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6-Cyano-7-nitroquinoxaline-2,3-dione as an excitatory amino acid antagonist in area CA1 of rat hippocampus

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1 A quantitative pharmacological investigation of the excitatory amino acid antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) has been made in area CA1 of rat hippocampal slices bathed in 1 mM Mg^{2+} containing medium.

2 At a concentration of 10 μM, CNQX reversibly antagonized responses to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), quisqualate and kainate; it produced a parallel shift in their log dose-response curves. Responses to N-methyl-D-aspartate (NMDA) were not antagonized by 10 μM CNQX (dose-ratio: 1.04 ± 0.06, $n = 3$).

3 Schild plots (constructed over the range 1–100 μM) yielded the following estimated pA_2 values, AMPA 5.8, quisqualate 5.9, and kainate 5.9. NMDA was antagonized by 100 μM CNQX, giving an apparent log K of 4.44 ± 0.06.

4 The slopes (±s.e.mean) of the Schild plots were for AMPA 0.84 ± 0.06, quisqualate 0.79 ± 0.04 and kainate 0.68 ± 0.07. These were all significantly less than unity.

5 Synaptic responses elicited by low frequency activation of the Schaffer collateral-commissural pathway were blocked completely by CNQX (10 μM) providing that a low stimulus intensity was used. With high intensity stimulation a small component remained that was blocked by the selective NMDA antagonist D-2-amino-5-phosphonovalerate (APV).

6 These results suggest that CNQX does not differentially affect the responses of CA1 neurones to AMPA, quisqualate and kainate. It does, however, depress responses to these agonists to a greater degree than it does responses to NMDA and it is a highly effective synaptic antagonist.

Introduction

Excitatory amino acids, such as L-glutamate, act on several types of receptor, three of which are commonly named after the agonists N-methyl-D-aspartate (NMDA), kainate and quisqualate (Watkins & Evans, 1981). The NMDA receptor has been well characterized pharmacologically due to availability of various classes of antagonists: selective competitive antagonists, such as D-2-amino-5-phosphonovalerate (APV) (Davies *et al.*, 1981), 'non-competitive' antagonists, such as phencyclidine (Anis *et al.*, 1983) and divalent cations, in particular Mg^{2+} (Ault *et al.*, 1980).

Recently, two quinoxalinediones have been reported to be the first potent and selective antagonists active at kainate and quisqualate receptors (Honoré *et al.*, 1987). These compounds displace binding of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), a quisqualate receptor ligand, and to a lesser extent kainate; they have little or no effect on binding to NMDA or other neurotransmitter receptor sites (Honoré *et al.*, 1988). They also antagonize kainate- and quisqualate-induced [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release from mouse cultured cortical neurones, with a lesser effect on release induced by NMDA (Drejer & Honoré, 1988). In electrophysiological experiments these substances depress responses to quisqualate and kainate to a greater extent than responses to NMDA (Honoré *et al.*, 1988; Blake *et al.*, 1988a; Neuman *et al.*, 1988; Fletcher *et al.*, 1988; Andreasen *et al.*, 1988a). They are also effective antagonists of synaptic responses (Blake *et al.*, 1988a; Fletcher *et al.*, 1988; Andreasen *et al.*, 1988b; Collingridge *et al.*, 1988a; Neuman *et al.*, 1988) and certain types of epileptiform activity (Fletcher *et al.*, 1988; McBain *et al.*, 1988) in the hippocampus and other regions of the brain (Fletcher *et al.*, 1988).

Despite the considerable recent interest in these compounds, there have been few quantitative pharmacological assessments of quinoxalinediones as

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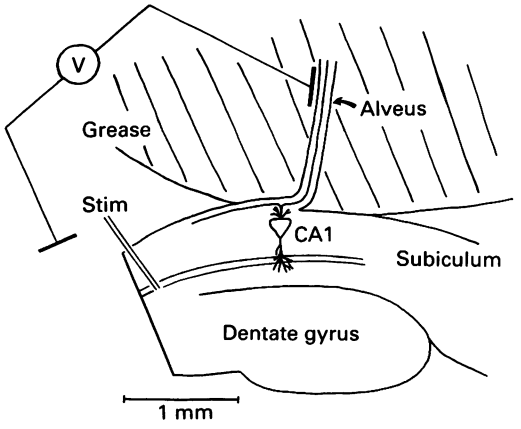

Figure 1 Schematic diagram of a rat hippocampal slice set up for grease gap recording. The potential difference was recorded (d.c.) between the alveus and the bathing medium. Stim = stimulating electrode.

excitatory amino acid antagonists. The purpose of the present investigation, therefore, has been to evaluate CNQX as an excitatory amino acid antagonist in the CA1 region of rat hippocampal slices; the region where most synaptic studies using CNQX have been performed.

