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Short communication

(−)-β-Cyclazocine is an antagonist of NMDA receptor-mediated responses and a potent neuroprotectant in rat cortical neurons

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Abstract

Microspectrofluorimetry and excitotoxicity experiments were performed to study the NMDA receptor-blocking and neuroprotective actions of (−)- and (+)-β-cyclazocine in cultured rat cortical neurons. (−)-β-Cyclazocine potently antagonized NMDA-induced $[Ca^{2+}]_i$ increases (IC_{50} = 220 nM) in neurons loaded with the Ca^{2+} fluorophore, fura-2. (−)-β-Cyclazocine was specific for NMDA receptor-mediated responses versus those mediated through non-NMDA receptors or voltage-activated Ca^{2+} channels. The agent was active against NMDA-induced neurotoxicity, even at 1 μM. In all experiments, the (+)-enantiomer was found to be considerably less potent than the (−)-enantiomer. These results indicate that (−)-β-cyclazocine is a specific NMDA receptor antagonist with potent neuroprotective properties in rat cortical neurons.

Keywords: NMDA receptor; NMDA (*N*-methyl-D-aspartate); Benzomorphan; Excitotoxicity

1. Introduction

Compounds active as functional antagonists at the *N*-methyl-D-aspartate (NMDA) receptor-ion channel complex, such as phencyclidine (PCP), MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate), and ketamine, are able to protect CNS neurons from glutamate- or NMDA-induced death in vitro (Weiss et al., 1986; Goldberg et al., 1988). In addition, there is evidence that compounds belonging to the opiate class of drugs can protect neurons from excitotoxic glutamate, quinolinate, or NMDA insults (Choi and Viseskul, 1988).

β-Cyclazocine, the *cis* stereoisomer of the benzomorphan, α-cyclazocine, has high affinity for both opiate and PCP receptors (IC_{50} against [^{3}H]Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol (DAMGO) binding = 15 ± 2 nM and IC_{50} against [^{3}H]PCP binding = 22 ± 1 nM; Todd et al., 1990). Drug discrimination studies using rats trained to bar-press for PCP have shown that these animals generalize to (−)-β-cyclazocine (Slifer and Balster, 1988). The electrophysiological actions of (−)- and (+)-β-cyclazocine have been investigated using extracellular recordings of field potential responses to NMDA (Church et al., 1991). Specifically, (−)-β-cyclazocine was found to inhibit responses to NMDA but not to quisqualate in both rat cortical wedges and rat spinal cord neurons, while (+)-β-cyclazocine was inactive against either type of response in both preparations. In order to further elucidate the mechanism by which (−)- and (+)-β-cyclazocine interact with the NMDA receptor-ion channel complex, we determined the effects of these agents on NMDA-evoked rises in intracellular free calcium ($[Ca^{2+}]_i$) using cultured rat cortical neurons loaded with the fluorescent dye, fura-2. This same preparation was used to assess the effects of (−)-β-cyclazocine on responses to the non-NMDA receptor agonists, kainate and AMPA, as well as responses mediated through high-voltage-activated Ca^{2+} channels. Finally, we tested the effects of (−)- and (+)-β-cyclazocine treatment on rat cortical neurons exposed to a toxic concentration of NMDA.

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2. Materials and methods

Primary cultures of rat cortical neurons were prepared from 18-day-old embryos according to the method of Banker and Cowan (1977). For the microspectrofluorimetric experiments, cells were plated onto 18-mm coverslips at a density of 10^5 cm^{-2}. In these neurons, free intracellular calcium ($[Ca^{2+}]_i$) responses to NMDA are fully expressed one week post-isolation and are stable at least until the second week in culture; therefore, studies were conducted 7–14 days following isolation. The procedures for fura-2 loading and $[Ca^{2+}]_i$ measurement have been described previously (Church et al., 1994). Briefly, cover slips containing neurons were loaded for 1.5 h with fura-2-AM (Molecular Probes, Eugene, OR, USA). The cover slips were washed, transferred to a chamber and perfused at a rate of 1.5 ml min^{-1} with a nominally Mg^{2+}-free solution containing, in mM, NaCl (136.5), KCl (3.0), NaH_2PO_4 (1.5), glucose (10.0), $CaCl_2$ (2.0), tetrodotoxin (0.0003), and Hepes (10.0), adjusted to pH 7.4 with NaOH. The NMDA co-agonist, glycine 2 μM, was also added to ensure saturation of strychnine-insensitive glycine sites. Rises in $[Ca^{2+}]_i$ were evoked by 20-s bolus applications of either 20 μM NMDA, 80 μM kainate, 40 μM AMPA, or 50 mM KCl (by substitution for NaCl), with a subsequent 15-s wash with perfusion medium. These concentrations were submaximal in these neurons and have generally been found to be equipotent as to elicitation of $[Ca^{2+}]_i$ increases, although the relative potency of these agonists has been found to vary somewhat between different culture batches. (−)- and (+)-β-cyclazocine were applied by superfusion. The dual-excitation ratio method, with subtraction of background fluorescence prior to ratio calculation, was used to measure $[Ca^{2+}]_i$. The total number of cells examined under given experimental conditions is referred to as n. Each concentration of each test compound was examined in at least 3 different cultures. Experiments were performed at room temperature (20–22°C).

