# Molecular Cloning, Expression, and Pharmacological Characterization of humEAA1, a Human Kainate Receptor Subunit

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Abstract: Kainate is a potent neuroexcitatory agent; its neurotoxicity is thought to be mediated by an ionotropic receptor with a nanomolar affinity for kainate. In this report, we describe the cloning of a cDNA encoding a human glutamate ionotropic receptor subunit protein from a human hippocampal library. This cDNA, termed humEAA1, is most closely related to rat and human cDNAs for kainate receptor proteins and, when expressed in COS or Chinese hamster ovary cells, is associated with high-affinity kainate receptor binding. We have successfully established cell lines stably expressing humEAA1. This is the first report of establishment of stable cell lines expressing a glutamate receptor subunit. The relative potency of compounds for displacing [3H]kainate binding of humEAA1 receptors expressed in these stable cell lines was kainate > quisqualate > domoate > L-glutamate  $\gg$  (RS)- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid > dihydrokainate > 6,7-dinitroquinoxaline-2,3-dione > 6-cyano-7nitroquinoxaline-2,3-dione. Homooligomeric expression of humEAA1 does not appear to elicit ligand-gated ion channel activity. Nevertheless, the molecular structure and pharmacological characterization of high-affinity kainate binding of the humEAA1 expressed in the stable cell line (ppEAA1-16) suggest that the humEAA1 is a subunit protein of a human kainate receptor complex. Key Words: Excitatory amino acid receptor-High-affinity kainate receptor-Human glutamate receptor subunit-cDNA cloning-Mammalian expression.

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Excitatory amino acid (EAA) receptors are found throughout the mammalian brain and represent the predominant transmitter receptors mediating synaptic excitation in the CNS. The majority of excitatory synapses in the CNS use glutamate as their neurotransmitter. Glutamate receptors play an important role in CNS physiology and pathology (Monaghan et al., 1989). Based on electrophysiological and pharmacological studies, these receptors have been classified into four main subclasses: N-methyl-D-aspartate (NMDA), (RS)- $\alpha$ -amino-3-hydroxy-5-methyl-4-isox-

azolepropionic acid (AMPA), kainate, and metabotropic types. Molecular studies have now demonstrated that these receptors are highly heterogeneous. At least six different G protein-coupled (metabotropic) glutamate receptors are known to exist, each as a single protein with seven putative membrane-spanning domains (Masu et al., 1991; Tanabe et al., 1992). Even more complex are the ionotropic glutamate receptors, which are heteromeric protein complexes with multiple subunits, each possessing four transmembrane regions, and arranged to form a ligandgated ion channel (Barnard and Henley, 1990). Based on molecular structure and selective agonist activation, subunit proteins for ionotropic glutamate receptors can be divided into NMDA, AMPA, and kainate types. The recent molecular cloning studies of EAA receptor subunits from each of the major subclasses have confirmed this classification. NMDA receptor protein subunits that exhibit homomeric or heteromeric channel activity when activated have recently been cloned from the rat (Moriyoshi et al., 1991; Monyer et al., 1992; Sugihara et al., 1992) and mouse (Kutsuwada et al., 1992; Meguro et al., 1992; Yamazaki et al., 1992). AMPA receptor proteins that have been cloned include rat GluR1, GluR2, GluR3, and GluR4. When expressed as homomeric or heteromeric complexes, they form ligand-gated cation-permeable ion channels that are activated by the agonists AMPA, kainate, or quisqualate (Hollman et al., 1989;

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Abbreviations used: 1S,3R-ACPD, 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, (RS)-α-amino-3-hydroxy-5-methyl4-isoxazolepropionic acid; CHO, Chinese hamster ovary; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EAA, excitatory amino acid; NMDA, N-methyl-D-aspartate; PCR, polymerase chain reaction.

Boulter et al., 1990; Keinanen et al., 1990). The human homologues of rat GluR1, designated GluH1 (Puckett et al., 1991) and HBGR1 (Sun et al., 1992). have also been cloned.

Rat GluR5 homomeric receptor channels show a very small response to glutamate only when its cDNA is expressed in *Xenopus* oocytes (Bettler et al., 1990). Using a mammalian expression system, Sommer et al. (1992) showed that rat GluR5 is capable of forming homomeric channels that can be gated by domoate, kainate, and glutamate. In addition, a rat kainate receptor protein (GluR6) has been identified, which forms homomeric receptor-operated ion channels that are activated by kainate, quisqualate, and glutamate but not AMPA (Egebjerg et al., 1991). Recently cloned rat GluR7 (Bettler et al., 1992) exhibits high sequence identity with glutamate receptor subunits GluR5 and GluR6. The structurally related proteins rat KA1 (Werner et al., 1991), rat KA2 (Herb et al., 1992), mouse  $\gamma$ 2 (Sakimura et al., 1992), and human EAA2 (Kamboj et al., 1992) have also been cloned. Rat GluR7, rat KA1, rat KA2, mouse  $\gamma$ 2, and human EAA2 exhibit [3H]kainate binding but when expressed in a homomeric manner do not exhibit any receptor-operated ion channel properties. Nevertheless, the molecular structure and binding pharmacology of these glutamate receptor subunits suggest that these subunits are a component of the heteromeric receptor complex. Rat KA2 (Herb et al., 1992) and mouse  $\gamma$ 2 (Sakimura et al., 1992) subunits have been shown to interact with the GluR6 subunit when coexpressed, resulting in new properties, such as AMPAsensitive channels, including the formation of agonist-sensitive channels.

The molecular cloning of these cDNAs has led to significant advances in our understanding of various subtypes of glutamate receptors. Human glutamate receptors are of great medical importance because of their postulated role in the mediation of learning and memory acquisition. Also, EAAs can be highly toxic to neurons, and dysfunction of this neurotransmitter system has been implicated in several neurological disorders, such as Alzheimer's disease, Huntington's chorea, epilepsy, Parkinson's disease, amyotrophic lateral sclerosis, AIDS encephalopathy, and dementia complex (Gasic and Hollman, 1992). The central involvement of EAA receptors in the human brain has prompted our efforts toward the isolation, cloning, and functional characterization of these receptors from human brain. Availability of these human brain cloned receptors would allow in vitro drug screening and drug design to discover and develop new receptor-specific drugs. Nonhuman mammalian models are not suitable for this purpose despite high homology of receptor sequences, as minute differences can cause dramatic pharmacological variation between species homologues of the same receptor (Oksenberg et al., 1992). Therefore, drug-receptor interactions should not be extrapolated from animal to human species without verification.

Here we report the cloning, molecular structure, expression in mammalian stable cell lines, and pharmacological characterization of a human kainate receptor protein, termed humEAA1. This humEAA1 appears to be a human homologue of rat KA1 (Werner et al., 1991) and exhibits [3H]kainate binding characteristics suggesting that it is a subunit protein of a human kainate receptor complex.