Methods

Experiments were performed on 400 μm slices obtained from adult female rats, prepared and set up for grease gap recording (at 27°C) as described previously (Blake *et al.*, 1988b). In all experiments, the CA3 region was removed to prevent effects recorded from CA1 being contaminated by agonist-induced effects in CA3. A stimulating electrode (Collingridge *et al.*, 1988b) was placed in stratum radiatum to elicit synaptic responses (Figure 1); stimuli comprised square wave pulses of 0.1 ms duration and were

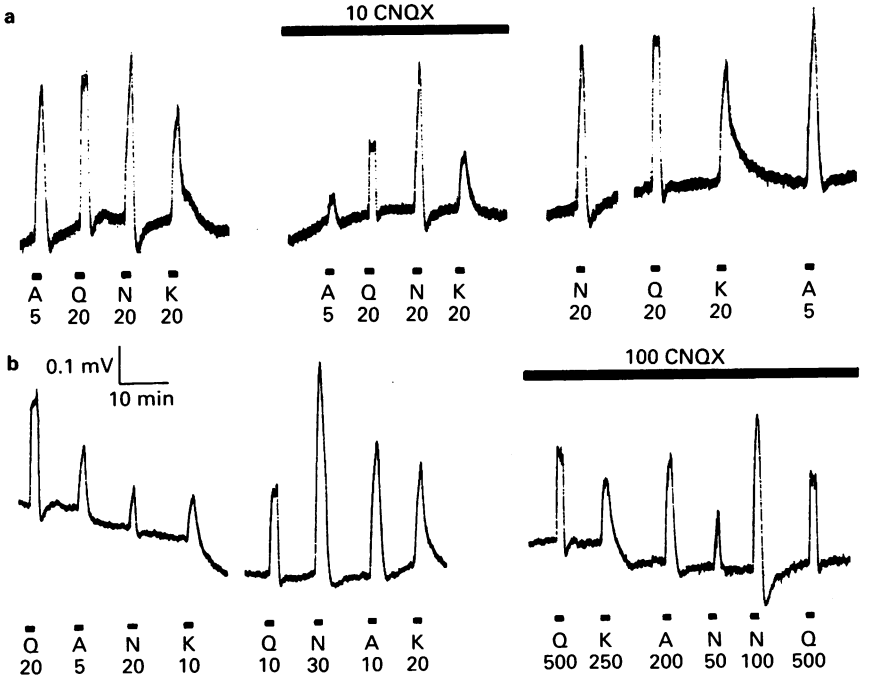

Figure 2 Antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, A), quisqualate (Q), N-methyl-D-aspartate (NMDA, N) and kainate (K) by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). (a) Responses to the agonists in control medium, in the presence of 10 μM CNQX and after washout of CNQX. The gaps in the record represent (from left to right) 20 min, 85 min and 10 min. CNQX 10 μM was perfused for 20 min and was washed out for 70 min before agonists were tested. (b) Responses to the agonists in control medium and in the presence of 100 μM CNQX. The gaps in the record both represent 30 min. CNQX 100 μM was perfused for 20 min before the agonists were tested.

Table 1 Apparent log affinity constants for CNQX against four different agonists at CA1 neurones of rats

CNQX (μM)	*AMPA*	*Quisqualate*	*Kainate*	*NMDA*
1	5.71 ± 0.03	5.91 ± 0.03	5.89 ± 0.07	—
10	5.81 ± 0.04	5.71 ± 0.06	5.72 ± 0.09	—
100	5.39 ± 0.06	5.50 ± 0.08	5.26 ± 0.03	4.44 ± 0.06*
10^+	5.83 ± 0.03	5.74 ± 0.06	5.51 ± 0.15	—

CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. NMDA = N-methyl-D-aspartate.
Data are presented as means ± s.e.mean for 3–6 slices. $^+$Experiments performed in Mg^{2+}-free medium (data calculated from dose-ratios presented in Blake *et al.*, 1988).
Apparent log *K* values for the different agonists were compared for each concentration of CNQX. Analyses of variance showed no significant differences between AMPA, kainate and quisqualate (1 μM: $F(2, 8) = 5.97$, $P > 0.05$; 10 μM: $F(2, 12) = 0.57$, $P > 0.05$; 100 μM: $F(2, 8) = 4.19$, $P > 0.05$; 10^+ μM: $F(2, 17) = 3.00$, $P > 0.05$.
Inclusion of NMDA in the statistical analysis (at 100 μM CNQX) yielded a highly significant effect ($F(3, 11) = 65.23$, $P < 0.0002$). * NMDA significantly different from the other agonists ($P < 0.001$; unpaired *t* tests).
– = no antagonism.

delivered at 0.033 Hz. The bathing medium comprised (mM): NaCl 124, $NaHCO_3$ 26, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1, D-glucose 10 (bubbled with 95% O_2, 5% CO_2) and was perfused at 1.5 ml min^{-1}. Antagonists were added to this perfusate; agonists were perfused as 2 ml aliquots. Only slices where electrical stimulation elicited a synaptic response of at least 1 mV were selected for study.

