Functional expression from cloned cDNAs of glutamate receptor species responsive to kainate and quisqualate

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The complete amino acid sequences of two mouse glutamate receptor subunits (GluR1 and GluR2) have been deduced by cloning and sequencing the cDNAs. *Xenopus* oocytes injected with mRNA derived from the GluR1 cDNA exhibit current responses both to kainate and to quisqualate as well as to glutamate, whereas oocytes injected with mRNA derived from the GluR2 cDNA show little response. Injection of oocytes with both the mRNAs produces current responses larger than those induced by the GluR1-specific mRNA and the dose-response relations indicate a positively cooperative interaction between the two subunits. These results suggest that kainate and quisqualate can activate a common glutamate receptor subtype and that glutamate-gated ionic channels are hetero-oligomers of different subunits.

Glutamate receptor; Kainate receptor; Quisqualate receptor; cDNA cloning; cDNA expression; Hetero-oligomer

1. INTRODUCTION

The glutamate receptor mediates excitatory transmission at many synapses in the mammalian central nervous system and is involved in plastic and pathological processes [1-3]. Pharmacological and electrophysiological studies have suggested that there are at least five classes of glutamate receptor [1]. The receptors for *N*-methyl-D-aspartate (NMDA), quisqualate, kainate and L-2-amino-4-phosphonobutyrate (AP4) are subtypes of glutamate-gated ionic channels, whereas the receptor for *trans*-1-amino-cyclopentyl-1,3-dicarboxylate (ACPD) is a G protein-coupled glutamate receptor. However, there has been no direct evidence for the presence of molecularly distinct glutamate receptor subtypes. Recently, Hollmann et al. [4] have reported the cloning, sequencing and expression of cDNA encoding a kainate subtype of the glutamate receptor from rat brain. The isolation and sequencing of cDNAs encoding kainate binding proteins from chicken [5] and frog [6] have also been described. In the present investigation, we have cloned and sequenced two cDNA species encoding glutamate receptor subunits from mouse cerebellum and have analyzed the functional properties of these subunits produced in *Xenopus* oocytes by expression of the cloned cDNAs.

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Abbreviations: NMDA, *N*-methyl-D-aspartate; AP4, L-2-amino-4-phosphonobutyrate; ACPD, *trans*-1-amino-cyclopentyl-1,3-dicarboxylate; PCR, polymerase chain reaction; γ-DGG, γ-D-glutamylglycine; GAMS, γ-D-glutamylaminomethylsulphonate; GDEE, glutamate diethylester; APV, D-(-)-2-amino-5-phosphonovaleric acid

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing of cDNAs*

Total RNA was extracted [7] from ICR mouse cerebella, and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography [8]. An oligo(dT)-primed cDNA library was constructed in phage λgt10 using poly(A)$^+$ RNA as template. Double-stranded cDNA prepared using the cDNA synthesis system (Bethesda Research Laboratories) was blunted by T4 DNA polymerase, methylated by *Eco*RI methylase, ligated with an *Eco*RI linker and cleaved by *Eco*RI. Size-selected fragments longer than ~1 kb pair, prepared by electrophoresis on 1.5% agarose gel, were ligated with λgt10. The screening of the cDNA library was effected by hybridization at 42°C in the presence of 30% formamide with a rat kainate receptor cDNA probe prepared as follows. Polymerase chain reaction (PCR) amplification was carried out using cDNA synthesized from rat cerebral poly(A)$^+$ RNA and synthetic oligodeoxyribonucleotides corresponding to nucleotide residues 1177-1201 and 2083-2107 of the rat GluR-K1 cDNA [4]. The 0.74-kb *Pst*I fragment from the PCR-amplified product was used as a probe. 21 positive clones randomly chosen were classified into two groups by restriction mapping and partial sequence analysis. cDNA inserts from representative clones of each class, carrying the entire protein-coding sequences of the glutamate receptor subunits GluR1 and GluR2, were subcloned into pBluescript SK(-) (Stratagene) to yield the plasmids pKCR30 and pKCR24, respectively. Nested deletions were made [9] and DNA sequencing was carried out on both strands by the dideoxy chain termination method [10]. Gaps and ambiguities were resolved by the use of appropriate synthetic primers. Oligodeoxyribonucleotides were prepared with an automatic DNA synthesizer (Applied Biosystems).

