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Quinoxaline Derivatives: Structure-Activity Relationships and Physiological Implications of Inhibition of *N*-Methyl-D-aspartate and Non-*N*-methyl-D-aspartate Receptor-Mediated Currents and Synaptic Potentials

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SUMMARY

The inhibitory potencies at excitatory amino acid (EAA) receptors of 11 quinoxaline derivatives were evaluated in two-electrode voltage-clamp recordings of *Xenopus* oocytes injected with rat cortex mRNA. Currents activated by kainate or (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) in *Xenopus* oocytes were inhibited competitively by all the quinoxaline derivatives, with apparent K_i values ranging from 0.27 to 300 μM against kainate and from 0.25 to 137 μM against AMPA. An excellent correlation was observed between inhibitory potencies of the quinoxaline derivatives against kainate and AMPA currents, in support of the contention that in this preparation these two agonists act at a single site. All 11 quinoxaline derivatives also inhibited current activated by the combination of glycine and *N*-methyl-D-aspartate (NMDA), apparently acting at the glycine site, and did so over a narrower range of apparent K_i values (0.37–8.1 μM). The correlation between the quinoxalines' kainate/AMPA potencies and their glycine/NMDA potencies was relatively weak. Thus, the quinoxaline derivatives were all good antagonists of glycine/NMDA currents and displayed a greater range of potencies against kainate and AMPA. The inhibitory effects of the six quinoxaline derivatives most potent in the *Xenopus* oocyte experiments were also tested against the excitatory postsynaptic field potential (EPSFP) recorded in the pyramidal cell dendritic field of the CA1 region of hippocampal slices after stimulation of the Schaffer collateral-commissural pathways. In slices superfused with "normal" medium (containing 1 mM Mg^{2+}), in which the EPSFP is mediated primarily by non-NMDA receptors, IC_{50} values correlated closely with the K_i values against kainate/AMPA obtained in oocyte experiments but were approximately 8-fold higher. Similarly, in slices superfused with nominally Mg^{2+}-free medium, in which the EPSFP is amplified due to a relief of the Mg^{2+} block of NMDA receptors, IC_{50} values correlated closely with the K_i values against glycine/NMDA obtained in oocyte experiments but were 60-fold higher. This comparison of results from the two experimental systems lends further support to the argument that hippocampal synaptic transmission is mediated postsynaptically by kainate/AMPA-type and NMDA/glycine-type EAA receptors that are pharmacologically indistinguishable from those expressed in mRNA-injected *Xenopus* oocytes. Furthermore, it suggests that EAA receptors *in situ* may be nearly saturated by high local concentrations of the endogenous ligands, a condition that would contribute substantially to the apparent non-NMDA receptor selectivity of certain quinoxaline derivatives.

Progress in the understanding of the physiology of EAA neurotransmission has been dependent upon the discovery of pharmacological agents that are able to act specifically upon the different EAA receptors (for review, see Ref. 1). Two QX derivatives, CNQX and DNQX, as well as DCHQXCA, have permitted considerable progress in this field by virtue of their relatively potent antagonism of certain EAA receptors. These compounds displace [^{3}H]AMPA, [^{3}H]kainate, and strychnine-insensitive [^{3}H]glycine binding (2–8) and antagonize electrical phenomena (2, 8–16) and neurotransmitter release (17, 18) induced by exogenous kainate and AMPA, as well as NMDA plus glycine, through an action at the glycine site of the NMDA receptor (8, 10, 14). Furthermore, numerous electrophysiological studies have shown that these molecules inhibit synaptic potentials thought to be mediated by EAAs (12–14, 19). In contrast, the quinoxalinediones have no effect on the metabotropic subtype of EAA receptor (for review, see Ref. 20).

In addition to CNQX, DNQX, DCHQXCA, and their parent

ABBREVIATIONS: EAA, excitatory amino acid; AMPA, (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; D-AP5, D-2-amino-5-phosphonopentanoic acid; NMDA, *N*-methyl-D-aspartate; 6ClQX, 6-chloroquinoxaline-2,3-dione; DCHQXCA, 6,7-dichloro-3-hydroxyquinoxaline-2-carboxylic acid; 6,7diClQX, 6,7-dichloro-quinoxaline-2,3-dione; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EPSFP, excitatory postsynaptic field potential(s); HQXCA, 3-hydroxyquinoxaline-2-carboxylic acid; 5NQX, 5-nitroquinoxaline-2,3-dione; 6NQX, 6-nitroquinoxaline-2,3-dione; ONQX, 5,6-dinitroquinoxaline-2,3-dione; MNQX, 5,7-dinitroquinoxaline-2,3-dione; QX, quinoxaline-2,3-dione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SAR, structure-activity relationships.

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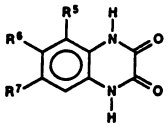
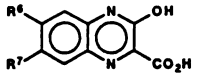
compounds QX and HQXCA (see Ref. 21), a variety of QX derivatives are now available that possess single substitutions (5NQX, 6NQX, and 6ClQX) or alternate double substitutions [ONQX, MNQX (see Ref. 3), and 6,7diClQX]. This set of compounds (Fig. 1) provides the opportunity to evaluate some (SAR) among the quinoxalines. This report describes experiments in which we examined the abilities of 11 quinoxaline





	R^5	R^6	R^7
QX	H	H	H
6ClQX	H	Cl	H
6,7diClQX	H	Cl	Cl
CNQX	H	CN	NO_2
5NQX	NO_2	H	H
6NQX	H	NO_2	H
ONQX	NO_2	NO_2	H
DNQX	H	NO_2	NO_2
MNQX	NO_2	H	NO_2

	R^6	R^7
HQXCA	H	H
DCHQXCA	Cl	Cl

Fig. 1. Structures of the quinoxaline derivatives used in this study.

