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A glutamate receptor channel with high affinity for domoate and kainate

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The non-NMDA family of glutamate receptors comprises a growing number of structurally related subunits (GluR-A to -D or -1 to -4; GluR-5, -6; KA-1). GluR-A to -D appear to constitute the major AMPA receptor subtypes but the functional and pharmacological characteristics of the other subunits are unresolved. Using a mammalian expression system we demonstrate here that homomeric GluR-5 receptors exhibit properties of a high affinity domoate (K_D ~2 nM) and kainate (K_D ~70 nM) binding site. For these receptors, the rank order of ligands competing with [^{3}H]kainate binding was domoate >> quisqualate ≈ glutamate >> AMPA ≈ CNQX. The respective receptor channels were gated in decreasing order of sensitivity by domoate, kainate, glutamate and AMPA. In contrast to recombinantly expressed GluR-A to -D channels, currents elicited at GluR-5 receptor desensitize channels to all agonists. This property is characteristic of currents in peripheral neurons on sensory ganglia. These findings suggest the existence of at least two distinct types of non-NMDA receptor channels, both gated by AMPA and kainate, but differing in pharmacology and current properties.

Key words: domoate binding/dorsal root ganglia/glutamate receptor/high-affinity kainate receptor/ion channel

Introduction

L-glutamate-gated cation channels show different properties and distributions in the central nervous system (Monaghan *et al.*, 1989). On the basis of specific agonists these channels have been termed NMDA, AMPA−low affinity kainate and high affinity kainate receptors. The latter two are collectively referred to as non-NMDA receptors. The molecular biology of non-NMDA receptors has uncovered a growing number of sequence-related subunits whose recombinant expression in homomeric and heteromeric assemblies sheds new light on the classification of these receptors.

It is now recognized that a family of four closely related subunits comprises the constituents of the AMPA−low affinity kainate subtype of glutamate-gated channels. As judged from the functional characteristics and widespread expression of its members in brain (Monyer *et al.*, 1991), this family appears to mediate the bulk of the fast excitatory glutamatergic neurotransmission. Receptors formed from these subunits or their combinations display high affinity binding of [^{3}H]AMPA (K_D ~5 nM) and a 1000-fold lower affinity to kainate (Keinänen *et al.*, 1990). Channels intrinsic to these receptors can be gated by AMPA, glutamate and also by kainate. However, current responses differ significantly when various agonists are used. Whereas AMPA- and glutamate-elicited currents exhibit fast rise and fade times, kainate-evoked currents do not desensitize. Futhermore, the ratio of peak to steady state component of glutamate- and AMPA-elicited currents is dependent on which of two modules, Flip or Flop, resides in the subunits (Sommer *et al.*, 1990). No such modulation with respect to desensitization is seen when kainate serves as agonist.

Recently, a specific position (Q/R site) within a putative channel-forming segment of the GluR-A to -D subunits has been identified as a critical determinant of mono- and divalent cation flow through AMPA receptor channels (Hume *et al.*, 1991; Verdoorn *et al.*, 1991). According to the gene sequences, this position should be occupied by a glutamine residue (Q). However, as a result of RNA editing the GluR-B subunit generally carries an arginine (R) at this position (Sommer *et al.*, 1991). This positively charged R residue dominates permeability and gating properties of AMPA channels formed in the presence of the GluR-B subunit (Hume *et al.*, 1991; Verdoorn *et al.*, 1991; Burnashev *et al.*, 1992).

The responsiveness of GluR-A to -D receptor channels to kainate suggests a blurred distinction between AMPA and low affinity kainate receptors. However, in brain there exist two high affinity binding sites for kainate (K_D values of ~5 and ~50 nM) which exhibit anatomical distributions distinct from AMPA binding sites (London and Coyle, 1979; Monaghan and Cotman, 1982; Unnerstall and Wamsley, 1983). Furthermore, in the peripheral nervous system, sensory ganglion neurons exhibit current responses that desensitize to kainate (Agrawal and Evans, 1986; Huettner, 1990). These observations indicate the existence of receptor channels with properties different from those formed of GluR-A to -D subunits. There are presently three subunit candidates for high affinity kainate receptors, GluR-5 (Bettler *et al.*, 1990), GluR-6 (Egebjerg *et al.*, 1991), and KA-1 (Werner *et al.*, 1991). KA-1 binds [^{3}H]kainate with a K_D of 5 nM, corresponding to one of the two high affinity sites in brain, but homomeric KA-1 receptors show no current response to kainate or other excitatory amino acids (Werner *et al.*, 1991). The GluR-5 and GluR-6 subunits share extensive sequence identity suggesting common properties. The respective homomeric receptor channels appear to exhibit different properties which might, however, be an artefact of expression. In the *Xenopus* oocyte, cRNA-