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2.2. *Expression of cDNAs*

The 3.2-kb *Eco*RI fragment from pKCR30 was cloned into the *Eco*RI site of pSP65 [11] to yield the plasmid pSPGR1, which carried the GluR1 cDNA in the same orientation as the SP6 promoter. The 3.2-kb *Sal*I-*Xba*I fragment from pKCR24 was ligated with the 3.0-kb *Xba*I-*Sal*I fragment from pSP64AX [12] to yield the plasmid pSPGR2. GluR1-specific and GluR2-specific mRNAs were synthesized in vitro [11] using *Hin*dIII-cleaved pSPGR1 and *Xho*I-cleaved pSPGR2, respectively, as templates. Transcription was primed with the cap dinucleotide G(5′)ppp(5′)G (0.5 mM) [13]. *Xenopus laevis* oocytes were injected with the GluR1-specific and/or the GluR2-specific mRNA; the concentration of the respective mRNAs was 0.1 or 0.2 $\mu g/\mu l$ and the average volume injected was ~50 nl per oocyte. The injected oocytes were incubated at 20°C for 3 days in modified Barth's medium [14] containing gentamycin (0.1 mg/ml). The follicular cell layer was removed [15] and whole-cell currents were recorded at −70 mV membrane potential and ~19°C with a conventional two-micropipette voltage clamp; the two pipettes were filled with 3 M KCl. The chamber was continuously perfused with normal frog Ringer's solution composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM Hepes-NaOH (pH 7.2). Glutamate receptor agonists were bath-applied for ~15 s; the NMDA solution applied contained 6 μM glycine.

3. RESULTS AND DISCUSSION

A cDNA library derived from mouse cerebellar poly(A)$^+$ RNA was constructed with the bacteriophage vector λgt10 and was screened by hybridization with a DNA fragment comprising part of the rat kainate receptor GluR-K1 cDNA [4]. Two classes of cDNA clones were isolated and sequence analysis of representative clones of each class, GluR1 and GluR2, revealed open reading frames that encode sequences of 907 and 883 amino acids, respectively (Figs. 1 and 2). The translational initiation site was assigned to the first ATG triplet that appears downstream of a nonsense codon found in-frame. Both the proteins contain a putative amino-terminal signal peptide, whose cleavage site was predicted by the method of von Heijne [16]. The proposed mature GluR1 and GluR2 proteins are composed of 889 and 862 amino acid residues with calculated M_r of 99,645 and 96,207, respectively. Fig. 3 shows the alignment of the deduced amino acid sequences of the GluR1 and GluR2 proteins, which exhibit 71% sequence identity with each other. As compared with the rat kainate receptor GluR-K1 [4] and the chick [5] and frog [6] kainate binding proteins, the GluR1 protein shows 99%, 37% and 38% sequence identity, respectively, and the GluR2 protein 71%, 39% and 39% sequence identity, respectively.

Analysis of the deduced amino acid sequences for local hydropathicity [18] suggests the presence of four putative transmembrane segments (M1–M4) in the GluR1 and GluR2 proteins. The hydrophobicity of segments M1, M3 and M4 is high enough to allow unambiguous assignment. Comparison of the hydropathicity profiles of GluR1 and GluR2 with those of the rat kainate receptor [4], the chick and frog kainate binding proteins [5,6] and the subunits of other neurotransmitter-gated ionic channels [19–21] leads to the assignment of segment M2, although its hydrophobicity is not very high. The structural characteristics observed suggest that the GluR1 and GluR2 proteins have the same transmembrane topology, with the amino-terminal region assigned to the extracellular side of the membrane, as proposed for the rat kainate receptor [4], the kainate binding proteins [5,6] and the subunits of neurotransmitter-gated ionic channels [19–21]; note that a region different from segment M2 was also proposed as a putative transmembrane segment of the rat kainate receptor [4]. This model is consistent with all the potential *N*-glycosylation sites [22] found in GluR1 and GluR2 (Fig. 3, marked with asterisks) being located on the extracellular side of the membrane. The region preceding segment M1 of GluR1 and GluR2 contains clusters of amino acid residues conserved in the rat kainate receptor and the kainate binding proteins (Fig. 3, underlined). These sites might be involved in ligand binding. Segment M2 of GluR1 and GluR2 is surrounded by negatively charged amino acid residues (residues 562, 566 and 586 of GluR1 and residues 566, 570 and 590 of GluR2). Interestingly, these residues are located at positions nearly equivalent to those of the three rings of negatively charged and glutamine residues that surround segment M2 of the nicotinic acetylcholine receptor subunits [23]. The three anionic rings, of which two are located near the cytoplasmic and extracellular mouths of the channel and the other is located between the two, forming a narrow channel constriction, have been identified as major determinants of the rate of ion transport through the acetylcholine receptor channel [23].