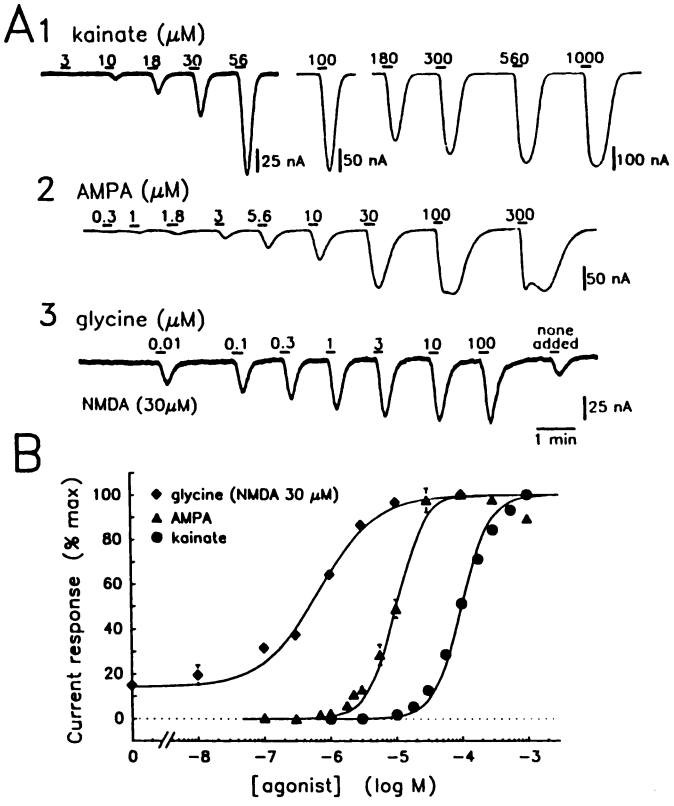


Fig. 2. Concentration dependence of the current responses to EAA analogues of three *Xenopus* oocytes injected with rat cortex mRNA. A, Chart recordings of current responses to kainate (3–1000 μM) (A1), AMPA (0.3–300 μM) (A2), and NMDA (30 μM) alone or with glycine (0.01–100 μM) (A3). Substances were bath-applied during the periods indicated by the *bars*. B, Plot of current responses as percentage of maximal response versus concentration of kainate (●), AMPA (▲), or glycine plus NMDA (30 μM) (◆). Data are mean + standard error of 3–15 determinations and were fit (*lines*) using the equation $I/I_{max} = \{1 + (EC_{50}/[agonist])^{n_H}\}^{-1}$, with the values of EC_{50} and n_H given in Table 1 and assuming 100 nM contaminating glycine.

TABLE 1

Maximal inward current amplitudes and EC_{50} values observed for the peak of responses to the EAA receptor analogues in *Xenopus* oocytes injected with rat cortex mRNA

The NMDA concentration was 30 μM for determination of glycine potency. The concentration of contaminating glycine was assumed to be 100 nM.

Agonist	*n*	Maximal current amplitude	EC_{50}	n_H
		nA	*μM*	
Kainate	15	−650 ± 120	100	2
AMPA	12	−74.5 ± 12.3	10	2
Glycine (+NMDA, 30 μM)	14	−29.2 ± 7.2	0.6	1

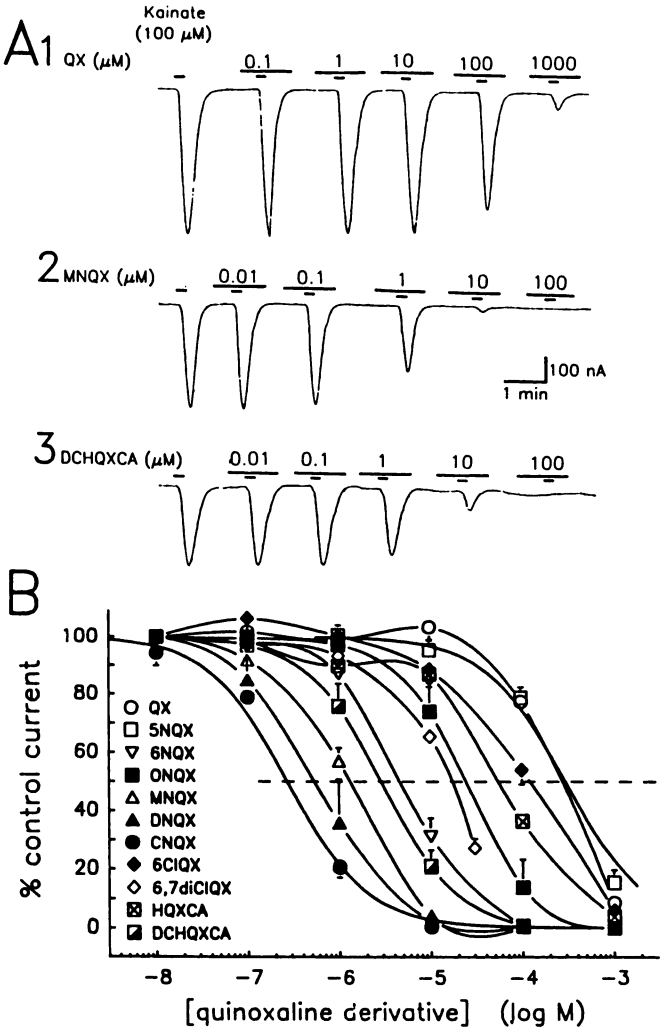


Fig. 3. Concentration dependence of the inhibition of kainate (100 μM) current by quinoxaline derivatives. A, Chart recordings of kainate current inhibition by QX (0.1–1000 μM) (A1), MNQX (0.01–100 μM) (A2), and DCHQXCA (0.01–100 μM) (A3), in three different oocytes. B, Plot of current responses as percentage of control response versus concentration of QX (○), 5NQX (□), 6NQX (▽), ONQX (■), MNQX (△), DNQX (▲), CNQX (●), 6ClQX (◆), 6,7diClQX (◇), HQXCA (⊠), or DCHQXCA (◪). Data are mean ± standard error of three to eight determinations. The *curves* were drawn for the most potent (CNQX) and least potent (5NQX) compounds using the equation $I/I_{max} = \{1 + ([antagonist]/IC_{50})^{n_H}\}^{-1}$, with the values of IC_{50} given in Table 2. For clarity, the other curves were drawn using a polynomial fitting routine and have no physical significance.

