Published as: *Neuron*. 1990 November ; 5(5): 569–581.

A Family of Glutamate Receptor Genes: Evidence for the Formation of Heteromultimeric Receptors with Distinct Channel Properties

Nobuki Nakanishi[1], **Neil A. Shneider**[1], and **Richard Axel**[1]

[1]Department of Biochemistry and Molecular Biophysics, Howard Hughes Medical, Institute College of Physicians and Surgeons, Columbia University New York, New York, 10032, USA

Summary

We have isolated two cDNA clones (GluR-K2 and GluR-K3) that share considerable sequence identity with the previously described glutamate receptor subunit, GluR-K1. The three glutamate receptor subunits show significant sequence conservation with the glutamine binding component of the glutamine permease of E. coli. Each of these clones encodes a channel responsive to both kainate and AMPA. The coexpression of GluR-K2 with either GluR-K3 or GluR-K1 results in the formation of channels whose current-voltage relationships differ from those of the individual subunits alone and more closely approximate the properties of kainate receptors in neurons. These observations indicate that the kainate/quisqualate receptors are encoded by a family of genes and are likely to be composed of hetero-oligomers of at least two distinct subunits.

Introduction

The amino acid glutamate mediates excitatory synaptic transmission in the vertebrate and invertebrate central nervous systems (Mayer and Westbrook, 1987). The diverse physiological actions of glutamate on central and peripheral neurons are likely to result from the presence of multiple glutamate receptor molecules with distinct ligand binding, ion selectivity, and conductance properties (Watkins and Evans, 1981; Dingledine et al., 1988). Pharmacological and electrophysiological studies have defined two broad classes of glutamate receptors, which can be distinguished by their response to selective agonists. The glutamate agonist N-methyl-o-aspartate (NMDA) activates large conductance channels (Nowak et al., 1984; Jahr and Stevens, 1987;Cull-Candy and Usowicz, 1987) that are permeable to monovalent cations and calcium (MacDermott et al., 1986). A second class of glutamate-responsive channels permeable only to monovalent cations (Mayer et al., 1987)

*Correspondence: Richard Axel, Ph.D., Columbia University, College of Physicians & Surgeons, 701 West 168th Street, New York, New York, 10032, USA, Tel: 212-305-6915, ra27@cumc.columbia.edu.

Note Added in Proof

After the completion of this manuscript, two studies describing the cloning of three new glutamate receptor cDNAs appeared (Keinanen et al., 1990, Science 249, 556–560; Boulter et al., 1990, Science 249, 1033–1037). GluR-B (Keinanen et al.) and GluR2 (Boulter et al.) correspond to GluR-K2. GluR-C (Keinanen et al.) and GluR3 (Boulter et al.) correspond to GluR-K3. Each of these glutamate receptor subunits exists in two alternatively spliced forms, designated "flip" and "flop" (Sommer et al., 1990, Science 249, 1580–1585). The GluR-K2 and GluR-K3 clones described in this manuscript correspond to the flip version of GluR-B and GluR-C, respectively.

can be activated by the agonists kainate or quisqualate, but not by NMDA. Quisqualate (and the more selective agonist α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate [AMPA]) elicits responses with time course and conductance properties that are different from those of kainate-evoked responses (Ishida and Neyton, 1985; Kiskin et al., 1986; Jahr and Stevens, 1987; Ascher and Nowak, 1988; Cull-Candy and Usowicz; 1989). However, selective antagonists that can distinguish kainate between quisqualate-activated currents have not been identified. Thus, it is not clear whether the two agonists activate the same or different receptors.

The diversity of responses to glutamate is reflected not only in the distinct electrophysiological properties evoked by different glutamate agonists, but also in the complexity of responses elicited by a single agonist. For example, in most neurons kainate activates channels permeable to monovalent ions (Mayer et al., 1987), but in a subpopulation of embryonic neurons, an increase in calcium permeability also accompanies the agonist-evoked current (Iino et al., 1990). Interestingly, this current shows a significant voltage dependence (Iino et al., 1990) that is distinct from the linear current-voltage relationship observed in most neurons (Mayer and Westbrook, 1984; Ascher and Nowak, 1988). This complexity is also observed at the level of single-channel recordings, which demonstrate that kainate activates multiple conductances (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987).

The diversity of responses elicited by glutamate could reflect the existence of multiple receptors with different combinations of shared subunits. It is also possible that receptor channel complexes with multiple binding sites exist, and multiple conformational states are associated with distinct channel properties (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987). One approach to understanding the complexity of glutamate receptors involves the cloning and characterization of the genes encoding the subunits of these receptors. Three different cDNAs encoding proteins capable of binding the glutamate agonist kainate have recently been cloned (Hollmann et al., 1989; Wada et al., 1989; Gregor et al., 1989). However, only one of these cDNAs, GluR-K1, has been shown to direct the synthesis of a kainate-responsive ion channel (Hollmann et al., 1989).

In initial studies, we cloned a functional GluR-K1 cDNA and examined the electrophysiological properties of the GluR-K1 receptor subunit in Xenopus oocytes. The current-voltage relationship of GluR-K1 differed markedly from that observed for the kainate-evoked current in most CNS neurons. These observations suggested that additional subunits may be required to reconstitute the properties of the native channel. We therefore isolated two homologous cDNAs from a library of cerebral cortex and hippocampal RNA. The primary structure of each of the proteins encoded by their cDNAs reveals an extracellular domain followed by four putative transmembrane domains characteristic of ligand-gated channels. Interestingly, homology is observed between the three glutamate receptor subunits and the binding component of the glutamine permease of E. coli. Each of the three glutamate receptor subunits alone can form a channel responsive to both kainite and quisqualate. The coexpression of heterologous pairs of receptor subunits results in the generation of kainite receptors with conductance properties that differ from those of the individual subunits alone and more closely approximate the electrophysiological properties

of kainite receptors in neurons. These data suggest that non-NMDA glutamate receptors are hetero-oligomers comprising at least two homologous, but distinct, subunits.

Results

Electrophysiological Properties of GluR-K1

In initial experiments, we examined the electrophysiological properties of the kainate receptors expressed in oocytes injected with RNA encoding GluR-K1 (Hollmann et al., 1989). Oligonucleotides prepared from the published sequence of GluR-K1 were used to isolate a functional GluR-K1 cDNA from a cDNA library of rat cerebral cortex and hippocampal RNA in the vector λ-ZAPII. Partial sequencing of this cDNA revealed a sequence identical to that previously described. This cDNA was rescued by in vivo excision into the RNA expression vector pBluescript, and in vitro synthesized RNA encoding GluR-K1 was expressed in Xenopus oocytes. Two-electrode voltage-clamp analysis of oocytes injected with GluR-K1 RNA reveals a smooth, inward current in response to kainate and the selective quisqualate agonist AMPA (Figure 1A), but not to NMDA. These responses are sensitive to the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Figure 1A), but insensitive to the NMDA inhibitor 2-amino-5-phosphonovaleric acid (APV; data not shown). These observations confirm that expression of GluR-K1 alone is capable of generating a functional channel that can be activated by kainate and quisqualate.

However, the current-voltage relationship of the GluR-K1 kainate response displays a pronounced voltage dependence distinct from that observed in CNS neurons (Figure 1B). Current-voltage curves obtained from oocytes injected with GluR-K1 reveal a pronounced inward rectification of kainate-gated currents at potentials more positive than −60 mV. At holding potentials between −150 and −70 mV, the relationship of the peak kainate current to voltage was linear. However, at more positive voltages, no current was detected and no reversal of the kainate-activated current was observed. This is in sharp contrast to the linear current-voltage relationship most often observed in CNS neurons (Mayer and Westbrook, 1984; Ascher and Nowak, 1988; Iino et al., 1990) and in oocytes injected with rat brain mRNA preparations (Verdoorn and Dingledine, 1988; Figure 1B). These observations suggest that although GluR-K1 is capable of generating a functional kainate receptor, additional subunits are required to reconstitute a receptor whose properties more closely resemble those commonly observed in CNS neurons. We therefore used the GluR-K1 cDNA as a hybridization probe at low stringency to isolate cDNAs that may encode other subunits of the kainate receptor.

Isolation and Characterization of GluR-K2 and GluR-K3

A cDNA library of rat cerebral cortex and hippocampal RNA was screened at low stringency using a 2.6 kb fragment of GluR-K1 spanning amino acids 14–882. Numerous hybridizing clones that share sequence identity with GluR-K1 were isolated. The deduced amino acid sequence of two full-length functional clones, GluR-K2 and GluR-K3, are presented here (Figure 2). The nucleotide sequences through the coding regions of GluR-K2 and GluR-K3 have been deposited with the GenBank-EMBL database. Examination of the nucleotide sequences reveals open reading frames encoding predicted proteins of 883 amino

acids for GluR-K2 and 888 amino acids for GluR-K3. Hydropathy analysis suggests that all three proteins initiate with a putative signal peptide and reveals several short hydrophobic stretches that could reflect transmembrane domains.