mRNAs specific for the GluR1 and GluR2 proteins were synthesized by transcription in vitro of the cloned cDNAs and were injected singly or together into *Xenopus* oocytes. After incubation for 3 days, the injected oocytes were tested for current responses to glutamate receptor agonists. Almost all oocytes injected with the GluR1-specific mRNA responded to 30 μM L-glutamate (Fig. 4A and Table I). This indicates that the GluR1 protein is a glutamate receptor subunit. As expected from the 99% sequence identity observed between GluR1 and the rat kainate receptor GluR-K1 [4], all injected oocytes responded to 100 μM kainate. Unexpectedly, however, 1 μM quisqualate also evoked a clear response in almost all oocytes. Three out of the 30 oocytes tested exhibited a small response to 100 μM L-aspartate. No detectable response was observed with 100 μM NMDA. None of 30 noninjected control oocytes responded to these glutamate receptor agonists.

The responsiveness of GluR1 both to kainate and to quisqualate is in contrast with the reported selectivity of the rat kainate receptor GluR-K1 for kainate [4]. Kainate evoked larger current responses than quisqualate, but quisqualate showed much higher apparent affinity than kainate as indicated by the effector concentrations required for half-maximal response (EC_{50}).

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CCTGAAG-----3'                                                                                                  2815
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Fig. 1. Nucleotide sequence of cloned cDNA encoding the mouse GluR1 protein and the deduced amino acid sequence. Nucleotide residues are numbered in the 5'- to 3'-direction, beginning with the first residue of the codon specifying the amino-terminal residue of the mature subunit and the preceding residues are indicated by negative numbers. Amino acid residues are numbered beginning with the amino-terminal residue of the mature subunit and the preceding residues are indicated by negative numbers. Numbers of the nucleotide and amino acid residues at the right-hand end of the individual lines are given. In the cDNA sequence, nucleotide 2815 is followed by a stretch of 11 dA residues, which may not represent the poly(A) tail because no conventional polyadenylation signal is found.