## MATERIALS AND METHODS

#### Isolation of cDNA for humEAA1

To isolate the cDNA for humEAA1 receptor subunit, the nucleotide sequences of the rat GluR1 receptor subunit (Hollmann et al., 1989), chicken kainate binding protein (Gregor et al., 1989), and frog kainate binding protein (Wada et al., 1989) were compared, to identify regions of homology capable of serving as primer annealing sites for polymerase chain reaction (PCR)-based amplification as described by Kamboj et al. (1992). The following oligonucleotide primers having nonhybridizing flanks bearing HindIII restriction sties were then synthesized:

# 5' GGGGTTTAAGCTTGAGCGT-

CGTCCTCTTCCTGGT 3'

#### 5' GGGGTTTAAGCTTGTGAAG-

# AACCACCAGACGCCG 3'

14714159, 1994, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1046j.1471-4159,1994.2010001x by University Of Georgia Libraries, Wiley Online Library on [22.082024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/ems-and-conditions) on Wiley Online Library for rules of use. OA articles are governed by the applicable Centerior Commons. Licroscope (1471-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1994, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159, 1271-4159

Using human hippocampal cDNA as template and the oligonucleotide primers and reaction conditions for PCR as described by Kamboj et al. (1992), the resulting PCR product having an expected nucleotide length (239 bp) was then purified following agarose gel electrophoresis and subcloned into the phagemid vector pTZ19 (Pharmacia). A comparison of the nucleotide sequence (amplified fragment) with the rat GluR1 revealed only  $\sim 60\%$  identity, indicating that a fragment from a novel human gene had been isolated.

To isolate the cDNA encoding the entire human receptor subunit, a bacteriophage λgt10-based library of human hippocampus cDNA (Clontech) was screened using a PCRgenerated, radiolabeled ( $[\alpha^{-32}P]dCTP$ ) version of the 239-bp amplification product. Of  $1 \times 10^6$  clones screened, probing identified 60 putative clones under the high-stringency hybridization conditions as described by Kamboj et al. (1992). DNA inserts were subcloned into the pTZ18 vector for DNA sequence analysis. Sequencing revealed one partial clone harboring, internally, a region with a nucleotide sequence identical to the sequence of the 239-bp probe. Because the cDNA library did not appear to contain a fulllength clone, an alternative human hippocampus cDNA library constructed in bacteriophage λZAPII (Stratagene) was screened by using a PCR-generated radiolabeled version of the subclone. Screening of  $1 \times 10^6$  clones of this library by hybridization under the stringency conditions described by Kamboj et al. (1992) led to the selection of 50 positive clones. For sequencing, pBluescript-SK phagemids carrying the inserts were excised. DNA sequence analysis identified two clones sharing a sequence overlap. One clone carrying a 2.2-kb insert and representing a 5' region of the open reading was designated pBS/RKLS181. The overlapping clone,