Most ligand-gated channels are thought to conform to a structure in which an N-terminal, extracellular, ligand binding domain is followed by four transmembrane regions (M1-M4). Assuming that the glutamate receptor subunits also adopt this configuration, we tentatively assigned the four transmembrane domains as depicted in Figure 2. The assignment of transmembrane domains M1 and M2 differ from that originally suggested for GluR-K1 and are in accord with the predicted transmembrane domains proposed for the chick and frog kainate binding proteins (KBPs; see Discussion). According to this assignment, the three glutamate receptor subunits (GluR-K1, GluR-K2, and GluR-K3) would contain a 525 amino acid N-terminal extracellular domain and a short (50–80 amino acid) C-terminal extracellular domain.

Overall, the three molecules share significant amino acid identity: GluR-K2 and GluR-K3 reveal 69% and 68% identity with GluR-K1, whereas GluR-K2 and GluR-K3 share slightly higher (73%) identity. Sequence conservation is most striking within the region encompassed by the putative transmembrane domains, where the three subunits share over 85% sequence identity. The extracellular N-terminal domains are less conserved, but nonetheless exhibit significant sequence identity; the three GluR-K subunits share about 50% sequence identity in this region. Comparison of the sequence of three GluR-K subunits with the two cloned KBPs suggests that these two sets of proteins are encoded by two related but distinct gene families. The overall identity between each of the GluR-K subunits, K1, K2, and K3, and the chick KBP, for example, is about 36%. Perhaps the most striking difference between the GluR-K1 subunits and the KBPs is the length of the N-terminal extracellular domain. Despite the fact that all five proteins bind kainate, the 525 residue extracellular domain of the GluR-K subunits is 370 amino acids longer than the 150 residue extracellular domain of the KBPs.

Kainate Receptors Are Homologous to the E. coli Glutamine Permease

A search of the GenBank-EMBL sequence database revealed an interesting homology between all five kainate receptor subunits (i.e., the GluR-K subunits and KBPs) and the periplasmic glutamine binding protein of E.coli (Nohno et al., 1986), an essential component of the glutamine permease (Figure 3). Glutamine is actively transported into E. coli by first binding to a highly specific, periplasmic glutamine binding protein (GlnH; Masters and Hong, 1981). The association of glutamine with GlnH is thought to elicit a conformational change that allows this protein to bind to components of the permease within the inner membrane, facilitating glutamine transport (for review, see Ames, 1986). Alignment of the N-terminal 100 amino acids of GlnH with residues 390–505 of the GluR-K subunits and residues 25–130 of the KBPs reveals about 20%–30% sequence identity and an additional 15% conservative substitutions with each of the five receptor subunits (Figure 3A). This region of homology that encompasses the bulk of the N-terminal extracellular domain of the KBP is likely to constitute the glutamate binding site.

We have identified a second region of sequence conservation encompassing the C-terminal 45 amino acids of GlnH and a cytoplasmic region connecting transmembrane domains M3 and M4 of the kainate receptor subunits (Figure 3B). This conserved region is thought to reside within the cell, forming a loop between M3 and M4. In contrast, the homologous region of GlnH resides within an extracellular protein secreted into the periplasmic space. The function of this conserved block of residues is unknown in both GlnH and the glutamate receptor subunits, and it is therefore difficult to assign a common function to this conserved domain.

GluR-K2 and GluR-K3 Generate Kainate-Sensitive Channels

The functional properties of GluR-K2 and GluR-K3 were examined by voltage-clamp analysis following the expression of the corresponding RNAs in Xenopus oocytes. When oocytes injected with GluR-K2 or GluR-K3 mRNA were exposed to kainate (100 μM), a smooth, inward current, similar in form to that observed with GluR-K1, was evoked (Figure 4A). However, the peak amplitude of the GluR-K2 response was considerably smaller than that observed with GluR-K1 or GluR-K3 (Table 1). At present we cannot determine whether the difference in the magnitude of the current responses elicited by the three different mRNAs results from differences in the number of kainite-responsive channels expressed in oocytes or from differences in the conductance or gating properties of the channels directed by these three receptor subunits.

The sensitivity of the channels encoded by GluR-K2 and GluR-K3 to other glutamate receptor agonists and antagonists was also investigated. Oocytes injected with either GluR-K2 or GluR-K3 mRNA showed no response to NMDA (40 μM; data not shown), but current responses were observed with the agonists glutamate (1 mM), quisqualate (10 μM), and AMPA (10 μM; Figure 4A). The response induced by these agonists resembles the inward current evoked by kainate. Both GluR-K2 and GluR-K3 responses to kainate and AMPA were blocked by CNQX (figure 4A). The peak amplitude of the AMPA response of each of the GluR-K subunits was significantly smaller than the corresponding kainate response. In glutamate-responsive neurons, AMPA elicits a very fast transient current peak that desensitizes quickly to a lower steady-state level (Ishida and Neyton, 1985; Kiskin et al., 1986). Our drug application system would not permit the detection of this rapid, transient current peak. The differences in amplitude between the AMPA and kainate responses we observe may result from the fact that with AMPA, we are measuring only the steady-state current following desensitization.

The current-voltage relationship of the GluR-K3 kainate response displays a pronounced voltage dependence similar to that observed with GluR-K1 (Figure 4C). At potentials between −150 and −70 mV, the relationship of the kainate-evoked current to voltage was linear. However, at more positive voltages, no current was detected and no reversal of the kainate-activated current was observed. The amplitude of the current with GluR-K2 remained low even at a potential of −130 mV, and it was therefore difficult to obtain a significant current-voltage relationship.

The dose-response curves for the activation of GluR-K1 and GluR-K3 with AMPA and kainate are shown in Figure 4B. The EC_{50} for kainate is 32 μM for GluR-K1 and 130 μM for

GluR-K3 (Table 1). The EC_{50} observed with AMPA is 1.3 μM for GluR-K1 and 36 μM for GluR-K3. The GluR-K2 response was too small to obtain an accurate dose-response curve. However, the GluR-K2 responses are evoked in the same concentration range as the other glutamate receptors. Hill coefficients calculated from kainate dose-response curves were 1.4 for GluR-K1 and 1.3 for GluR-K3 (Table 1), suggesting possible cooperative activation by ligand binding to more than one subunit of the receptor complex. Taken together, these data demonstrate that each of the three GluR-K subunits can form a ligand-gated ion channel responsive to the glutamate agonists kainate and AMPA.

Glutamate Receptors with Combinations of Different Subunits

We presume that the kainate channels produced upon expression of the GluR-K subunits alone in Xenopus oocytes result from the formation of oligomers. However, the current-voltage relationships of these kainate responses differ dramatically from those observed in most CNS neurons. Therefore, we next expressed all possible combinations of the three glutamate receptor subunits to ask whether these subunits are capable of forming specific hetero-oligomeric receptors whose electrophysiological properties more closely resemble those of the kainate receptors observed in vivo.

In initial experiments, oocytes were co-injected with RNA from GluR-K1 and GluR-K3. These oocytes display a smooth, inward current response to kainate, AMPA (Figure 5A, trace a), and L-glutamate (data not shown); no current response was evoked by NMDA (data not shown). The kainate and AMPA responses were blocked by the non-NMDA antagonist CNQX (Figure 5A, trace a), but not by the NMDA inhibitor APV (data not shown). The dose-response curve reveals an EC_{50} of 55 μM for kainate and 24 μM for AMPA (Figure 5B; Table 1). The current-voltage relationship of this response displays the same voltage dependence as that observed upon injection of either GluR-K1 or GluR-K3 alone (Figure 5C). This kainate-activated channel rectifies inwardly such that no outward current was detected even at positive holding potentials as high as +50 mV. At negative voltages from −150 to −70 mV, the current-voltage curve is linear and the peak current amplitudes approximate the sum of the kainate current responses obtained with GluR-K1 and GluR-K3 alone (Table 1). These data suggest that the coinjection of GluR-K1 and GluR-K3 results in either the expression of two discrete homo-oligomers or the expression of hetero-oligomers whose functional properties closely resemble those of the corresponding homo-oligomers.

The receptors expressed after coinjection of GluR-K1 + GluR-K2 or GluR-K3 + GluR-K2 exhibit similar pharmacological properties but strikingly different electrophysiological properties from those observed upon expression of the corresponding homo-oligomers. Oocytes expressing these two combinations of receptor subunits exhibit an inward current in response to kainate and AMPA that was blocked by CNQX (Figure 5A, traces b and c). No response was observed with NMDA, nor did APV block the responses observed with kainate (data not shown). The dose-response curve for the receptors formed upon coinjection of GluR-K1 and GluR-K2 revealed a mean EC_{50} of 110 μM for kainate and 2.2 μM for AMPA (Figure 5B; Table 1). A similar concentration dependence was observed upon coinjection of the combination of GluR-K3 and GluR-K2, with mean EC_{50} values of 110 μM and 19 μM for kainate and AMPA, respectively. Initial evidence for the formation of

hetero-oligomers emerged from an examination of the magnitude of the kainate-evoked currents in oocytes expressing combinations of GluR-K3 + GluR-K2 receptor subunits (Table 1). In oocytes expressing GluR-K2 alone, kainate routinely elicits a weak 1–5 nA current at −70 mV, whereas in cells expressing GluR-K3, kainate evokes a current of 1120 ± 626 nA. Significant augmentation of the kainate-evoked current to 2400 ± 766 nA is observed upon coinjection of GluR-K2 and GluR-K3. Although the amplitude of the responses may vary in different batches of oocytes, the ratio of current amplitudes for the different combinations of subunits remains constant.