In the neuroprotection experiments, cortical neuronal cultures were exposed to 1 mM NMDA, in the presence or absence of (−)-β-cyclazocine or (+)-β-cyclazocine, for 24 h. In all cases 1 μM glycine was included in the medium; some culture wells had only glycine added, with no NMDA or β-cyclazocine, in order to assess cell loss during the procedure. All treatments were performed in parallel within the same culture batch. Cultures were then stored in an incubator containing 5% CO_2 mixed with humidified room air at 37°C. Cell mortality was assessed 24 h later by trypan blue exclusion (Paul, 1975). The results for each well were calculated as the number of viable neurons remaining after 24 h, divided by the number counted before the challenge. The number of separate cultures used in the study is referred to as N; errors refer to the S.E.M.

3. Results

In studies with fura-2-loaded cortical neurons, applications of 20 μM NMDA induced mean rises in $[Ca^{2+}]_i$ of 360 ± 60 nM from a baseline of 75 ± 6 nM (n = 531). This rise in $[Ca^{2+}]_i$ began within seconds after addition of NMDA into the chamber and persisted for about 30 s following washout. Return to baseline Ca^{2+} levels generally was complete at approximately 3 min following washout. (−)-β-Cyclazocine attenuated NMDA-evoked rises in $[Ca^{2+}]_i$ with an IC_{50} of 220 nM (Fig. 1A).

In the presence of (−)-β-cyclazocine, several applications of NMDA were necessary in order to obtain the maximum attainable reduction of NMDA-evoked rises in $[Ca^{2+}]_i$ for a given drug concentration (see Fig.

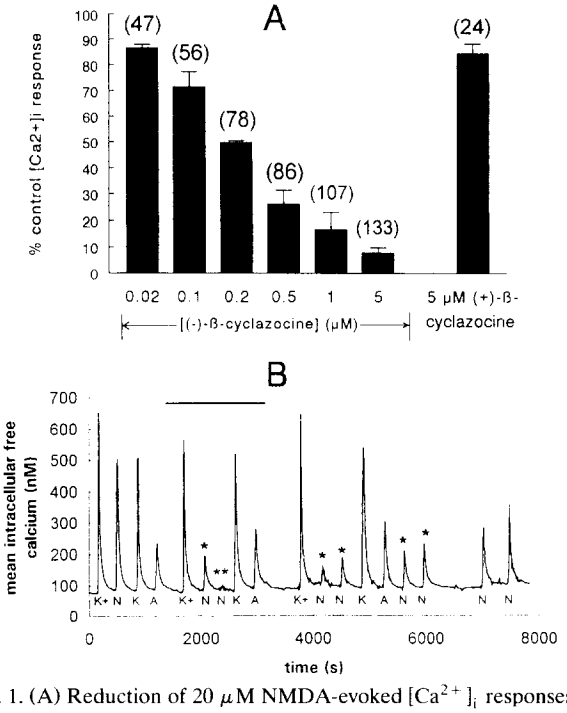

Fig. 1. (A) Reduction of 20 μM NMDA-evoked $[Ca^{2+}]_i$ responses by β-cyclazocine in fura-2-loaded cortical neurons. Values shown are the steady state means of measurements taken from three different cortical culture batches for each concentration tested, corrected for baseline $[Ca^{2+}]_i$ and normalized to control responses with 20 μM NMDA only; error bars refer to the S.E.M. for the normalized values. The n for each treatment is indicated in parentheses above each bar. (B) Selectivity of 5 μM (−)-β-cyclazocine. $[Ca^{2+}]_i$ responses to 50 mM potassium (K^+), 20 μM NMDA (N), 80 μM kainate (K), and 40 μM AMPA (A) are shown for control, during superfusion of 5 μM (−)-β-cyclazocine (solid bar), and after washout of (−)-β-cyclazocine. Responses in the presence of (−)-β-cyclazocine were 86.8%, 30.8%, 8.2%, 109.6%, and 106.9% of control for K^+, N, N, K, and A, respectively. This trace is the mean of responses from 27 neurons measured simultaneously. Significant difference from control: $^*P < 0.05$; $^{**}P < 0.01$ by Student's t-test.