-60 -1	
AGGCCCGGGGTCTCGGGCGCCTTTGGTGTGCTTCCTGCGTGGCTCGTGATGGTCGCCTGCAGCCCGCACTCCTTGAGGATCGCTGCTATC MetProArgValSerAlaProLeuValLeuLeuProAlaTrpLeuValMetValAlaCysSerProHisSerLeuArgIleAlaAlaIle -20 -1 1	30 10
TTGGACGACCCCATGGAGTGCAGCAGAGGGGAGCGGCTCTCCATCACCCTGGCCAAGAACCGCATCAACCGCGCTCCTGAGAGGCTGGGC LeuAspAspProMetGluCysSerArgGlyGluArgLeuSerIleThrLeuAlaLysAsnArgIleAsnArgAlaProGluArgLeuGly	120 40
AAGGCCAAGGTCGAAGTGGACATCTTTGAGCTTCTCAGAGACAGCGAGTACGAGACTGCAGAAACCATGTGTCAGATCCTCCCCAAGGGG LysAlaLysValGluValAspIlePheGluLeuLeuArgAspSerGluTyrGluThrAlaGluThrMetCysGlnIleLeuProLysGly	210 70
GTGGTCGCTGTCCTCGGACCATCGTCCAGCCCAGCCTCCAGCTCCATCATCAGCAACATCTGTGGAGAGAGGAGGTCCCTCACTTCAAA ValValAlaValLeuGlyProSerSerSrrProAlaSerSerSerIleIleSerAsnIleCysGlyGluLysGluValProHisPheLys	300 100
GTGGCCCCAGAGGAGTTCGTCAAGTTCCAGTTCCAGAGATTCACAACCCTGAACCTCCACCCAGCAACACTGACATCAGCGTGGCTGTA ValAlaProGluGluPheValLysPheGlnPheGlnArgPheThrThrLeuAsnLeuHisProSerAsnThrAspIleSerValAlaVal	39 <b>0</b> 130
${\tt GCTGGGATCCTGAACTTCTTCAACTGCACCACCGCCTGCCT$	480 160
${\tt CAATTCCTTATCTCCAAGGACACGCTGTCCGTCCGCATGCTGGATGACACCCGGGACCCCACCCCGCTCCTCAAGGAGATCCGGGACGACG1nPheLeuIleSerLysAspThrLeuSerValArgMetLeuAspAspThrArgAspProThrProLeuLeuLysGluIleArgAspAsp$	570 190
AAGACCGCCACCATCATCCACCCCAACGCCTCCATGTCCCACCACCATCTCCTGAAGGCAGCCGAACTTGGGATGGTGTCAGCCTAT LysThrAlaThrileIleIleHisAlaAsnAlaSerMetSerHisThrIleLeuLeuLysAlaAlaGluLeuGlyMetValSerAlaTyr	660 220
${\tt TACACATACATCTTCACTAATCTGGAGTTCTCACTCCAGAGAACGGACAGCCTTGTGGATGATCGTGTCAACATCCTGGGATTTTCCATT\\ {\tt TyrThrTyrIlePheThrAsnLeuGluPheSerLeuGlnArgThrAspSerLeuValAspAspArgValAsnIleLeuGlyPheSerIle} \\$	750 250
$\label{thm:condition} TTCAACCAATCCCATGCTTTCCTTCCAAGAGTTTGCCCAGAGCCTCAACCAGTCCTGGCAGGAGAACTGTGACCATGTGCCCTTCACTGGGCACAACCAGTCTGTGACCATGTGCCCTTCACTGGGCACAACCAGTCTGACCATGTGACCATGTGCCCTTCACTGGGCACAACCAGAACTGTGACCAAGAACTGTGACCATGTGCCCTTCACTGGGCACAACCAGAACTGTGACCATGTGCCCTTCACTGGGCACAACCAGAACTGTGACCATGTGCCCTTCACTGGGCACAACCAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGCAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGCAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACCATGTGCCCTTCACTGGGAGAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTGTGACAACTAACT$	840 280
$\tt CCTGCGCTCTCCTCGGCCCTGCTGTTTGATGCTGTCTATGCTGTGTGATGCGGTGCAGGAGATGAACCGGAGCCAAGAGATCGGCGTGPTOAlaLeuSerSerAlaLeuLeuPheAspAlaValTyrAlaValValThrAlaValGlnGluLeuAsnArgSerGlnGluIleGlyVal$	930 310
${\tt AAGCCCTTGTCCTGCGGCCCAGATCTGGCAGCACGGCACCAGCCTCATGAACTACCTGCGCATGGTAGAATTGGAAGGTCTTACCLysProLeuSerCysGlySerAlaGlnfleTrpGlnHisGlyThrSerLeuMetAsnTyrLeuArgMetValGluLeuGluGlyLeuThrCagaCagaCagaCagaCagaCagaCagaCagaCagaCag$	1020 340
${\tt GGCCACATTGAATTCAACAGCAAAGGCCAGAGGTCCAACTACGCTTTGAAAATCTTACAGTTCACAAGGAATGGTTTTCGGCAGATCGGCGCGGGHis IleGluPheAsnSerLysGlyGlnArgSerAsnTyrAlaLeuLysIleLeuGlnPheThrArgAsnGlyPheArgGlnIleGly$	1110 370
${\tt CAGTGGCACGTGGCAGGGGCCTCAGCATGGACAGCCTCTATGCCTCCAACATCTCGGACACCTCTCTCAACACCACCCTGGTCGTCGInTrpHisValAlaGluGlyLeuSerMetAspSerHisLeuTyrAlaSerAsnIleSerAspThrLeuPheAsnThrThrLeuValValCalCalCalCalCalCalCalCalCalCalCalCalCa$	1200 400
lem:lem:lem:lem:lem:lem:lem:lem:lem:lem:	1290 430
$\label{thm:coccost} ATGCTCAAGGAGCTGGCAGAGATCCTCCGATTCAACTACAAGATCCGCCTGGTTGGGGATGGCGTGTACGGCGTTCCCGAGGCCAACGGCCMetLeuLysGluLeuAlaGluIleLeuArgPheAsnTyrLysIleArgLeuValGlyAspGlyValTyrGlyValProGluAlaAsnGlyAspGlyValTyrGlyValProGluAlaAsnGlyAspGlyValTyrGlyValProGluAlaAsnGlyAspGlyValTyrGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyValProGluAlaAsnGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAspGlyAs$	1380 460
ACCTGGACGGAATGGTCGGGGAGCTGATCGCTAGGAAAGCAGATCTGGCTGTGGCAGGCCTCACCATTACAGCTGAACGGGAGAAGGTGTATTCPThrGlyMetValGlyGluLeuIleAlaArgLysAlaAspLeuAlaValAlaGlyLeuThrIleThrAlaGluArgGluLysVal	1470 490
$\label{thm:control} ATTGATTTCTCTAAGCCATTCATGACTCTGGGAATTAGCATTCTTTACCGCATTCATATGGGACGCAAACCCGGCTATTTCTCCTTCCT$	1560 520
${\tt GACCCATTTCTCCGGGGGTCTGGCTCTTCATGCTTCTAGCCTATCTGGCCGTCAGCTGTGTCCTCTTCCTGGTGGCTCGGTTGACGCCCASpProPheSerProGlyValTrpLeuPheMetLeuLeuAlaTyrLeuAlaValSerCysValLeuPheLeuValAlaArgLeuThrPro$	1650 550
${\tt TACGAGTGGTACAGCCCACACCCATGTGCCCAGGGCCGGTGCAACCTCCTGGTGAACCAGTACTCCCTGGGCAACAGCCTCTGGTTTCCGTyrGluTrpTyrSerProHisProCysAlaGlnGlyArgCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAlaGlnGlyArgCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAlaGlnGlyArgCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnSerLeuTrpPheProMisProCysAsnLeuLeuValAsnGlnTyrSerLeuGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyAsnGlyA$	1740 580
$\label{thm:constraint}  GTCGGGGGGTTCATGCACCCTCGCCCCTTGTCCACCCGCTGTGTCAGTGGGGGTCTGGTGGGGATTCACGCTGCGGGGGGGG$	1830 610
lem:lem:lem:lem:lem:lem:lem:lem:lem:lem:	1920 640
eq:GACCAGACCGCCATTGAATATGCCACAATTCACCGAGGCTCCAGCATGACCTTCTTCCAAAATTCCCGCTACCAGACCTACCAACGCATGASpGlnThrAlalleGluTyrGlyThrIleHisGlyGlySerSerMetThrPhePheGlnAsnSerArgTyrGlnThrTyrGlnArgMet	2010 670
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CTCCTGGAATCCACCATGAACGAGTACTATCGGCAGCGAAACTGCAACCTCACTCA	2190 730
$\label{thm:control} ATTGGCATGCCAGTTCGGCTTTTTCCGGGACGAGTTTGATCTGGCCATTCTCCAGCTGCAGGAGAACAACCGCCTGGAGATCCTGAAGIleGlyMetProValGlySerValPheArgAspGluPheAspLeuAlaIleLeuGlnLeuGlnGluAsnAsnArgLeuGluIleLeuLys$	2280 760
CGCAAATGGTGGGAAGGAGGGAAGTGCCCCAAGGAGGAGAAGATCACAGAGCTAAAGGCCTGGGAATGGAGAATATTGGTGGAATCTTTGTG ArgLysTrpTrpGluGlyGlyLysCysProLysGluGluAspHisArgAlaLysGlyLeuGlyMetGluAsnIleGlyGlyIlePheVal	2370 790
${\tt GTTCTTATTTGTGGCTTAATCGTGGCCATTTTTATGGCTATGTTGGAGTTTTTATGGACTCTCAGACACTCAGAAGCAACTGAGGTGTCC} \\ {\tt ValleuIleCysGlyLeuIleValAlaIlePheMetAlaMetLeuGluPheLeuTrpThrLeuArgHisSerGluAlaThrGluValSer} \\ {\tt GalleuIleCysGlyLeuIleValAlaIlePheMetAlaMetLeuGluPheLeuTrpThrLeuArgHisSerGluAlaThrGluValSer} \\ {\tt GalleuIleCysGlyLeuIleValAlaIleCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysGlyCysG$	2460 820
GTCTGCCAGGAGATGGTGACCGAGCTGCGCAGCATTATCCTGTGTCAGGACAGTATCCACCCCGGCGGCGGGGGGGG	2550 850
${\tt CCCCGGCCCCCGATCCCCGAGGGGGCGCGGCGGCGGCGGCGGCGACGCTCAGCAAGCGGAAGCTGTGCGGGGGAGGGGAGCCCGACCAGCCGACCAGCCGAGCCGACCAGCAG$	2640 880
$\label{thm:constraint} CTCGCGAGAGACTGGCGGGAGGGCCGCGGGCTGCACGCAC$	2730 910
CTGCGGGCACGCCGTCGCCCGCAGCGAGGAGAGCCTGGAGTTGGAGAAAACCACCAACAGCAGCGAGCCCGAGTAG 2811 LeuArgAlaArgProSerProAlaArgSerGluGluSerLeuGluTrpGluLysThrThrAsnSerSerGluProGluEnd 936	

FIG. 1. Nucleotide sequence of the cDNA encoding the humEAA1 subunit and its deduced amino acid sequence. Nucleotides are numbered in the 5'- to 3'-terminal direction, starting with the first nucleotide of the codon for the putative amino-terminal residue of the mature subunit. Nucleotides -1 to -60 encode a putative signal peptide. Numbers of the nucleotide and amino acid residues are given to the right of each line.