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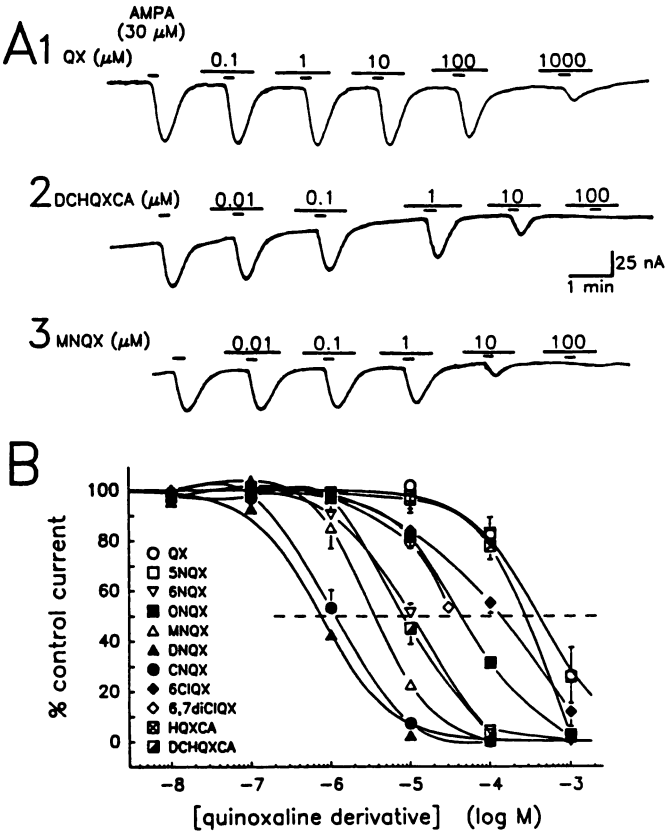

Fig. 4. Concentration dependence of the inhibition of AMPA (30 μM) current by quinoxaline derivatives. A, Chart recordings of AMPA current inhibition by QX (0.1–1000 μM) (A1), DCHQXCA (0.01–100 μM) (A2), and MNQX (0.01–100 μM) (A3), in three different oocytes. B, Plot of current responses as percentage of control response as a function of concentration of QX (○), 5NQX (□), 6NQX (▽), ONQX (■), MNQX (△), DNQX (▲), CNQX (●), 6ClQX (♦), 6,7diClQX (◇), HQXCA (⊠), or DCHQXCA (◪). Data are mean ± standard error of three to six determinations. The *curves* were drawn for the most potent (DNQX) and least potent (QX and 5NQX) compounds using the equation $I/I_{max} = \{1 + ([antagonist]/IC_{50})^{nH}\}^{-1}$, with the values of IC_{50} given in Table 2. For clarity, the other curves were drawn using a polynomial fitting routine and have no physical significance.

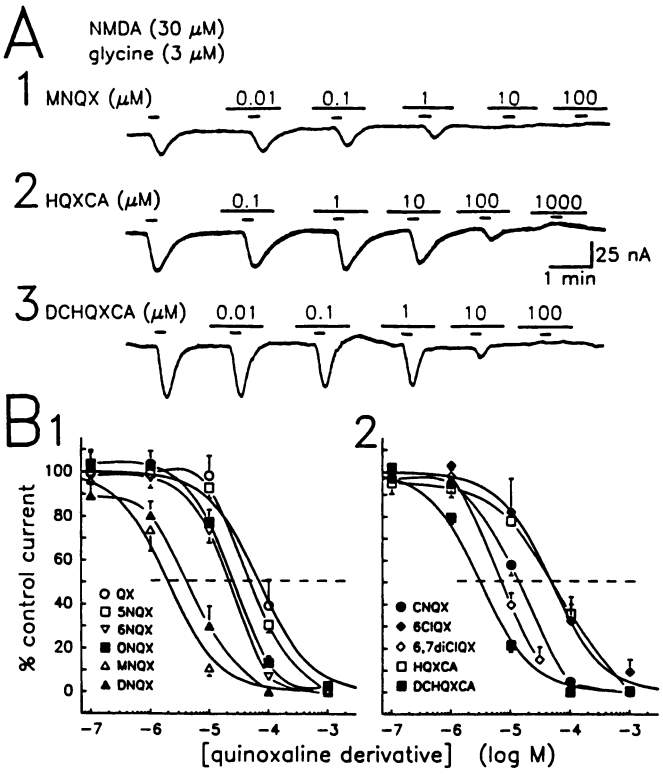

Fig. 5. Concentration dependence of the inhibition by quinoxaline derivatives of current activated by glycine (3 μM) plus NMDA (30 μM). A, Chart recordings of glycine/NMDA current inhibition by MNQX (0.01–100 μM) (A1), QX (0.1–1000 μM) (A2), and DCHQXCA (0.01–100 μM) (A3), in three different oocytes. B, Plot of current responses as percentage of control response as a function of concentration of QX (○), 5NQX (□), 6NQX (▽), ONQX (■), MNQX (△), DNQX (▲), CNQX (●), 6ClQX (♦), 6,7diClQX (◇), HQXCA (□), or DCHQXCA (■). Data are mean ± standard error of three to seven determinations. The *curves* were drawn for the most potent (MNQX and DCHQXCA) and least potent (QX and 6ClQX) compounds using the relationship $I/I_{max} = \{1 + ([antagonist]/IC_{50})^{nH}\}^{-1}$, with the values of IC_{50} given in Table 2. For clarity, the other curves were drawn using a polynomial fitting routine and have no physical significance.

derivatives to inhibit 1) currents induced by kainate, AMPA, and NMDA plus glycine in voltage-clamped *Xenopus* oocytes expressing EAA receptors after injection of mRNA from rat cortex and 2) EPSFP recorded in the dendritic field of CA1 pyramidal neurons in hippocampal slices after stimulation of the Schaffer collateral-commissural pathways.

Materials and Methods

Xenopus Oocyte Experiments

Preparation of partially purified poly(A)$^+$ mRNA. mRNA was prepared from the cerebral cortex of male Wistar rats (100–150 g) by the guanidium thiocyanate/phenol/chloroform single-step method (22). Poly(A)$^+$ mRNA was isolated using oligo(dT)-cellulose chromatography.

Preparation and injection of *Xenopus* oocytes. A section of ovary was removed from a *Xenopus* under cold anaesthesia through a small abdominal incision. Individual stage V and VI oocytes (23) were dissected manually and stored on ice in Barth's solution (24) until injection of mRNA within 24 hr. The follicular cells were not removed. Individual oocytes were injected with 50 nl of an aqueous solution containing 50 ng of poly(A)$^+$ mRNA. After injection, the oocytes were incubated at 18° for 2–3 days, to allow expression. The oocytes were then stored at 6–8°; current responses to kainate, AMPA, and glycine/NMDA were thus maintained for as long as the oocytes remained viable.

Experimental protocol. Electrical recordings were performed in a Plexiglass bath at room temperature (18–22°). The volume of the bath was approximately 200 μl, and a medium flow rate of 4 ml/min was used. Recordings of kainate and AMPA currents were carried out in OR2 medium (25) of composition (in mM) NaCl, 82.5; KCl, 2.5; $CaCl_2$, 1; $MgCl_2$, 1; NaH_2PO_4, 1; HEPES, 5; pH 7.4. For recordings of NMDA/glycine currents, $MgCl_2$ was omitted from the medium and the concentration of $CaCl_2$ was raised to 2 mM.