More convincing evidence for the formation of functional hetero-oligomers derives from the analysis of the current-voltage relationships of the kainate receptors formed upon expression of GluR-K1 + GluR-K2 or GluR-K3 + GluR-K2 (Figure 5C). In contrast to the inward rectification observed upon expression of the individual receptor subunits alone, coexpression of GluR-K2 with either GluR-K1 or GluR-K3 resulted in kainate responses whose current-voltage relationships were linear in the range of −1SO to +SO mV. The reversal potentials of the kainate response observed with either pair of subunits was 0 to −S mV, consistent with the values observed in neurons (Mayer and Westbrook, 1984; Ascher and Nowak, 1988) and in oocytes injected with cerebral cortex and hippocampal mRNA (Figure 1B). The electrophysiological and pharmacological properties of receptors formed upon coexpression of GluR-K1 + GluRK2 + GluR-K3 are similar to those obtained with GluR-K1 + GluR-K2 or GluR-K3 + GluR-K2 (Figure SA, trace d; Figures SB and SC).

These data provide electrophysiological evidence that both GluR-K1 and GluR-K3 can form functional hetero-oligomers with GluR-K2. Moreover, the properties of these hetero-oligomeric receptors more closely resemble the conductance properties observed for kainate receptors in vivo. These data suggest that the native kainate receptor is a multimer consisting of at least two different subunits.

Discussion

Putative Structure of the Kainate Receptor Subunits

The sequences of five kainate binding proteins have been determined: GluR-K1, GluR-K2, GluR-K3, and two KBPs from frog and chick (Figure 2). The sequence of each of these proteins is consistent with a configuration in which an N-terminal ligand binding domain is followed by four transmembrane domains, which participate in the formation of an ion channel. This basic structure has been suggested for the acetylcholine (Noda et al., 1982; Claudio et al., 1983), γ-aminobutyric acid (GABA; Schofield et al., 1987), and glycine (Grenningloh et al., 1987) receptor subunits, and in the case of the acetylcholine receptor, it is supported by a large number of structure-function analyses (Mishina et al., 1985; Kao and Karlin, 1986).

There are, however, striking differences between the family of kainate receptor subunits and the other ligand-gated channels. First, the N-terminal extracellular domain of the GluR-K family is large, 525 amino acids, compared with about 230 residues for the acetylcholine receptor and 225 residues for the GABA receptor subunits. In contrast, the frog and chick KBPs reveal a short, 150 amino acid N-terminal extracellular domain that shares about 45%

identity with the corresponding region of the GluR-K family of proteins. If we presume that this 150 amino acid stretch constitutes the kainate binding domain, then a question arises as to the function of the 370 N-terminal amino acids of the GfuR-K subunits.

A second property peculiar to the kainate receptor subunits emerges from a consideration of the distribution of conserved residues throughout the length of the molecule. For other ligand-gated channels, maximal sequence conservation is observed through the regions encompassing the putative transmembrane domains, but significant divergence is observed in the regions separating the individual transmembrane segments (Numa, 1989; Levitan et al., 1988). This is most apparent in the cytoplasmic loop separating M3 and M4, which shows significant sequence and length diversity. In contrast, this region is of fixed length and shows greater than 80% sequence identity in the GluR-K family. This striking conservation unique to the kainate receptors suggests that this cytoplasmic loop may be structurally constrained to permit contact with additional molecules within the receptor complex.

One important point concerns the assignment of putative transmembrane domains. From a comparison of the sequences of the GluR-K subunits, we favor the placement of M1 and M2 suggested for the KBPs, as depicted in Figure 2. The proper identification of M2 is of particular interest, since biochemical and genetic evidence strongly suggests that the M2 helices from neighboring subunits of the acetylcholine receptor line the channel pore (Giraudat et al., 1986; Obertür et al., 1986; Imoto et al., 1986). In the putative M2 domain of GABA and glycine, as well as the acetylcholine receptor subunits, small polar or neutral residues repeat in every fourth position such that they are capable of forming a continuous ridge on the helical surface (Unwin, 1989). This ridge of small polar amino acids is flanked by a ridge of large hydrophobic residues. This conserved distribution would be consistent with one suggested gating mechanism in which the open pore is lined mainly by small residues. Channel closure would result from helical displacement, in which the larger hydrophobic ridge now replaces the smaller residues. The putative M2 transmembrane domains of all five kainate binding proteins in the alignment shown in Figure 2 maintain this conserved distribution of small polar and hydrophobic residues. This structural conservation in the chick and frog KBPs suggests that these molecules may also function as components of an ion channel in association with other subunits

If our assignment of M2 is correct, then the regions immediately flanking M2 in the GluR-K subunits are likely to form the channel entrance. In the acetylcholine receptor, M2 in all subunits is flanked on its C-terminal extracellular face by negatively charged glutamate or aspartate residues, which may form an anionic ring (Imoto et al., 1988). On the N-terminal cytoplasmic side there are two such anionic rings. Mutations that reduce the net negative charge in each of these rings dramatically reduce channel conductance. In the three GluR-K receptor subunits the short region between M1 and M2 (which presumably flanks the pore on the cytoplasmic face) contains 8 glutamate or aspartate residues (Figure 2). A single aspartate flanks M2 on its extracellular face. It is of interest that the region between M1 and M2 is highly divergent in the GluR-K subunits. Furthermore, the chick and frog KBPs have an 11 amino acid deletion in the region between M1 and M2 that reduces their net negative charge by 2. If this region is a component of the cytoplasmic entrance of the channel pore,

then the presence of multiple, conserved, negatively charged residues interspersed among highly divergent residues in the GluR-K subunits may, in part, be responsible for the multiple conductance states evoked by kainate in neurons.

It is striking that a 100 amino acid stretch within the N-terminal extracellular domains of the five glutamate receptor subunits (GluR-K subunits and KBPs) shares significant sequence conservation with the binding subunit of the glutamine permease of E. coli (Figure 3A). Given the structural similarities of the two ligands and the fact that one common essential function of these six proteins is ligand binding, it is likely that the region of conservation comprises the ligand binding site. Therefore, we suggest that the ligand binding site resides within residues 395–510 of the GluR-K subunits and within the homologous regions (residues 22–130) of the KBPs.

The observation that significant sequence homology is maintained over billions of years of evolution suggests that the conserved residues may constitute the actual site of contact for ligand or may be essential to maintain the structural integrity of the binding site. Although the three-dimensional structure of the glutamine binding protein has not been determined, analysis of the structures of related binding proteins of bacteria (including the L-arabinose binding protein, the sulfate binding protein, the Leu/IleNal binding protein, the D-galactose binding protein, and the leucine-specific binding protein) reveals very similar domains and tertiary structures (Quiocho, 1990). Each structure is composed of two distinct globular domains (Gilliland and Quiocho, 1981). The ligand binds within a cleft between the domains (Newcomer et al., 1979), eliciting a conformational change thought to facilitate the association between the binding protein and the corresponding membrane-bound transport proteins (Mao et al., 1982). The neurotransmitter glutamate may therefore recognize a similar structure within the extracellular domain of the glutamate receptor.

The glutamine binding protein and the glutamate-activated ion channel share the property of ligand binding, but exhibit other divergent properties. Ligand binding to GlnH facilitates binding to the glutamine transporter, whereas ligand binding to the neurotransmitter receptors, GluR-K, opens a channel permeable to small ions. It is possible that the glutamine/glutamate binding domain emerged early in evolutionary time and evolved, perhaps by exon shuffling, as a component of several different families of genes that bind glutamate but carry out distinct cellular functions. In the case of the glutamate receptor genes, exons encoding a primitive glutamate binding domain may have become associated with sequences encoding an ion channel to create a glutamate-gated channel. A similar situation is illustrated by the cGMP-activated ion channel recently cloned from retina (Kaupp et al., 1989). Although clearly related to a superfamily of voltage-gated ion channels Jan and Jan, 1990), this protein reveals a putative cGMP binding domain homologous to cAMP and cGMP binding regions of proteins of totally unrelated function in both prokaryotes and eukaryotes. Thus, the binding motifs and perhaps even the conformational changes elicited by binding and essential for the gating of ligand-activated ion channels evolved from genes that serve distinct functions in prokaryotes.

Functional Diversity of Kainate Receptors

The presence of a family of genes encoding different glutamate receptor subunits may provide a molecular basis for the diversity of responses to glutamate observed in neurons. Single-channel recordings in rat cerebellar and hippocampal neurons indicate that kainate activates channels with multiple conductances (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987). Kainate elicits predominantly small-amplitude channel openings (less than 1 pS, 8pS, and 1S-18 pS) and large-amplitude openings of 38 pS at a much lower frequency. We have isolated three genes encoding functional kainate receptor subunits that are capable of associating to form at least five distinct kainite-responsive channels. The presence of a family of kainate receptor subunits along with the ability of these subunits to associate with one another provides a combinatorial mechanism to generate a large number of different receptor complexes, each exhibiting distinct electrophysiological properties. This combinatorial mechanism has been demonstrated in vivo for the muscle acetylcholine receptor subunits (Mishina et al., 1986) and has been suggested by subunit mixing experiments with the neuronal acetylcholine receptor (Wada et al., 1988), the GABA receptor (Levitan et al., 1988), and the different subunits of the voltage-sensitive potassium channel (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990).