mediated homomeric GluR-5 expression led to a barely detectable conductance change upon superfusion with high concentrations of glutamate (Bettler *et al.*, 1990), whereas GluR-6 channels could be effectively gated by glutamate and kainate, but not by AMPA (Egebjerg *et al.*, 1991). Notably, the kainate-evoked GluR-6 currents were desensitized in the presence of this agonist in a manner reminiscent of similarly evoked currents measured in primary sensory afferent neurons (Huettner, 1990). The affinity of kainate to GluR-5 and -6 receptors has not been determined.

We analysed GluR-5 as one member of this latter receptor family with respect to both pharmacology and current properties. Our data suggest that subunits of this family constitute receptor channels of unknown subunit complexity and composition with high affinity for domoate and kainate.

Results

Molecular heterogeneity of the GluR-5 subunit

Ionotropic glutamate receptors are structurally related and share amino acid sequences around their putative transmembrane regions (Hollmann *et al.*, 1989; Boulter *et al.*, 1990; Keinänen *et al.*, 1990; Moriyoshi *et al.*, 1991). Polymerase chain reaction (PCR)-mediated DNA amplification (Mullis and Faloona, 1987) with oligonucleotide primers specific for these conserved sequences (Keinänen *et al.*, 1990) and rat brain-derived cDNA as a template, produced DNA fragments with partial coding sequences for an array of homologous receptor polypeptides, including GluR-A to -D. The PCR-generated DNA fragments served as convenient probes for the isolation of the corresponding full-length cDNAs from rat brain cDNA libraries. One cDNA species isolated in this manner was identical in sequence to the previously published GluR-5 cDNA (Bettler *et al.*, 1990).

The first description of the GluR-5 subunit reported the existence of two molecular forms differing in the presence (GluR-5-1) or absence (GluR-5-2) of a 15 amino acid insert located in the predicted large N-terminal extracellular domain, 177 residues proximal to the first putative transmembrane region (Bettler *et al.*, 1990). Our analysis of cloned cDNAs and PCR products indicates the existence of additional molecular forms of the GluR-5 polypeptide (Figure 1). Specifically, GluR-5 cDNAs differ in regions encoding sequences distal to the putative fourth transmembrane region, indicating that the encoded subunit can exist in one of three variants with respect to C-terminal amino acid sequences. The simplest mechanistic explanation for the different C-termini is alternative splicing, given that all GluR-5 cDNAs shared distal 3′ untranslated nucleotide sequences.

Furthermore, as a result of RNA editing (Sommer *et al.*, 1991), the putative channel-forming region of GluR-5 harbours either a glutamine (Q) or arginine (R) residue in