All experiments were performed using a two-electrode voltage clamp, with a third electrode placed in the bath as voltage reference. Glass microelectrodes (approximately 1 MΩ) were filled with 3 M KCl. Holding potential was usually adjusted to −60 mV, although no appreciable difference in the actions of the quinoxaline derivatives was evident for holding potentials over the range of −100 to −40 mV (see Refs. 8 and 15). Current responses were recorded on a chart recorder.

EAA receptor agonists were bath-applied for 15 sec, and responses were evaluated as the peak of inward current. Longer applications of AMPA or glycine/NMDA induced current responses of equal amplitude. Increasing the duration of kainate (100 μM) application from 15 to 60 sec increased current by only 5–10%, indicating that the average concentration of agonist surrounding the oocyte at 15 sec was within

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TABLE 2

Inhibition by quinoxaline derivatives of currents activated by kainate (100 μM), AMPA (30 μM), and NMDA (30 μM/glycine (3 μM) in *Xenopus* oocytes injected with mRNA from rat cortex and of EPSFP recorded in the CA1 region of rat hippocampal slices after stimulation of Schaffer collaterals

IC_{50} values were derived from inhibition curves constructed with data from a minimum of three experiments at each drug concentration. K_i estimates were derived for kainate and AMPA, assuming inhibition through the equipotent interaction of a single molecule of antagonist with either of two sites on the channel and using the formula $K_i = IC_{50} \times (EC_{50}/[agonist])$, and for glycine, using the formula $K_i = IC_{50}/\{1+([agonist]/EC_{50})\}$ and assuming antagonist action at a single glycine site (cases VI and I, respectively, in Ref. 39). The EC_{50} values used in the calculations were kainate, 100 μM; AMPA, 10 μM; and glycine, 0.6 μM; the concentration of contaminating glycine was assumed to be 100 nM.

Quinoxaline derivative	*Xenopus* oocytes						Hippocampal slices	
	Kainate		AMPA		NMDA/glycine			
	IC_{50}	K_i	IC_{50}	K_i	IC_{50}	K_i	1 mM Mg^{2+} (non-NMDA), IC_{50}	0 Mg^{2+} (NMDA/glycine), IC_{50}
	μM							
QX	270	270	410	137	42	6.8		
5NQX	300	300	410	137	50	8.1		
6NQX	4.4	4.4	10	3.3	22	3.6	40	130
ONQX	23	23	42	14	24	3.9	130	>300
MNQX	1.2	1.2	3.4	1.1	2.2	0.37	6.9	20
DNQX	0.53	0.53	0.75	0.25	4.1	0.66	2.8	80
CNQX	0.27	0.27	1.2	0.40	13	2.1	2.9	130
6ClQX	120	120	130	43	42	6.8		
6,7diClQX	15	15	32	11	7.0	1.1		
HQXCA	54	54	260	87	50	8.1		
DCHQXCA	2.9	2.9	8	2.7	3.1	0.50	12	38

5–10% of the stated concentration. For routine evaluation of quinoxaline inhibitory potency, the agonists were applied at the following concentrations: kainate, 100 μM; AMPA, 30 μM; glycine, 3 μM; and NMDA, 30 μM. Because the agonist EC_{50} values were determined under identical conditions, any difference between the stated agonist concentration and the "true" average agonist concentration surrounding the oocyte is of no consequence to the calculation of quinoxaline K_i values. In these experiments, the quinoxaline derivatives were bath-applied for 30–45 sec before and after the application of agonist, as well as during agonist application. In control experiments (data not shown), inhibition by CNQX, DNQX, QX, or HQXCA of the current evoked by kainate, AMPA, or glycine/NMDA was maximal well within this 30-sec pretreatment period.

Hippocampal Slice Experiments

Transverse hippocampal slices (500 μm) were prepared with a tissue chopper (McIlwain) from the brains of male Wistar rats (150–250 g). Slices were initially incubated for 45 min in nominally Ca^{2+}-free medium containing 10 mM Mg^{2+} (26). The slices were subsequently stored in normal medium of composition (in mM) NaCl, 120; KCl, 5; $CaCl_2$, 1; $MgSO_4$, 1; KH_2PO_4, 1.3; $NaHCO_3$, 20; HEPES, 10; glucose, 10; adjusted to pH 7.35 and bubbled with O_2/CO_2 (95%/5%) at room temperature.

Experiments were performed on submerged slices at 35°. EPSFP were recorded in the dendritic field of the CA1 region, using NaCl (3 M)-filled glass electrodes, after electrical stimulation in the stratum radiatum with a bipolar tungsten electrode. Single square stimuli (50–100 μA, 20 μsec) were applied every 30 sec. Protocol generation, data acquisition, and data analysis were performed using an A-D converter (Labmaster DMA; Scientific Solutions) and interface (TL-1; Axon Instruments) and a microcomputer equipped with an appropriate software package (pCLAMP; Axon Instruments). Data were digitized at 1–5 kHz. EPSFP amplitude was evaluated by measurement of the peak voltage of the negative wave with respect to the base-line voltage.

Slices were superfused with either the normal medium described above or Mg^{2+}-free medium, from which the $MgSO_4$ was omitted. As previously described (see Refs. 12, 13, 19, 27, and 28), in normal medium the EPSFP was mediated primarily by non-NMDA receptors (insensitive to 10 μM D-AP5), whereas in Mg^{2+}-free medium there appeared to be a prominent NMDA receptor-mediated component of the synaptic response (inhibited by 10 μM D-AP5). The quinoxaline derivatives were bath-applied for periods of 8–10 min in either normal or Mg^{2+}-free medium, to evaluate their actions on the non-NMDA receptor and NMDA receptor responses, respectively.

Drug Preparation

Kainate, NMDA, and glycine (all from Sigma) and AMPA (RBI) were prepared as aqueous stock solutions (20–300 mM), stored at −20°, and diluted in the appropriate medium immediately before use.

The quinoxaline derivatives (see Fig. 1 for structures) were purchased from Tocris Neuramin and stored at −20° as 30–100 mM stock solutions in dimethylsulfoxide. All were soluble when subsequently diluted to 10 nM to 1 mM in the experimental media, with the exception of 6,7diClQX, which was not soluble at concentrations greater than 30 μM. The maximal final concentration of dimethylsulfoxide (1%) had no effect in either experimental system (data not shown).

