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Excitatory Amino Acid Receptors Expressed in *Xenopus* Oocytes: Agonist Pharmacology

TODD A. VERDOORN[1] and RAYMOND DINGLEDINE

Department of Pharmacology and Neurobiology Curriculum, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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SUMMARY

The properties of excitatory amino acid (EAA) receptors transplanted into *Xenopus* oocytes were investigated by voltage clamp 48 hr to 5 days after oocytes had been injected with mRNA isolated from rat brain. The application of EAA agonists to mRNA-injected cells, but not to uninjected or water-injected cells, produced several different inward currents, two of which are characteristic of neuronal EAA receptors. Currents with properties expected from activation of *N*-methyl-D-aspartate (NMDA) receptors were evoked by L-glutamate (EC_{50} = 2.3 μM), D-aspartate (10 μM), L-aspartate (13 μM), NMDA (31 μM), and ibotenate (35 μM). Inward currents activated by these agonists were blocked by Mg^{2+} in a voltage-dependent manner and antagonized by 10–50 μM D-2-amino-5-phosphonovaleric acid (D-APV). The D-APV block was not voltage dependent. A second type of inward current was produced by kainate, domoate, (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and L-glutamate. This smooth inward current was insensitive to Mg^{2+} and D-APV. L-Glutamate and domoate were equipotent for activating this current (EC_{50} = 14 μM) whereas kainate was less potent (98 μM). The kainate potency was somewhat voltage dependent, inasmuch as the EC_{50} was 33% lower when measured at +38 mV than when measured at −60 mV in the same cells. Quisqualate (50 μM) and AMPA (50 μM) drastically reduced the kainate current, suggesting these agonists also interact with this receptor. Some mRNA preparations encoded only receptors for the kainate response, which argues for distinct NMDA and non-NMDA receptors. A third type of inward current was produced by quisqualate. This current, consisting of oscillating and smooth components, was carried by chloride and not evoked by AMPA, suggesting it is not likely caused by activation of the conventional neuronal quisqualate receptor. The utility of the oocyte preparation for quantitative pharmacological studies of EAA receptors is discussed.

Neuronal receptors for the EAA are becoming increasingly important for the understanding of normal and pathological brain function. Three types of EAA receptors are thought to exist in the mammalian brain, named originally according to the agonists kainate, quisqualate, and NMDA (1). Neuronal NMDA receptors are clearly distinguished by their voltage-dependent block by Mg^{2+} (2, 3) and by a variety of potent and selective competitive antagonists (4) and open channel blockers (5, 6). The characterization of kainate and quisqualate receptors had progressed more slowly due to the paucity of selective antagonists, and for these receptors the correspondence between binding and physiological studies is incomplete. For example, radioligand binding studies in isolated neuronal membranes (7) and autoradiography in brain sections (8, 9) have demonstrated the existence of a binding site for kainate with a dissociation constant in the 5–10 nM range. However, with the possible exception of a single study (Ref. 10; but see Ref. 11), kainate concentrations of 1–10 μM or higher have been required to open cation channels in neuronal membranes (12–17). The situation is further complicated by the recent suggestion that one type of quisqualate receptor may modulate inositide phosphate metabolism (18, 19). Detailed pharmacological studies of the kainate and quisqualate receptors would help resolve the issue of whether neuronal depolarization is mediated by one or two non-NMDA receptors.

Quantitative pharmacological studies of EAA receptor/ion channel complexes are difficult in neurons for a number of reasons. In intact tissue preparations (e.g., brain slices), tortuous diffusion pathways and uptake processes limit knowledge of drug concentrations in equilibrium with receptors. Further, the consequences of depolarization induced by the application of agonists to highly interconnected, heterogenous neuronal tissue (e.g., K^+ release into interstitial space, synaptic activa-

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[1] Present address: Max-Planck-Institut fur Biophysikalisch Chemie, D-3400 Gottingen, Federal Republic of Germany.

ABBREVIATIONS: EAA, excitatory amino acids; NMDA, *N*-methyl-D-aspartate; SDS, sodium dodecyl sulfate; MBS, modified Barth's solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; D-APV, D-2-amino-5-phosphonovaleric acid; AMPA, (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; $MeSO_4$, methyl sulfate.

tion of neighboring cells) may distort agonist dose-response curves. Single channel studies of neurons in culture can, in principle, overcome these problems, but are difficult to maintain for the time needed to complete dose-response experiments. A novel preparation suitable for quantitative pharmacological studies is the *Xenopus* oocyte injected with brain mRNA. This cell is large, hardy, and spherical, allowing for adequate voltage clamp and accurate measurements of drug-induced currents. Drug-induced membrane currents should be linearly proportional to the number of activated receptors for receptors coupled directly to ion channels, thus eliminating the effects of spare receptors. Diffusion and uptake problems are minimized so that the ionic and drug concentrations at the receptors can be controlled and manipulated.

Xenopus oocytes injected with rat or chick brain mRNA translate and express functional receptors for a variety of neurotransmitters (20–22). Inward currents produced by glutamate, kainate, quisqualate, and NMDA have been described in oocytes injected with rat brain mRNA (19, 21, 23, 24). The oocyte translation system has also been used to demonstrate the functionality of cloned cDNAs for a number of neurotransmitter receptors (25–29), and for structure-activity studies of the nicotinic acetylcholine receptor (30, 31). The use of the oocyte translation system for receptor reconstitution makes it essential to evaluate whether the properties of transplanted or cloned receptors differ from those of the native receptors in neurons.

In this report we characterize the responses to a variety of EAA agonists in oocytes injected with native rat brain mRNA. The pharmacological and physiological properties of the responses evoked by EAA agonists are shown to be similar to those found in neurons, with the exception of currents induced by quisqualate. Preliminary reports of some of this work have appeared (32–34).

Methods

Primary cell culture. Primary cultures were prepared from whole brains (minus cerebellum) of 18 to 19 day prenatal rats as previously described for hippocampal primary culture (35). Cells were plated into polylysine-coated 75-cm^2 flasks, fed with serum-containing medium, and cultured for 7 to 10 days before harvesting for RNA extraction.