Perhaps the property that differs most strikingly among the GluR-K receptors we have generated is the voltage dependence of the kainate responses. Whole-cell, voltage-clamp recordings from a majority of CNS neurons demonstrate a linear current-voltage relationship for the kainate-evoked currents (Mayer and Westbrook, 1984; Ascher and Nowak, 1988; Iino et al., 1990). In one study, however, inward rectification of a kainate channel was described for a subpopulation of cultured embryonic neurons (Iino et al., 1990). Another example of inward rectification of a kainate channel was described in a study of oocytes injected with cerebellar mRNA (Randle et al., 1988). These voltage-dependent kainate responses reverse at 0 mV, and significant outward current is observed at more positive voltages. In our study, the putative homo-oligomeric channels formed by the GluR-K1 and GluR-K3 subunits, also exhibit striking inward rectification (Figure 1B; Figure 4C); however, no reversal of these kainate responses was observed, and no significant outward current was detected even at +SO mV. Coexpression of GluR-K1 or GluR-K3 with GluR-K2, however, results in the formation of hetero-oligomeric kainate receptors that show no voltage dependence of activation (Figure SC). At present, we do not know whether the inward rectification observed with GluR-K1 or GluR-K3 is a consequence of ion block or an intrinsic voltage-dependent property of a subset of kainate receptors. Whatever the mechanism, the voltage-dependent properties of the homo-oligomeric kainate receptors can be dramatically altered when the subunits associate to form hetero-oligomers. Although our data indicate that GluR-K2 can associate with either GluR-K1 or GluR-K3, we do not know whether these receptor subunits are coordinately expressed in the same neuron. It is therefore possible that the hetero-oligomers we have formed in vitro do not reflect native hetero-oligomers in vivo.

One property shared by the five receptors formed by the GluR-K subunits is the consistent ability to respond to both kainate and AMPA. In neurons, AMPA and quisqualate elicit responses that differ from those evoked by kainate. Quisqualate elicits a fast, transient

current peak that desensitizes rapidly, whereas kainate most often activates a nondesensitizing current (Ishida and Neyton, 1985; Kiskin et al., 1986). Moreover, these two agonists preferentially activate different subpopulations of single-channel conductances Jahr and Stevens, 1987; Ascher and Nowak, 1988; Cull-Candy and Usowicz, 1989). One interpretation of these observations is that there exist distinct glutamate receptors capable of binding either kainate or quisqualate. However, the two agonists cross-desensitize (Kiskin et al., 1986; O'Brien and Fischbach, 1986; O'Dell and Christensen, 1989), and there are no selective antagonists that can distinguish kainate from quisqualate-activated currents. Thus, despite the different electrophysiological responses evoked by kainate and quisqualate, these results suggest that the two agonists may activate the same receptors. Our data indicate that all the receptor combinations we have examined can be activated by both kainate and AMPA, thus providing physical evidence that kainate and AMPA bind and activate the same receptor subtypes.

Thus, the complexity of electrophysiological properties exhibited by glutamate receptors appears to be mirrored by an equivalent complexity in the genes encoding these receptors. This physical and functional diversity of excitatory amino acid receptors may provide a mechanism for activity-dependent processes of CNS neurons, including temporal integration, rhythmic firing, and synaptic plasticity.

Experimental Procedures

Isolation and Characterization of GluR-K Clones

Total RNA was isolated using RNAzol (Cinna/Biotex; Chomczynski and Sacchi, 1987) from hippocampus and cerebral cortex of 3- to 5-week-old rats (Sprague-Dawley; Hilltop). Poly(A)+ RNA was selected by passing total RNA through an oligo(dT)-cellulose spin column (Pharmacia), and a directional cDNA library was constructed in the bacteriophage expression vector λ-ZAPII using the Stratagene cDNA synthesis kit (Stratagene). To isolate a full-length cDNA clone encoding GluR-K1, two oligonucleotides were synthesized according to the published sequence, corresponding to nucleotides −95 to −45 and 2641 to 2690 of GluR-K1 (Hollman et al., 1989). These oligonucleotides were end labeled with T4 polnucleotide kinase in the presence of (γ-^{32}P] ATP and used as a hybridization probe to screen 1×10^6 independent cDNA clones. Bluescript plasmids were rescued from positive GluR-K1 clones by in vivo excision and partially sequenced by the chain termination method (Sanger et al., 1987) to confirm their identity.

To isolate homologs of GluR-K1 from the same library, plaque hybridization on nylon membranes (MSI) was performed in 20% formamide, 1 M NaCl, 10% dextran sulfate, 50 mM Tris-HCI (pH 7.5), 1% SDS at 42°C using the coding region of GluR-K1 as probe. After hybridization, filters were washed in 20% formamide, 1 M NaCl, 50 mM Tris-HCI (7.5), 1% SDS at 42°C. Positive plaques were grouped following high-stringency hybridization using cDNA inserts prepared from each clone as hybridization probes. Nucleotide sequencing of representatives of each group identified GluR-K2 and GluR-K3 as homologs of GluR-K1.

Nucleotide and Deduced Amino Acid Sequence Analysis

Nucleotide sequences were assembled using the IBI MacVector 3.0 (Pustell, 1988) program. Deduced amino acid sequences of GluR-K subunits were compared with protein sequences translated from nucleotide sequences stored in the GenBank (Release 63.0) and EMBL (Release 19) databases using the FASTA (Pearson and Lipman, 1988) algorithm. The extent of protein sequence similarity was calculated using the PAM250 matrix (Dayhoff et al., 1983). To evaluate the significance of similarity scores, randomly shuffled sequences that preserved local sequence composition were generated using the RDF2 (Pearson and Lipman, 1988) program.

Synthesis of GluR-K Transcripts In Vitro

pGluR-K DNAs were linearized with Xhol endonuclease, followed by digestion with proteinase K (100 μg/ml) and phenol-chloroform-isoamyl alcohol extractions. Transcription reactions (50 μl) contained 40 mM Tris-HCI (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 30 U of placental ribonuclease inhibitor (Promega), 0.5 mM (each) ATP, CTP, UTP, and GTP, 1.0 μCi of [^{3}H]UTP (1000 Ci/mmol; Amersham), 1 mM GpppG (Pharmacia), 10 U of 13 RNA polymerase (Bethesda Research Laboratories), and 2 μg of linearized plasmid DNA and were incubated at 37°C for 60 min. Template DNA was digested with DNAase I (Pharmacia), and RNA transcripts were purified by three precipitations in ammonium acetate and ethanol. Concentrations of synthesized RNA were estimated by calculating the percentage of label incorporated and were confirmed by agarose-formaldehyde gel electrophoresis and ethidium bromide staining.

Expression in Oocytes

Oocytes were harvested from adult Xenopus laevis under anesthesia (0.1% aminobenzoic acid ethyl ester; Sigma) and manually dissected into groups of 10–20 cells. The follicle layers were removed by incubation in calcium-free ND-96 (82.5 mM NaCl, 2.0 mM KCI, 1.0 mM $MgCl_2$, 1 mg/ml streptomycin; Specialty Media) containing collagenase type 1A (2 mg/ml; Sigma). Oocytes were maintained at 18°C in Barth's saline containing 2% Ficoll, penicillin, and streptomycin (Specialty Media). Media were exchanged daily in the 2–5 days before electrophysiological recordings. Cells were typically injected with 50 μl of RNA sample. For voltage-clamp recordings, oocytes were placed in a continuous-flow chamber perfused with oocyte Ringer solution at room temperature. All drugs were introduced through this system by switching the perfusion line inlet. Kainate, AMPA, quisqualate, NMDA (Cambridge Research), and L-glutamate (Sigma) were each added to the standard bath solution. The antagonists CNQX and APV (Cambridge Research) were also included for some experiments. For all experiments involving NMDA, 5 μM glycine was added to the perfusing solution. The oocytes were voltage-clamped with the use of a two-electrode voltage clamp (Axoclamp 2A) having a virtual ground and remote switchable headstage. Membrane currents were recorded through the virtual ground and filtered at 10 Hz. Responses were recorded on a Gould chart recorder and stored on an IBM PC AT using pClamp software (Axon) for electrophysiological data acquisition and analysis. Electrodes had 2–5 MΩ resistance and were filled with 3 M KCI.

