 (7 nmol/2.5 μl icv), a noncompetitive NMDA receptor antagonist, with succinate (1 μmol/2.5 μl icv). The co-administration of MK-801 protected against succinate-induced increase of total protein carbonylation and decrease of locomotor activity. These results suggest the involvement of NMDA receptors in these effects of succinate, which may of particular relevance for succinate-accumulating conditions, such as SDH inhibitors intoxication and inherited SDH deficiencies.

Theme: Disorders of the nervous system.
Topic: Neurotoxicity

Keywords: Locomotor behavior; Free radical; MK-801; Succinate dehydrogenase; Malonate; 3-Nitropropionic acid

1. Introduction

Succinate is the endogenous substrate of succinate dehydrogenase (SDH), which is active in the tricarboxylic acid cycle and the electron transport chain during ATP synthesis [1,16]. This dicarboxylic acid accumulates in some inborn errors of the metabolism, such as complex II deficiency, malonic and methylmalonic acidemias. Succinate accumulation in malonic and methylmalonic acidemias is probably due to the competitive inhibition of SDH by malonate [14,16,26] and methylmalonate [11,14,24,38,41]. In fact, it has been shown that succinate accumulation reaches nearly 150 μM in the urine of malonic acidemic patients [31], and cerebral concentrations range between 0.5 and 8.3 mM in the white matter of patients with complex II deficiency [6]. Interestingly, white matter cerebral damage in these patients proved to be more extensive in those presenting intermediate

Abbreviations: ANOVA, analysis of variance; AP5, D-2-amino-5-phosphonovaleric acid; ATP, adenosine triphosphate; DHBA, dihydroxybenzoic acid; DNPH, 2,4-dinitrophenylhydrazine; fEPSPs, field excitatory post-synaptic potentials; icv, intracerebroventricular; MK-801, (+)-5-methyl-10,11-dihydroxy-5-dibenzo (a,b) cycloheptene-5,10-imine or dizocilpine; NMDA, *N*-methyl-D-aspartate; SDH, succinate dehydrogenase; 3-NPA, 3-nitropropionic acid; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TBARS. thiobarbituric acid reactive substances; MDA, malondialdehyde

* Corresponding author. Fax: +55 55 3220 8241.
E-mail address: cf.mello@smail.ufsm.br (C.F. Mello).

0006-8993/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.brainres.2005.05.053

cerebral succinate concentrations (0.5–1.3 mM) than in those presenting high cerebral succinate concentrations (3.7–8.3 mM).

A recent study from Roehrs et al. [33] may have shed some light on this apparent paradox. In that study it was demonstrated that succinate, at low concentrations (0.3–1 mM), increases neuronal post-synaptic excitatory potentials (fEPSPs) in hippocampal slices from adult rats by NMDA-mediated mechanisms. Conversely, high concentrations of succinate (3–10 mM) decrease fEPSPs and reverse the increase of fEPSPs induced by low concentrations of succinate [33]. The excitatory action of succinate was confirmed in vivo, since it induced convulsions in mice, which were prevented by the co-administration of MK-801 (an NMDA receptor antagonist). The authors suggested that succinate might be acting as a partial NMDA agonist, or at two distinct sites with opposite electrophysiological implications. The findings by Roehrs et al. [33] explain why high concentrations of succinate (above the mM range) attenuate or reverse the neurotoxic action of malonate and methylmalonate [3,10,17,34,42] but causes convulsions, at comparatively low doses, by NMDA-mediated mechanisms, in mice.

The activation of NMDA receptors is though to be associated with generation of reactive species [21,27,28,37, 39,43], which have been recognized as important mediators of tissue injury in several neurodegeneration models [2,15,20,35,36], including those in which succinate accumulation has been demonstrated [19] or is a strong possibility, such as competitive SDH inhibitors exposure [13,23,24]. In line with this view, intrastriatal malonate increases 2,3 and 2,5-DHBA, 3-nitrotyrosine and peroxynitrite generation [25] and methylmalonic acid increases lipoperoxidation [13,23,24]. However, until the present moment, no study has addressed whether succinate accumulation causes oxidative damage per se. Therefore, in this study we investigated whether succinate causes lipoperoxidation and protein carbonylation, and if NMDA receptors are involved in the succinate-induced oxidative damage.

2. Materials and methods

2.1. Chemicals

All reagents were acquired from Sigma (St. Louis, MO, USA), except thiobarbituric acid (TBA), which was obtained from Merck (Darmstadt, Germany). Succinate and other solutions injected were prepared in 0.9% NaCl (saline).

2.2. Animals

Adult male Swiss albino mice (30–40 g), maintained in a 12-h dark/12-h light cycle at controlled temperature (22 *T* 1 °C), with free access to tap water and standard laboratory chow (Guabi, Santa Maria, RS, Brazil) were used. All experimental protocols were conducted in accordance with National and International legislation (guidelines of the Brazilian College of Animal Experimentation (COBEA) and U.S. Public Health Services Policy on Humane Care and Use of Laboratory Animals-PHS Policy), and with the approval of the Ethical Committee for animal research of the Federal University of Santa Maria (protocol number 0120256/2002-19). The number of animals used was kept to a minimum by using planned statistical analyses at predefined stages of the experiments. Adequate measures were taken to minimize pain and discomfort.

2.3. Behavioral effects of succinate on mice

Free hand intracerebroventricular (icv) injections into the lateral ventricles of the conscious mice were made using a 30-gauge needle attached to a 10-μl Hamilton syringe (3 mm of the needle tip exposed) according to Clark et al. [8] by an experimenter who was not aware of the pharmacological treatment. 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 (icv) confirmed by needle track verification with a PZO MST131 stereomicroscope. Only data from animals with the needle track aiming the lateral ventricle were considered. Animals were injected (icv) with 5 μl of succinate (0.7, 1.0 and 1.7 μmol) or 0.9% NaCl and, immediately thereafter, individually placed in a round open-field (35 cm of internal diameter), which had its floor divided into 10 areas of equal size. During 10 min the number of areas crossed, the number of rearing responses and the total time spent in immobility were recorded manually. The involvement of NMDA receptors in the succinate-induced neurochemical and behavioral alterations was assessed by co-injecting the animals with 7.0 nmol MK-801 and 1.0 μmol succinate in 2.5 μl plus 2.5 μl volumes in the same syringe, separated by an air bubble. The animals were immediately transferred to the open field and observed for 10 min, as described above.

2.4. TBARS assay ex vivo

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their forebrain removed. Tissues were homogenized in 10 volumes (w/v) of 10 mM Tris–HCl, pH 7.4, containing sodium dodecyl sulfate (SDS, 0.10%—final concentration) using a glass homogenizer and TBARS content was estimated in a medium containing 0.2 ml of brain homogenate, 0.1 ml of 8.1% SDS, 0.4 ml of acetic acid buffer (500 mM, pH 3.4) and 0.75 ml of 0.81% thiobarbituric acid (TBA). The mixture was finally made up