RNA extraction. RNA was prepared from rat brain and cultured neurons by a variation of the method of Chirgwin *et al.* (36). All glassware was baked at 350° for 4 hr to destroy ribonucleases. Young rats (approximately 150 g) were decapitated and their brains (minus cerebellum) were homogenized (Brinkman Polytron, one pass, setting 4) at 20° in 16 ml/g of tissue of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium lauryl sarcosine, 0.1% Sigma Antifoam A, and 0.56% β-mercaptoethanol. When cultured cells were used the flasks were rinsed once with minimum essential medium and then 5 ml per flask of the guanidinium solution was added to dissolve the cells without physical homogenization. In either case the resulting mixture was centrifuged at 10,000 × *g* for 10 min at 10° (Beckman Model J2-21 centrifuge with Beckman JA-17 rotor) and the supernatant was layered onto a solution of 5.7 M cesium chloride, 25 mM sodium acetate (pH 5.0) that had previously been treated with 0.01% diethyl pyrocarbonate. The RNA was pelleted by centrifugation at 83,000 × *g* for 18 hr at 20° (Beckman L8 ultracentrifuge and SW-28 rotor). The supernatant was removed, the RNA was dissolved in sterile 10 mM Tris·HCl (pH 7.2), 1 mM EDTA, and 0.5% SDS, and then residual proteins were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). One-twentieth volume of sterile 4 M sodium acetate (pH 5.5) and 2.5 volumes of 95% ethanol were added to the aqueous phase and the RNA was precipitated overnight at −20°.

Poly(A)$^+$ RNA was selected by one round of oligo(dT) cellulose chromatography (37). Before use, the oligo(dT) cellulose was prepared according to the procedures recommended by the manufacturer. The total RNA was collected from ethanol by centrifugation for 30 min at 10,000 × *g* at 0°. The pellet was washed once with 70% ethanol, lyophilized to remove most of the residual fluid, and dissolved in sterile binding buffer (0.5 M lithium chloride, 10 mM Tris base, pH 7.0, 1 mM EDTA, 0.025% SDS). The RNA was bound to the oligo(dT) by shaking for 15 min at 20°, then the suspension was loaded into sterile Dispocolumns (Bio-Rad, Richmond, CA) and the columns were washed at 20° with approximately 10 bed volumes of binding buffer before the poly(A)$^+$ RNA was eluted with sterile elution buffer (10 mM Tris·HCl, pH 7.0, 1 mM EDTA, 0.01% SDS) at 68°. Fractions (1.5 ml) were collected and the absorbence at 260 nm of each fraction was measured to identify fractions in which the RNA eluted. Fractions that contained significant amounts of RNA were precipitated with 95% ethanol. The final washed pellets were dissolved in sterile water to a concentration of 0.8–1.2 mg of RNA per ml and stored in single-use aliquots at −70° until injection into the oocytes.

Handling, injection, and culture of oocytes. Female *Xenopus* were anesthesized by immersion in water containing 0.625% tricaine methanesulfonate and a small incision was made in the lower abdomen. One or more lobes of ovary were removed into the culture solution [MBS] containing (in mM):88 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 10 HEPES, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, and 0.91 $CaCl_2$, and supplemented with 0.01 mg/ml each of penicillin and streptomycin). The abdominal musculature and then the skin of the frog was sutured. The frog was allowed to heal for at least three weeks before more oocytes were removed. Oocytes were separated from the ovary manually with forceps or by two different enzymatic treatments. In one protocol small pieces of ovary were placed in MBS in which the Ca^{2+} salts were replaced with NaCl plus Sigma type I or IA collagenase (2 mg/ml) and shaken for 45 min to 1 hr until the oocytes fell freely from the ovary. This collagenase treatment was shown by histological examination to remove the follicle cell layer that surrounds manually dissected oocytes. Alternatively, in more recent experiments ovarian lobes were placed in normal MBS containing neutral dispase (2 mg/ml) and shaken for 1 to 1.5 hr until the oocytes fell freely from the ovary. The treated oocytes were then placed in MBS made hypertonic by the addition of 50 mM extra NaCl or 50 mM sucrose. The oocytes shrunk over the next 10–15 min allowing the follicle cell layer to be removed manually with fine forceps. Follicular or defolliculated oocytes were then ready for injection with the mRNA solution. The dispase procedure resulted more consistently in healthy defolliculated oocytes.

Glass capillary tubes that fit the metal plunger of a Drummond micropipetter (1–10 μl size) were pulled to a fine tip with a vertical microelectrode puller and the tip of the pipette was broken to an inside diameter of 10–20 μm. The injection pipettes were back-filled with paraffin oil and placed on the metal plunger. A rubber O-ring at the seat of the glass pipet effectively sealed the system against air leaks. The mRNA solution was drawn up into the tip by turning the plunger back. Each oocyte was injected with 50–75 nl of RNA solution or water, then placed in approximately 100 μl of culture solution in a 96-well dish and cultured for 24–48 hr before electrophysiological experiments were begun. The oocytes remained viable for up to 5 days after their removal from the frog.

Electrophysiology. Oocytes were placed in a small recording chamber (volume approximately 500 μl) and continuously perfused at a flow rate of 5 ml/min with MBS supplemented with 1 mM $CaCl_2$. Oocytes were impaled with microelectrodes filled with 3 M CsCl or 3 M KCl, which was sometimes supplemented with 0.1 M K-EGTA. The composition of the pipette solution had no detectable effect on the measured values reported in these experiments. Oocytes with input resistances of 0.3 MΩ or greater were voltage clamped with one or two microelectrodes (Axoclamp 2A; Axon Instruments, Burlingame, CA) at

a holding potential of −60 or −80 mV. For single electrode clamping the electrodes typically had resistance between 0.6 and 2 MΩ whereas those used for two-electrode clamping ranged from 2 to 5 MΩ. Drugs were dissolved in the perfusion solution and applied by gravity perfusion with a dead time of 9 sec. Solution changes were normally completed within 40 sec. Current and voltage signals were recorded on FM tape and a chart recorder, and digitized by an IBM AT computer for later analysis. For digitizing, the current signals were low-pass-filtered with an 8-pole Bessel filter at 10–250 Hz, which was no more than one half the digitizing frequency.

Current-voltage curves were constructed by either stepping the command voltage from −60 mV to various levels and measuring the current at each level or by ramping the command voltage from −80 mV to about +40 mV over 2 sec. With this rate of voltage change the capacitive currents associated with the voltage ramp were small. In both cases the current was plotted as a function of the measured voltage. Current-voltage relationships for drugs were determined by subtracting the curve obtained at rest from that obtained near the peak of the agonist response. The chord conductance of agonist currents, $G_{(V)}$, at membrane potential V, was calculated by the following equation:

$$G_{(V)} = I_V/(V - V_{rev}) \quad (1)$$

where I_V is the agonist current measured at V, and V_{rev} is the reversal potential of the agonist current.

Full concentration-response curves for agonists were obtained by applying sequentially increasing concentrations of the drug for a time sufficient to produce a maximum current at each concentration. The agonist was then washed out and at least 5 min were allowed to elapse before the next concentration of agonist was applied. The difference between the maximum and the baseline current was measured. An example of the construction of such a curve for one oocyte is seen in Fig. 1. All other curves were obtained in a similar manner. Each curve was fitted to the logistic equation:

$$\text{current} = \text{maximum current}/[1 + (EC_{50}/[\text{agonist}])^n] \quad (2)$$

(where n represents the Hill slope coefficient) by a nonlinear least squares curve-fitting program. The 95% confidence interval of the concentrations causing half-maximal response (EC_{50}) was calculated as the log of the drug concentration. Curves for the figures represent the average of 3 to 18 replicate experiments and in each cell 7 to 11 agonist concentrations were used to define the concentration-response relationship.