The current-voltage relationship of agonist-evoked responses was determined by continuously applying ligand while the membrane potential was stepped through a series of voltages. Leak current was determined by performing the same series of voltage steps in the absence of ligand. The difference between these two currents at each potential was taken as the agonist-activated current. Individual voltage steps were of 2 s duration, separated by 3–8 s intervals. Typically, steps were made from an initial voltage of −70 mV, covering the voltage range of −150 mV to +50 mV in 10 mV intervals. Peak currents were sampled 1.7 s after the start of each voltage step. Dose-response curves were fitted by a form of the Michealis-Menten equation:

$$I=I_{max}/(1+(EC_{50}/A)^{h})$$

Where I is the measured amplitude of evoked current, I_{max} is the maximum response, EC_{50} is the effector concentration causing half-maximal response, A is the agonist concentration, and the exponent h is the Hill coefficient determined from a Hill plot of the same data. EC_{50} values were determined by the least squares fit of the concentration-response curves to this equation.

Acknowledgments

We wish to acknowledge Adriana Nemes for her contributions to DNA sequence analysis; Amy B. MacDermott and Andreas Kyrozis for their help with electrophysiological data aquisition and analysis; Andrew Furley for his help with sequence comparison and database search. We thank A. B. MacDermott, Steve Siegelbaum, and Thomas Jessell for their critical reading of the manuscript. We are grateful to Phyllis Kisloff for her help in the preparation of the manuscript. This work was supported by the Howard Hughes Medical Institute (R. A. and N. N.) and by a predoctoral fellowship from the Dean's Office, Columbia University (N. A. S.).

References

Ames GF-L. Bacterial periplasmic transport systems: structure, mechanism, and evolution. Annu. Rev. Biochem. 1986; 55:397–425. [PubMed: 3527048]

Ascher P, Nowak L. Quisqualate and kainate-activated channels in mouse central neurons in culture. J. Physiol. 1988; 399:227–245. [PubMed: 2457088]

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 1987; 162:156–159. [PubMed: 2440339]

Christie MI, North RA, Osborne PB, Douglass J, Adelman JP. Heteropolymeric potassium channels expressed in Xenopus oocytes from cloned subunits. Neuron. 1990; 4:405–411. [PubMed: 2317379]

Claudio T, Ballivet M, Patrick J, Heinemann S. Nucleotide and deduced amino acid sequence of Torpedo cafifornica acetylcholine receptor gamma subunit. Proc. Natl. Acad. Sci. USA. 1983; 80:1111–1115. [PubMed: 6573658]

Cull-Candy SG, Usowicz MM. Multiple-conductance channels activated by excitatory amino acids in cerebellar neurons. Nature. 1987; 325:525–528. [PubMed: 2433594]

Cull-Candy SG, Usowicz MM. On the multiple-conductance single channels activated by excitatory amino acids in large cerebellar neurons of the rat. J. Physiol. 1989; 415:555–582. [PubMed: 2484210]

Dayhoff MO, Barker WC, Hunt LT. Establishing homologies in protein sequences. Meth. Enzymol. 1983; 97:524–545. [PubMed: 6855599]

Dingledine R, Boland LM, Chamberlin NL, Kawasaki K, Kleckner NW, Traynelis SF, Verdoorn TA. Amino acid receptors and uptake systems in the mammalian central nervous system. CRC Crit. Rev. Neurobiol. 1988; 4:1–96.

Gilliland GL, Quiocho FA. Structure of the L-arabinose-binding protein from Escherichia coli at 2.4 A resolution. J. Mol. Biol. 1981; 146:341–362. [PubMed: 7021859]

Giraudat J, Dennis M, Heidmann T, Chang J-Y, Changeux J-P. Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: serine-262 of the delta subunit is labeled by [3H]chlorpromazine. Proc. Natl. Acad. Sci. USA. 1986; 83:2719–2723. [PubMed: 3085104]

Gregor P, Mano I, Maoz I, McKeown M, Teichberg VI. Molecular structure of the chick cerebellar kainate-binding subunit of a putative glutamate receptor. Nature. 1989; 342:689–692. [PubMed: 2480525]

Grenningloh G, Rienitz A, Schmitt B, Methfessel C, Zensen M, Beyreuther K, Gundelfinger ED, Betz H. The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. Nature. 1987; 328:215–220. [PubMed: 3037383]

Hollmann M, O'Shea-Greenfield A, Rogers SW, Heinemann S. Cloning by functional expression of a member of the glutamate receptor family. Nature. 1989; 342:643–648. [PubMed: 2480522]

Iino M, Ozawa S, Tsuzuki K. Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurons. J. Physiol. 1990; 424:151–165. [PubMed: 1697342]

Imoto K, Methfessel C, Sakman B, Mishina M, Mori Y, Konno T, Fukuda K, Kurasaki M, Bujo H, Fujita Y, Numa S. Location of the delta subunit region determining ion transport through the acetylcholine receptor channel. Nature. 1986; 324:670–674. [PubMed: 2432430]

Imoto K, Busch C, Sakman B, Mishina M, Konno T, Nakai J, Bujo H, Mori Y, Fukuda K, Numa S. Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. Nature. 1988; 335:645–648. [PubMed: 2459620]

Isacoff EY, Jan YN, Jan LY. Evidence for the formation of heteromultimeric potassium channels in Xenopus oocytes. Nature. 1990; 345:530–534. [PubMed: 2112229]

Ishida AT, Neyton J. Quisqualate and L-glutamate inhibit retinal horizontal-cell responses to kainate. Proc. Natl. Acad. Sci. USA. 1985; 82:1837–1841. [PubMed: 2858853]

Jan LY, Jan YN. A super family of ion channels. Nature. 1990; 345:672. [PubMed: 1694264]

Jahr CE, Stevens CF. Glutamate activates multiple single channel conductances in hippocampal neurones. Nature. 1987; 325:522–525. [PubMed: 2433593]

Kao PN, Karlin A. Acetylcholine receptor binding site contains a disulfide crosslink between adjacent half-cystinyl residues. J. Biol. Chem. 1986; 261:8085–8088. [PubMed: 3722144]

Kaupp UB, Niidome T, Tanabe T, Terada S, Bonigk W, Stuhmer W, Cook NJ, Kangawa K, Matsuo H, Hirose T, Miyata T, Numa S. Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. Nature. 1989; 342:762–766. [PubMed: 2481236]

Kiskin NI, Krishtal OA, Tsyndrenko AY. Excitatory amino acid receptors in hippocampal neurons: kainate fails to desensitize them. Neurosci. Lett. 1986; 63:225–230. [PubMed: 2869459]

Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Kohler M, Fujita N, Rodriguez HF, Stephenson A, Darlison MG, Barnard EA, Seeburg P. Structural and functional basis for GABAA receptor heterogeneity. Nature. 1988; 335:76–79. [PubMed: 2842688]

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ, Barker JL. NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons. Nature. 1986; 321:519–522. [PubMed: 3012362]

Mao B, Pear MR, McCammon JA, Quiocho FA. Hinge-bending in L-arabinose-binding protein. J. Biol. Chem. 1982; 257:1131–1133. [PubMed: 7035444]

Masters PS, Hong J-S. Genetics of the glutamine transport system in Escherichia coli. J. Bacteriol. 1981; 147:805–819. [PubMed: 6115851]

Mayer ML, Westbrook GL. Mixed-agonist action of excitatory amino acids on mouse spinal cord neurons under voltage clamp. J. Physiol. 1984; 354:29–53. [PubMed: 6148411]

Mayer ML, Westbrook GL. The physiology of excitatory amino acids in the vertebrate central nervous system. Prog. Neurobiol. 1987; 28:197–276. [PubMed: 2883706]

Mayer ML, MacDermott AB, Westbrook GL, Smith SJ, Barker JL. Agonist- and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured with arsenazo Ill. J. Neurosci. 1987; 7:3230–3244. [PubMed: 2444678]

Mishina M, Tobimatsu T, Imoto K, Tanaka K-1, Fujita Y, Fukuda K, Kurasaki M, Takahashi H, Morimoto Y, Hirose T, Inayama S, Takashashi T, Kuna M, Numa S. Location of functional regions of acetylcholine receptor a-subunit by site-directed mutagenesis. Nature. 1985; 313:364–369. [PubMed: 3838201]

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature. 1986; 321:406–411. [PubMed: 2423878]

Newcomer ME, Miller DM, Quiocho FA. Location of the sugar-binding site of L-arabinose-binding protein. J. Biol. Chem. 1979; 254:7529–7533. [PubMed: 381285]

Noda M, Takahashi H, Tanabe T, Toyosato M, Furutani Y, Hirose T, Asai M, Inayama S, Miyata T, Numa S. Primary structure of a subunit precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. Nature. 1982; 299:793–797. [PubMed: 6182472]

Nohno T, Saito T, Hong J-S. Cloning and complete nucleotide sequence of the Escherichia coli glutamine permease operon (gin HPQ). Mal. Gen. Genet. 1986; 205:260–269.

Nowak L, Bregestovski P, Ascher P, Herbert A, Prochiantz A. Magnesium gates glutamate-activated channels in mouse central neurones. Nature. 1984; 307:462–465. [PubMed: 6320006]

Numa S. A molecular view of neurotransmitter receptors and ionic channels. Harvey Leet. 1989; 83:121–165.