			1	ST(	NAT. DESTIN					•		
	HUMAN RAT HUMAN	EAA1 KA1 EAA2	,	MERSTVLIQP KIISPVLSNL	MPRVSAPLVL MPRVSAPLVL MPAELLLL	LPAWLVMVAC LPAWLLMVAC LIVAFASPSC	431 431 432	MLKELAEILR MLRELAELLP	FNYKIRLVGD FPYRLRLVED	GVYGVP.EAN GLYGAP.EPN	GTWTGMVGEL GTWTGMVGEL GSWTGMVGEL	IARKADLAVA INRKADLAVA
	MOUSE RAT RAT	γ2 GluR5 GluR6	. м	MERSTVLIQP KIISPVLSNL	MPAELLLL GLWTRDTSWT VFSRSIKVLL	LIVAFANPSC LLYFLCYILP CLLWIGYSQG	432 436 436	LLKELSNILG LLRELSTILG	FLYDVKLVPD FTYEIRLVED	GKYGAQND.K GKYGAQDDVN	GSWTGMVGEL GEWNGMVREL GOWNGMVREL	IDHRADLAVA IDHKADLAVA
	RAT RAT	GluR7 GluR1			MPYIFAFF	CTGFLGAVVG	<b>438</b> <b>4</b> 24	LLKELAHILG LAAEIAKHVG	YSYRLEIVSD	GKYGAQDD.K GKYGARDPDT # ##	GOWNGMVREL RAWNGMVGEL # ### ##	VYGRADVAVA
1	SPRS	LRIA	AILDDP	MECSRGERLS MECSRGERLS	ITLAKNRINK	APERLGKAKV	480	GLTITAEREK	VIDESKPEMT	LGISILYRVE	MGRKPGYFSF MGRRPGYFSS	LDPFSPGVWL LDPFSPGVWL
1	. QVLSS	LRMA	AILDDQ AILDDQ	TVCGRGERLA TVCGRGERLA . EPVNVEELA	LALAREQING LALAREQING	IIEVPAKARV IIEVPAKARV	481	AFTITAEREK	VIDESKPEMT	LGISILYRVH	MGRKPGYFSF MGRKPGYFSF NGTNPGVFSF	LDPFSPAVWL
1	VHTT	LRFG	GIFEYVESG. GIFEYADGPN	AQVINAEEHA	FRFAVNTINR FRFSANIINR	NRTLLPNTTL NRTLLPNTTL	486 487	PLAITYVREK PLTITHVREK	VIDESKPEMT AIDESKPEMT	LGISILYRKP LGVSILYRKP	NGTNPGVFSF NGTNPSVFSF	LNPLSPDIWM LNPLSPDIWM
				ERAA				## ## TM1	<del></del>	** **	QKSKPGVFSF	* * *
45	EVDIFE	LLRD	SEYETAETMC	QILPKGVVAV QILPKGVVAV	LGPSSSPASS	SIISNICGEK	530	<b>FMLLAYLAVS</b>	CVLFLVARLT	PYEWYSPB	PCAQGRCNLL PCAQGRCNLL PCLRARPHIL	VNQYSLGNSL
46 47	TYDIOR	LORD	SQYETTDTMC DSFEASRRAC	QILPKGVVSV DQLALGVAAL	LGPSSSPASA FGPSHS.SSV	STVSBICGER SAVQSICNAL	531 535	FMLLAYLAVS YVLLACLGVS	CVLFLAARLS CVLFVIARFT	PYEWYNPB	PCLRARPHIL PCNPD.SDVV	ENQYTLGNSL ENNFTLLNSF
46 49	TYDIQE	INLY	DSFEASKRAC DSFEATKKAC	DOLSLGVAAI DOLALGVVAI	FGPSHS.SSA FGPSQG.SCI	NAVQSICNAL NAVQSICNAL	536 537	YVLLAYLGVS	CVLFVIARFS	PYEWYDPH	PCNPD.SDVV PCNPG.SEVV	ENNETLLNSE
	*		* *		**	. *		* ** TM2	<del>***</del> *	** *	EGRDOTTSDO -TM3	-} -}
95	EVPHFF	VAPE	EFVRFQLQRF	TTLNLHPSNT TTLNLHPSNT ASVSLYPSNE	DISVAVAGIL	NFFNCTTACL	578	WFPVGGFMQQ	GSTIAPRALS	TRCVSGVWWA	FTLIIISSYT FTLIIISSYT FTLIIISSYT	<b>ANLAAFLTVO</b>
96 96	EVPHIC	VGPE TRWK	ETPRLQYLRF HPS.VDSRDL	ASVSLYPSNE FYINLYPDYA	DVSLAVSRIL AISRAVLDLV	KSFNYPSASL LYYNWKTVTV	579	WFPVGGFMQQ	GSEVMPRALS	TRCVSGVWWA	FTLIIISSYT FTLIIISSYT	ANLAAFLIVO
98	EVPHIC	LRWK	HHP.LDNKDT	FYVSLYPDFS FYVNLYPDYA	SLSHAILDLV	QSLKWRSATV	583 584	WFGVGALMRQ WFGMGSLMQQ	GSELMPKALS GSELMPKALS	TRIVGGIWWF TRIIGGIWWF	FTLIIISSYT FTLIIISSYT	anlaafltve Anlaafltve
				VLQLRP	*		5/4	** * * *	# ##	* * **	#########	*****
145	ICAKAE	CLLN	LEKLLR. QFL	ISKOTLSVRM ISKOTLSVRM ISKETLSVRM	LD.DTRDPTP	LLKEIRDDKT	628	RMEVPIESVD	DLADQTAIEY	GTIRGGSSMT	FFQNSRYQTY FFQNSRYQTY	<b>QRMWNYMY</b> SK
146	ICAKAE	CLLR	LEELVR.GFL	ISKETLSVRM RYNIKIRIRO	LD.DSRDPTF	LLKEIRDDKV	629	RMEVPVESAD	DLADQTNIEY	GTIRAGSTMT	FFQNSRYQTY FFQNSRYQTY FFKKSKISTY	<b>QRMWNYMQSK</b>
147	VYDDST	GLIR	LQELIM, APS	RYNLRLKIRQ RYNIRLKIRQ	LPIDSDDSRP	LLKEMKRGRE	633 634	RMESPIDSAD RMESPIDSAD	DLAKQTKIEY DLAKQTKIEY	GAVEDGATMT GAVKDGATMT	FFKKSKISTY FFKKSKISTF	DKMWAFMSSR EKMWAFMSS.
		*	*	KOWQVTAVNI		*	624		MARQTEIAY		ffrrsklavf	## # #