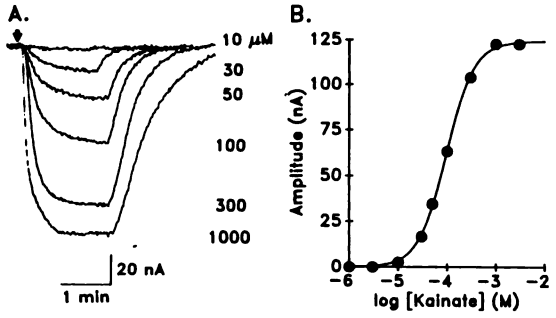

Fig. 1. An example of a complete concentration response curve for kainate in a single mRNA-injected oocyte. This oocyte had been injected with approximately 50 ng of rat brain mRNA 3 days before the experiment. The oocyte had a resting membrane potential of −56 mV and input resistance measured at the resting potential of 0.7 MΩ. A, A series of six superimposed inward currents produced by the application of kainate concentrations denoted on the *right*. The drugs were dissolved in the perfusion solution and applied at the time indicated by the *arrow*. The perfusion of the drug was continued until the current reached a steady state for each individual concentration. Five minutes separated the application of each concentration. B, The amplitudes of the currents are plotted as a function of log of the kainate concentration. The *smooth curve* was drawn from the fit of the data to the logistic equation (Eq. 2).

Materials. Guanidinium thiocyanate was obtained from Fluka Chemical Corp. (Ronkonkoma, NY), cesium chloride was from Gallard and Schlesinger (Carle Place, NY) phenol and neutral dispase (Type 1) were from Boerhinger-Mannheim (Indianapolis, IN), and oligo(dT) cellulose was from Collaborative Research (Waltham, MA). NMDA, D-APV, domoate, quisqualate, ibotenate, and AMPA were purchased from either Cambridge Research Biochemicals (Valley Stream, NY) or Tocris Neuroamin (Buckhurst Hill, England) whereas the other reagents and drugs used were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents used in the preparation of mRNA were used exclusively for that purpose.

Results

These results were obtained from 584 oocytes that met the criterion of resting input resistance of 0.3 MΩ or greater. The average resting membrane potential and input resistance of 516 oocytes injected with mRNA was −54 ± 1 mV and 0.91 ± 0.02 MΩ, respectively. Water-injected or uninjected control oocytes had an average membrane potential of −49 ± 1 mV and input resistence of 0.92 ± 0.07 MΩ (N = 68). All oocytes were tested with agonists at least 48 hr after injection, but in some mRNA-injected oocytes there was no agonist response until 72 hr post-injection. The numbers reported include all cells tested between 48 hr and 5 days after injection.

NMDA current. We have previously reported that rat brain NMDA receptors expressed in *Xenopus* oocytes have properties similar to NMDA receptors found on neurons (24). Oocytes injected with five separate mRNA preparations responded reproducibly to applications of NMDA (130 of 165 tested) or other NMDA agonists. The mean inward current elicited at −60 mV by 100 μM NMDA was −54 ± 4 nA (N = 130) in the absence of Mg^{2+} and with 3 μM added glycine in the perfusion solution. Eighty-five of 87 oocytes injected with the best mRNA preparation responded to 100 μM NMDA with a mean inward current of −68 ± 2 nA (N = 85). Under these conditions, seven other mRNA preparations failed to induce any response to 100 μM NMDA (less than 5 nA) in 31 oocytes tested. Four of the seven negative mRNA preparations induced the expression of non-NMDA receptors, however. Similarly, uninjected or water-injected oocytes showed no NMDA responses at NMDA concentrations up to 300 μM (N = 21).

Further experiments were conducted to examine more closely the conductance mechanism activated by NMDA in oocytes. NMDA receptors are blocked in a voltage-dependent manner by Mg^{2+} in neurons (2, 3) and in oocytes (24). We used this unique characteristic of NMDA receptors to determine the extent to which other EAA agonists activate the NMDA conductance in oocytes. The currents evoked by EC_{50} concentrations of the agonists L-glutamate, L-aspartate, D-aspartate, and ibotenate were all blocked by Mg^{2+} in a voltage-dependent manner (Fig. 2). We quantified the extent of the voltage-dependent Mg^{2+} block by calculating the ratio of the chord conductances measured from the current-voltage plots at −60 and −30 mV in the presence and absence of Mg^{2+} (Table 1). A ratio of 1.0 indicates no voltage dependence whereas ratios less than 1.0 reflect a higher membrane conductance at the more depolarized (−30 mV) level. Notice that the conductance ratios in the presence of Mg^{2+} were similar (0.11–0.15) for NMDA, L-aspartate, D-aspartate, and ibotenate, but the mixed action of L-glutamate at NMDA and non-NMDA receptors was manifested by a higher ratio (0.22).

We have previously reported that the NMDA chord con-

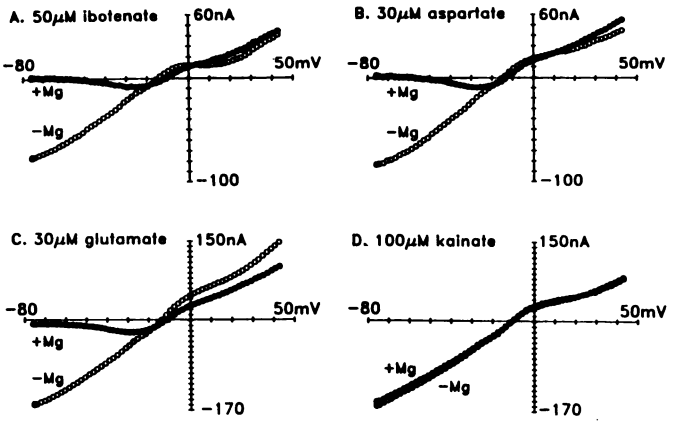

Fig. 2. Current-voltage relationships for ibotenate (A), L-aspartate (B), L-glutamate (C), and kainate (D) in the presence and absence of 1 mM Mg^{2+} in the same mRNA-injected oocyte. This cell had been injected with 50 ng of mRNA 5 days previously and had a resting membrane potential and input resistance of −65 mV and 1.1 MΩ, respectively. Mg^{2+} exerted a voltage-dependent block of currents induced by NMDA receptor agonists but not of those induced by kainate.

ductance in the depolarizing limb of the current-voltage curve was lower in the absence of Mg^{2+} than in its presence (24). This feature of the NMDA conductance, which was shared by L-aspartate and ibotenate (Fig. 2) as well as L-glutamate and D-aspartate, has also been seen in cultured mouse spinal cord neurons (38). To investigate the possibility that NMDA activates the Cl^- channel endogenous to the oocyte (39–41), 44 mM of the Cl^- in the perfusion solution was replaced with impermeant methylsulfate ions ($MeSO_4$). If NMDA opened only a Cl^- channel, a shift in the measured reversal potential of +16 mV would be expected. However, in six oocytes the reversal potential in normal chloride-containing MBS was −4 ± 2 mV whereas in $MeSO_4$ MBS it was − 3 ± 2 mV. These data suggest that the NMDA channel in oocytes is not appreciably permeable to Cl^-, although a small amount of activation of the endogenous Ca^{2+}-dependent Cl^- channel cannot be ruled out. Detailed characterization of the NMDA current-voltage relationship and the ionic selectivity of the oocyte NMDA channel is currently in progress.