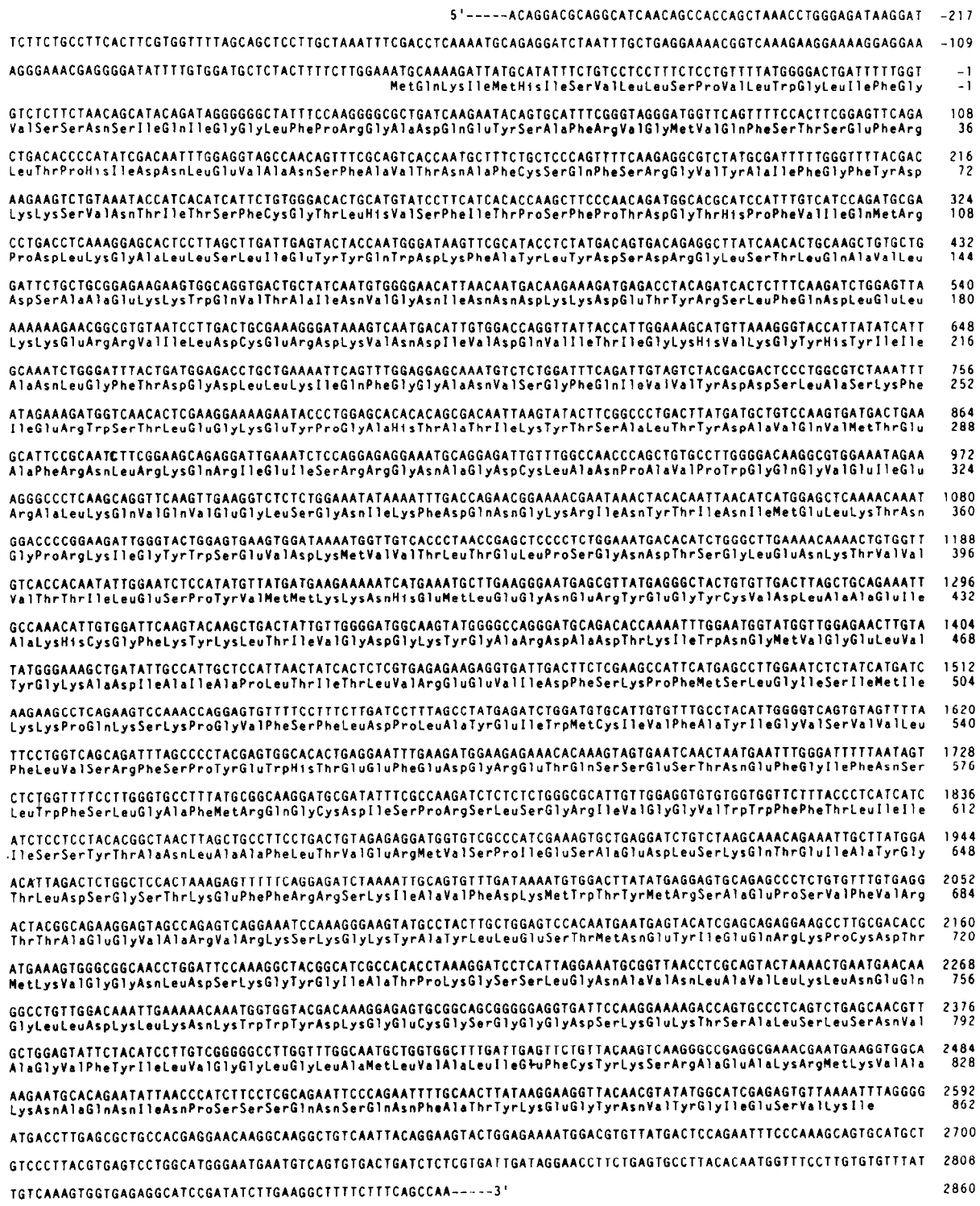

Fig. 2. Nucleotide sequence of cloned cDNA encoding the mouse GluR2 protein and the deduced amino acid sequence. For numbering of nucleotide and amino acid residues, see the legend to Fig. 1.

The EC_{50} values obtained from the dose-response curves were 9.2 μM, 36 μM and 0.15 μM for L-glutamate, kainate and quisqualate, respectively (Fig. 4C). The Hill coefficients calculated from these curves were 0.74, 1.1 and 0.83 for L-glutamate, kainate and quisqualate, respectively.

The effects of various glutamate receptor antagonists were examined at a concentration of 1 mM (Table II). The response of 100 μM kainate in oocytes injected with the GluR1-specific mRNA was suppressed by kynurenate, a glutamate receptor antagonist with broad specificity, and was inhibited to some extent by γ-D-