Results

***Xenopus* oocyte experiments.** Inward current responses to kainate (100 μM), AMPA (30 μM), and glycine (3 μM)/NMDA (30 μM) were recorded in 75 oocytes from 14 series of injections of rat cortical mRNA. The concentration-response relationship for each agonist was examined in 12–15 oocytes (Fig. 2). The maximal peak inward currents and concentrations activating half-maximal inward current (EC_{50}) for each agonist are listed in Table 1. The EC_{50} values obtained were close to those of previous reports (15, 29–36). Kainate induced a smooth noninactivating current at all concentrations, whereas clear inactivation of AMPA-induced current was observed with concentrations greater than 100 μM (see Refs. 36 and 37). However, the ratio of peak to steady state current observed with high concentrations of AMPA (peak:steady state ≃ 0.1) was approximately 100-fold less than that observed with fast perfusion systems and isolated neurons (peak:steady state ≃ 10; see Ref. 38). Furthermore, kainate current was typically 5–10-fold greater than peak AMPA current in our experiments, a ratio close to that observed for steady state kainate and AMPA

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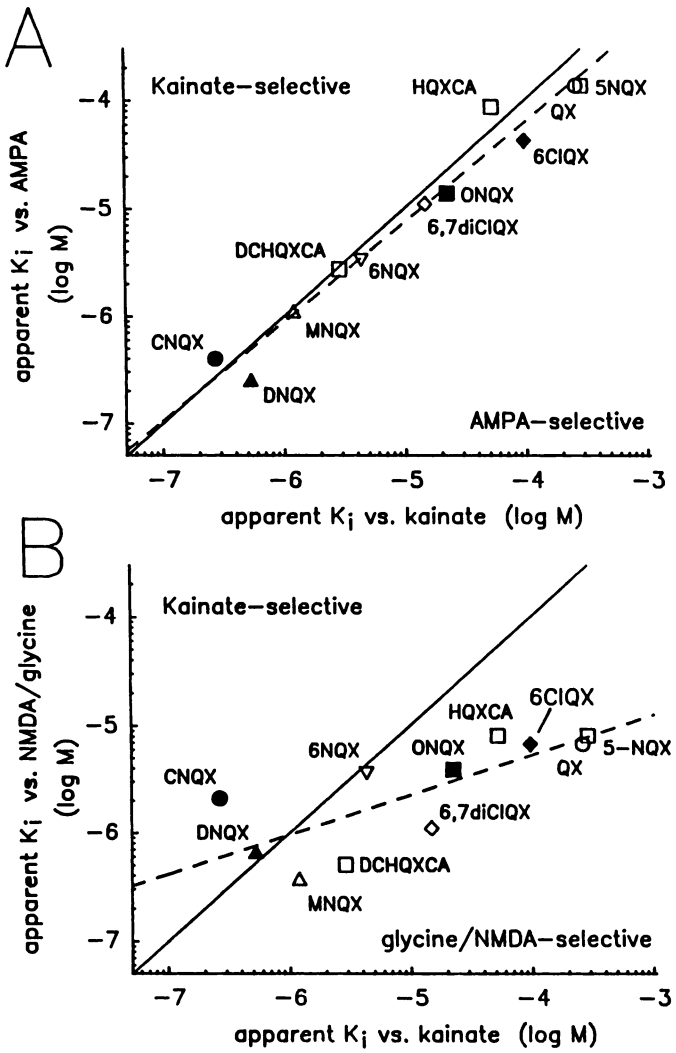

Fig. 6. Comparison of apparent K_i values for the inhibition by quinoxaline derivatives of kainate-, AMPA-, and NMDA/glycine-induced current in mRNA-injected *Xenopus* oocytes. A, Kainate versus AMPA; B, kainate versus glycine/NMDA. K_i values were derived from experimental IC_{50} values as in Table 2. *Dashed lines*, least-squares regression fits of the data having slopes s and correlation coefficients r of $s = 0.92$ and $r = 0.97$ (A) and $s = 0.37$ and $r = 0.77$ (B). *Solid lines*, equipotency.

currents in fast perfusion systems (38). Similarly, inactivation of glycine/NMDA current was rarely observed. These results indicate that the ligand-receptor interactions are near equilibrium at all times during the perfusion of drugs.

Quinoxaline derivatives, bath-applied before (for 30–45 sec), during, and after application of the agonists, induced concentration-dependent, rapidly reversible, inhibition of EAA currents in mRNA-injected *Xenopus* oocytes. In control experiments (data not shown), we confirmed that 1, 10, and 100 μM CNQX or DCHQXCA induced parallel rightward shifts of the concentration-response relationships for kainate and glycine (with [NMDA] constant at 30 μM; see Refs. 8, 14, and 15), as well as for AMPA. Schild plots of these data yielded pA_2 values and slopes (mean ± standard error), respectively, for CNQX of 6.3 ± 0.1 and 1.12 ± 0.05 versus kainate, 6.5 ± 0.1 and 0.97 ± 0.04 versus AMPA, and 5.7 ± 0.3 and 1.10 ± 0.26 versus glycine/NMDA. For DCHQXCA the values were 5.3 ± 0.01 and 1.06 ± 0.15 versus kainate, 5.5 ± 0.2 and 1.01 ± 0.09 versus AMPA, and 6.1 ± 0.2 and 1.23 ± 0.12 versus glycine/NMDA. The pA_2 values were in good agreement with the K_i values obtained from the routine experiments in which the quinoxaline derivatives were tested at five concentrations against a single concentration of EAA agonist (see below). The slopes of the Schild plots were near unity, indicating that the inhibition by the quinoxaline derivatives is competitive in nature. However, in the presence of 100 μM CNQX or DCHQXCA, submaximal glycine/NMDA responses were observed even with 300 μM glycine, leaving open the possibility of a component of inhibition at a site other than the glycine site (see Refs. 8 and 15). Furthermore, and in confirmation of previous reports (10, 15), inhibition of glycine/NMDA current by CNQX and DCHQXCA displayed noncompetitive kinetics with respect to NMDA (data not shown).