In contrast to the voltage-dependent block of NMDA currents by Mg^{2+} (2, 3) and ketamine (6), the competitive antagonist D-APV has been shown to block NMDA receptors in a voltage-independent manner in neurons (6). This is also the case in oocytes (Fig. 3). D-APV did not significantly alter the measured reversal potential of the NMDA current (+1 ± 5 mV in 10 μM D-APV versus −2 ± 2 mV in control, $N = 5$), nor did it exert a voltage dependent block in the absence of Mg^{2+} ($G_{(-60)}/G_{(-30)} = 1.09 \pm 0.08$ with D-APV and 1.03 ± 0.02 without D-APV, $N = 5$). This indicates that the mechanism of D-APV block in oocytes is similar to that in neurons.

We have tested the effects of D-APV on the amplitudes of currents evoked by approximately EC_{50} concentrations of various NMDA receptor agonists. Examples of antagonism by D-APV of currents induced by L-aspartate, L-glutamate, NMDA, and ibotenate are shown in Fig. 4. D-APV also significantly reduced the current evoked by D-aspartate. The extent to which 10 μM D-APV blocked approximately EC_{50} concentrations of

TABLE 1
Summary of receptor properties

Receptor	Agonist	EC_{50} (95% confidence interval)[a]	Hill coefficient[a]	Reversal potential	Conductance Ratio[b]		Inhibition by 10 μM D-APV[c]
					−Mg	+Mg	
		μM		mV			%
NMDA	NMDA	31 (26–36) ($N = 18$)	1.33 ± 0.06[d] ($N = 18$)	−7 ± 1 ($N = 21$)	0.95 ± 0.02 ($N = 21$)	0.15 ± 0.04[d] ($N = 8$)	78 ± 4 ($N = 12$)
	L-Glutamate	2.3 (1.7–3.0) ($N = 3$)	1.60 ± 0.25 ($N = 3$)	−10 ± 4 ($N = 7$)	0.77 ± 0.26 ($N = 7$)	0.22 ± 0.02[d] ($N = 7$)	70 ± 4 ($N = 3$)
	L-Aspartate	13 (8.2–20) ($N = 4$)	0.95 ± 0.09 ($N = 4$)	−13 ± 2 ($N = 7$)	0.85 ± 0.04[d] ($N = 7$)	0.11 ± 0.03[d] ($N = 7$)	85 ± 4 ($N = 3$)
	D-Aspartate	10 (8.4–12) ($N = 4$)	1.28 ± 0.12 ($N = 4$)	−13 ± 3 ($N = 5$)	0.76 ± 0.06[d] ($N = 5$)	0.12 ± 0.02[d] ($N = 5$)	70, 77 ($N = 2$)
	Ibotenate	35 (22–58) ($N = 4$)	1.10 ± 0.19 ($N = 4$)	−16 ± 3 ($N = 3$)	0.84 ± 0.08 ($N = 3$)	0.12 ± 0.04[d] ($N = 3$)	89 ± 1 ($N = 3$)
Non-NMDA	Kainate	98 (76–125) ($N = 13$)	1.58 ± 0.13[d] ($N = 13$)	−8 ± 1 ($N = 31$)	0.93 ± 0.02[d] ($N = 16$)	0.90 ± 0.02[d] ($N = 17$)	3 ± 2 ($N = 6$)
	L-Glutamate	14 (8.4–23) ($N = 4$)	1.14 ± 0.05 ($N = 4$)	−12 ± 1 ($N = 4$)	ND[e]	0.96 ± 0.05 ($N = 4$)	0, 0 ($N = 2$)
	Domoate	14 (13–15) ($N = 4$)	1.47 ± 0.07[f] ($N = 4$)	−7 ± 1 ($N = 4$)	ND	1.02 ± 0.04 ($N = 4$)	ND
	AMPA	ND	ND	−7 ± 4 ($N = 9$)	0.75 ± 0.06[d] ($N = 9$)	ND	6 ± 6 ($N = 3$)
Oscillating	Quisqualate	NF[g]	NF	−5 ± 2 ($N = 13$)	0.65 ± 0.04[d] ($N = 13$)	0.48 ± 0.07[d] ($N = 4$)	ND

[a] Values were determined by the least squares fit of the concentration-response curves to Eq. 2. The 95% confidence intervals were calculated as the log of the EC_{50} values and appear in parentheses next to the EC_{50} values.
[b] Chord conductances were calculated according to Eq. 1 at −60 and −30 mV and the ratio $G_{(-60)}/G_{(-30)}$ is reported. Agonist currents with no voltage dependence over this range of membrane potentials will have ratios near 1.0 whereas ratios lower than 1.0 indicate some block at more negative membrane potentials. Ratios slightly lower than 1 for some NMDA agonists in the absence of Mg^{2+} may have been caused by residual Mg^{2+} (measured by atomic absorbtion spectroscopy to be ≤1 μM) or by some activation of the voltage-dependent Cl^- conductance (see values for quisqualate).
[c] Values are the percentage of agonist current blocked by 10 μM D-APV. The agonists were tested at approximately EC_{50} concentrations.
[d] Significantly different from 1.0 ($p < 0.001$).
[e] ND, not determined.
[f] Significantly different from 1.0 ($p < 0.01$).
[g] NF, the data did not fit well to Eq. 2 and no parameters were estimated.

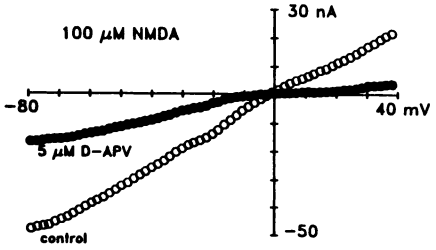

Fig. 3. Current-voltage relationships for 100 μM NMDA in the presence and absence of 5 μM D-APV in an oocyte that had been injected with 75 ng of mRNA 3 days previously. The experiment was conducted in Mg^{2+}-free MBS containing 3 μM added glycine. Notice the reduction by D-APV of the NMDA-induced current at all voltages tested with no change in the reversal potential or the voltage dependence of the response. The membrane potential was −51 mV and the input resistance was 1.0 MΩ.