193	ATILLE	LANAS	MSHTILLKAA	ELGMVSAYYT ELGMVSAYYT ELGMTSAFYK	YIFTNLEFSL	ORMOSLVOOR	678	QPSVFVKSTE	EGIARVLN	SNYAFLLEST	MNEYYRORN. MNEYYRORN.	CNLTQIGGLL
194 194	STIIII FYVIFE	CSHE	ISHLVLRKAS TAAEILKQIL	ELGMTSAFYK FMGMMTEYYH	YILTTMDFPI YFFTTLDLFA	LHLDGIVEDS LDLELYRYSG	679 682	QPSVFVKSTE OOSALVKNSD	EGIARVLN EGIORVLT	SRYAFLLEST TDYALLMEST	MNEYHRRLN. MNEYHRRLN. SIEYVTQRN.	CNLTQIGGLL CNLTQIGGFI
196	FRIIFE	CSHT	MAAQILKQAM	AMGMMTEYYR AMGMMTEYYR KLEKNGIGYR	FIFTTLDLYA	LDLEPYRYSG	683 683	RQSVLVKSNE KPSALVKNNE	EGIQRVLT EGIQRTLT	SDYAFLMEST ADYALLMEST	TIEFVTORN. TIEYITORN.	CNLTQIGGLI CNLTQIGGLI
		•	*	₫ QSLNQSWQEN				#	##	** * **	MNEYIEQRKP	* **
243 244	VNILGE SNILGE	SIFN	QSHAFFQEFS TSHPFYPEFV	QSLNQSWQEN RSLNMSWREN	CEASTY	TGPALSSALL LGPALSAALM	725 725	DTKGYGIGMP	VGSVFRDEFD	LAILQLQENN	RLEILKRKWW RLEILKRKWW RLEILKRKWW	EGGKCP
244	VNMTGE	RLLN	IDNPHVSSII	RSLNMSWREN EKWSMERLQA EKWSMERLQA	PPRPETGLLD	GMMTTEAALM	726 729	DEKGYRUGTP	LGSPFRDEIT	LAILQLQEEG	RLEILKRKWW	EGGRCP
246	VNLTGF	RILN	VIAZVRYNOV	EKWSMERLQA QQWRTSDSRD	APRAESGLLD	GVMMTDAALL	730	DSKGYGIGTP	MGSPYRDKIT	IAILQLQEED	TTDKTKNKAM KTHIWKEKAM KTHWWKEKAM	RGSGCP
289	# #		VOELNR	.sqeigvkpl	SCGSAOIWOR	## GTSLMNYLRM		* *** *	** *	* * * * TM4	_ # _ # ### 	
289 290	FDAVYA FDAVHV	VVTA VVVSA	VQELNR VRELNR	.SQEIGVKPL .SQEIGVKPL	SCGSAQIWQH ACTSANIWPH	GTSLMNYLRM GTSLMNYLRM	771	KEEDHRAKGL	GMENIGGIFV	VLICGLIVAI	FMAMLEFLWT FMAMLEFIWT FVAVMEFIWS	LRHS.EASEV
294	DDAVYN	ATAV	SH R	.SQEIGVKPL .ASQLTVSSL .FPOMTVSSL	OSERHKPWRL.	GPREMNI.IKE	772 775	KEEDHRAKGL EEDSKEASAL	GMENIGGIFV GVENIGGIFI	VLICGLIIAV VLAAGLVLSV	FVAVMEFIWS FVAIGEFLYK	TRRSAESEEV SRKNNDVEQC
296 276		VSVC MAEA	YQR FQSLRRQRID	.FPOMTVSSL .APOMTVNSL ISRRGNAGDC	QCHRHKAWRF LANPAVPWGQ	GGRFMNFIKE GIDIQRALQQ	776 776 774	EEESKEASAL EEENKEASAL GDSKDKTSAL	GVQNIGGIFI GIQKIGGIFI SLSNVAGVFY	VLAAGLVLSV ILIGGLGLAM	FVAVGEFLYK LVAVGEFLYK LVALIEFCYK	SKKNAQLEKR LRKTAEREQR SRSESKRMKG
334	* *	TGHI	EFN.SKGQRS	NYALKILQFT	RNGFRQIGQW	HVAEGLSMD.		*	•	* **	# ## AVPPPRPPI.	
335	VEYDGL	TGRV	EFN.SKGQRT	NYALKILOFT NYTLRILEKS NYTLRILEKS	RQGBREIGVW	YSNRTLAMN.	820 822	SVCQEMM SVCQEML	TELRSIILCQ QELRHAVSCR	DNIHPRRRRS KTSRSRRRR	GGLPPQPPV. PGGPSRALLS	LEERRPR LRAVREMRLS
336 336	ARWDGI AHWEGI	TGRI	TFNNTDGLRK TFNKTNGLRT	DFDLDIISLK DFDLDVISLK	EEGTKKIGIW EEGLEKIGTW	NSNSGLNMTD DPASGLNMTE	825	LSFNAIM	EELGISLKNO	KKLKKKSRTK	PGGPSRALLS GKSSFTSILT	CHORRTORKE
338	AQWEGL	TGRI	VENKTSGLRT	DFDLDIISLK NYTLHVIEMK	EDGLEKVGVW	SPADGLNITE	826 824	SFCSTVA FCLIPQQSIN	DEIRFSLTCQ EAIRTSTLPR	RRLKHKPQPP NSGAGASGGG	LL MMVKTDAVIN GSGENGRVVS	METFNDRRLP QDFPKSMQSI
	. SHLYA	SNIS	DTLFNTTLVV	TTILENPYLM	LKGNHQEMEG		863	GTATLSNGKL	CGAGEPDQLA	ORLAGEAALV	ARGC	.THIRVCPEC
383 383	.ATTLE	INLS	QTLANKTLVV QTLANKTLVV	TTILENPYUM TTILENPYUM TTILENPYUM TTILEEPYUM TTILEEPYUM TTILEEPYUM TTILEEPYUM TTILEEPYUM TTILEEPYUM	RRPNFQGLSG RRPNFQALSG	NERFEGFCVD NERFEGFCVD	869 869	NGKLYSAGAG	GDAGSAHGGP	ORLLDDPGPP	SGARPAAPTP GGPRPQAPTP	CTHVRVCQEC
386 388	SOKGKP	ANIT	DSLSNRSLIV DSLTNRSLIV	TTILEEPYVL TTILEEPFVM	FKKSDKPLYG FRKSDRTLYG	NDRFEGYCID NDRFEGYCID	872	TVA				
374	ATDAQA	.GGDN	SSVQNRTYIV	TTILEDPYVM	LKKNANQFEG †	NDRYEGYCVE	873 874	PSMSHSSGMP	LGATGL	***		
							906	PALPGPAGST	VASAQRGEPG	LEWEKTTNSS VGQDHQQQRA	EPE	
							919	RRIGALRASG	AGAPPRGLGV	PALATSPPRP	RPGPAGPREL RPGPTGPREL	AEHE