Oberthur W, Muhn P, Baumann H, Lottspeich F, Wittmann-Liebold B, Hucho F. The reaction site of a non-competitive antagonist in the delta subunit of the nicotinic acetylcholine receptor. EMBO J. 1986; 5:1815–1820. [PubMed: 3758027]

O'Brien RJ, Fischbach GD. Characterization of excitatory amino acid receptors expressed by embryonic chick motoneurons in vitro. J. Neurosci. 1986; 6:3275–3283. [PubMed: 2430076]

O'Dell TJ, Christensen BN. A voltage-clamp study of isolated stringray horizontal cell non-NMDA excitatory amino acid receptors. J. Neurophysiol. 1989; 61:162–172. [PubMed: 2563761]

Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA. 1988; 85:2444–2448. [PubMed: 3162770]

Pustell JM. Interactive molecular biology computing. Nucl. Acids Res. 1988; 16:1813–1820. [PubMed: 3353222]

Quiocho FA. Atomic structure of periplasmic binding proteins and the high-affinity active transport system in bacteria. Phil. Trans. R. Soc. (Lond.) B. 1990; 326:341–351. [PubMed: 1970641]

Randle JC, Vernier P, Garrigues A-M, Brault E. Properties of the kainate channel in rat brain mRNA injected Xenopus oocytes: ionic selectivity and blockage. Mol. Cell. Biochem. 1988; 80:121–132. [PubMed: 2459596]

Ruppersberg JP, Schroter KH, Sakmann B, Stocker M, Sewing S, Pongs O. Heteromultimeric channels formed by rat brain potassium-channel proteins. Nature. 1990; 345:535–537. [PubMed: 2348860]

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 1977; 74:5463–5467. [PubMed: 271968]

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg P, Barnard EA. Sequence and functional expression of the GABAA receptor shows a ligand-gated receptor super-family. Nature. 1987; 328:221–227. [PubMed: 3037384]

Unwin N. The structure of ion channels in membranes of excitable eel Is. Neuron. 1989; 3:665–676. [PubMed: 2484344]

Verdoorn TA, Dingledine R. Excitatory amino acid receptor expressed in Xenopus oocytes: agonist pharmacology. Mol. Pharmacol. 1988; 34:298–307. [PubMed: 2901662]

Wada K, Ballivet M, Boulter J, Connolly J, Wada E, Deneris ES, Swanson LW, Heinemann S, Patrick J. Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. Science. 1988; 240:330–334. [PubMed: 2832952]

Wada K, Dechesne J, Shimasaki S, King RG, Kusano K, Buonanno A, Hampson DR, Banner C, Wenthold Rj, Nakatani Y. Sequence and expression of a frog brain complementary DNA encoding a kainate-binding protein. Nature. 1989; 342:684–689. [PubMed: 2556640]

Watkins JC, Evans RH. Excitatory amino acid transmitters. Annu. Rev. Pharmacol. Toxicol. 1981; 21:165–204. [PubMed: 6112965]

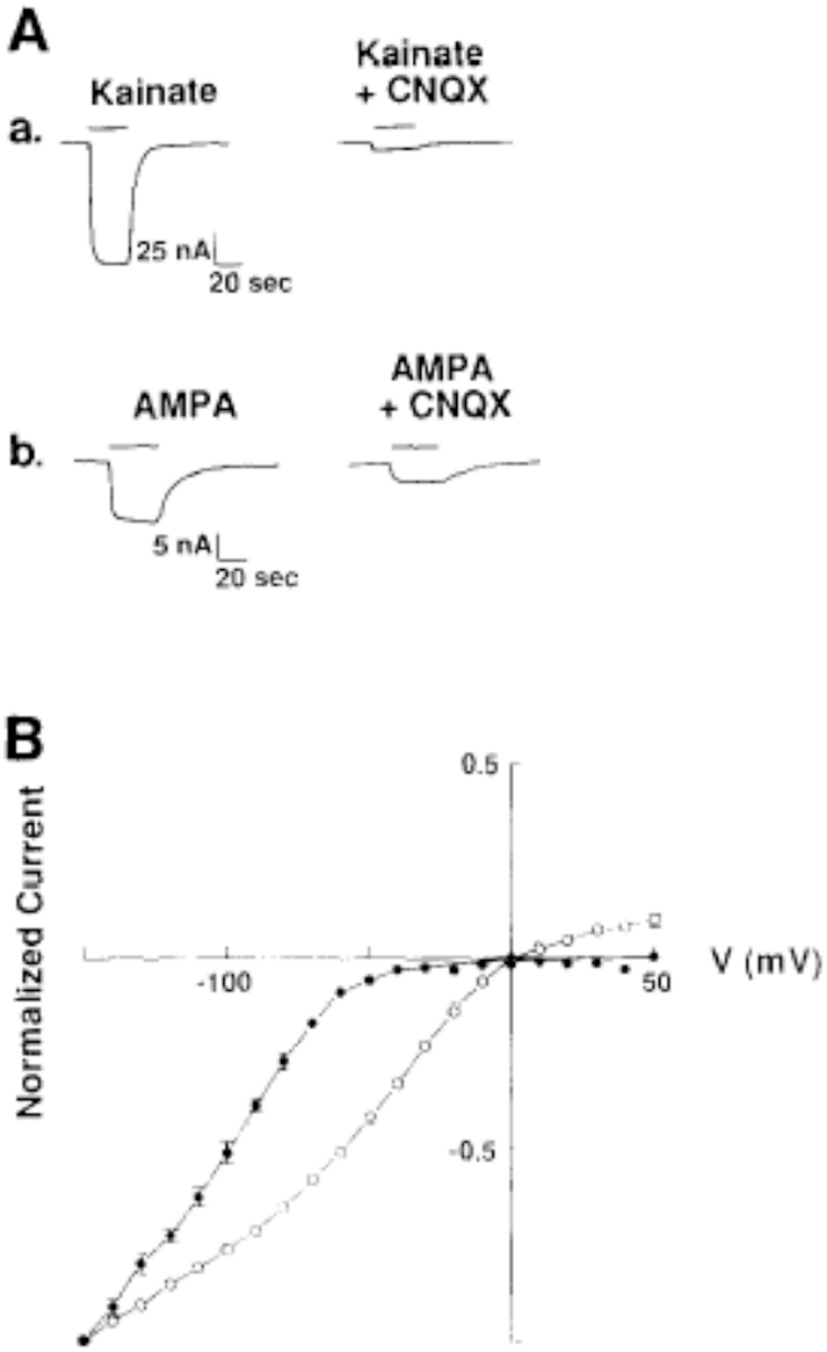

Figure 1. Expression of Functional Kainate/AMPA-Responsive Channels in Xenopus Oocytes after Injection of Synthetic GluR-K1 mRNA

(A) Trace a: Voltage-clamp recording from a single Xenopus oocyte injected with 3 ng of synthetic GluR-K1 RNA. Downward deflections indicate inward currents. The oocyte membrane potential was maintained at −100 mV, and kainate (100 μM) was applied by superfusion for the duration indicated by the bars. The antagonist CNQX (10 μM) reversibly blocked the GluR-K1 kainate response (recovery not shown). Trace b: Current traces obtained from the same egg after application of 10 μM AMPA for the duration indicated. The response was reversibly blocked by CNQX.

(B) Current-voltage plot of the kainate (200 μM)-evoked response of oocytes injected with 3 ng of GluR-K1 (closed circles) or 50 ng of rat cerebral cortex and hippocampal poly(A)$^+$ mRNA (open circles). Responses were normalized to current values evoked at a holding potential of −150 mV. Each point represents the mean and standard error of kainate-evoked currents obtained from 4–7 oocytes.

Figure 2. Alignment of the Deduced Ammo Acid Sequences of the Rat GluR-K2 and GluR-K3 cDNAs with Published Sequences of GluR-K1, Chick KBP, and Frog KBP

The predicted amino-terminal residue of the mature protein is numbered 1, and the preceding residues are indicated by negative numbers. Boxed amino acid residues are those identical in all three of the GluR-K subunits. The four proposed transmembrane domains (M1-M4) are indicated by solid bars. The two sets of arrows denote the borders of two regions of the GluR-K subunits and the KBPs that bear homology to E. coli GlnH (see Figure 3). Smaller residues conserved within the putative M2 region are shaded. Triangles

indicate the position of negatively charged residues flanking the M2 regions of the GluR-K subunits.

The deduced amino acid sequence for GluR-K1 is from Hollmann et al. (1989); that for chick KBP, from Gregor et al. (1989); and that for frog KBP, from Wada et al. (1989).

The DNA sequences of the GluR-K2 and GluR-K3 cDNAs are available on request and have been deposited with the GenBank-EMBL database under accession numbers X54655 and X54656, respectively.