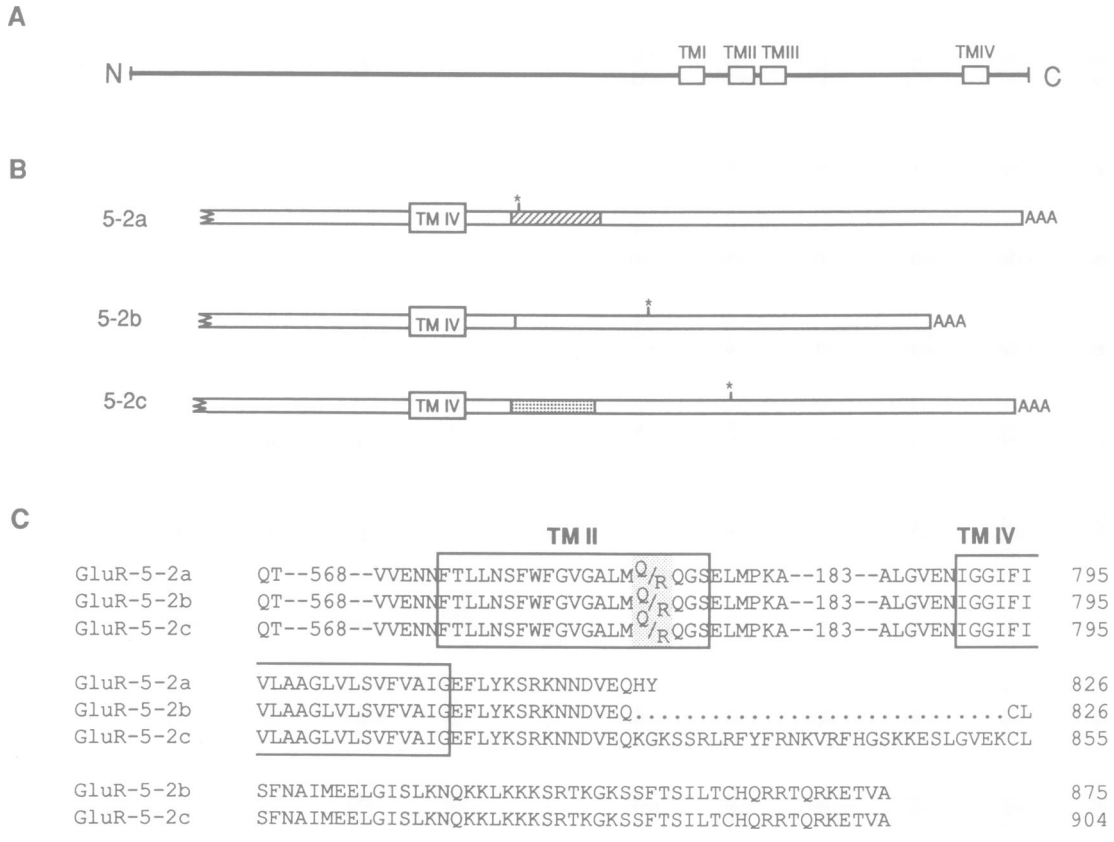

Fig. 1. Sequence heterogeneity of GluR-5. (**A**) Symbolizes the structure of a glutamate receptor subunit. Putative transmembrane regions are depicted as open boxes. (**B**) The 3′ portion of GluR-5 mRNA. Sequence variation at the 3′ end is symbolized by insertion of differently shaded boxes for GluR-5-2a and -c, compared with the published sequence of GluR-5-2b (Bettler *et al.*, 1990). Positions of stop codons are marked by vertical bars and asterisks. (**C**) Shows the sequence variability for GluR-5 based on its amino acid sequence. Membrane spanning regions II and IV are boxed. The heterogeneity (Q/R site) in TMII is shaded. Amino acid numbering refers to the splice variant GluR-5-2 starting with the first amino acid of the mature polypeptide.

a particular position (Q/R site) of its second transmembrane region. Analysis of our cloned GluR-5 cDNA sequences indicates that the amino acid substitution in the Q/R site seems to occur independently of either C-terminal variability or N-terminal insertion. The potentially more revealing analysis of *in situ* expression patterns in brain sections of the various molecular forms of GluR-5 was precluded by low mRNA levels and the single nucleotide exchange underlying Q/R site editing (Sommer *et al.*, 1991).

Pharmacological profile of recombinantly expressed homomeric GluR-5 receptors

The GluR-5 subunit was transiently expressed in cultured 293 cells (Pritchett *et al.*, 1989) and cell membranes were subjected to filter binding assays using [^{3}H]kainate as the ligand. Expression of C-terminal variants did not affect the pharmacological behaviour of GluR-5. However, 5- to 10-fold higher B_{max} values were obtained with the shortest C-terminal form and hence, this subunit variant was used in all studies. Similarly, a substitution in Q/R site residues did not influence binding properties as evidenced by a com-

