Quinoxalinediones: Potent Competitive Non-NMDA Glutamate Receptor Antagonists

TAGE HONORÉ,* STEVE N. DAVIES, JØRGEN DREJER, ELIZABETH J. FLETCHER, POUL JACOBSEN, DAVID LODGE, FLEMMING E. NIELSEN

The *N*-methyl-D-aspartate (NMDA)–subtype of glutamate receptors has been well described as a result of the early appearance of NMDA antagonists, but no potent antagonist for the "non-NMDA" glutamate receptors has been available. Quinoxalinediones have now been found to be potent and competitive antagonists at non-NMDA glutamate receptors. These compounds will be useful in the determination of the structure-activity relations of quisqualate and kainate receptors and the role of such receptors in synaptic transmission in the mammalian brain.

EXCITATORY AMINO ACIDS SUCH AS glutamate are major neurotransmitters in the mammalian central nervous system (*1, 2*). From electrophysiological (*3, 4*) and binding (*5*) studies there appear to be at least three major subtypes of receptors mediating the postsynaptic action of glutamate. Several classes of antagonists are available for the NMDA subtype (*6*), but there have been no potent and selective antagonists that both displace the binding of quisqualate or kainate, or both, and reduce their excitatory actions on central neurons. We report here two novel and potent non-NMDA receptor antagonists, 6,7-dinitroquinoxaline-2,3-dione (DNQX, FG 9041) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, FG 9065) (Fig. 1). These non-NMDA receptor antagonists, DNQX and CNQX, inhibit [^{3}H]AMPA ([^{3}H]α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) binding to quisqualate receptors at submicromolar concentrations. They are one-fifth as effective at kainate receptors, and are considerably weaker at other binding sites that include a range of neurotransmitter receptors. The compounds also selectively block the excitatory action of quisqualate and kainate on spinal neurons with little or no effect on that of NMDA.

Binding experiments were performed at 4°C with extensively washed rat cortical membranes (*7*). We used [^{3}H]kainate (5 n*M*) binding in 50 m*M* tris-citrate buffer to examine kainate receptors (*8*), and [^{3}H]AMPA (5 n*M*) binding in 30 m*M* tris-HCl buffer with 2.5 m*M* $CaCl_2$ and 100 m*M* potassium thiocyanide for quisqualate receptors (*7*). We studied NMDA receptors by using [^{3}H]3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) binding (5 n*M*) in 30 m*M* tris-HCl buffer with 2.5 m*M* $CaCl_2$ (*9*). Binding was selectively inhibited at the quisqualate receptor by DNQX and CNQX (Ferrosan Research Division). These results and those of some standard agonists and antagonists are shown in Table 1. The concentrations that inhibit 50% of [^{3}H]AMPA binding (IC_{50}) for DNQX and CNQX were 500 and 300 n*M*, respectively, with Hill coefficients close to unity, thus indicating no noncompetitive or cooperative interactions. The affinities of DNQX and CNQX were comparable to the affinity of L-glutamate, but were approximately one-tenth as effective as quisqualate and AMPA. Both compounds were about one-fifth as effective at kainate binding sites, 2.0 and 1.5 μ*M*, respectively, and very weak at [^{3}H]CPP binding sites ($IC_{50} \geq 25$ μ*M*).

The compounds were also relatively inactive ($IC_{50} > 25$ μ*M*) at the following binding sites: [^{3}H]spiperone in the presence of the dopamine agonist (±)-2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) (5-HT receptors), [^{3}H]WB 4101 (α-noradrenergic receptors), [^{3}H]QNB (muscarinic receptors), [^{3}H]spiperone and [^{3}H]SCH 23390 (dopamine D1 and D2 receptors), [^{3}H]naloxone (opiate receptors), [^{3}H]flunitrazepam (benzodiazepine receptors), [^{3}H]GABA (γ-aminobutyric acid receptors), and [^{3}H]strychnine (glycine receptor). We found CNQX was inactive at phencyclidine (PCP) sites on the NMDA-coupled ionophore as measured with [^{3}H]*N*-(1-(2-thienyl)cyclohexyl)piperidine (TCP) binding, whereas DNQX was found to be a weak inhibitor (IC_{50} = 2.9 μ*M*).

We also examined changes in the excitability of neurons in the spinal cord of

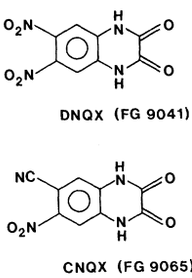

Fig. 1. Chemical structures of DNQX and CNQX.

