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In vivo assessment of kynurenate neuroprotective potency and quinolinate excitotoxicity

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Summary. Three complementary questions related to the kynurenine pathway and excitotoxicity were addressed in this study: (i) Which extracellular levels of quinolinic acid (QUIN) may be neurotoxic? (ii) Which extracellular levels of kynurenic acid (KYNA) may control excessive NMDA-receptor function? (iii) Can "anti-excitotoxic" levels of KYNA be reached by inhibition of kynurenine-3-hydroxylase (i.e. inhibition of QUIN synthesis and shunts of kynurenine metabolism toward KYNA)? Multifunctional microdialysis probes were used in halothane anaesthetised rats to apply NMDA or QUIN directly to the brain, with or without co-perfusion of KYNA, to record the resulting local depolarisations, and to monitor changes in dialysate KYNA after kynurenine-3-hydroxylase inhibition. QUIN produced concentration-dependent depolarisations with an estimated EC_{50} (i.e. concentration in the perfusion medium) of 1.22 mM. The estimated ED_{50} for KYNA inhibition of NMDA-responses was 181 μM. Kynurenine-3-hydroxylase inhibition (Ro-61-8048, 100 mg/kg i.p.) increased dialysate KYNA 11 times (to 33.8 nM) but without any reduction of NMDA-responses. These data challenge the notion that extracellular accumulation of endogenous QUIN may contribute to excessive NMDA-receptor activation in some neurological disorders, and the suitability of kynurenine-3-hydroxylase inhibition as an effective anti-excitotoxic strategy.

Keywords: Amino acids – Kynurenine pathway – Kynurenic acid – Quinolinic acid – NMDA-receptor – Excitotoxicity – Microdialysis

Introduction

Quinolinic (QUIN) and kynurenic (KYNA) acids are two metabolites of the kynurenine pathway, with opposite actions on glutamate-receptors (Stone, 1993; Botting, 1995). Quinolinate is an agonist of NMDA-receptors and, therefore, the marked rise in cerebral QUIN concentrations that is

associated with inflammatory neurological diseases could be detrimental to the survival of neurones in these conditions (Alberati-Giani et al., 1996; Heyes et al., 1997). Beagles et al. (1998) have demonstrated that QUIN levels in the extracellular fluid increased from 0.11 to 7.3 μM during endotoxin-induced CNS inflammation, and prolonged exposure to submicromolar concentrations of QUIN was toxic to organotypic cultures of rat corticostriatal system (Whetsell and Schwarcz, 1989). However, QUIN is a relatively weak NMDA-receptor agonist, 10^3 less potent than glutamate when assayed on hippocampal neurons in the absence of Mg^{2+} (QUIN EC_{50} = 2.3 mM; Patneau and Mayer, 1990), and millimolar concentrations of QUIN were necessary to cause acute degeneration of rat striatal neurones *in vivo* (Schwarcz et al., 1983).

Kynurenate is an antagonist of all ionotropic glutamate-receptors with preferential affinity for the NMDA-receptor glycine site (Stone, 1993), and intracerebral administration of exogenous KYNA protected against excitotoxins (Taber et al., 1996; Foster et al., 1984). Accordingly, increasing the extracellular concentration of this kynurenine metabolite in the brain could be a pertinent alternative to the administration of exogenous NMDA-receptor antagonists for the treatment of neurological disorders suspected to involve excessive NMDA-receptor activation. KYNA accumulation in the brain can be achieved by inhibition of kynurenine-3-hydroxylase, which reduces QUIN formation and shunts kynurenine metabolism toward KYNA (Pellicciari et al., 1994; Speciale et al., 1996). The administration of kynurenine-3-hydroxylase inhibitors protected DBA/2 mice against audiogenic convulsions (Russi et al., 1992; Carpenedo et al., 1994), but whether this anti-convulsant action was linked to KYNA accumulation is still unclear. Indeed, the extracellular levels of KYNA measured after administration of the most potent kynurenine-3-hydroxylase inhibitors reported so far (Speciale et al., 1996; Röver et al., 1997) are well below the ED_{50} for KYNA blockade of the NMDA-receptor glycine site (15 μM; Kessler et al., 1989).