Drugs

Drugs were stored frozen in stock solutions of 3 mM KCl. N-methyl-D-aspartate, quisqualate and kainate were obtained from Sigma. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and D-2-amino-5-phosphonovalerate were provided by Dr J.C. Watkins (Dept. Pharmacology, University of Bristol) and 6-cyano-7-nitroquinoxaline-2,3-dione was provided by Dr T. Honoré (Ferrosan Group).

Results

The four excitatory amino acids AMPA, kainate, quisqualate and NMDA produced dose-dependent depolarizations of CA1 neurones as described previously (Blake *et al.*, 1988*b*). CNQX depressed responses to all four agonists (Figure 2) in a reversible manner (Figure 2a). Maximal effects of CNQX were seen within 20 min (Figure 2b) but full recovery varied between 70 and 180 min. Of the four agonists tested NMDA was affected by far the least.

In the example illustrated in Figure 2a, doses were chosen to give roughly equal submaximal depolarizations. CNQX (10 μM) depressed responses to AMPA to a greater extent than responses to either kainate or quisqualate. However, this greater depression need not represent real differential antagonism since the dose-response curves of the various agonists do not have the same slopes (Blake *et al.*, 1988b).

To quantify the antagonism by CNQX two methods were adopted to generate dose-ratios. In the first method, full dose-response curves for each agonist were constructed using one agonist per slice. CNQX (10 μM) produced a parallel shift in the log dose-response curve to AMPA ($n = 2$; Figure 3), kainate ($n = 2$) or quisqualate ($n = 1$). In the second method (Figure 2b) four agonists were tested on the same slice; responses in the presence of CNQX (1, 10 or 100 μM) were bracketed between two control

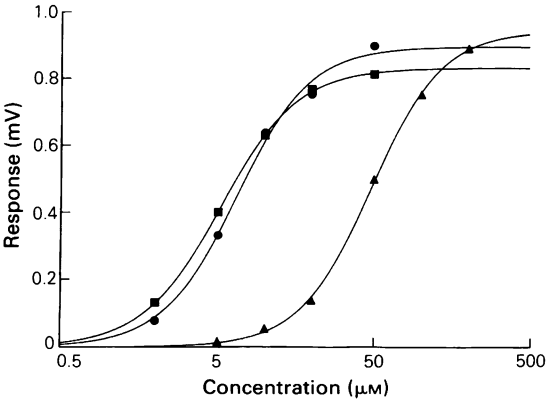

Figure 3 Log dose-response curve for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in control medium (●), in 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-containing medium (▲) and following washout of CNQX (■). Agonists were applied 30–100 min after the start of the perfusion of CNQX and 70–140 min after the start of the washout of CNQX. The EC_{50} values of each plot are 6.6, 47.0 and 5.2 μM.