Table 1. Inhibitory activity of selected glutamate antagonists and agonists. Rat cortical membranes were prepared as described in (*7*). The final pellet was homogenized in buffer (50 volumes per gram of original tissue) and used for binding assays. Portions (0.5 ml) in triplicate were incubated for 1 hour at 0°C with [^{3}H]kainic acid [New England Nuclear (NEN); 60.0 Ci/mmol], 30 min at 0°C with [^{3}H]AMPA (NEN; 27.6 Ci/mmol), and 30 min at 0°C with [^{3}H]CPP (Tocris; 20 Ci/mmol) in the absence or presence of 0.6 m*M* L-glutamate for determination of specific binding. Free and bound radioactivity were separated by filtration through Whatman GF/C glass fiber filters followed by three washes with 5 ml of ice-cold buffer. The IC_{50} values were obtained from a Hill analysis of the effect of at least four different concentrations of the inhibitor. Results shown are means ± SEM of three determinations, otherwise results are means of two determinations. Abbreviations: GAMS, γ-D-glutamylaminomethylsulfonic acid; D-APV, D-2-aminophosphonovaleric acid; MK-801, (+)-10,11-dihydro-5-methyl-5-H-dibenzo[*a,d*]cycloheptene (*5, 11*); GDEE, glutamic acid diethylester; and —, not tested.

Compound	Inhibition of binding (IC_{50}) to rat cortical membranes (μ*M*)		
	[^{3}H]AMPA	[^{3}H]Kainate	[^{3}H]CPP
DNQX	0.50 ± 0.10	2.0 ± 0.10	40
CNQX	0.30 ± 0.15	1.5 ± 0.30	25
GAMS	>100	>1000	90
GDEE	>100	400	100
Pentobarbital	>1000	>1000	>1000
D-APV	>500	>5	0.075 ± 0.010
CPP	450	—	0.050 ± 0.025
MK-801	>1000	>1000	>100
Ketamine	>100	—	>100
Quisqualate	0.025 ± 0.005	0.20 ± 0.01	40
AMPA	0.045 ± 0.005	45	>100
Kainate	20	0.0045 ± 0.0010	>100
NMDA	>200	—	15
L-Glutamate	0.60 ± 0.05	0.25 ± 0.01	0.20 ± 0.10

T. Honoré, J. Drejer, P. Jacobsen, F. E. Nielsen, Ferrosan Research Division, DK-2860 Soeborg, Denmark. S. N. Davies, E. J. Fletcher, D. Lodge, Royal Veterinary College, London NW1 OTU, United Kingdom.

*To whom correspondence should be addressed.

pentobarbitol-anesthetized rats in response to excitatory amino acids and potential antagonists (*10*). Briefly, action potentials from single neurons were recorded with the 3.6*M* NaCl-filled center barrel of seven-barreled microelectrodes; the outer barrels contained drugs to be ejected by electrophoresis. Once a neuron was located, approximately equal and submaximal increases in firing rates were obtained by cyclical ejection of the agonists to be tested before administration of an antagonist.

Administration of DNQX and CNQX to 15 cells reduced the responses to quisqualate and kainate approximately equally, but had little effect on the response to *N*-methyl-DL-aspartate (NMA) (Table 2 and Fig. 2). The time course of the effects on quisqualate and kainate differed. The response to kainate decreased rapidly, whereas the response to quisqualate decayed more slowly, but to a similar level. Recovery was always rapid. Background firing rate was not reduced by DNQX and CNQX in the three spontaneously active cells. On another 11 cells that were tested for response to the putative transmitters L-glutamate and L-aspartate, DNQX was very effective in reducing responses to quisqualate and L-aspartate but less effective against L-glutamate and NMA responses (Table 2).

This result suggests that L-glutamate did not excite these rat spinal neurons via quisqualate or kainate receptors. Interestingly, both competitive (*1, 3*) and ketamine-like noncompetitive (*10*) NMDA antagonists exhibit the same relative selectivity on L-aspartate and L-glutamate responses as reported here for DNQX.

Combinations of ketamine and DNQX in doses that blocked responses to NMA and quisqualate abolished the response to L-aspartate completely, whereas the response to glutamate was only reduced by 65 ± 14% (SD, $n = 6$). Such results may suggest that aspartate excites neurons via the same receptors that mediate responses to NMDA, quisqualate, and kainate, but that glutamate has an additional mechanism for exciting spinal neurons (*11*).