carrying a 3.1-kb insert and representing the remaining 3' region of the open reading frame, was designated pBS/RKLS161. These overlapping clones were used to construct a full-length cDNA containing the entire open reading frame.

# Expression of humEAA1 in mammalian cells

For transient expression in mammalian cells, cDNA coding for the human EAA1 receptor was incorporated into the mammalian expression vector pcDNA1 (Invitrogen). COS1 cells were transfected with 8 µg of DNA (as pcDNA1-humEAA1) per 10<sup>8</sup> COS cells, by diethylaminoethyl-mediated DNA transfection, and were treated with chloroquine essentially as described by Kamboj et al. (1992).

For stable expression in mammalian cells, cDNA coding for the human EAA1 receptor was incorporated into the mammalian expression vector pRC/CMV (Invitrogen). Chinese hamster ovary (CHO) cells were transfected with 3  $\mu$ g of DNA (as pRC/CMV-humEAA1) per  $5 \times 10^5$  CHO cells, by the calcium phosphate-DNA coprecipitation procedure, as described by Sambrook et al. (1989). Cells resistant to neomycin were selected in 10% fetal bovine serum-supplemented  $\alpha$ -minimal essential medium containing G418. Individual colonies of G-418-resistant cells were isolated  $\sim 2-3$  weeks later and then propagated for northern blot analysis and for subsequent use in ligand binding assays. A cell line stably expressing humEAA1, designated as ppEAA1-16, was established and propagated for further ligand binding studies.

# [3H]Kainate binding

Binding studies were performed essentially as described by Kamboj et al. (1992). Frozen CHO cells from the stable cell line expressing the human EAA1 subunit were lysed, by suspension in ice-cold purified water, and then centrifuged for 20 min at 50,000 g. The resulting membrane pellets were frozen at -80°C for at least 24 h. For binding assays, to remove any endogenous glutamate, the membrane preparations were washed by resuspending in >100 volumes of 50 mM Tris-HCl buffer (pH 7.5 at 5°C) and centrifuged for 10 min at 50,000 g.

[³H]Kainate binding experiments (including displacement studies using nonradioactive competitive ligands) were performed by incubating washed membranes (100–150 µg of protein per sample) with [³H]kainate (5 nM) in the same buffer as used for washing, in a total volume of 1 ml. L-Glutamate (1 mM) was used to define nonspecific binding. The binding reaction was performed in an ice bath for 60 min after addition of the membrane suspension. Bound ligand was separated from free ligand by rapid filtration through Whatman GF/B filters. The saturation and Scatchard data were analyzed with the aid of the computer software program INPLOT (Graph Pad, San Diego, CA, U.S.A.).

#### Materials

AMPA, kainic acid, NMDA, domoic acid, quisqualic acid, dihydrokainate, 6,7-dinitroquinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) were purchased from Tocris Neuramin (Essex, U.K.). L-Glutamate (disodium salt) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

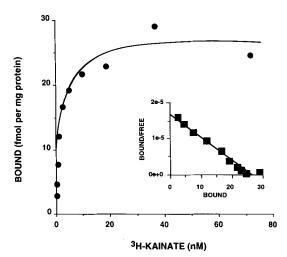
## RESULTS

The PCR-mediated DNA amplification, using human hippocampal cDNA as template, the oligonucleotide primers, and reaction conditions for PCR as described by Kamboj et al. (1992), resulted in generation of an amplified product having an expected nucleotide length (239 bp). A comparison of the nucleotide sequence (amplified fragment) with the rat GluR1 (Hollmann et al., 1989) revealed only  $\sim 60\%$  identity, indicating that a fragment from a novel human gene had been isolated. A human brain cDNA library derived from hippocampus was screened using this PCR-generated probe.

Two overlapping cDNA clones encoding a human glutamate receptor subunit, designated as humEAA1, were isolated and subjected to DNA sequence analysis. The nucleotide sequence analysis of the cloned cDNA revealed an open reading frame encoding 956 amino acid residues (Fig. 1). An analysis of the deduced amino acid sequence of humEAA1 shows that the amino-terminus has a stretch of hydrophobic amino acids, serving as a putative leader sequence. The first 20 amino acids are likely to be cleaved off to form the mature protein, which is predicted to start with a serine residue at the amino-terminus (von Heijne, 1986). The proposed mature protein consists of 936 amino acids, with a calculated molecular weight of 105,080.

The AMPA/kainate/NMDA receptor subunits are thought to conform to a structure in which a large amino-terminal extracellular domain is followed by a region containing four transmembrane domains (TM1-TM4). The locations of these transmembrane domains in the humEAA1 protein sequence are similar to those proposed for various subunits of AMPA/kainate/NMDA receptors (Keinanen et al., 1990) (Fig. 2). Based on this assignment, humEAA1 consists of a 526-amino-acid N-terminal extracellular domain, followed by a region containing four putative transmembrane domains (TM1, spanning residues

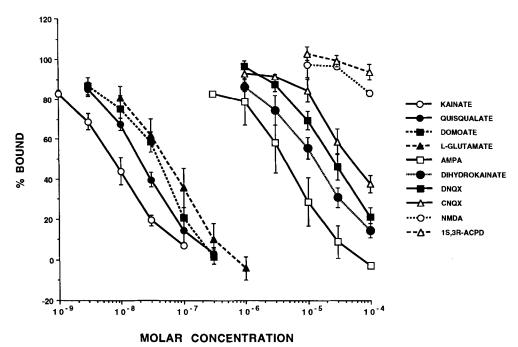
FIG. 2. Alignment of the deduced amino acid sequence of the human EAA1 receptor subunit with seven published rat, mouse, and human glutamate receptor subunits. The sequences of rat GluR1, rat GluR5, rat GluR6, rat GluR7, rat KA1, mouse γ2, human EAA2, and the human EAA1 polypeptide were aligned with the aid of the computer program Pileup [the sequence analysis software package by Genetics Computer Group, Inc. (Devereux et al., 1984)]. Dotted lines indicate gaps introduced for better alignment. Asterisks indicate positions at which the identical amino acid is found. All polypeptide sequences are numbered from the proposed mature N-terminus. The predicted signal peptide sequences and transmembrane regions TM1–TM4 are marked. Solid circles indicate potential N-linked glycosylation sites in the humEAA1; solid triangles indicate calmodulin-dependent protein kinase type II consensus phosphorylation sites (30) in the predicted intracellular domains (between TM1 and TM2 and between TM3 and TM4).



**FIG. 3.** Representative saturation and Scatchard (**inset**) plots of [<sup>3</sup>H]kainate binding to humEAA1-expressing stable cell line CHO cell membranes. Washed membranes were incubated with [<sup>3</sup>H]-kainate in the absence (total binding) or presence (nonspecific binding) of 1 m*M* L-glutamate in a 1-ml volume at 0°C for 1 h. Bound ligand was separated from free ligand by rapid filtration. Data are expressed as amount of specific [<sup>3</sup>H]kainate bound (total minus nonspecific).