1B). Therefore, the blockade of NMDA receptor-mediated responses by (−)-β-cyclazocine is dependent on the presence of agonist, as has been found for the open-channel NMDA blockers ketamine, phencyclidine, and MK-801 (MacDonald et al., 1991). In this respect, cortical cultures that had been superfused with drug for periods greater than 1 h required the same number of agonist applications to reach the steady state reduction of responses as did cultures that had been exposed for only 5 min (not shown).

Due to limited supplies of the drug, only one concentration (5 μM) of (+)-β-cyclazocine was tested. The right-hand bar in Fig. 1A shows that this concentration produced relatively little antagonism of NMDA-evoked responses.

Fura-2-loaded cortical neurons were also used to assess the selectivity of (−)-β-cyclazocine against responses mediated via NMDA receptors, non-NMDA excitatory amino acid receptors, and voltage-gated Ca^{2+} channels. Fig. 1B shows the effects of 5 μM (−)-β-cyclazocine on responses of a set of cortical neurons to applications of 20 μM NMDA, 80 μM kainate, and 40 μM AMPA. In addition, the effects of 5 μM (−)-β-cyclazocine to applications of 50 mM K^+, which permit Ca^{2+} entry through voltage-activated Ca^{2+} channels (see Church et al., 1994), are shown. The mean steady state response to NMDA during superfusion of 5 μM (−)-β-cyclazocine was 7.8 ± 2.1% of the control, while the mean responses to kainate, AMPA, and K^+ were 94.5 ± 2.2%, 99.4 ± 0.3%, and 102.0 ± 2.2% of the control, respectively (n = 71).

Cortical cultures exposed to 1 mM NMDA showed a large reduction in the number of surviving neurons (55.6 ± 3.8% reduction with NMDA plus 1 μM glycine relative to control cultures with just 1 μM glycine; N = 7). The majority of cells present 24 h following NMDA treatment had rough, discontinuous plasma membranes, increased somal volumes, cytoplasmic vacuoles, and a 'bath-chain' appearance in their axonal processes. When only glycine was included in the bathing medium, with no added NMDA, the neurons present 24 h later appeared healthy and morphologically similar to those observed before the treatment. Following treatment with 1 μM (−)-β-cyclazocine many neurons had retained the smooth, healthy appearance characteristic of pretreatment observations. The results are summarized in Fig. 2, which shows that the (−)-enantiomer significantly protected cortical neurons from NMDA-induced damage at a concentration of 1 μM and afforded close to full protection at 10 μM. In the latter case the cultured cells closely resembled those not exposed to NMDA. However, little or no neuroprotection was observed when 1 μM of the (+)-enantiomer was tested (Fig. 2). At a higher concentration (10 μM) (+)-β-cyclazocine afforded some degree of neuroprotection, although slightly less than that achieved with 1 μM (−)-β-cyclazocine.

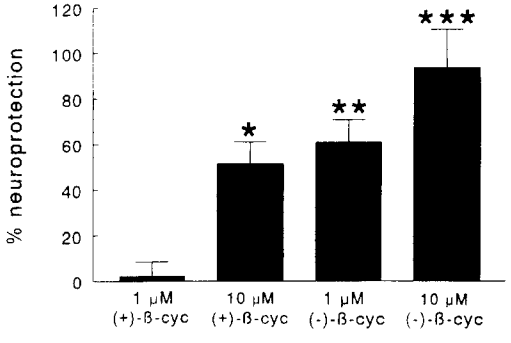

Fig. 2. Stereospecific neuroprotection by β-cyclazocine. The percentage of neurons rescued from 1 mM NMDA-induced neuronal degeneration by either (−)-β-cyclazocine or (+)-β-cyclazocine, measured at 24 h, is shown. Values were corrected for cell losses during the procedure (as estimated from control cultures where no NMDA or β-cyclazocine was added) and normalized to the mean values obtained from cultures where only 1 mM NMDA was added. All treatments were performed in parallel with seven different culture batches; each treatment was replicated 3 times within a given culture batch. Error bars indicate S.E.M. for the culture batches. Statistics performed were a one-way analysis of variance followed by a Tukey test. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$.

4. Discussion

The results of this study show that the (−)-enantiomer of β-cyclazocine is a selective and highly potent antagonist of NMDA receptor-mediated responses in central nervous system neurons. It is the most potent NMDA receptor antagonist in the benzomorphan series known to date; its IC_{50} value (220 nM) against NMDA-evoked responses is lower than values for compounds from other classes, with only MK-801 showing greater potency against NMDA-induced $[Ca^{2+}]_i$ rises (IC_{50} ~ 2 nM; Dayanithi et al., 1995).