Examples of routine experiments in which the potencies of the quinoxaline derivatives were determined are shown for kainate (Fig. 3A), AMPA (Fig. 4A), and glycine/NMDA (Fig. 5A). All the quinoxaline derivatives inhibited the currents completely, with the exception of 6,7diClQX, whose limited solubility precluded its application at sufficiently high concentrations. The inhibition data were well fit by a single-site model for the drug-receptor interaction (Figs. 3B, 4B, and 5B). For kainate and AMPA the inhibition curves were widely spread over more than 3 orders of magnitude, and the potencies of the quinoxaline derivatives against the two agonists were very similar. The rank order of potency was CNQX ≃ DNQX > MNQX > DCHQXCA ≃ 6NQX > 6,7diClQX ≃ ONQX > 6ClQX ≃ HQXCA > QX ≃ 5NQX. In contrast, for glycine/NMDA the inhibition curves were restricted to a comparatively small concentration range (approximately 20-fold). The rank order of potency was MNQX > DCHQXCA > DNQX > 6,7diClQX > CNQX > 6NQX ≃ ONQX > QX ≃ 5NQX ≃ HQXCA ≃ 6ClQX.

Half-inhibitory concentrations (IC_{50} values) for the actions of the 11 quinoxaline derivatives on the EAA agonist-induced currents were estimated from the inhibition curves and are listed in Table 2. In addition, Table 2 lists the K_i values calculated using equations formulated by Cheng and Prusoff (39) and the concentrations and EC_{50} values for kainate, AMPA, and glycine used in these experiments, assuming the inhibition to be competitive for each of the quinoxaline derivatives. For kainate and AMPA, we have assumed that the equipotent interaction of a single antagonist molecule with either of two sites is sufficient to inhibit receptor/channel current activation dependent upon simultaneous occupation of both sites by two agonist molecules (case VI; Ref. 39). Comparison of the K_i values for inhibition of kainate- and AMPA-induced current (Fig. 6A) shows that the quinoxaline derivatives are essentially equipotent against the two agonists (regression slope = 0.92, correlation coefficient $r = 0.97$). In contrast, only weak correlation of potencies against kainate and glycine/NMDA was evident (slope = 0.37, $r = 0.77$; Fig. 6B).

Hippocampal slice experiments. The EPSFP evoked by stimulation in the stratum radiatum of hippocampal slices was recorded in the dendritic field of the CA1 region (Fig. 7). In normal medium containing 1 mM Mg^{2+}, the EPSFP was of short duration (10–20 msec at half-maximal amplitude) and was relatively insensitive to D-AP5 (data not shown), indicating that it is mediated primarily by non-NMDA receptors. In Mg^{2+}-free medium, the EPSFP was prolonged (duration, 100–200 msec) by the appearance of a second component that was

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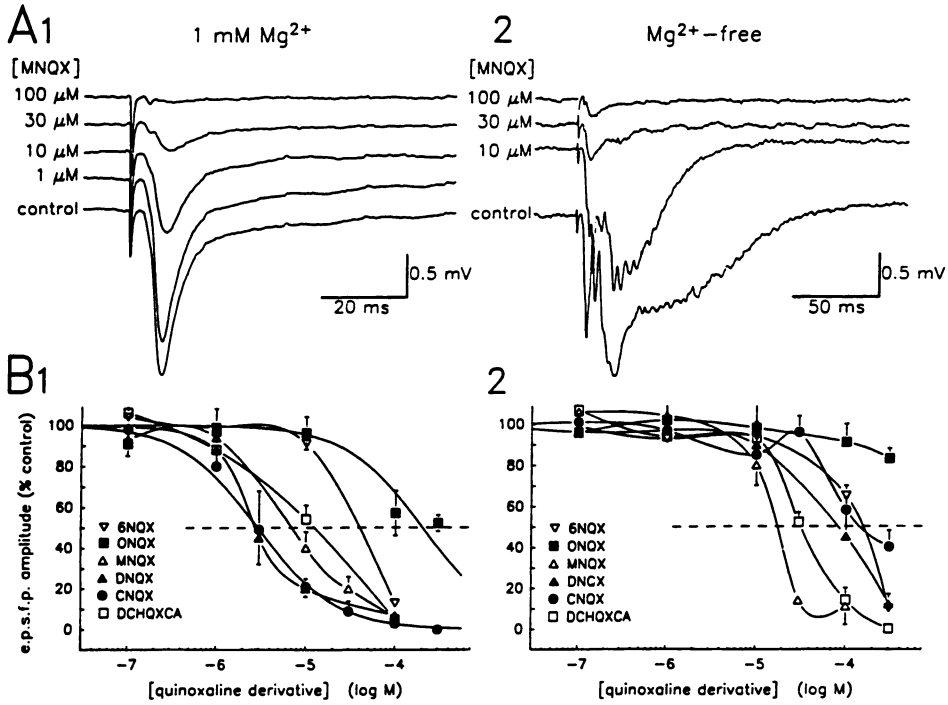

Fig. 7. Inhibition of EPSFP evoked in the dendritic field of the CA1 region of hippocampal slices after electrical stimulation of the stratum radiatum. A1, EPSFP mediated predominantly by non-NMDA receptors in a slice superfused with medium containing 1 mM $MgCl_2$ before (control) and after bath application of MNQX (1, 10, 30, and 100 μM). A2, Prolonged EPSFP mediated predominantly by NMDA receptors in a slice superfused with medium containing no added $MgCl_2$ before (control) and after bath application of MNQX (10, 30, and 100 μM). B, Plots of EPSFP amplitude as percentage of control response in medium containing 1 mM Mg^{2+} (B1) or no added Mg^{2+} (B2), as a function of concentration of 6NQX (▽), ONQX (■), MNQX (△), DNQX (▲), CNQX (●), or DCHQXCA (□). Data are mean ± standard error of three to nine determinations. In B1, the curves were drawn for the most potent (CNQX) and least potent (ONQX) compounds using the equation $I/I_{max} = \{1 + ([antagonist]/IC_{50})^{n_H}\}^{-1}$, with the IC_{50} values given in Table 2. For clarity, the other curves in B1 and all the curves in B2 were drawn using a polynomial fitting routine and have no physical significance.

sensitive to D-AP5 (data not shown), indicating that it is mediated primarily by NMDA receptors.

The inhibitory effects of the six quinoxaline derivatives that were most potent in the oocyte experiments were also tested against the EPSFP recorded in the presence and absence of Mg^{2+} (Fig. 7A). In 1 mM Mg^{2+}-containing medium, the concentration-dependent inhibition of the EPSFP was consistent with a single site of action (Fig. 7B1). The rank order of potency was CNQX ≃ DNQX > MNQX > DCHQXCA > 6NQX > ONQX. In Mg^{2+}-free medium, the EPSFP was inhibited at considerably higher concentrations of all the compounds; ONQX induced only slight (20%) inhibition at 300 μM, the highest concentration tested. In Mg^{2+}-free medium, the inhibition curves appeared to be steeper than in 1 mM Mg^{2+}-containing medium (Fig. 7B2) and the rank order of potency also differed, MNQX > DCHQXCA > DNQX > CNQX ≃ 6NQX ≫ ONQX. The inhibition induced by high concentrations of antagonists was complete within 2–3 min. Recovery from (washout of) the effects of the quinoxalinedione derivatives was generally very slow (>30 min) in 1 mM Mg^{2+} medium, although somewhat faster reversal of the inhibition of the slow component of the EPSFP in Mg^{2+}-free medium was often observed, presumably because these molecules are highly lipophilic. Notably, the effects of the hydroxyquinoxaline-2-carboxylate derivative DCHQXCA were usually fully reversible in <15 min.