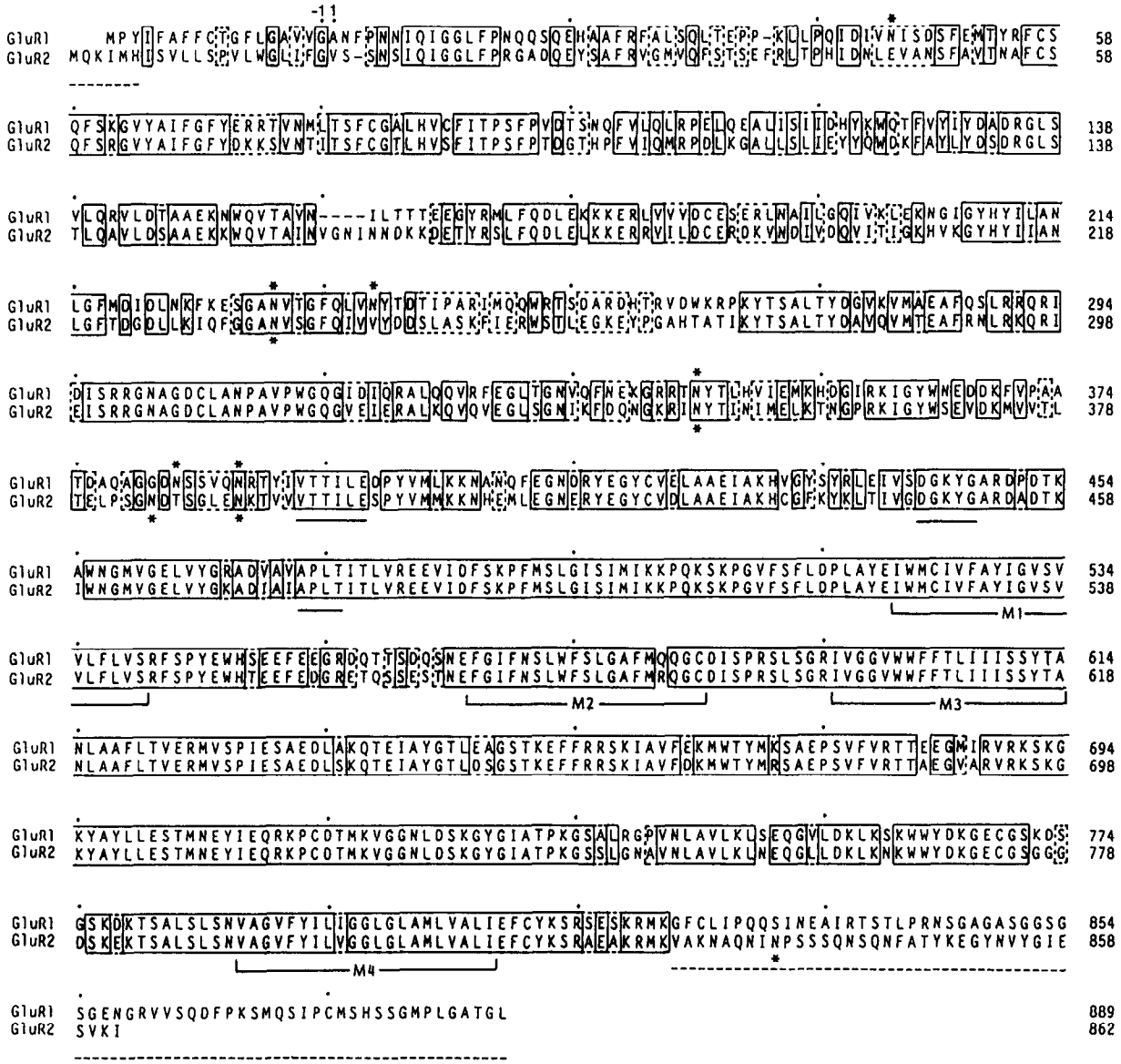

Fig. 3. Alignment of the deduced amino acid sequences of the GluR1 and GluR2 proteins. Sequence comparison was performed using GENETYX software (SDC Corp. Inc.). Numbers of the amino acid residues at the right-hand end of the individual lines are given. Sets of identical amino acid residues in the homologous region (residues −15 to 822 of GluR1 and residues −15 to 826 of GluR2) are enclosed by solid lines, and sets of conservative residues [17] by broken lines. The non-homologous regions are indicated by dashed lines beneath the sequences. The asparagine residues as potential *N*-glycosylation sites are marked with asterisks. The putative transmembrane segments (M1–M4) are indicated. Clusters of amino acid residues conserved among GluR1, GluR2, rat GluR-K1 [4] and the chick [5] and frog [6] kainate binding proteins are underlined.

glutamylglycine (γ-DGG) and γ-D-glutamylaminomethylsulphonate (GAMS), reported to act preferentially on kainate and NMDA receptors [24] and on kainate and quisqualate receptors [25], respectively. Glutamate diethylester (GDEE), thought to be selective for quisqualate receptors [26], showed essentially no inhibitory effect, but exhibited weak agonist activity. The NMDA receptor antagonist D-(−)-2-amino-5-phosphonovaleric acid (APV) slightly inhibited the response to kainate.

Oocytes injected with the GluR2-specific mRNA exhibited no detectable response to 30 μM L-glutamate, 1 μM and 100 μM quisqualate, 100 μM L-aspartate and 100 μM NMDA (Table I). One of the 30 oocytes tested showed a marginal response to 100 μM kainate (Table I). Similar results were obtained when the mRNA was injected in a 5-fold higher concentration.