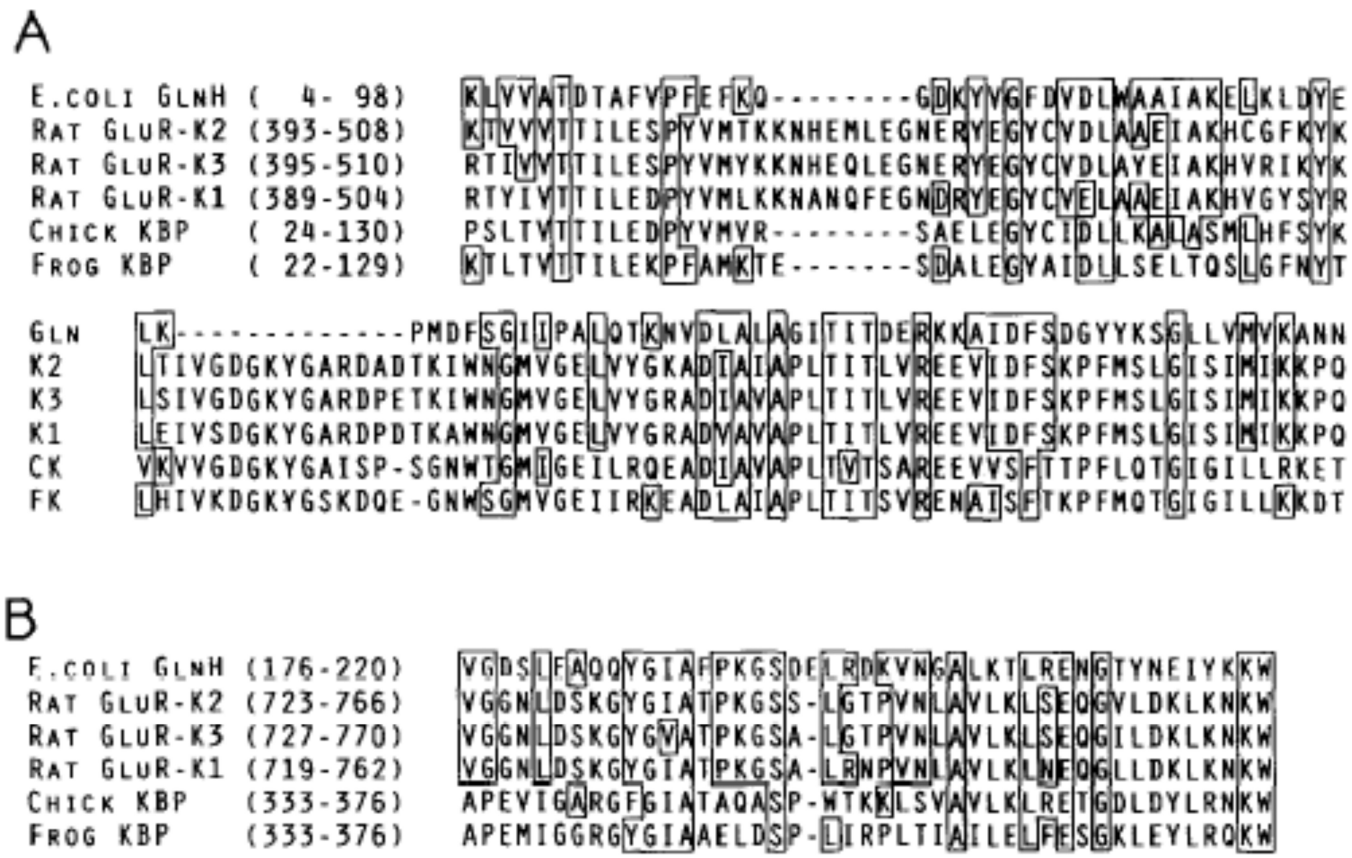

Figure 3. Sequence Homology between E. coli GlnH, GluR-K Subunits, and Two KBPs

(A) Sequence homology between E. coli GlnH (amino acid residues 4-98; Nohno et al., 1986), GluR-K subunits, and two KBPs within the region indicated by the first set of arrows in Figure 2. Exact positions in each protein are shown by numbers in parentheses. Boxed amino acid residues represent identity between GlnH and at least one of the GluR-K subunits or KBPs. In this region, sequence identity between GlnH and each of the kainate receptor subunits is 33% for GluR-K2, 30% for GluR-K3, 30% for GluR-K1, 21% for chick KBP, and 29% for frog KBK. The alignment shown in this figure gives a similarity score that is 12.7 standard deviations above the mean value of those obtained from the alignments of 200 random shuffles of the GluR-K1 sequence (amino acids 389-504) with GlnH. Two gaps were introduced to maximize identities.

(B) Sequence homology between amino acid residues 176 and 220 of E. coli GlnH, GluR-K subunits, and two KBPs in the region indicated by the second set of arrows in Figure 2. In this region, sequence identity between GlnH and each of the kainate receptor subunits is 44% for GluR-K2, 42% for GluR-K3, 47% for GluR-K1, 29% for chick KBP, and 27% for frog KBP. The alignment shown in this figure gives a similarity score that is 12.0 standard deviations above the mean value of those obtained from the alignments of 200 random shuffles of the GluR-K1 sequence (amino acids 719-762) with GlnH.

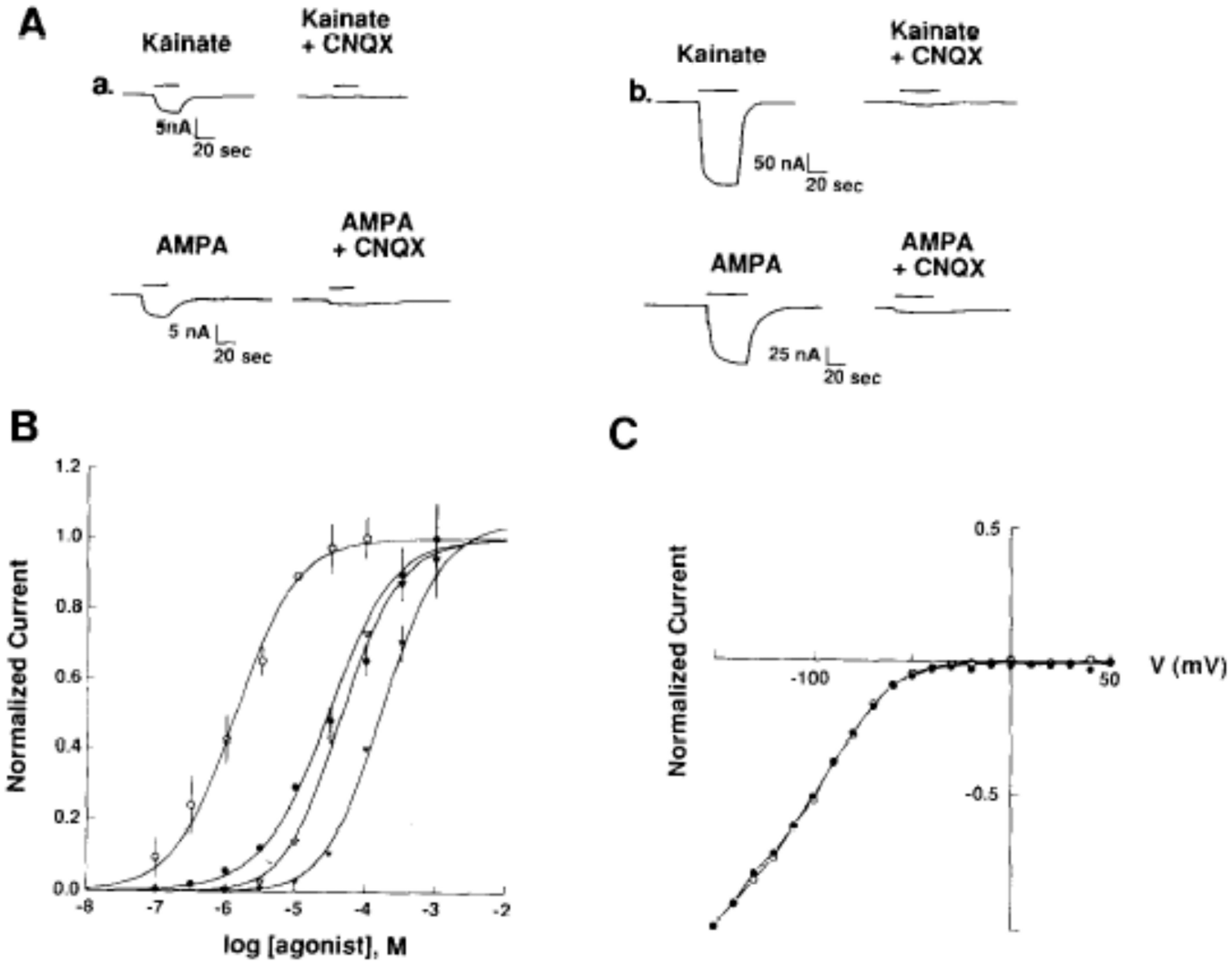

Figure 4. GluR-K2 and GluR-K3 Generate Kainate- and AMPA-Sensitive Channels when Expressed Alone in Xenopus Oocytes

(A) Inward current responses are shown to kainate (100 μM) and AMPA (10 μM) 4–5 days after injection of 10 ng of GluR-K2 (trace a) or 3 ng of GluR-K3 (trace b) mRNA. Membrane potentials were held at −110 mV for GluR-K2. For GluR-K3, the egg was clamped at −70 mV (kainate) and −100 mV (AMPA). All kainate and AMPA responses were inhibited by the antagonist CNQX (10 μM). The same egg injected with either GluR-K2 or GluR-K3 mRNA was used to record the kainate- and AMPA-evoked currents shown in trace a (GluR-K2) and trace b (GluR-K3).