The IC_{50} values estimated from the inhibition curves are listed in Table 2. In Fig. 8, these IC_{50} values are compared with the estimates of K_i obtained in the oocyte experiments. Good correlations were observed for the sensitivities to each of the quinoxaline derivatives of the EPSFP in 1 mM Mg^{2+} versus kainate-induced current (Fig. 8A; slope = 0.93, r = 0.97) and for those of the EPSFP in Mg^{2+}-free medium versus NMDA/glycine-induced current (Fig. 8B; slope = 0.90, r = 0.90). The IC_{50} values in 1 mM Mg^{2+} were on average 8-fold higher than the K_i values estimated in the oocyte experiments with kainate or AMPA, whereas in Mg^{2+}-free medium the IC_{50} values were on average 60-fold higher than the K_i values estimated in the oocyte experiments with NMDA/glycine.

Discussion

We have chosen two established experimental systems in which to compare the EAA antagonist potencies and efficacies of a series of 11 quinoxaline derivatives. Our goal was to determine the abilities of the quinoxaline derivatives to interact with, and to discriminate between, the glycine site of the NMDA/glycine receptor/channel complex and non-NMDA receptor/channel sites. Furthermore, we wished to address the question of the relationship between the sites at which kainate and AMPA act to activate current.

Our results are in good agreement with results from a number of experimental systems, concerning the quinoxaline derivatives for which data are available (CNQX, DNQX, MNQX, 6,7diClQX, HQXCA, and DCHQXCA; see the introduction). In particular, we confirm that these quinoxaline derivatives act competitively with respect to kainate and glycine (14, 15), as well as AMPA, in mRNA-injected *Xenopus* oocytes and that they inhibit both non-NMDA and NMDA receptor-mediated components of the CA1 EPSFP (13, 40). This study extends these findings with data on a series of as yet untested quinoxaline derivatives.

Biological considerations. The 11 compounds displayed very close correlation between their apparent K_i values against kainate-induced current and those against AMPA-induced current. This provides further pharmacological support (see Refs. 11, 15, 31, 34, 38, and 41–43) for the arguments, from molecular biological approaches (44–46), that these two agents activate current through actions at a similar or identical site on a single receptor/channel complex. The similarity is all the more compelling when one considers the differences in activity against the NMDA/glycine receptor/channel complex. There was weak correlation between potency versus kainate/AMPA and po-

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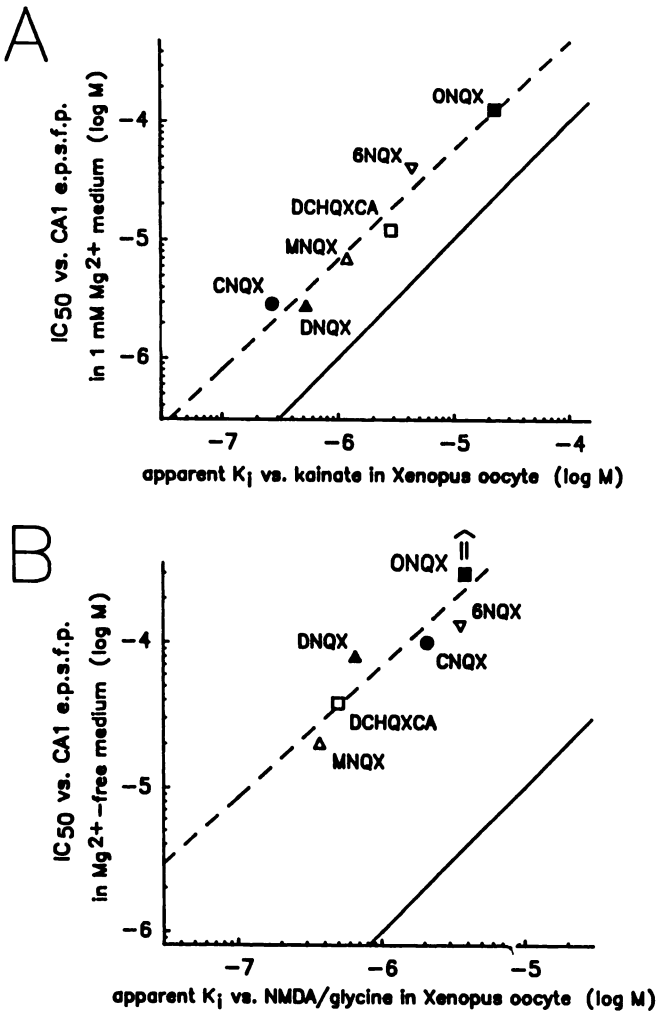

Fig. 8. Plots comparing the inhibitory potencies and rank order for the quinoxaline derivatives. In A, the apparent K_i values against kainate-induced current in *Xenopus* oocytes are compared with the IC_{50} values against the non-NMDA component of the hippocampal CA1 EPSFP recorded in 1 mM Mg^{2+}-containing medium. In B, the apparent K_i values against glycine/NMDA-induced current in *Xenopus* oocytes are compared with the IC_{50} values against the NMDA component of the hippocampal CA1 EPSFP recorded in Mg^{2+}-free medium. In each plot, the *solid line* indicates equipotency, whereas the *dashed line* represents the least-squares linear regression fit of the data. In A, the regression line (slope = 0.93, r = 0.97) is shifted to approximately 8-fold higher concentrations on the *ordinate*. In B, the regression line (slope = 0.90, r = 0.90) is shifted to approximately 60-fold higher concentrations on the *ordinate*.

tency versus glycine/NMDA, indicating that these sites are structurally distinct. An interesting observation is that eight of the 11 molecules tested displayed apparent K_i values reflecting higher affinity for the glycine site of the NMDA receptor than for the kainate/AMPA site. Thus, although appropriate substitution can produce a molecule with reasonable kainate/AMPA selectivity, the quinoxaline derivatives are generally better glycine site ligands.