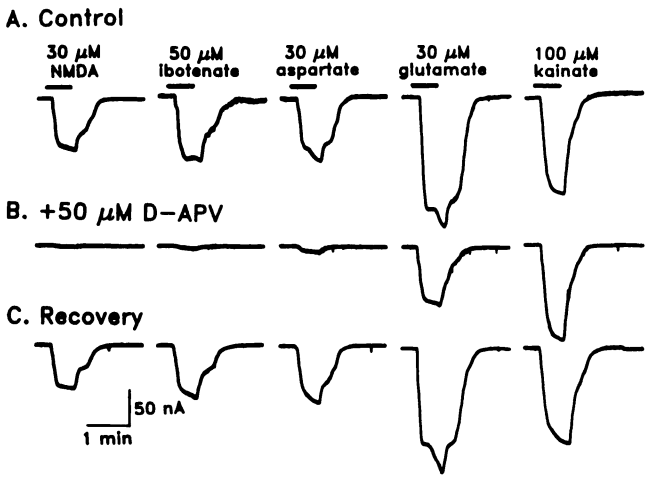

Fig. 4. The effect of 50 μM D-APV on NMDA and non-NMDA responses in a single oocyte injected with 50 ng of rat brain mRNA 5 days previously. A, Inward current responses to the application of NMDA, ibotenate, L-aspartate, L-glutamate, and kainate at a holding potential of −60 mV. The drugs were applied during the times denoted by the *bars*. B, Applications of the same agonists in the presence of 50 μM D-APV. C, Recovery of the responses 10 min after washout of D-APV. This cell had a resting membrane potential of −65 mV and an input resistance of 1.1 MΩ.

the various agonists is shown in Table 1. D-APV exerts a competitive block of NMDA receptors in oocytes with 100 μM D-APV producing a 100-fold parallel shift to the right of the NMDA concentration-response curve (42).

Full concentration-response curves were constructed for NMDA, L-glutamate, L-aspartate, D-aspartate, and ibotenate. The averages of these curves are shown in Fig. 5. The fit of the data to the logistic equation was good. With the exception of NMDA, the Hill coefficients were somewhat variable among the population of oocytes tested but were not significantly different from 1.0 (Table 1). L-glutamate was the most potent of the agonists when tested under conditions favoring NMDA activation (no Mg^{2+} and 3 μM added glycine) followed by D-aspartate, L-aspartate, NMDA, and ibotenate.

Non-NMDA smooth current. Smooth inward currents activated by kainate, domoate, L-glutamate, and AMPA were observed regularly in oocytes injected with 16 separate mRNA preparations. Fifteen other mRNA preparations were tested that induced no detectable response to kainate at concentrations up to 1 mM (number of oocytes = 77). Uninjected or water-injected control oocytes also showed no kainate response

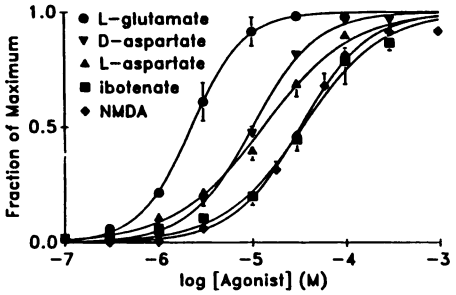

Fig. 5. Concentration-response relationships for agonists that activate the NMDA receptor in mRNA-injected oocytes. The holding potential was −60 mV. All curves were constructed under conditions that favor the activation of NMDA receptors (no Mg^{2+} and 3 μM glycine). The *points* represent the means of 3 to 18 separate oocytes. The *curves* are drawn from the fits of the data to the logistic equation. The maximum currents ranged from 20 to 247 nA.

at up to 3 mM kainate (n = 57). Of the oocytes injected with positive mRNA, 260 out of 323 responded to application of 100 μM kainate with a mean current at −60 mV of −63 ± 4 nA. Oocytes injected with the best mRNA preparation invariably (34 out of 34 tested) responded to 100 μM kainate with a mean current of −120 ± 12.5 nA.

This smooth inward current maintained the same level throughout long drug applications (tested up to 1 min in the case of kainate and AMPA), suggesting that this current does not readily desensitize. The reversal potential of the current was −5 to −10 mV (Table 1) and the kainate reversal potential was not changed when 44 mM of the Cl^- was replaced with $MeSO_4$ (V_{rev} = −9 ± 1 mV in $MeSO_4$ versus −8 ± 1 mV in control, N = 13). These data suggest that kainate opens a nonselective cation channel in the oocytes rather than the endogenous Cl^- channel.

This current was different from the NMDA response in a number of ways. Kainate and AMPA responses were insensitive to D-APV at 10 or 50 μM (Fig. 4; Table 1), as were currents evoked by L-glutamate under conditions in which NMDA receptor activation was suppressed (1 mM Mg^{2+}, no added glycine). In further contrast to currents activated by NMDA agonists, Mg^{2+} did not block the kainate current nor did it affect the kainate chord conductance in the depolarizing limb of the current-voltage curve (Fig. 2). Additionally glycine appears to be required for NMDA receptor activation in oocytes (43), but robust currents were evoked by kainate, L-glutamate, AMPA, and domoate in the absence of glycine.

Full concentration-response curves for three agonists that activate this current are shown in Fig. 6 and the EC_{50} values are reported in Table 1. L-Glutamate was more potent than kainate under conditions in which most of the NMDA responses were blocked (1 mM Mg^{2+} and no added glycine). Domoate was equipotent with L-glutamate. Kainate concentrations of 1 μM or lower elicited less than 0.5% of the maximum kainate current in 13 oocytes injected with positive mRNA. All three curves fit well with the logistic equation. The estimated Hill coefficients were greater than one for kainate and domoate whereas L-glutamate had a Hill coefficient close to one. Note that in these conditions L-glutamate was 6-fold less potent than when it was tested under conditions that favor NMDA receptor activation.

At a concentration of 50 μM, AMPA induced small inward currents (mean amplitude at −60 mV = −10 ± 2 nA; N = 12)

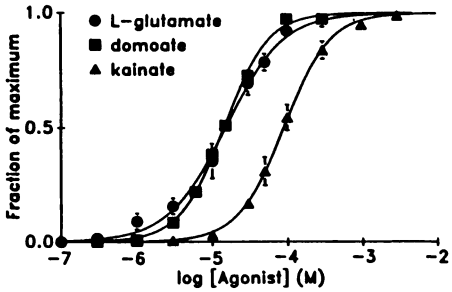

Fig. 6. Concentration-response relationships for agonists that activate the non-NMDA smooth current in mRNA-injected oocytes. These experiments were conducted at −60 mV under conditions in which most of the NMDA responses are blocked (1 mM Mg^{2+} and no added glycine). The *points* for kainate are the average of 13 cells whereas those for L-glutamate and domoate are the means of four oocytes. Maximum currents ranged from 27 to 508 nA.