The purpose of our *in vivo* studies was to address the following questions:

- Which extracellular levels of QUIN produce local depolarisation of brain cells, indicative of possible, acute neurotoxicity?
- Which extracellular levels of KYNA may be required to control excessive NMDA-receptor function?
- Can these potentially anti-excitotoxic KYNA levels be reached with the novel kynurenine-3-hydroxylase inhibitor, 3,4-dimethoxy-*N*-[4-(3-nitrophenyl)thiazol-2-yl]benzene-sulfonamide (Ro-61-8048; Röver et al., 1997)?

Material and methods

Microdialysis probes incorporating an electrode allowed us to apply a multidisciplinary approach to individual brain regions; i.e. they were used to administer NMDA or QUIN locally to the brain area under study (with or without co-perfusion of KYNA), to record the resulting local depolarisations, and to monitor changes in dialysate KYNA after kynurenine-3-hydroxylase inhibition.

All experiments were performed with male, Lister Hooded rats (weight 240–340 g; Harlan UK, Blackthorn, U.K.) with food and water available ad libitum. Animal procedures were in strict accordance with the British Home Office guidelines and specifically licensed under the Animals (Scientific Procedures) Act 1986. Anaesthesia during surgical preparation and subsequent experimental procedures was maintained with halothane (2.5% and 1.5–1.75%, respectively) in O_2:N_2O (30:70), with the animal breathing spontaneously. To minimise any possible interference of halothane anaesthesia with the processes under study, once the surgical procedure had been completed, its concentration in the breathing mixture was kept to a minimum (i.e. $\geq$1.5%). The depth of anaesthesia was carefully controlled by continuous monitoring of the electro-encephalogram (EEG) and arterial blood pressure. A femoral artery was catheterised for arterial blood pressure monitoring, and a vein for induction of cardiac arrest upon completion of the experiment. Body temperature was maintained at 37.5–38°C throughout the experiment.

Intracerebral microdialysis

Multifunctional microdialysis probes (ME-H2; Applied Neuroscience, London) (Obrenovitch et al., 1993; 1994) were implanted into the dorsolateral striatum (coordinates: 0.8 mm anterior to bregma, 3 mm lateral, and 6 mm deep from the cortical surface; Paxinos and Watson, 1986) for the Ro-61-8048 study, and in the frontal cortex for all other experiments (co-ordinates: 1.2 mm anterior to bregma, 2.5 mm lateral, and 2 mm deep). Unless otherwise stated, microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF) (composition in mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26; pH 7.3 adjusted with 1 M NaOH) at 1 μl/min. At least 2 hours of stabilisation were allowed after probe implantation. The extracellular direct current (d.c.) potential was derived from the potential between the electrode built into the probe and a chlorided silver reference electrode placed under the scalp, and recorded as described previously (Obrenovitch et al., 1993; 1994).

Quinolinic acid study

Increasing concentrations of QUIN (1, 2, 3 mM) were perfused through the microdialysis probe for 2 min, followed by 30 min of recovery after the 1 mM QUIN challenge, and by 40 min of recovery after the 2 and 3 mM stimuli (Fig. 1).

Exogenous KYNA and NMDA-induced depolarisations

Nine separate depolarisations were produced by perfusion of 150 μM NMDA through the microdialysis probe for 2 min, each followed by 20 min of recovery (Fig. 2). This concentration of NMDA in the perfusion medium is close to the apparent EC_{50} in this preparation (126 μM), and NMDA-induced responses of this type were concentration-dependently blocked by the glycine site NMDA-receptor antagonist, L-701,324 (Obrenovitch et al., 1997). Ten min after the 2nd NMDA application, increasing concentrations of KYNA (0.03, 0.1, 0.3, 0.5 or 1 mM) were perfused for 22 min, starting 10 min before each NMDA challenge. The last two NMDA stimuli were control (i.e. NMDA in normal aCSF) to test the reversibility of KYNA inhibition.