A comparison between the activities of the quinoxaline derivatives on rat cerebral cortex receptors expressed in *Xenopus* oocytes and those of the hippocampal CA1 pyramidal cells in slices is also instructive. There was a good correlation between the relative potencies of the six most potent quinoxaline derivatives against electrical phenomena in oocytes and slices for similar conditions [kainate/AMPA current versus the EPSFP in 1 mM Mg^{2+} medium ("non-NMDA receptor-mediated") and glycine/NMDA current versus the EPSFP in Mg^{2+}-free medium ("NMDA receptor-mediated")]. However, the IC_{50} values of the quinoxaline derivatives in the slices exceeded their K_i values by 8-fold in 1 mM Mg^{2+} medium and by 60-fold in Mg^{2+}-free medium. Several explanations of these observations are possible. First, the potency differences could result from the expression of different receptors in the two systems. This explanation is unlikely, however, because the correlation regression slopes near unity argue strongly that the same receptors are expressed in the two systems.

Second, potency differences may, in part, be due to incomplete access of the drugs to the synaptic receptors. However, the six molecules tested vary considerably in their water and lipid solubilities and the time-courses of their inhibition and the washout of this effect. Nevertheless, the potencies of all six molecules in oocyte and slice experiments, determined after sufficiently long periods of application, were closely correlated. This indicates that access to the site of action was not an important limiting factor. Another observation that must be accounted for is that the potency differences between experimental systems were much greater for action at the glycine site of the NMDA receptor (approximately 60-fold) than for action at the kainate/AMPA receptor (approximately 8-fold). In order to explain this discrepancy on the basis of limited drug access, one must postulate that synaptic NMDA/glycine and kainate/AMPA receptors are located differently on CA1 neurons and that NMDA/glycine receptors are more 'protected' from exogenous substances.

A third explanation is that the ratios between the estimates of K_i in oocytes and IC_{50} in slices reflect, at least in part, the synaptic concentrations of the endogenous ligands. High concentrations of endogenous ligand would compete with antagonists for the receptors and reduce their apparent potency. In order to estimate the effective synaptic concentration of a putative endogenous ligand acting on a receptor, one must assume that equilibrium conditions are established at the peak of the EPSP in the hippocampal CA1 region. This assumption cannot presently be confirmed with certainty. However, the 8-fold and 60-fold potency differences for kainate/AMPA and glycine/NMDA receptor responses, respectively, may be tentatively interpreted as indicating that the ratio of the synaptic concentration of endogenous ligand to its EC_{50} is lower for the kainate/AMPA receptor ligand than for the glycine site ligand. Thus, we would conclude, as have other authors on the basis of studies using CNQX, DNQX, and kynurenic acid derivatives to antagonize NMDA responses in the spinal cord or cortex (10, 47, 48), that the synaptic concentrations of endogenous glycine (or other glycine site ligand) are saturating. Glutamate (or other endogenous ligand) would be present at lower concentrations relative to its EC_{50}. Alternatively, because it may be present for only a short time in the synapse during an EPSP, glutamate may compete less effectively with antagonist. Thus, although quinoxaline derivatives display, at best, moderately higher affinity for kainate/AMPA receptors (8-fold in the case of CNQX), the greater saturation of the glycine site of NMDA receptors in intact synapses would confer a greater degree of kainate/AMPA selectivity (45-fold for CNQX).

Structure-activity relationship. The *Xenopus* oocyte experiments show that the parent compound QX is a moderately potent glycine/NMDA antagonist (K_i = 8.1 μM) and 10-fold

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less potent kainate/AMPA antagonist. Single nitro substitutions at positions 5 or 6 of QX have quite different effects on the potency of the molecules as EAA antagonists. The 5-nitro substitution does not alter potency. In contrast, the 6-nitro substitution yields a compound that is 100-fold more potent than QX against kainate and AMPA, although only marginally more potent than QX (3-fold) against glycine/NMDA. Thus, 6NQX is a moderately potent but unselective antagonist of NMDA and non-NMDA receptors. Dinitro substitutions yield molecules that possess much greater potency than QX but limited selectivity between NMDA and non-NMDA receptors. Thus, DNQX is potent and slightly kainate/AMPA selective and MNQX is potent and slightly glycine/NMDA selective. Curiously, for ONQX potency is only moderately increased versus kainate/AMPA, whereas potency is almost unchanged versus glycine/NMDA. Either steric constraints or charge density differences could explain these results (see below).

We confirm the potency and selectivity of CNQX for kainate/AMPA receptors. It is unfortunate that other cyano-substituted QX derivatives are not yet available so that the influence of this substitution could be more extensively studied.

In contrast to the 6-nitro substitution, the single 6-chloro substitution of QX had little effect on potency or selectivity. This is quite unlike the effects of single halide substitutions in kynurenic acid to yield 7-chlorokynurenic acid (49) or in indole-2-carboxylic acid to yield 5-fluoro-indole-2-carboxylic (50), which increase potency and selectivity for the glycine site of the NMDA receptor. However, the 6,7-dichloro substitution of QX increases NMDA/glycine and kainate/AMPA potency approximately 10-fold (see also Ref. 48). Similarly, the 6,7-dichloro substitution of HQXCA, yielding DCHQXCA, substantially increases potency. In light of the recent reports (48, 51, 52) that 5,7-dichlorokynurenate is a still more potent and selective glycine site antagonist, it would be interesting to know the activities of 7-chloro-HQXCA, 5,7-dichloro-QX, or 5,7-dichloro-HQXCA.

The different effects of nitro and chloro substitutions of QX can be interpreted in terms of the differing properties of these electron-withdrawing groups. Indeed, nitro substitution of an aromatic ring entails a strong mesomeric and inductive electron-withdrawing effect, whereas chloro substitution is endowed with a mesomeric electron-donating and an inductive electron-withdrawing effect (53). Because mesomeric effects are transmitted over much longer distances than inductive effects, one can predict that nitro and chloro substitutions will have opposite effects on the level of acidity and charge density of the quinoxalinedione. Apparently such a difference is of paramount importance in the interaction of the molecule with the kainate/AMPA receptor/channel protein and of somewhat lesser importance for the glycine site of the NMDA/glycine receptor/channel protein. In addition, 5,6-dinitro and 6,7-dinitro substitutions will affect the two pseudo-2-quinolone functions to approximately the same extent, whereas 5,7-dinitro substitution will profoundly influence only one quinolone function, leaving the other more or less unaffected. The effects of the substitutions on the acidity of each of the two quinolone functions and the relationship between this acidity and EAA receptor potency will be examined in separate report.

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