To determine whether quisqualate or kainate receptors, or both, mediate synaptic responses in the spinal cord, we tested the effects of DNQX and compared them with those of ketamine on dorsal horn neurons. These neurons were excited by quisqualate and NMA that was ejected by electrophoresis and by synaptic inputs after electrical stimulation of the receptive field on the skin. With 32 separate administrations of DNQX and ketamine, which reduced quisqualate and NMA responses, respectively, neither drug had any significant effect on synaptic inputs. When ketamine and DNQX were combined to totally block responses to NMA and quisqualate, synaptic responses were reduced by 36 ± 14%. We could not determine if this means that (i) synaptic inputs are mediated by receptors other than quisqualate, kainate, and NMDA receptors; (ii) the synapses are too distally placed on the dendrites; or (iii) the subsynaptic concentration of the endogenous transmitter is too great for its action to have been affected by the doses of antagonist used.

Our results demonstrate a clear preference of DNQX and CNQX for non-NMDA receptors with about equal potencies of the compounds in blocking responses induced by quisqualate and kainate. However, in the binding experiments DNQX and CNQX were relatively weak inhibitors of [^{3}H]kainate binding (in the absence of Ca^{2+}, that is, the Ca^{2+}-sensitive high-affinity kainate binding site) as compared to the inhibitory activity in the [^{3}H]AMPA binding assay. This may indicate that under physiological conditions, only the Ca^{2+}-insensitive kainate binding sites that are the quisqualate receptors (*8*) may be of functional relevance. Alternatively, the specificity of the glutamate receptor subtypes could be different in cortex and spinal cord.

The development of these new and potent non-NMDA antagonists opens the possibility for extensive studies of the quisqualate and kainate receptor subtypes. DNQX and CNQX will be useful probes to study the role of these receptors in synaptic transmission in the mammalian brain and may offer

Table 2. Reduction of excitation of rat spinal neurons. The experiments were performed as in Fig. 2. Results are given as mean percentage change in peak firing rate ± SD. Abbreviations are as in Table 1.

Compound	Number of cells	ΔPeak firing rate (%)				
		NMA	Kainate	Quisqualate	L-Glutamate	L-Aspartate
DNQX	7	14 ± 5	68 ± 20	54 ± 23	—	—
CNQX	7	−1 ± 5	56 ± 23	48 ± 27	—	—
DNQX	8	13 ± 13	68 ± 15	64 ± 26	—	—
GDEE	8	10 ± 30	−31 ± 26	22 ± 18	—	—
CNQX	3	14 ± 25	83 ± 29	56 ± 12	—	—
GAMS	3	3 ± 6	69 ± 53	31 ± 17	—	—
DNQX	11	18 ± 19	—	62 ± 15	15 ±20	70 ± 15