Ro-61-8048 study

This novel kynurenine-3-hydroxylase inhibitor was administered as a homogenous suspension (0.5 ml per 100 g rat weight), prepared by homogenisation in 0.1% (v/v)

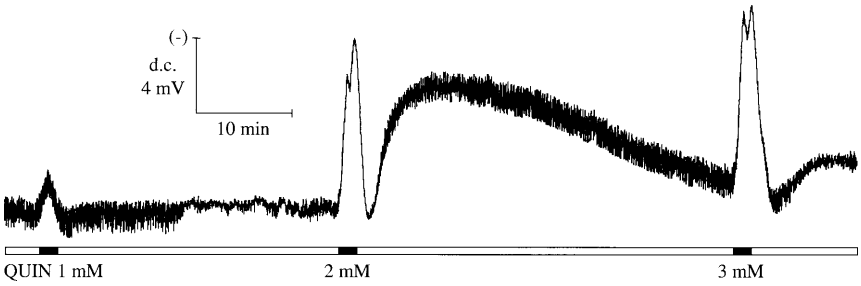

Fig. 1. Representative changes in the d.c. potential produced by quinolinic acid (*QUIN*) in the rat frontal cortex, *in vivo*. Increasing concentration of QUIN were applied for 2 min via a microdialysis probe, and the resulting depolarisation recorded precisely at the site of drug application

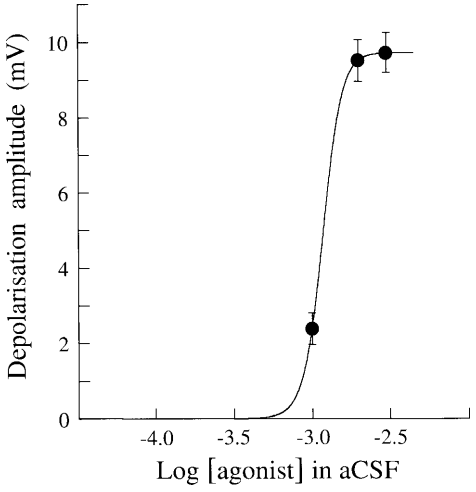

Fig. 2. Logarithm concentration-response curve obtained for quinolinic acid (QUIN) applied via a microdialysis probe implanted into the rat frontal cortex. Data (•, mean ± SEM) are from 7 experiments. The estimated EC_{50} (i.e. QUIN concentration in the aCSF producing half-maximal response) was 1.22 ± 0.03 mM. Note the very steep log[concentration]-response relationship

Tween-80/water, followed with sonication (Röver et al., 1997). Animals received 100 mg/kg Ro-61-8048 (i.p.) 14 min after the second NMDA challenge (see next). Twelve separate NMDA-stimuli were applied (200 μM NMDA in perfusion medium for 2 min), each followed by 28 min of recovery. For determination of changes in extracellular KYNA levels, 12 consecutive 30-min dialysate samples were collected starting 2 min before the first NMDA application. KYNA was determined by reverse-phase HPLC with fluorescence detection (Swartz et al., 1990; Röver et al., 1997). Briefly, 50 μl samples (from 30 μl dialysate mixed with 30 μl aCSF) were injected with an autosampler (717plus, Waters, Milford, MA, USA) into the HPLC column (Spherisorb S3 ODS2, 4.6 mm × 125 mm; 3 mm particles, PhaseSep, Waters). Isocratic elution of KYNA was achieved with 50 mM sodium acetate/4.5% (v/v) acetonitrile (pH 6.2 adjusted with glacial acetic acid)

at a flow rate of 1 ml/min (HPLC pump 422; Kontron, Watford, U.K.) at ambient temperature. Fluorimetric detection of KYNA was enhanced by zinc acetate (0.5 M) delivered post-column at 1 ml/min (510 HPLC pump, Waters). Fluorescence detection wavelengths were 344 and 390 nm (bandwidth 18 nm) for excitation and emission, respectively (F-1050 Fluorescence Spectrophotometer, Merck-Hitachi, Darmstadt, Germany). Acquisition and processing of the chromatograms were performed with the Millenium 2010 Chromatography Manager (Millipore Corp., Waters, Milford, MA), and KYNA concentrations determined as peak-area measurements against external standards.