The observed blockade of NMDA-evoked rises in $[Ca^{2+}]_i$ in the fura-2-loaded cortical neurons likely reflects the action of (−)-β-cyclazocine at the PCP site associated with the NMDA ion channel. The affinity of (−)-β-cyclazocine for opioid receptors (Todd et al., 1990) was unlikely to contribute to the antagonism of NMDA responses since morphine and naloxone have been previously found to be ineffective against NMDA-evoked rises in $[Ca^{2+}]_i$ (Church et al., 1994). Certain features of the antagonism of NMDA-evoked $[Ca^{2+}]_i$ responses by (−)-β-cyclazocine, most notably its agonist dependence, are consistent with open-channel block of the ion channel coupled to the NMDA receptor. Use dependence of blockade, where the maximal reduction of NMDA receptor-mediated responses is built up over several agonist applications, is a common feature among PCP-like drugs that block open NMDA ion channels (Church and Lodge, 1990; MacDonald et al., 1991). Electrophysiological studies inves-

tigating the actions of (−)-β-cyclazocine on NMDA receptor-mediated activity would be required to determine the specific nature of the open-channel block.

The neuroprotective properties of (−)-β-cyclazocine were demonstrated by its actions to protect cortical neuronal cultures from damage induced by exposure to a toxic level of NMDA. Currently, only MK-801, with a concentration of 0.3 μM producing 50% neuroprotection, has been found to be more potent to protect neurons against NMDA-mediated neurotoxicity (Goldberg et al., 1988). PCP itself has been found to produce 50% neuroprotection at 1 μM (Goldberg et al., 1988), whereas the present results indicate that 1 μM (−)-β-cyclazocine provides 60% protection against NMDA-induced damage.

The stereospecificity of the neuroprotective and NMDA receptor antagonist effects of β-cyclazocine were qualitatively consistent with the findings in previous studies with this agent, including binding to the PCP site in rat brain membranes (Todd et al., 1990), antagonism of NMDA receptor responses in rat spinal neurons in vivo and in rat cortical wedges in vitro (Church et al., 1991), and discriminative stimulus properties (Slifer and Balster, 1988). In addition, the higher potency of the (−)-isomer over the (+)-isomer is qualitatively similar to that seen with the optical isomers of β-cyclazocine's geometric isomer, α-cyclazocine (Mendelsohn et al., 1984; Martin and Lodge, 1988; Church and Lodge, 1990), although the separation of potencies is greater with the β-cyclazocine enantiomers than with the α-cyclazocine enantiomers. It is interesting to note that with most other NMDA channel blockers, including ketamine (Lodge et al., 1982), SKF-10,047 (*N*-allylnormetazocine) (Martin and Lodge, 1988), and the PCP analogue, 3-methyl-phencyclidine (PCMP; Martin and Lodge, 1988), it is the (+)-enantiomers that are the most potent. The greater potency of the (−)- over the (+)-enantiomer seen for β-cyclazocine is a relatively uncommon feature of chiral uncompetitive NMDA receptor antagonists and is a property shared only by α-cyclazocine and pentazocine (Mendelsohn et al., 1984; Church and Lodge, 1990).

The results demonstrating the efficacy of 10 μM (+)-β-cyclazocine in the neuroprotection assay were somewhat unexpected, given (+)-β-cyclazocine's weak antagonism of NMDA-evoked $[Ca^{2+}]_i$ increases in the fura-2-loaded neurons. While this compound has demonstrated affinity for μ-opioid receptors, the (+)-stereochemistry with β-cyclazocine is even less favourable, relative to that of the (−)-enantiomer, for opioid-site binding than for PCP site effects (Todd et al., 1990). Competition with (+)-[^{3}H]*N*-allylnormetazocine binding has been shown to favour the (+)-isomer of β-cyclazocine over the (−)-isomer, indicating the former is the more potent ligand at σ receptors (Martin et al., 1984). On the other hand, no binding studies showing the affinity of β-cyclazocine for other receptors, ion channels, or enzymes have been reported in the literature. The possibility that (+)-β-cyclazocine can produce neuroprotection via sites distinct from either PCP or opioid receptors, such as σ receptors (or, alternatively, that the neuroprotective action of (−)-β-cyclazocine is hampered by a hitherto unidentified mechanism), remains available for future investigation.

The present results indicate that (−)-β-cyclazocine selectively blocks NMDA ion channels relative to its effects on non-NMDA ion channels and high-voltage-activated Ca^{2+} channels. Since the clinical management of any pathology involving a given neurochemical system demands that pharmacological agents specific for affecting that particular system be used, the high selectivity of (−)-β-cyclazocine to decrease responses to NMDA suggests that this agent has potential for the therapy of disease states associated with NMDA receptor-mediated activity. To our knowledge, no studies testing the effects of (−)-β-cyclazocine on stroke-related brain damage in vivo have been published to date. Therefore the utility of (−)-β-cyclazocine as a neuroprotectant in the treatment of hypoxic/ischaemic CNS damage (e.g. stroke) and neurodegenerative disorders, such as amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's chorea, is a promising area of future research.

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