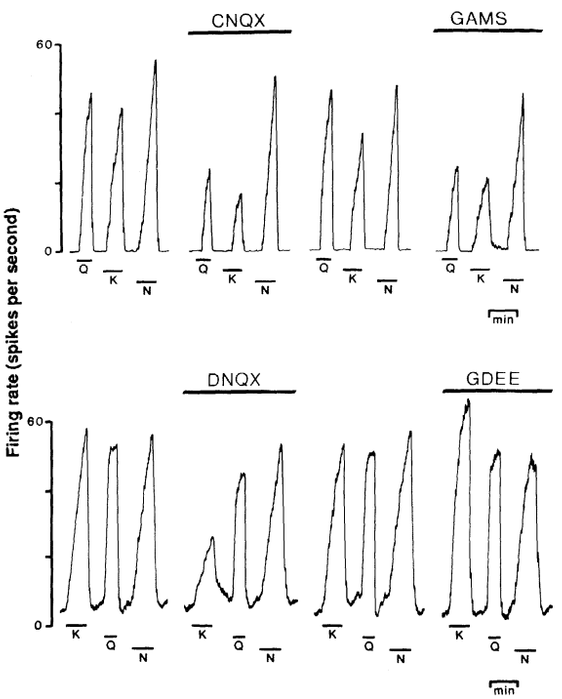

Fig. 2. Differential effect of CNQX and DNQX on responses of two dorsal horn neurons to the electrophoretic ejection of glutamate analogs. In the upper row of rate meter recordings, control responses to quisqualate (Q, 21 nA from a 5 m*M* in 200 m*M* NaCl solution), kainate (K, 44 nA from a 5 m*M* in 200 m*M* NaCl solution), and NMA (N, 35 nA from a 200 m*M* solution) are shown in the first group of responses. In this neuron, ejection of CNQX (20 nA from a 1 m*M* in 200 m*M* CaCl solution; *p*H 9.8) reduced the increase in firing rate by quisqualate and kainate about equally (second group of responses). Recovery is shown 4 min after we stopped the ejection of CNQX (third group of responses). On this same cell, GAMS (20 nA from an undiluted 200 n*M* solution) gave a similar reduction of quisqualate and kainate responses (fourth group of responses), indicating that GAMS is considerably less potent than CNQX. The lower row of traces are from another neuron excited by kainate (60 nA), quisqualate (54 nA), and NMA (44 nA) (first group of responses). DNQX (5 nA from a 5 m*M* in 200 m*M* NaCl solution) reduced the response to kainate to a greater extent than that to quisqualate or to NMA (second group of responses). Recovery (third group of responses) was obtained in 5 min. Subsequent administration of GDEE (80 nA from an undiluted 200 m*M* solution) had little effect on the responses to quisqualate and NMA, but reversibly enhanced that to kainate (fourth group of responses). Scale bar, 1 min.

important leads to the therapeutic potential of non-NMDA antagonists.

REFERENCES AND NOTES

1. J. C. Watkins and R. H. Evans, *Annu. Rev. Pharmacol. Toxicol.* **21**, 165 (1981).
2. H. McLennan, *Prog. Neurobiol. (NY)* **20**, 251 (1983); D. Lodge, Ed., *Excitatory Amino Acids in Health and Disease* (Wiley, New York, 1988).
3. H. McLennan and D. Lodge, *Brain Res.* **169**, 83 (1979).
4. J. Davies, R. H. Evans, A. A. Francis, J. C. Watkins, *J. Physiol. (Paris)* **75**, 641 (1979).
5. A. C. Foster and G. E. Fagg, *Brain Res. Rev.* **7**, 103 (1984); D. T. Monaghan, V. R. Holets, D. W. Toy, C. W. Cotman, *Nature* **306**, 176 (1983); J. T. Greenamyre, J. M. Olsen, J. B. Penney, A. B. Young, *J. Pharmacol. Exp. Ther.* **233**, 254 (1985).
6. T. J. Biscoe *et al.*, *Eur. J. Pharmacol.* **45**, 315 (1977); J. Davies, A. A. Francis, A. W. Jones, J. C. Watkins, *Neurosci. Lett.* **21**, 77 (1981); D. Lodge and N. A. Anis, *Br. J. Anaesth.* **56**, 1143 (1984); A. M. Thomson, D. C. West, D. Lodge, *Nature* **313**, 479 (1985); J. Davies *et al.*, *Brain Res.* **382**, 169 (1986).
7. T. Honoré and M. Nielsen, *Neurosci. Lett.* **54**, 27 (1985).
8. T. Honoré, J. Drejer, M. Nielsen, *ibid.* **65**, 47 (1986).
9. ———, J. C. Watkins, H. J. Olverman, *Eur. J. Pharmacol.* **136**, 137 (1987).
10. N. A. Anis, S. C. Berry, N. R. Burton, D. Lodge, *Br. J. Pharmacol.* **79**, 565 (1983).
11. L. Alberto, O. Goldberg, V. I. Teichberg, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3250 (1981); J. Davies and J. C. Watkins, *Brain Res.* **327**, 113 (1985).

12. The close collaboration with Squibb Institute for Medical Research, Princeton, NJ, is highly appreciated.

9 March 1988; accepted 25 May 1988

An *E. coli* Promoter That Regulates Transcription by DNA Superhelix-Induced Cruciform Extrusion

MARSHALL S. Z. HORWITZ AND LAWRENCE A. LOEB

DNA can form structures other than the Watson-Crick double helix. The potential contributions to gene regulation from one such structure have been investigated by assembling a promoter capable of adopting cruciform base-pairing. Transcription from this promoter by RNA polymerase in vitro was repressed as the cruciform was extruded by increasing negative DNA supercoiling. Transcription in vivo was induced as supercoiling was relaxed by growth in conditions that inhibit DNA gyrase. A DNA conformational change is therefore capable of regulating the initiation of transcription.