Oocytes injected with both the GluR1-specific and the GluR2-specific mRNA displayed larger current responses to 30 μM L-glutamate, 100 μM kainate and 1 μM quisqualate than those injected with the GluR1-specific mRNA alone (Fig. 4B and Table I); note the difference in vertical scale between Fig. 4A and B. The additive effect was most prominent for the response to 100 μM kainate. Thus, the glutamate receptor produced in oocytes by injection of both the GluR1-specific and

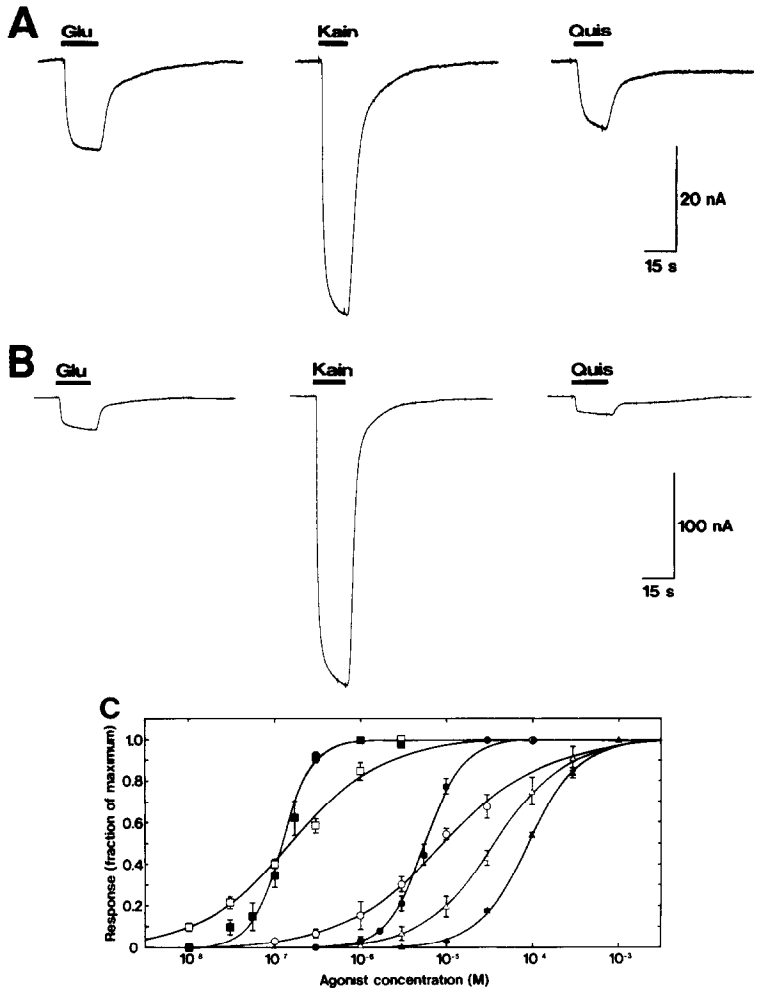

Fig. 4. Functional properties of the GluR1 and GluR2 proteins expressed in *Xenopus* oocytes. A and B, Whole-cell currents activated by bath application of 30 μM L-glutamate (Glu), 100 μM kainate (Kain) and 1 μM quisqualate (Quis) in an oocyte injected with the GluR1-specific mRNA (0.1 μg/μl) alone (A) or with both the GluR1-specific and the GluR2-specific mRNA (0.1 μg/μl each) (B). Membrane potential, −70 mV. Inward current is downward. The duration of agonist application is indicated by bars without taking into account the dead-space time in the perfusion system (~1 s). C, Dose-response curves for inward currents activated by L-glutamate (circles), kainate (triangles) or quisqualate (squares) in oocytes injected with the GluR1-specific mRNA (0.2 μg/μl) alone (open symbols) or with both the GluR1-specific and the GluR2-specific mRNA (0.1 μg/μl each) (closed symbols). Each point represents the mean ± SD of measurements on 4–11 oocytes at −70 mV membrane potential. The theoretical curves have been drawn according to the equation $I = I_{max}/[1 + (EC_{50}/A)^n]$, where I represents the current response, I_{max} the maximum response, A the concentration of agonist, and n the Hill coefficient. The maximum current responses were 14–48 nA (○), 16–70 nA (△), 6–22 nA (□), 7–36 nA (●), 112–313 nA (▲) and 5–19 nA (■).