Drugs/Chemicals

Ro-61-8048 was a generous gift from Dr. A. M. Cesura (Hoffmann-La Roche Ltd., Basel, Switzerland). NMDA, QUIN and KYNA were purchased from Sigma Chemicals (Poole, U.K.). Drug solutions were prepared from 5 or 10 mM stock solutions in aCSF, with pH adjusted to 7.3. All drug solutions were freshly prepared on the day of the experiment. All HPLC solvents were of HPLC grade (HiPerSolv for HPLC) from Merck/BDH (Poole, U.K.). Other chemicals were of analytical grade.

Data analysis and presentation

The magnitude of NMDA- and QUIN-responses was accurately determined by measuring the height of each individual response after display and magnification on the screen of the computer used for data analysis. In Figs. 1 and 3, the polarity of the d.c. potential was defined so that QUIN and NMDA-induced depolarisations produce upwards voltage deflection. To facilitate comparison of the data in Fig. 3, the d.c. potential sequences were aligned by setting the 2-min period preceding the NMDA-challenge to 0 mV. All values in Results are mean ± SEM. The estimated EC_{50} (i.e. agonist, QUIN concentration in the aCSF producing half-maximal response) and ED_{50} (i.e. KYNA concentration in the aCSF producing 50% inhibition of NMDA-induced depolarisation) were determined from the log concentration-response relationships fitted by the Gauss-Newton method (Horne and Simmonds, 1989). Statistical analysis for individual comparison was by Student's paired *t* test.

Results

Quinolinic acid-induced depolarisations

QUIN applied to the rat frontal cortex produced clear and very reproducible concentration-dependent depolarisations, with a concentration threshold of 0.5 to 1 mM (Fig. 1). As it is shown in Fig. 2, the log[QUIN]-response relationship was remarkably steep, with 2 mM QUIN already producing a near-maximal response, and an estimated EC_{50} of 1.22 mM (i.e. concentration in the perfusion medium). The responses were short lasting with a steep onset, but the applications of 2 and 3 mM QUIN were followed, consistently by a marked and prolonged negative shift of the d.c. potential (Fig. 1) suggesting that the region under study had been subjected to a severe insult. The responses to 2 and 3 mM QUIN often showed a double peak (Fig. 1), possibly reflecting the initiation of a wave of spreading depression (i.e. propagating, transient disruption in transmembrane ionic gradients).

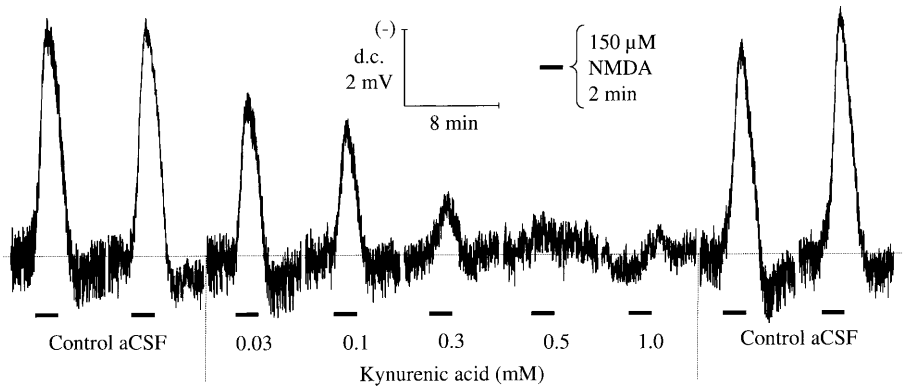

Fig. 3. Representative changes in the d.c. potential produced by NMDA, with or without co-application of KYNA, in the rat frontal cortex, *in vivo*. Nine separate depolarisations were produced by perfusion of 150 μM NMDA through the microdialysis probe for 2 min, each followed by 20 min of recovery. Increasing concentrations of KYNA (0.03, 0.1, 0.3, 0.5 or 1 mM) were perfused for 22 min, starting 10 min before each NMDA application (3rd to 7th). The first and last 2 NMDA stimuli were used as control (NMDA in aCSF)