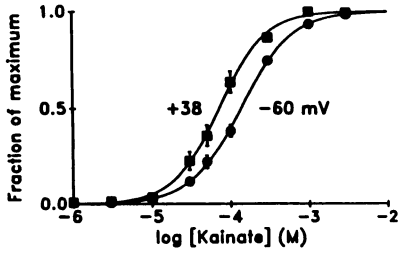

Fig. 7. Voltage dependence of kainate potency. Kainate concentration-response curves were constructed while the command voltage was continuously stepped from −60 +38 mV. At −60 mV the amplitude of the kainate-induced inward current was measured whereas at +38 mV the current was outward. The *points* on these curves represent the mean ± standard error of four cells. The kainate potency was voltage dependent, but the slope and the maximum conductance were not.

in mRNA-injected oocytes. In uninjected oocytes 50 μM AMPA produced no detectable current ($N = 5$), but 200 μM produced an average inward current at −60 mV of −12 ± 3 nA ($N = 3$). It appears that at high concentrations AMPA has nonspecific actions in oocytes, therefore concentration-response curves were not generated for this agonist.

The potency of kainate depended on the membrane potential of the oocyte. Complete concentration response curves were constructed as the membrane potential was stepped repeatedly between −60 and +38 mV. The EC_{50} for kainate was 72 (43–123) μM at +38 mV and 138 (100–190) μM at −60 mV (Fig. 7). This difference was significant ($p < 0.05$, two-tailed paired t test; $N = 4$). The maximum kainate conductance at −60 and +38 mV was the same in these four cells as estimated from the measured current and the mean kainate reversal potential obtained in other cells. Likewise, membrane potential affected only the EC_{50} and not the Hill coefficient of the logistic equation (Eq. 2).

Quisqualate. Quisqualate elicited an inward current at −60 mV in mRNA-injected oocytes. This current, which was also activated occasionally by 10–30 μM L-glutamate or by 30–50 μM ibotenate, could not be readily attributed to an action at known EAA receptor/ion channel complexes. In some cells 10–30 μM quisqualate produced oscillatory inward currents superimposed on a smooth inward component (see Fig. 8A), whereas in others only the smooth current was seen. The magnitude of the response was variable, with rapid and often complete desensitization after the first application of 10–30 μM quisqualate. Eight mRNA preparations encoded receptors for this quisqualate response; 69 of 102 oocytes injected with these mRNAs and tested with 30 μM quisqualate responded with an average peak current at −60 mV of −22 ± 7 nA. Oocytes ($N = 49$) injected with six other mRNA preparations had no detectable response to 30 μM quisqualate and in 32 uninjected or water-injected control oocytes 30–50 μM quisqualate produced no current.

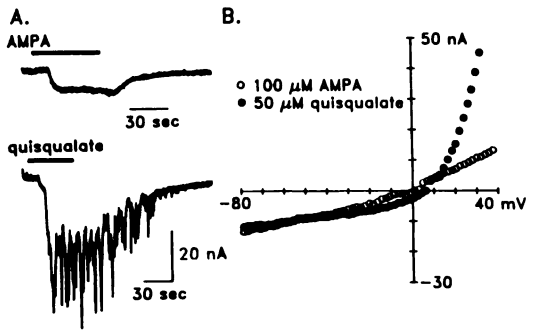

Fig. 8. Quisqualate and AMPA produce different types of currents in mRNA-injected oocytes. A, Examples of currents induced by 100 μM AMPA (*top*) and 50 μM quisqualate (*bottom*) in an mRNA-injected oocyte at a holding potential of −60 mV. AMPA was applied first during the time indicated by the *bar*, followed by quisqualate 5 minutes later. This cell had a membrane potential of −52 mV and an input resistance of 0.5 MΩ and had been injected with 50 ng of mRNA 2 days before the experiment. B, Current-voltage relationships for smooth currents activated by 100 μM AMPA and 50 μM quisqualate in a different oocyte. This oocyte had been injected with 75 ng of mRNA 3 days previously and had a membrane potential of −36 mV and an input resistance of 0.4 MΩ. In this cell the response to quisqualate had no obvious oscillations. Note the differences between the two curves, particularly at depolarized membrane potentials.

The oscillatory component of the quisqualate current was similar in appearance to the currents produced by the activation of the endogenous muscarinic acetylcholine receptor (40) or the serotonin receptor in mRNA-injected oocytes (21). The muscarinic response (39, 40) and the quisqualate current (19) are due to a Ca^{2+}-dependent Cl^- conductance endogenous to the oocyte (41) and perhaps mediated by receptor-coupled production of inositol trisphosphate and subsequent mobilization of cytoplasmic Ca^{2+}. Indeed, the reversal potential of smooth (four cells) and oscillating currents (two cells) evoked by quisqualate was shifted from −5 ± 2 mV to +11 ± 3 mV ($N = 6$) when 44 mM of the Cl^- was replaced with $MeSO_4$. The expected shift for a Cl^- channel under these conditions is +16 mV, indicating that virtually all of the quisqualate current, whether smooth or oscillatory in appearance, is carried by Cl^-. The quisqualate current also showed large outward rectification at depolarized membrane potentials (Fig. 8B), which is characteristic of the Ca^{2+}-activated Cl^- channel in native oocytes (41). The mean ratio of the quisqualate chord conductances measured at +20 mV and −70 mV was 4.2 ± 0.9 ($N = 13$). Although the Ca^{2+} dependence of this current is well known (39, 41), we found that omission of EGTA from the recording electrodes had no effect on the amplitude or desensitization of the quisqualate response.

AMPA, a quisqualate analogue that binds potently to the quisqualate receptor in neuronal membranes (9, 44), was ineffective at activating the Cl^- current in any mRNA-injected oocytes. Current oscillations were never observed in response to 10–200 μM AMPA, even in cells that responded to quisqualate with oscillations (Fig. 8A). Moreover, the AMPA current-voltage relationship was linear ($G_{(+20)}/G_{(-70)} = 1.1 \pm 0.3$, $N = 8$) even in cells that showed strongly rectifying smooth quis-

qualate currents (Fig. 8B). In further contrast to the quisqualate response, the AMPA reversal potential was not affected by the replacement of 44 mM Cl^- with $MeSO_4$ (−12 ± 5 mV under control conditions versus −15 ± 6 mV in $MeSO_4$, N = 4). Therefore, even at high concentrations (200 μM), AMPA did not activate the quisqualate-induced Cl^- conductance.