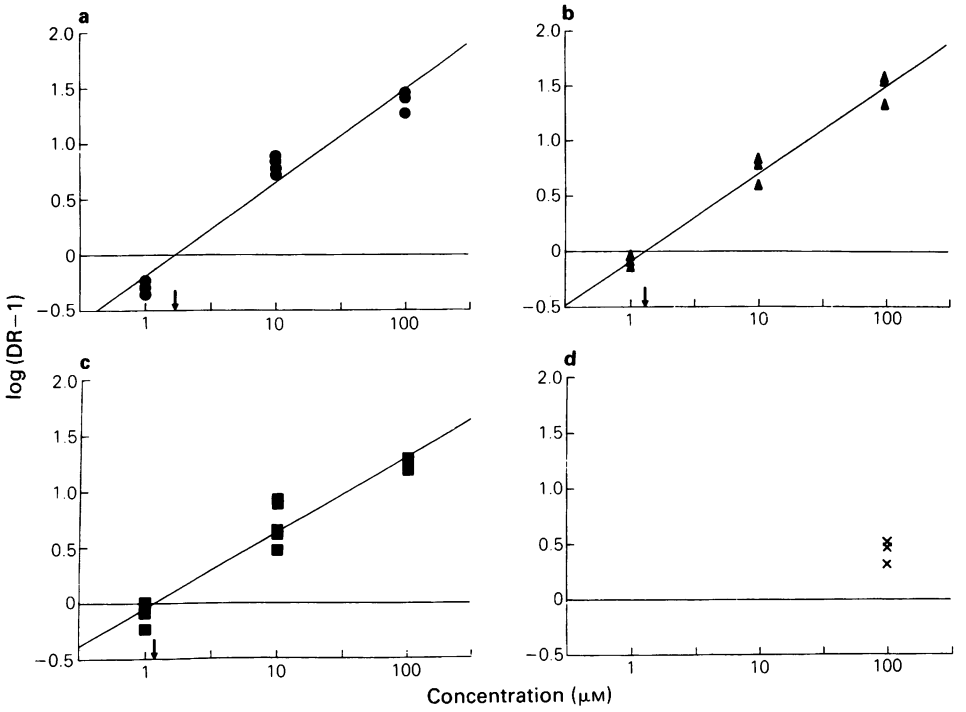

Figure 4 Schild plots for antagonism of (a) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (●), (b) quisqualate (▲), (c) kainate (■) and (d) N-methyl-D-aspartate (NMDA) (×) by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The estimated pA_2 values (indicated by arrows) for AMPA, quisqualate and kainate are 5.8, 5.9 and 5.9, respectively. For each agonist, every point was obtained from a separate slice and the lines were fitted by linear regression. The regressions for AMPA, kainate and quisqualate are highly significant ($P < 0.001$) and their slopes do not differ significantly from one another ($P > 0.05$) but they are significantly less than unity ($P < 0.05$).

responses (on the linear region of the log dose-response plots (Blake *et al.*, 1988b)) (Figure 2b). Both methods produced similar dose-ratios for 10 μM CNQX (see Figure 4). From the dose-ratios, apparent log K values were calculated for each antagonist concentration (Table 1) and Schild plots were also constructed (Figure 4). The respective pA_2 values were for AMPA 5.8, quisqualate 5.9 and kainate 5.9. Responses to NMDA were only affected by 100 μM CNQX (Figure 4); at 10 μM the mean ± s.e.mean dose-ratio was 1.04 ± 0.06 ($n = 3$). When all data are considered together, there was no difference in the antagonism of responses to AMPA, quisqualate and kainate, whereas NMDA was antagonized to a much lesser extent.

The slopes (±s.e.mean) of the Schild plots were for AMPA 0.84 ± 0.06, quisqualate 0.79 ± 0.04 and kainate 0.68 ± 0.07; these were all significantly ($P < 0.05$) less than unity.

In 5 slices, the effect of 10 μM CNQX upon synaptic responses evoked by two or more stimulus intensities was examined. When low intensities (approximately twice threshold for evoking a response) were used CNQX completely abolished the response (Figure 5a). However, when higher stimulus strengths were used (4–8 times threshold) a small component remained in CNQX; this was reversibly blocked by 10–50 μM APV (Figure 5b).

Discussion

The present data are consistent with the suggestion that CNQX is a moderately potent antagonist at excitatory amino acid receptors of the non-NMDA type but is only a weak NMDA antagonist (Honoré *et al.*, 1988). The estimated pA_2 values (and slopes of the Schild equations) are similar to those derived from the study of Fletcher *et al.* (1988) using rat neocortical wedges; their values were for quisqualate 6.2 (0.8), for kainate 5.5 (0.8) and for NMDA 4.5. The reason for the shallow Schild slopes in both of these studies is not known. It is interesting that kynurenate also produces a shallow Schild slope for