527-546, inclusive; TM2, spanning residues 571-589; TM3, spanning residues 600-618; and TM4, spanning residues 785-805), and, finally, an extracellular carboxy-terminal domain of 131 amino acid residues.

The predicted human EAA1 polypeptide shares significant amino acid identity with rat and human glutamate receptor subunits: GluR1, 35.3%; GluR2, 35.4%; GluR3, 35.5%; GluR4, 34.4%; GluR5, 41.8%; GluR6, 44.2%; GluR7, 41.6%; KA1, 94.2%; KA2, 69.0%; humEAA2, 69.0%; and NMDAR1, 25.2% (see Hollman et al., 1989; Keinanen et al., 1990; Bettler et al., 1990, 1992; Egebierg et al., 1991; Morivoshi et al., 1991; Werner et al., 1991; Herb et al., 1992; Kamboj et al., 1992). The human EAA1 has  $\sim 37\%$  amino acid sequence identity with the cloned chicken kainate binding protein (Gregor et al., 1989) and frog kainate binding protein (Wada et al., 1989). Sequence conservation is most striking within the region encompassed by the transmembrane domains, where various cloned AMPA/kainate receptor subunits share >50% sequence identity with the human EAA1 receptor subunit. This would predict that the humEAA1 polypeptide is a glutamate-gated ion channel receptor subunit. The human EAA1 receptor subunit shares ~94% amino acid identity with rat KA1 (Werner et al., 1991) and appears to be a human homologue of rat KA1. The human EAA1 receptor subunit differs most strikingly from this rat receptor in the proposed carboxy-terminal extracellular domain (Fig. 2). A stretch of 40 amino acid residues at the extreme carboxy-terminus, starting at residue Cys<sup>896</sup>, has no significant identity with the corresponding region in rat KA1. Also, the proposed mature rat KA1 protein is composed of 935 amino acid residues. whereas the predicted human EAA1 mature protein is



**FIG. 4.** Displacement curves of [ $^3$ H]kainate binding to humEAA1-expressing stable cell line CHO cell membranes by EAA analogues. Washed humEAA1-expressing stable cell line CHO cell membranes were incubated with [ $^3$ H]kainate (5 nM). Nonspecific binding was determined with 1 mM  $\bot$ -glutamate. Data are mean  $\pm$  SE (bars) values, expressed as percentages of specific [ $^3$ H]kainate binding from three experiments, performed in triplicate.

**TABLE 1.** Affinities of EAA analogues for [<sup>3</sup>H]kainate binding to humEAA1 receptors expressed in CHO cells from a stable cell line (ppEAA1-16)

Compound	$K_{i}$ (n $M$ )	Hill coefficient
Kainate	$2.29 \pm 0.43$	$0.936 \pm 0.042$
Ouisqualate	$6.43 \pm 1.36$	$1.036 \pm 0.043$
Domoate	$10.5 \pm 2.22$	$1.111 \pm 0.073$
L-Glutamate	$18.3 \pm 6.59$	$1.147 \pm 0.077$
AMPA	$1.970 \pm 1.260$	$1.093 \pm 0.068$
Dihydrokainate	$3.840 \pm 983$	$0.859 \pm 0.139$
DNOX	$8.490 \pm 2.550$	$0.961 \pm 0.013$
CNOX	$17,200 \pm 4,490$	$0.920 \pm 0.043$
1S.3R-ACPD	>100,000	_
NMDA	>100,000	_

Data are mean  $\pm$  SE values of three separate experiments, each performed in triplicate. [ $^3$ H]Kainate binding was conducted with a radioligand concentration of 5 nM. Reactions were initiated by addition of washed membrane suspensions, and samples were incubated on ice in a 1-ml volume per sample for 1 h. Protein content per tube was  $\sim 150 \ \mu g$ . Reactions were terminated by rapid filtration through Whatman GF/B filters.

composed of 936 amino acid residues. The human EAA1 subunit has eight potential N-glycosylation sites within the proposed amino-terminal extracellular domain, which are also present in rat KA1. The human EAA1 subunit has one potential N-glycosylation site within the proposed carboxy-terminal extracellular domain, and this site is not present in rat KA1. The proposed intracellular domains between TM1 and TM2 and between TM3 and TM4 contain consensus phosphorylation sites for Ca<sup>2+</sup>-calmodulin-dependent protein kinase type II and protein kinase C (Kemp and Pearson, 1990). These enzymes have been suggested to play an important role in the induction and maintenance of long-term potentiation (Kennedy, 1989). The conservation of some cysteine residues and some putative N-glycosylation and phosphorylation sites in human EAA1 indicates structural features similar to the previously described glutamate receptor subunits (Fig. 2).

Human EAA1-specific mRNA was transcribed in vitro and injected into *Xenopus* oocytes, to test whether this subunit can form a homooligomeric ion channel. We did not record any responses to the application of glutamate receptor agonists in a large number of oocytes tested. We did not detect any indication of ion channel activity when humEAA1 RNA was analyzed in combination with RNA encoding the humEAA2 subunit (H. Sudan and P. N. R. Usherwood, personal communication).

CHO cell lines stably expressing humEAA1 were established as described in Materials and Methods. This is the first report describing successful establishment of stable cell lines expressing a glutamate receptor subunit. The binding of selective EAA ligands to washed and dialyzed membranes prepared from the CHO cells stably expressing humEAA1 was exam-

ined, and high-affinity binding of [3H]kainate was found. In saturation analysis experiments (three experiments), [ ${}^{3}H$ ]kainate bound with a  $K_{D}$  of 2.3  $\pm$  0.37 nM and a  $B_{\rm max}$  of 20.8  $\pm$  4.0 fmol/mg of protein. Figure 3 shows a representative saturation curve and Scatchard plot from these experiments. When the NMDA receptor ligand [3HlCGS-19755 (10 nM)] (Murphy et al., 1988) or the AMPA receptor ligand  $[^3H]AMPA (5 nM) (Honoré et al., 1982)$  was used, no specific binding was observed (data not shown). Kainate was the most potent displacer of [3H]kainate (5 nM) binding, followed by quisqualate, domoate, and then L-glutamate (Fig. 4). The  $K_i$  values for these compounds were in the nanomolar range (Table 1). AMPA, dihydrokainate, and the quinoxalinedione AMPA receptor antagonists, DNQX and CNQX, exhibited affinity constants for this receptor in the micromolar range. NMDA, as well as the selective metabotropic (G protein-coupled) EAA agonist 1S.3R-ACPD, did not affect [3H]kainate binding at up to 100  $\mu M$  (Fig. 4 and Table 1). The relative potency of compounds in displacing [3H]kainate binding was kainate > quisqualate > domoate > L-glutamate ≥ AMPA > dihydrokainate > DNQX > CNQX (Fig. 4). This pharmacological profile is similar to that of the cloned humEAA2 (Kamboj et al., 1992) receptor subunit profile, which was kainate > quisqualate > domoate > L-glutamate > DNQX > dihydrokainate > CNQX > AMPA; however, humEAA1 has considerably lower affinities for dihydrokainate, DNQX, and CNOX.