Exogenous KYNA and NMDA-induced depolarisations

Co-perfusion of KYNA with NMDA concentration-dependently inhibited NMDA-induced depolarisation, with a concentration theshold of 0.03 to 0.1 mM (Fig. 3). The estimated ED_{50} (i.e. concentration in the perfusion medium) for KYNA inhibition of NMDA-responses was 181 $\pm$ 9 μM (n = 6). The last 2 NMDA stimuli, carried out with normal aCSF, showed that the inhibitory action of KYNA was completely reversible.

Effects of Ro-61-8048 on dialysate KYNA and NMDA-induced depolarisation

Ro-61-8048 (100 mg/kg i.p.) increased progressively the dialysate level of KYNA in the rat striatum, from 3.0 $\pm$ 0.9 (basal level) to 33.8 $\pm$ 8.0 nM (n = 6) 4-h post-treatment (Fig. 4). However, this 11-fold increase in extracellular KYNA was not associated with any reduction in the amplitude of NMDA-induced depolarisations (data not shown). Possible interference of the drug vehicle (0.1% Tween-80 in water) with the studied variables were ruled out in separate, control experiments.

Discussion

As our study with Ro-61-8048 was dedicated to the field of kynurenine pathway pharmacology, and most of the previous studies with quinolinic acid were performed in the striatum, corresponding experiments were carried out in this brain region. All the other experiments were performed in the cortex because they were part of another research programme related to the genesis

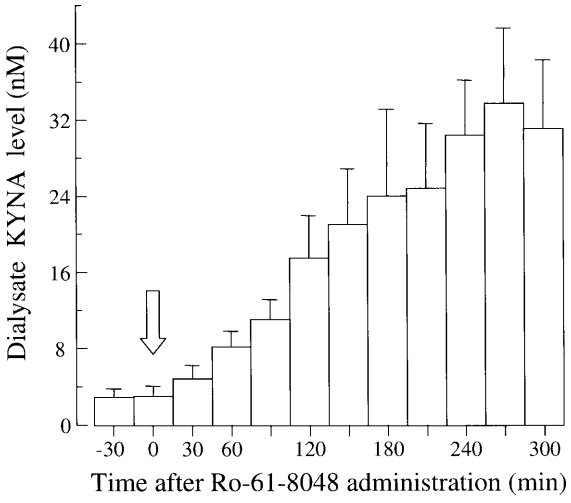

Fig. 4. Effects of kynurenine-3-hydroxylase inhibition on dialysate levels of KYNA in the rat striatum. Ro-61-8048 was administered at the dose of 100 mg/kg i.p. (vertical block arrow)

and pharmacology of *cortical* spreading depression. Perfusion of NMDA through a microdialysis probe produces similar responses in cerebral cortex and striatum, with regard to both pattern and magnitude (EC_{50} = 126 and 188 μM for cortex and striatum, respectively; the latter value was taken from Obrenovitch et al., 1994).

Can accumulation of endogenous QUIN become excitotoxic?

Since the first report that intrastriatal microinjection of QUIN produces selective neuronal death (Schwarcz et al., 1983), numerous studies have confirmed that the application of large amount of exogenous QUIN (e.g. 0.5–1 μl of 30 mM QUIN; Harris et al., 1998) is excitotoxic. However, whether or not the accumulation of *endogenous* QUIN associated with some neurological disorders (e.g. neuroinflammation) can reached excitotoxic levels is far from established. On the one hand, QUIN levels in the extracellular fluid increased from 0.11 to 7.3 μM during endotoxin-induced CNS inflammation (Beagles et al., 1998). On the other hand, estimations of the extracellular QUIN concentrations after an injection of 200 nmol (i.e. common amount used to induced striatal neurodegeneration) indicated a peak level of 13.7 mM at 10–20 min post-injection, which declined to 1.2 mM by 2 h (Bakker and Foster, 1991). When brains were treated with large amounts of QUIN (600 nmol), the lesion remained local around the injection site, i.e. it did not include neuronal loss in distant structures as with kainate-induced striatal lesions (Schwarcz et al., 1983; Zaczek et al., 1980).