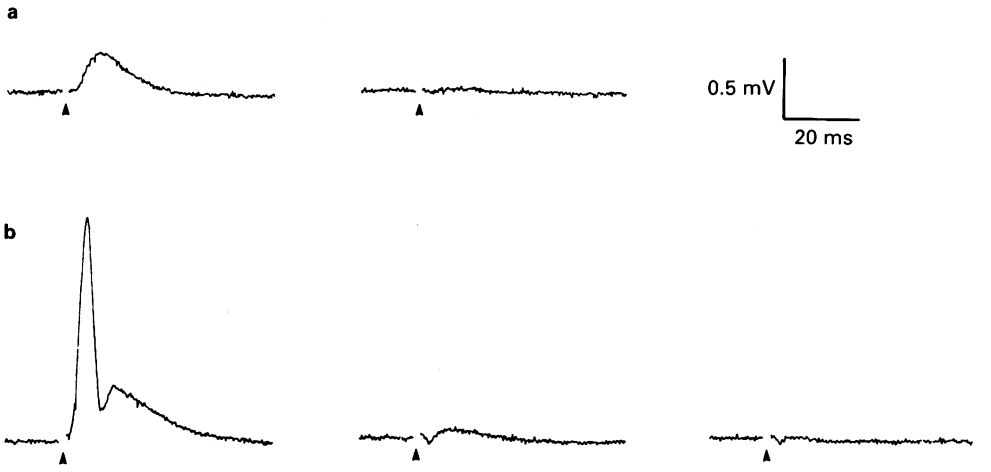

Figure 5 The effect of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on synaptic responses evoked by stimulation of the Schaffer collateral-commissural pathway. Records in (a) were obtained with 20 V (twice threshold) and in (b) with 60 V stimulus intensities. They show (from left to right) control records, the effects of 10 μM CNQX (applied for 20–25 min) and the effect of the addition of 10 μM APV for 7 min (at 60 V). All traces are averages of 5 successive records; stimulus artefacts have been blanked and times of stimulation are indicated by arrowheads.

antagonism of responses to kainate and quisqualate whereas 1-(*p*-bromobenzoyl)-piperazine-2,3-dicarboxylate gives a Schild slope of unity (Evans *et al.*, 1987). One explanation for these data is that the antagonism of non-NMDA receptor ligands by CNQX and kynurenate is not (entirely) competitive.

One difference compared with the study of neocortical wedges was that we were unable to obtain any consistent differentiation in the antagonism of responses to AMPA, quisqualate and kainate. This also contrasts with binding studies where CNQX was 5 times more potent at displacing AMPA than kainate from rat neocortical membranes (Honoré *et al.*, 1988). It is possible, however, that the kainate receptor recorded in the present electrophysiological experiments is different from that determined in binding assays. The simplest interpretation of our results is that AMPA, kainate and quisqualate are all acting (predominantly at least) on the same receptor (see also Kemp *et al.*, 1987; Fletcher *et al.*, 1988). This receptor may be the one responsible for the generation of the fast e.p.s.p. in the CA1 region of the hippocampus as suggested by both the present and previous studies (Neuman *et al.*, 1988; Collingridge *et al.*, 1988a; Blake *et al.*, 1988a; Andreasen *et al.*, 1988b).

In a number of investigations, CNQX has been evaluated as an antagonist on the basis of the percentage depression of responses induced by single doses of agonist. In spinal cord *in vivo* kainate and quisqualate responses were depressed about equally (Honoré *et al.*, 1988), while in hippocampal slices kainate has been found to be either the more sensitive (Andreasen *et al.*, 1988a) or the less sensitive (Neuman *et al.*, 1988) of the two agonists to the action of CNQX. In neocortical wedges, agonists were depressed in the order AMPA > kainate > quisqualate (Fletcher *et al.*, 1988). However, as exemplified by Figure 2a, it is not possible to draw accurate conclusions about the selectivity of CNQX when percentage depressions (rather than dose-ratios) are measured, since the dose-response curves of the agonists have very different slopes (and additionally for quisqualate, a smaller maximum response) (Blake *et al.*, 1988b).

The effect of 10 μM CNQX as an excitatory amino acid antagonist in the CA1 region was essentially the same in the present study (in 1 mM Mg^{2+}) as that reported previously in Mg^{2+}-free medium (Blake *et al.*, 1988a). This shows that the antagonism of non-NMDA receptor ligands by CNQX is not influenced by Mg^{2+} (up to 1 mM). At 10 μM, CNQX has no significant effect on responses to NMDA, in either the presence or absence of Mg^{2+}. In the hippocampus this concentration of CNQX totally blocks fast e.p.s.ps evoked by low stimulus strengths, as reported previously (Blake *et al.*, 1988a). However, in agreement with intracellular studies (Collingridge *et al.*, 1988a; Andreasen *et al.*, 1988a), at high stimulus strengths a CNQX-resistant component is seen; this can be blocked by APV.

Very recently it has been reported that CNQX blocks responses to NMDA via an action at the allosteric glycine site (Birch *et al.*, 1988). As a consequence the ability of CNQX to block responses to NMDA will depend on the glycine concentration. It

is generally believed that in brain slices, glycine is present at supersaturating levels for this site; this will increase the effective selectivity of the antagonist.

In summary, CNQX is sufficiently selective in area CA1 of hippocampal slices to block non-NMDA receptor-mediated synaptic components completely whilst having no detectable effect on synaptic or agonist-induced responses mediated via NMDA receptors.

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