(B) Dose-response curves of GluR-K3 for kainate (closed triangles) and AMPA (closed circles) compared with those of GluR-K1 for kainite (open triangles) and AMPA (open circles). Responses were normalized to average current values evoked at concentrations of 300 μM AMPA and 3 mM kainate. Each point represents the mean and standard error of agonist-evoked currents obtained from 3–6 oocytes, voltage-clamped at −70 mV. EC_{50} values and Hill coefficients derived from curves are listed in Table 1. Curves were fitted as described in Experimental Procedures.

(C) Current-voltage plot of kainate (200 μM) currents evoked in oocytes injected with GluR-K3 RNA (3 ng) (open circles) compared with that obtained from oocytes injected with GluR-K1 RNA (3 ng) (closed circles). Responses were normalized to current values evoked at −150 mV. Each point represents the mean and standard error of kainate-evoked currents obtained from 4–6 oocytes.

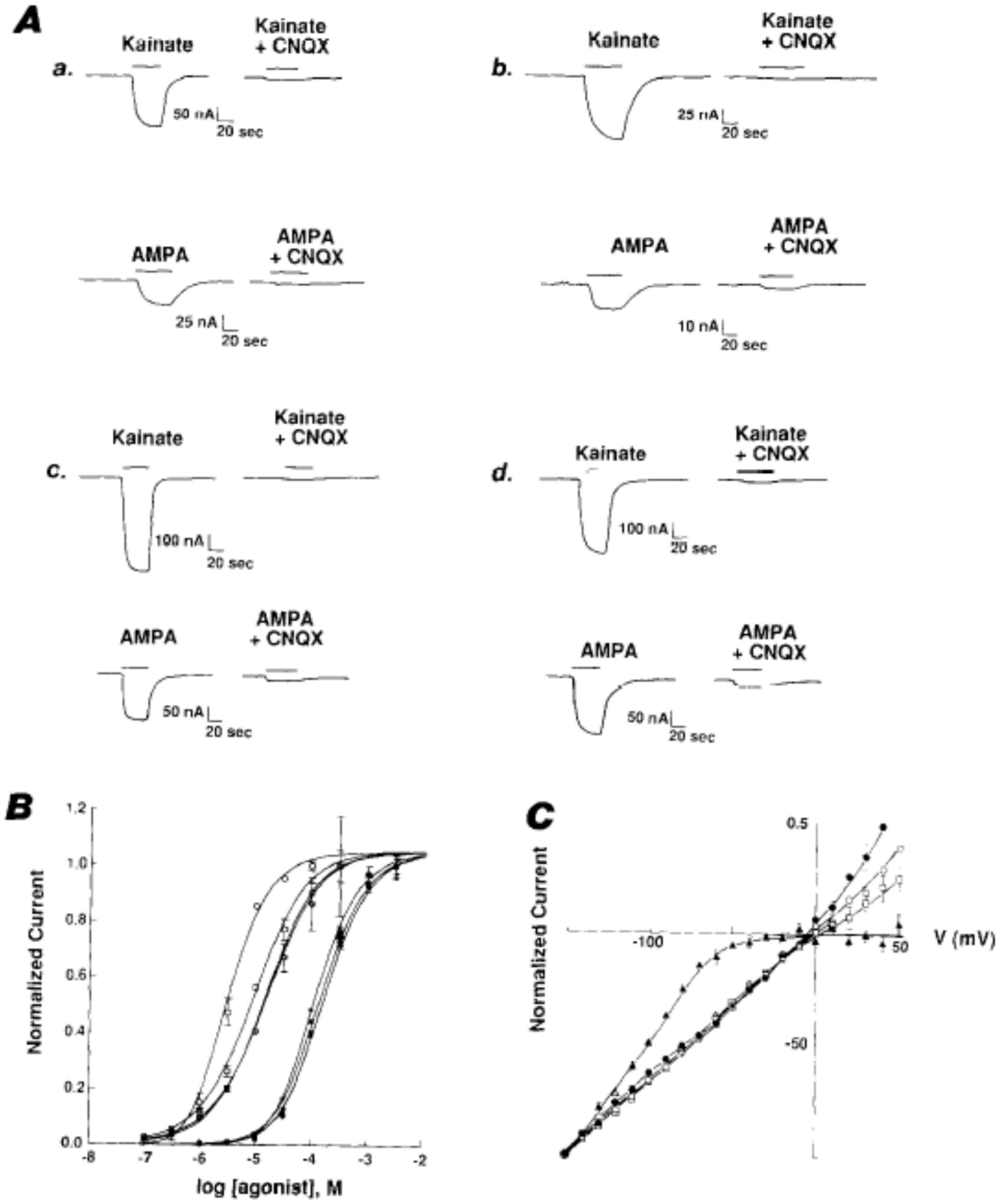

Figure 5. Combinations of GluR-K Subunits Form Distinct Channels when Expressed in Xenopus Oocytes

(A) Current traces recorded from single oocytes 4–5 days after injection with GluR-K1 + GluR-K3 (trace a), GluR-K1 + GluR-K2 (trace b), GluR-K3 + GluR-K2 (trace c), or GluR-K1 + GluR-K2 + GluR-K3 (trace d) mRNA. Oocytes were injected with 3 ng of RNA from each subunit (6 ng total for paired combinations, 9 ng for GluR-K1 + GluR-K2 + GluR-K3). Membrane potentials were held at −70 mV, except for the GluR-K1 + GluR-K2 AMKA (−100 mV) and GluR-K1 + CluR-K2 + GluR-K3 kainate (d, 50 mV) responses. Kainate (100 μM) or AMPA (10 μM) was applied through the bath for the durations indicated by the bars.

Responses were blocked by CNQX (10 μM). For all subunit combinations, the same egg was used to record the kainate- and AMPA-evoked responses shown.

(B) Kainate dose-response curves for GluR-K1 + GluR-K3 (closed triangles), GluR-K1 + GluR-K2 (closed circles), GluR-K3 + GluR-K2 (closed diamonds), and GluR-K1 + GluR-K2 + GluR-K3 (closed squares) together with AMPA dose-response curves for GluR-K1 + GluR-K3 (open triangles), GluR-K1 + GluR-K2 (open circles), GluR-K3 + GluR-K2 (open diamonds), and GluR-K1 + GluR-K2 + GluR-K3 (open squares). For each curve, responses were normalized to average current values evoked at concentrations of 300 μM AMPA and 3 mM kainate. Each point represents the mean and standard error of agonist-evoked currents obtained from 4–6 oocytes and recorded at a holding potential of −70 mV. EC_{50} values and Hill coefficients derived from these curves are listed in Table 1.

(C) Current-voltage plots of kainate currents evoked in oocytes injected with GluR-K1 + GluR-K3 (closed triangles), GluR-K1 + GluR-K2 (closed circles), GluR-K3 + GluR-K2 (open squares), or GluR-K1 + GluR-K2 + GluR-K3 (open circles). Oocytes were injected with 3 ng of each subunit RNA. Responses were normalized to current values evoked at −150 mV. Each point represents the mean and standard error of kainate-evoked currents obtained from 4–7 oocytes.

Table 1

Functional Properties of GluR-K Subunits Expressed in Xenopus Oocytes

Combination of Subunit-Specific GluR-K mRNAs	Peak Amplitudes (nA)		EC_{50} (μM)		Hill Coefficient (Kainate)	Voltage Sensitivity (Kainate)
	Kainate	AMPA	Kainate	AMPA		
GluR-K1	323 ± 183 (12)	26 ± 13 (10)	32 (5)	1.3 (3)	1.4	0
GluR-K2	2 (3)	1 (3)	-	-	-	-
GluR-K3	1120 ± 626 (12)	212 ± 112 (11)	130 (6)	36 (6)	1.3	0
GluR-K1 + GluR-K3	775 ± 413 (10)	168 ± 84 (7)	55 (4)	24 (4)	1.8	0
GluR-K1 + GluR-K2	374 ± 177 (10)	69 ± 30 (7)	110 (3)	2.2 (3)	1.4	1.1
GluR-K3 + GluR-K2	2400 ± 746 (10)	680 ± 376 (9)	110 (9)	19 (5)	1.4	1.0
GluR-K1 + GluR-K2 + GluR-K3	2191 ± 844 (11)	413 ± 109 (6)	100 (4)	13 (3)	1.3	1.0

Xenopus oocytes were injected with 3 ng of each subunit-specific GluR-K RNA (6 ng total for paired combinations, 9 ng for GluR-K1 + GluR-K2 + GluR-K3). Peak Amplitudes represent mean current responses (±SD) to bath application of 100 μM kainate or 30 μM AMPA. Membrane potential was voltage-clamped at −70 mV. Numbers in parentheses represent the number of oocytes (N) used to calculate mean amplitudes. Six other oocytes injected with GluR-K2 RNA showed no detectable response to either kainate or AMPA. EC_{50} values and Hill coefficients were calculated as described in Experimental Procedures. The voltage sensitivity was quantified as the ratio of the slope conductances at −10 mV and −100 mV obtained from the current-voltage plots presented in Figures 1B, 4C, and 5C.