Although quisqualate seemed to be quite potent at activating the Cl^- current, the large degree of desensitization likely obscured its true potency. Concentration-response curves for quisqualate were bell-shaped (Fig. 9), probably due to rapid desensitization at higher concentrations. It was not possible to fit the data to the logistic equation so EC_{50} values were not determined. The threshold concentration of quisqualate was 0.1–0.3 μM whereas maximum responses were elicited by 3–10 μM quisqualate.

Interactions between agonists. Different agonists were applied in combination to determine whether evoked currents were strictly additive. AMPA and quisqualate significantly reduced the response to 100 μM kainate (Fig. 10). The current induced by 100 μM kainate was reduced by 70 ± 5% by 50 μM AMPA (N = 3), whereas responses to 30 μM NMDA were unaffected by AMPA (N = 3). Quisqualate at 50 μM was even more effective at blocking kainate currents, reducing the response to 100 μM kainate by 97 ± 1% (N = 3). Thus, in accord with other reports (12, 17, 45), significant interactions occur between the currents induced by kainate and agonists thought to be specific for quisqualate receptors.

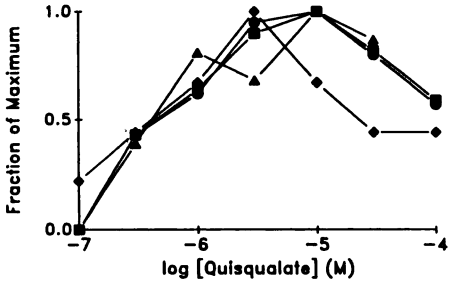

Fig. 9. Fractional responses to quisqualate plotted as a function of quisqualate concentration. Each different *symbol* represents a separate mRNA-injected oocyte that was exposed to sequentially increasing concentrations of quisqualate. These data could not be fit to the logistic equation because of the decreased response at higher quisqualate concentrations. The maximum current ranged from 18 to 52 nA.

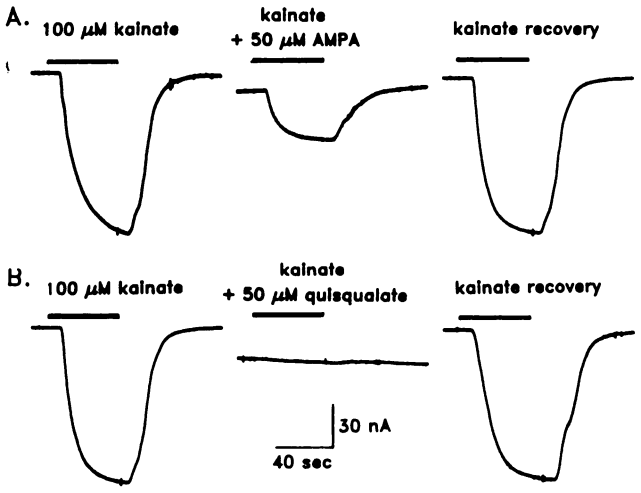

Fig. 10. Block of kainate currents by AMPA and quisqualate in mRNA-injected oocytes. A, Kainate (100 μM) was applied during the time indicated by the *bar* before, during, and after perfusion with 50 μM AMPA. The difference in the baseline current represents the magnitude of the current caused by AMPA itself. B, Responses to 100 μM kainate before, during, and after perfusion with 50 μM quisqualate. Here again the lower baseline of the quisqualate trace indicates the magnitude of the quisqualate-induced current in this particular oocyte. The holding potential for both A and B was −60 mV.

Receptor density and agonist EC_{50} values. In some systems the potency of a drug increases as receptor density increases, as predicted by certain formulations of occupancy theory (46). However, in mRNA-injected oocytes there was no consistent correlation between the maximum current and the EC_{50} for any of the agonists tested. The correlation coefficients ranged from −0.64 to 0.77 for the eight agonist responses tested. The number of open receptor/channel complexes per cell can be estimated from the maximum agonist-induced current and the single channel conductances. The main conductance state estimated for the NMDA-activated channel in neurons is 50 pS, whereas for kainate-activated channels this estimate is 5 pS (15, 16, 47, 48). The maximum NMDA currents ranged from 20 to 247 nA in different oocytes, which corresponds to 10,000 to 100,000 open channels per cell, whereas non-NMDA currents were derived from approximately 100,000 to 2,000,000 open channels per cell. The actual number of functional receptor/channel complexes is likely to be much higher depending on the maximum open channel probability at high agonist concentrations.

Discussion

The oocyte as a pharmacological tool. These results show that the oocyte is a useful tool for quantitative pharmacological investigation of neuronal EAA receptors. The ease of impalement and hardiness of this cell allow prolonged experiments to be performed on a single oocyte. The effects of uptake and diffusion on the concentrations of drugs at the receptors are minimized and agonist potency is reproducible from cell to cell. We cannot, however, rule out the possibility that agonist currents may show some desensitization with our relatively slow method of applying drugs. In properly voltage-clamped oocytes the magnitude of agonist-induced currents should be linearly proportional to the number of activated receptors, assuming that the EAA receptors are directly coupled to ion channels. Thus, spare receptors resulting from tissue-dependent response amplification mechanisms may not be present for EAA receptors in this preparation. We have used these useful features to produce full concentration-response curves for a variety of EAA agonists. The determination of antagonist potencies in oocytes is in progress (42) and should produce additional information concerning the properties of neuronal EAA receptors and drugs that interact with them. The possibility exists that the pharmacological properties of receptors expressed in oocytes may be altered by different post-translational processing of the receptor proteins, the lipid environment of the oocyte membrane, or oocyte-specific proteins. Therefore, wherever possible we have attempted to draw comparisons between the properties of neuronal and oocyte EAA receptors in order to determine the relevance of data obtained in oocytes. No qualitative or quantitative differences between EAA receptors in neurons and oocytes have emerged yet, although more subtle tests than those used here may well reveal receptor properties unique to oocytes.

The usefulness of the oocyte system also extends to structural

studies. The structural basis of the different EAA receptors has become an important issue with the finding that similar channels with multiple conductances are activated by NMDA, quisqualate, and kainate (15, 16). We have identified mRNA preparations that encode receptors mediating the non-NMDA smooth current in the absence of any detectable NMDA receptors. The lack of response to NMDA was not due to inability of the oocyte to synthesize NMDA receptors inasmuch as the same batch of oocytes could respond to NMDA when injected with other mRNAs. This indicates that the distinct properties of EAA receptors are not likely due to different post-translational processing of the same mRNA product by endogenous oocyte enzymes and suggests that the mRNAs encoding these two receptors may not be identical. The possibility cannot be ruled out, however, that mRNA preparations that do not produce functional NMDA receptors may be deficient in an mRNA encoding specific post-translational processing enzymes. More complete structural information awaits the isolation and characterization of cDNA clones for the EAA receptors. The oocyte system is uniquely useful for the demonstration of functionality of cloned receptors coupled to ion channels and has been an important tool in the cloning of a number of such proteins (25, 27–29). The data presented here show that the oocyte system is appropriate for the screening and identification of clones encoding the EAA receptors as well.