Table I

Whole-cell currents activated by glutamate receptor agonists in *Xenopus* oocytes injected with either or both of the GluR1-specific and the GluR2-specific mRNA

mRNA injected	Whole-cell currents activated (nA)				
	30 μM L-glutamate	100 μM kainate	1 μM quisqualate	100 μM L-aspartate	100 μM NMDA
GluR1	11 ± 15 (29/30)	29 ± 23 (30/30)	9 ± 13 (28/30)	2 ± 1 (3/30)	ND (0/30)
GluR2	ND (0/30)	1 (1/30)	ND (0/30)	ND (0/30)	ND (0/30)
GluR1, GluR2	29 ± 26 (30/30)	267 ± 191 (30/30)	15 ± 14 (30/30)	2 (2/30)	ND (0/30)

Oocytes were injected with the GluR1-specific mRNA (0.1 μg/μl) or the GluR2-specific mRNA (0.1 μg/μl) or both (0.1 μg/μl each). Inward currents activated by each agonist were recorded at −70 mV membrane potential. Data are given as mean ± SD when indicated; the means have been calculated only for the responsive oocytes. Numbers in parentheses indicate the number of responsive oocytes relative to the number of oocytes tested. Our detectable limit was ~1 nA. ND, not detectable

TABLE II

Sensitivities to antagonists of the GluR1 protein expressed alone or together with the GluR2 protein

Antagonist added (1 mM)	Response to 100 μM kainate (%)	
	GluR1	GluR1, GluR2
None	100	100
Kynurenate	25 ± 5 (4)	10 ± 0.7 (3)
γ-DGG	55 ± 1 (4)	42 ± 3 (4)
GAMS	54 ± 2 (4)	39 ± 2 (3)
GDEE	98 ± 2 (5)	97 ± 3 (4)
APV	82 ± 2 (4)	73 ± 0.9 (4)

Oocytes were injected with the GluR1-specific mRNA (0.2 μg/μl) alone or with both the GluR1-specific and the GluR2-specific mRNA (0.1 μg/μl each). Inward currents activated by 100 μM kainate in the presence and absence of 1 mM antagonist were recorded at −70 mV membrane potential. Data are given as means ± SD; numbers in parentheses indicate the number of oocytes tested. The current responses to 100 μM kainate were 5–54 nA for the oocytes injected with the GluR1-specific mRNA alone and 18–151 nA for the oocytes injected with both the GluR1-specific and the GluR2-specific mRNA. No current response was detected when atagonist alone was applied, except that GDEE evoked a small response in the oocytes injected with the GluR1-specific mRNA alone (6 ± 4% of the response to 100 μM kainate, $n = 5$)

the GluR2-specific mRNA is more selective for kainate than that produced by injection of the GluR1-specific mRNA alone. The EC_{50} values were 5.7 μM, 91 μM and 0.12 μM for L-glutamate, kainate and quisqualate, respectively (Fig. 4C). The values for L-glutamate and quisqualate are similar to those obtained for GluR1 expressed alone, whereas the value for kainate is somewhat larger. The Hill coefficients calculated were 2.0, 1.5 and 2.4 for L-glutamate, kainate and quisqualate, respectively. These values, especially those for L-glutamate and quisqualate, are larger than those obtained for GluR1 expressed alone (close to unity), indicating a positively cooperative interaction between the GluR1 and GluR2 proteins. The increase in channel activity and cooperativity, together with the amino acid sequence similarity, suggests that GluR2 is also a glutamate receptor subunit. The response to 100 μM kainate in oocytes injected with both the GluR1-specific and the GluR2-specific mRNA was inhibited by kynurenate, γ-DGG, GAMS and APV (Table II). The extents of inhibition were somewhat larger than those observed for GluR1 expressed alone. GDEE exhibited essentially no effect.

The present investigation demonstrates a molecular species of glutamate receptor which is responsive both to kainate and to quisqualate. This supports the view that kainate and quisqualate may activate a common receptor subtype [27–29]. Our results also show that co-expression in *Xenopus* oocytes of the two glutamate receptor subunits GluR1 and GluR2 produces higher channel activity and cooperativity, as compared with the GluR1 subunit expressed alone. This suggests that glutamate-gated ionic channels, like other neurotransmitter-gated ionic channels, are hetero-oligomers of different subunits, although the combination of GluR1 and GluR2 may not necessarily represent a native receptor.

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