In our study, QUIN produced a concentration-dependent depolarisation with an estimated EC_{50} of 1.22 mM (Figs. 1, 2). If we assume that around 1/10 of this concentration was that to which the surrounding tissue was exposed

(During et al., 1989), then the extracellular concentration threshold for QUIN-induced excessive excitation is still >15 times higher than the QUIN levels measured in the immune-activated brain (Beagles et al., 1998). Together, all these data suggest strongly that, although increased synthesis of QUIN from activated microglia and invading macrophages can lead to a 10–100-fold increase in its extracellular levels, the latter remain far below the concentration of QUIN necessary to produce excessive NMDA-receptor activation and subsequent neuronal death. A pronounced, increased sensitivity of the inflammed nervous tissue would be necessary for QUIN accumulation to become detrimental to neuronal survival in this condition. This rationale also contradicts the speculations that QUIN may be an endogenous excitotoxin in alcohol withdrawal, hepatic encephalopathy and glutaric aciduria (Heyes, 1987; Morgan, 1991; Bergqvist et al., 1996).

Increased extracellular KYNA concentration and control of excessive NMDA-receptor function

At least 100 μM KYNA had to be perfused through a microdialysis probe implanted into the frontal cortex to attenuate the responses to 150 μM NMDA (Fig. 3) or 2 mM QUIN (data not shown). If one assumes 10–20% delivery ratio through the microdialysis probe, these results agree with the ED_{50} for KYNA blockade of the NMDA-receptor glycine site (15–40 μM; Kessler et al., 1989; Danysz et al., 1989), where KYNA activity is far higher than its unspecific competitive antagonists actions on glutamate-binding sites (Stone, 1993). Our data also agree with the concentration of KYNA required to block electrically evoked potentials in the CA1 field of hippocampal slices (Scharfman, 2000). In our study, Ro-61-8048 (100 mg/kg i.p.) increased the striatal dialysate levels of KYNA from 3 to 34 nM 4 h post-injection, and this change was not associated with any reduction of NMDA-responses measured precisely within the same region. Röver et al. (1997) found that the same drug, administered orally at the dose of 42 mg/kg, increased dialysate KYNA in the hippocampus of non-anaesthetised rats from 11.0 to 82.5 nM. Another potent kynurenine-3-hydroxylase inhibitor, (*R*,*S*)-3,4-dichlorobenzoylalanine (FCE 28833A) increased hippocampal dialysate KYNA from 2.4 to 193 nM, when administered i.p. at the dose of 400 mg/kg (Speciale et al., 1996). Even in the latter case, and after correction for microdialysis recovery, the increased extracellular level of KYNA appears one order of magnitude less than the ED_{50} for KYNA blockade of the NMDA-receptor glycine site. Therefore, inhibition of kynurenine-3-hydroxylase, by itself, appears unsuitable for the control of excessive NMDA-receptor function. The fact that administration of kynurenine-3-hydroxylase inhibitors showed anticonvulsant activity in the DBA/2 mouse model (Russi et al., 1992; Carpenedo et al., 1994) and protected against D-amphetamine potentiation of NMDA excitotoxicity (Poeggeler et al., 1998) may reflect either interference of KYNA with other molecular target(s) or drug actions unrelated to the kynurenine pathway. The possibility that, in some brain regions, neurones may express NMDA-receptor channels

with a subunit composition favouring glycine sensitivity cannot be ruled out. However, the subunit that is essential for the relevant cation channel formation (i.e. NR1) was clearly demonstrated to include a glycine binding site with nM affinities for reference glycine site antagonists (Miyazaki et al., 1999).

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