NMDA receptor. NMDA receptors expressed in oocytes retained the properties of their neuronal counterparts. The currents induced by NMDA in oocytes were blocked in a voltage-dependent manner by Mg^{2+} and potentiated by glycine in a dose-dependent fashion (24, 43). Others have shown that currents mediated by NMDA receptors in oocytes are also reduced by Zn^{2+} and a number of uncompetitive NMDA channel blockers (49). D-APV exerted a competitive block in oocytes (42) that was independent of voltage in both neurons (6) and oocytes, indicating that this antagonist has a similar mechanism of action in both cell types. The reversal potential of the NMDA current in oocytes is similar to those reported in neuronal preparations (47, 50), although a detailed study is needed to compare the ionic selectivity of the open NMDA channel in neurons and oocytes.

L-Glutamate, D-aspartate, L-aspartate, and ibotenate were also found to activate NMDA receptors. At concentrations of 30–50 μM or less, most of the current induced by these agonists is mediated by NMDA receptor activation as judged by the degree of Mg^{2+} block at −60 mV, by the degree of block produced by 10 μM D-APV, and by their requirement for glycine. However, at higher concentrations it is clear that L-glutamate activates a non-NMDA receptor as well (see below), and ibotenate and L-glutamate induced the oscillating Cl^- current in some cells. L-Glutamate is known to be a mixed agonist in neurons (51). In contrast, Schild analysis of the antagonism of currents produced by NMDA and L-aspartate provide strong evidence for the selectivity of these compounds for NMDA receptors at agonist concentrations up to 15 mM (42).

The relative potencies of agonists in oocytes under conditions that favor NMDA receptor activation (L-glutamate > D-aspartate > L-aspartate > ibotenate = NMDA) compare favorably with the relative IC_{50} values of these compounds for inhibiting specific binding of various radioligands to NMDA receptors in neuronal membranes (9, 52–54). The absolute potencies of these agonists in oocytes are approximately 10-fold lower than those found in binding assays but consistent with the concentrations necessary to activate NMDA receptors in isolated neurons or membrane patches (12–17, 47).

Non-NMDA receptor. Currents activated by kainate, AMPA, domoate, and L-glutamate (in the presence of 1 mM Mg^{2+} and the absence of added glycine) are not mediated by NMDA receptors because they are not blocked by D-APV or Mg^{2+} (Figs. 2 and 4; Table 1) and not potentiated by glycine.[2] Domoate and L-glutamate are the most potent agonists for this current, which is likely caused by the opening of a nonselective cation channel.

The pharmacological properties of this agonist current are not indicative of the high affinity kainate binding site found in neuronal membranes. Binding studies on neuronal membranes (7) or brain tissue sections with autoradiography (8, 9) have largely confirmed the existence of separate kainate and quisqualate binding sites. However, these compounds show significant cross-reactivity, and there are inconsistencies between binding and physiological studies.

Two lines of evidence suggest that the receptor mediating the non-NMDA smooth current in mRNA-injected oocytes does not correspond to the nanomolar kainate binding site. First, in oocytes as in neurons the potencies of the agonists for this receptor are 1000-fold lower than those predicted from binding studies. The affinity of kainate for binding sites labeled by [^{3}H]kainate or L-[^{3}H]glutamate is 6–80 nM in neuronal membranes (7, 9), which is close to the concentration (50 nM) required to cause spontaneous interictal bursts in hippocampal slices (11). In most cases (but see Ref. 10), however, much higher concentrations (at least 1 μM) are necessary before kainate-activated membrane currents can be detected (12, 15–17, 48). Indeed, the effective concentrations of kainate are much closer to the affinity of kainate for the quisqualate binding site than for the high affinity kainate binding site (7, 55). Second, the relative potencies of the non-NMDA agonists (L-glutamate = domoate > kainate) do not match those of the high affinity kainate binding site (domoate > kainate > L-glutamate) (7). Rather, the ratio of EC_{50} values for L-glutamate and kainate in oocytes (0.14) is very similar to their inhibitory potency ratios versus [^{3}H]AMPA binding (0.09–0.16) (41, 56), whereas at the nanomolar [^{3}H]kainate binding site this ratio ranges from 10 to 72 (7). Selective antagonists that would positively identify the receptor(s) mediating kainate and quisqualate currents are not yet available. One attractive explanation for discrepancies between binding and physiological studies is that the kainate-activated currents in neuronal and oocyte membranes may be caused by activation of the quisqualate receptor as defined by binding studies.

Further supporting this conclusion is the observation that AMPA and quisqualate inhibit kainate-induced currents in oocytes, indicating that these agonists interact with the same receptor that kainate does. These results, which are also seen in neuronal preparations (12, 17, 47), raise the following question: if kainate currents are due to the activation of the quisqualate receptor why are AMPA and quisqualate such poor agonists? The current induced by AMPA, although much smaller, is very similar to the kainate current. It is not blocked by Mg^{2+} or D-APV and the AMPA channel is not appreciably permeable to Cl^-. Thus, AMPA may be a partial agonist at this

[2] N. W. Kleckner and R. Dingledine, unpublished observation.

receptor in oocytes. In contrast, to NMDA, kainate, and AMPA, most of the quisqualate current in oocytes is due to the opening of Cl^- channels, and therefore quisqualate does not appear to be an agonist at either NMDA or non-NMDA receptors. The quisqualate response mediated by conventional EAA receptors in oocytes may desensitize rapidly enough to preclude detection with our relatively slow method of applying agonists.

Interestingly, kainate is 3 to 5 times more potent at activating inward currents in oocytes than in isolated neurons (13, 14). Also interesting is the observation that the potency of kainate is dependent on the oocyte membrane potential. Kainate is more potent at positive than at negative voltages, which might be explained by an influence of membrane potential on the confirmation of the receptor molecule.

Quisqualate. The properties of the quisqualate-activated chloride conductance in mRNA-injected oocytes are unlike those of conventional quisqualate receptors. The receptor mediating this current is coupled to a conductance mechanism endogenous to the oocyte (40, 41) that increases production of inositol trisphosphate and subsequent mobilization of intracellular Ca^{2+} (19). This receptor is not likely to correspond to [^{3}H] AMPA binding sites in neuronal membranes (9, 56) because 30–200 μM AMPA does not activate the Cl^- conductance. Interestingly, quisqualate and ibotenate increase inositol triphosphate turnover in cerebellar slices from young rats (18). This effect does not appear to be mimicked by AMPA in hippocampal slices.[3] Thus it is likely that quisqualate is acting at a novel site which may correspond to a new neuronal EAA receptor (19).

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Send reprint requests to: Raymond Dingledine, Department of Pharmacology, CB 7365, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.