THE OCCASIONAL DEPARTURE OF DNA from the Watson-Crick double helix suggests a mechanism for the control of gene expression. A variety of secondary structures, including left-handed helices (*1*), heteronomous DNA (*2*), anisomorphic DNA (*3*), loops (*4*), and cruciforms (*5*), have been postulated to regulate transcription through conformational changes in the DNA template. To survey the extent to which DNA secondary structure may influence transcription initiation, we have characterized the interaction of *Escherichia coli* RNA polymerase with a promoter that contains a DNA sequence with the potential to fold into a cruciform structure.

The plasmid pX (Fig. 1A) contains a 50-bp inverted repeat, composed mostly of A-T base pairs, spanning from −23 to +27, with respect to the transcription start site, +1. Each repeated unit contains a "−10" consensus sequence. The most upstream of the units is spaced to align with a "−35" sequence to complete the elements of a promoter (*6*), the start site of which is located near the center of the inverted repeat. The promoter directs transcription of the gene *tet*, which confers resistance to tetracycline. The control plasmid pO (Fig. 1B) contains a 50-bp direct repeat composed of the same units as in pX. The two plasmids differ by an inversion from +6 to +21. Since the sequence of the repeated unit (Fig. 1C) nearly approximates an inverted repeat, the net sequence difference between pX and pO is merely eight A-T transversions. Cruciform extrusion could occur in pX when intrastrand DNA base-pairing replaces interstrand base-pairing within the inverted repeat. Because cruciform extrusion relieves superhelical stress, the cruciform is expected to become the stable species at high negative superhelical densities (*7*).

Two-dimensional gel electrophoresis (*7*) was used to confirm the ability of pX DNA to adopt a cruciform structure in vitro (Fig. 2A). A distribution of topoisomers was prepared by partial relaxation of physiologically supercoiled plasmid DNA (*8*). In the first dimension, topoisomers that had a negative superhelical density sufficient to extrude cruciforms were retarded in mobility by a change in linking difference equal to the change in twist accompanying the helical unwinding of cruciform formation. The addition of an intercalator to the second dimension positively supercoiled the DNA, which removed the cruciform and revealed an arc of spots corresponding to topoisomers that extruded the cruciform during electrophoresis in the first dimension. Topoisomers of pX with a linking difference of −14 or less (superhelical density, σ, ≤ -0.03) were retarded by 5.2 turns (equal to the anticipated length of the cruciform, 50 bp, assuming 10.5 bp per turn). This cruciform was further mapped to the expected location at the *tet* promoter by sensitivity to the single-strand–specific S1 nuclease (*9*). The pO DNA does not form such secondary structure as determined by electrophoresis (Fig. 2B), and by insensitivity to S1 nuclease (*9*).

We assessed the influence of the cruciform on the promotion of in vitro transcription by *E. coli* RNA polymerase. For some promoters negative supercoiling facilitates the helical unwinding of open complex formation (*10, 11*). Reactions performed with plasmid DNA templates of different superhelical densities (Fig. 3A) indicate that transcription from the promoter in pX did initially increase with supercoiling, but was then repressed at the negative superhelical densities at which the cruciform is extruded. By contrast, transcription from the promoter in pO increased fairly uniformly with negative supercoiling.

The degree of supercoiling at which this cruciform is extruded in vitro is within the

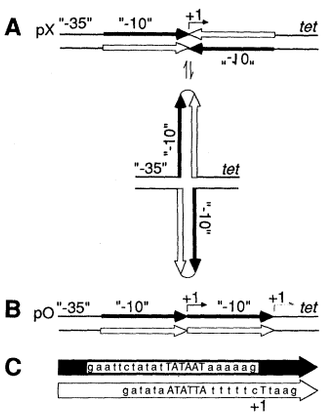

Fig. 1. Promoters studied. (**A**) The inverted repeat of the promoter in pX, illustrating potential cruciform base-pairing. (**B**) The direct repeat of the promoter in pO. A secondary start site is represented in stippled print. (**C**) The sequence of each repeat, contained on an Eco RI restriction fragment obtained from pAT12 by the technique of random selection (*22*). The "−10" sequence and starting base (template strand) are in uppercase. The promoters were constructed by ligation of this fragment into the Eco RI site of pBdEC, a derivative of pBR322 modified by deletion of the *tet* promoter from Eco RI to Cla I (*22*). Both pX and pO contain a β-lactamase gene.

The Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, SM-30, University of Washington, Seattle, WA 98195.