# **DISCUSSION**

We have isolated a new member of the human EAA receptor gene family, humEAA1, that has a nanomolar affinity for kainate. Human EAA1 has ~35% amino acid sequence identity with the cloned rat AMPA (GluR1-GluR4) receptor subunits and  $\sim 37\%$ amino acid sequence identity with the cloned chicken kainate binding protein (Gregor et al., 1989) and frog kainate binding protein (Wada et al., 1989). It has higher sequence identity with the cloned rat kainate [GluR5-GluR7 (42-44%)] receptor subunits and has even higher sequence identity with the cloned rat, mouse, and human high-affinity kainate (KA1, 94%; γ2, KA2, and humEAA2, 69%) receptor subunits. The human EAA1 receptor subunit appears to be a human homologue of rat KA1. It differs most strikingly from the rat KA1 (Werner et al., 1991) receptor subunit in the proposed carboxy-terminal extracellular domain. A stretch of 40 amino acid residues at the extreme carboxy-terminus, starting at residue Cys<sup>896</sup>, has no significant identity with the corresponding region in rat KA1. There are only five of 40 amino acid residues that are identical in this region. The functional significance of this difference is not known at present. Also, the proposed mature rat KA1 protein is composed of 935 amino acid residues, whereas the predicted mature humEAA1 protein is composed of 936 amino acid residues. The deduced amino acid sequence of human EAA1 is entirely consistent with the proposed structure of subunits of ligand-gated ion channels, which is based on four membrane-spanning  $\alpha$ -helices following an extracellular amino-terminal domain. Sequence conservation between humEAA1 and other glutamate receptor subunits is most striking within the region encompassed by the transmembrane domains, and also there is conservation of some cysteine residues and some putative N-glycosylation and phosphorylation sites in humEAA1; this would predict that the humEAA1 polypeptide is a glutamate-gated ion channel receptor subunit.

We did not detect ion channel activity when humEAA1 RNA was injected into Xenopus oocytes either singly or in combination with RNA encoding the humEAA2 subunit. The electrophysiological results suggest that either the expression of human EAA1 in *Xenopus* is poor or that humEAA1 encodes a subunit that requires at least one additional subunit, other than humEAA2, to form a fully functional receptor-activated ion channel. Absence of homooligomeric channel activity has been reported for other subunits of the EAA receptor family, such as the kainate binding subunit protein from chick brain (Gregor et al., 1989) or frog brain (Wada et al., 1989), rat GluR7 (Bettler et al., 1992), rat KA1 (Werner et al., 1991), rat KA2 (Herb et al., 1992), mouse  $\gamma$ 2 (Sakimura et al., 1992), and humEAA2 (Kamboi et al., 1992). However, the rat KA2 subunit has been shown to interact with rat GluR5 and GluR6 subunits to produce new properties, such as AMPA-sensitive channels, including the formation of agonist-sensitive channels (Herb et al., 1992). The functional data that was shown for mouse  $\gamma^2$  (enhanced kainate-induced currents when coexpressed with mouse GluR6) also strengthen the contention that these proteins are subunits of heteromeric kainate ionotropic EAA receptor complexes (Sakimura et al., 1992).

However, humEAA1 has pharmacological characteristics that strongly suggest it is a subunit protein for a kainate-type of ionotropic EAA receptor. Human EAA1 receptor protein expressed in the stable cell line exhibited high-affinity [ ${}^{3}$ H]kainate binding with a  $K_{\rm D}$ of  $2.3 \pm 0.37$  nM. This value is in close agreement with  $K_D$  values for the high-affinity [ $^3$ H]kainate binding sites found in brain membranes (Monaghan et al., 1989) and also with [3H]kainate binding site on cells expressing rat KA1 (Werner et al., 1991). Although two high-affinity [ ${}^{3}$ H]kainate binding sites ( $K_{\rm D}$  of 5 and 50 nM) have been identified using brain tissues (Monaghan et al., 1989), we observed only one [3H]kainate binding site on cells expressing humEAA1. The rank order of displacement affinities was kainate > quisqualate > domoate > L-glutamate > AMPA dihydrokainate > DNQX > CNQX ≫ NMDA = 1S, 3R-ACPD. This is similar to that of the rank order of potency that was reported for the recently

described humEAA2 (Kamboj et al., 1992) receptor subunit, which was kainate > quisqualate > domoate > L-glutamate > DNQX > dihydrokainate > CNQX > AMPA  $\geqslant$  NMDA = 1S.3R-ACPD, but humEAA1 has considerably lower affinities for dihydrokainate, DNOX, and CNOX. The humEAA1 pharmacological profile is also similar to the rank order of potency that was reported for the recently described rat KA1 receptor (Werner et al., 1991). Like humEAA1, rat KA1 also exhibits high-affinity [3H]kainate binding but does not appear to support channel activity when expressed as a homomeric receptor complex. Furthermore, the pharmacology of [3H]kainate binding displacement was also similar to that observed when channel activity was studied using the rat GluR6 receptor clone (Egebjerg et al., 1991). Agonist potency at the rat GluR6 receptor was kainate > quisqualate > L-glutamate > AMPA, and the quinoxalinedione AMPA antagonist CNQX had a  $K_i$  of 4  $\mu M$ . [3H]Kainate binding of humEAA1 exhibited the same rank order of agonist potency; however, it has a considerably lower  $K_i$  value for CNQX (17.2  $\mu M$ ). The affinity of CNQX for the human EAA1 was about fourfold lower than that for the rat GluR6 receptor protein but was  $\sim$ 33-fold lower than that for cloned AMPA receptors. For example, the  $K_i$  value for CNQX using the rat GluR1 receptor was 0.519  $\mu M$ (Dawson et al., 1990). This is consistent with previous evidence that quinoxalinedione AMPA antagonists can block the electrophysiological effects of kainate and are selective but not specific for AMPA receptors (Honoré et al., 1988). The pharmacological profile of humEAA1 differs from that of the rat GluR6 and rat GluR7 subunits. Human EAA1 has a 30-40-fold higher affinity for kainate than the rat GluR6 and rat GluR7 (Bettler et al., 1992). Human EAA1 has a lower affinity for domoate than kainate, whereas rat GluR6 and rat GluR7 have higher affinities for domoate than kainate (Bettler et al., 1992).

HumEAA1 has the characteristics of a subunit of a human high-affinity kainate receptor, based on its molecular structure and binding pharmacology. The successful cloning of the humEAA1 cDNA should therefore lead to a better understanding of the molecular nature of the high-affinity kainate receptors and their role in normal and diseased human CNS. Because drug-receptor interactions should not be extrapolated from animal to human species without verification (Oksenberg et al., 1992), the availability of a human high-affinity kainate receptor subunit would allow for drug screening and drug design of receptor-selective drugs based on human receptors.

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