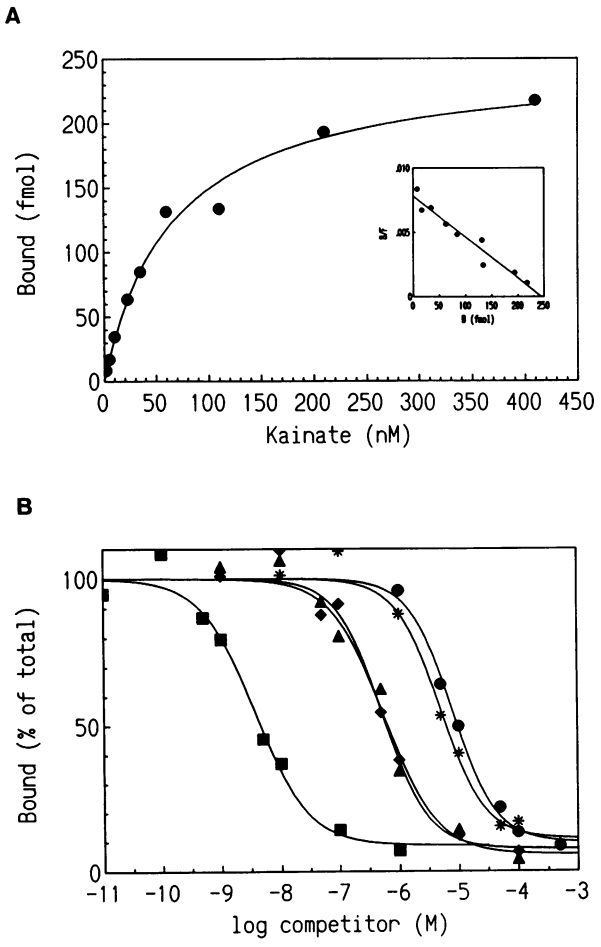

Fig. 2. Ligand binding properties of GluR-5-2a(R) expressing cells. A saturation isotherm of [^{3}H]kainate with a Scatchard plot (inset) of a representative experiment is shown in (**A**). Each data point corresponds to ~2 × 10^6 cells and is averaged from duplicate measurements. The calculated K_D value is 67 nM, the maximum binding corresponds to ~70 000 binding sites per cell. (**B**) Competition curves reflecting displacement of 50 nM [^{3}H]kainate by unlabelled domoate (■), quisqualate (♦), glutamate (▲), AMPA (●) and CNQX (*) were obtained using mean values from three independent experiments. Results are expressed as percentage of maximal binding.

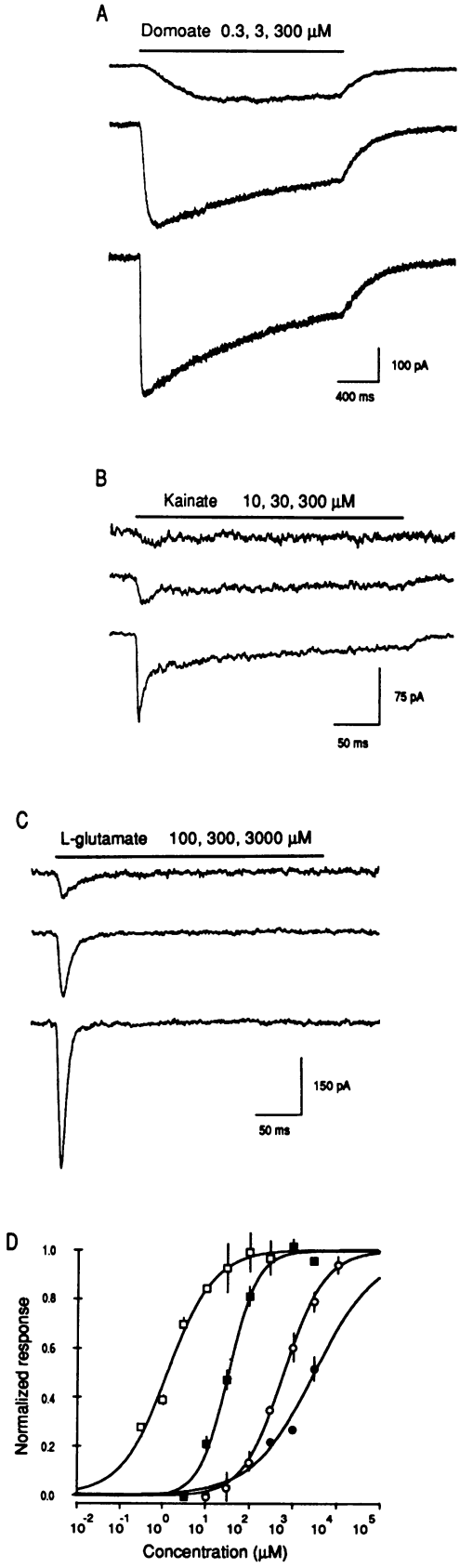

Fig. 3. Electrophysiological properties of GluR-5-2a receptor channels. Shown are examples of inward currents evoked by rapid application of domoate (**A**), kainate (**B**) and L-glutamate (**C**) at −60 mV in a cell expressing the non edited form of the receptor. (**D**) shows normalized dose response curves of peak currents to domoate (□), kainate (■), L-glutamate (○), and AMPA (●).

parison of heteromeric GluR-5(Q)/GluR-5(R) receptors with homomeric GluR-5(R) receptors (not shown). From binding isotherms such as the one shown in Figure 2A, a K_D of 73.3 ± 19.2 nM (n = 6) was determined for [^{3}H]kainate. This value approximates that established for the lower of the two high affinity kainate binding sites in brain (Hampson *et al.*, 1987).

Inhibition binding constants (K_i values) of [^{3}H]kainate for various agonists were computed from competition binding curves (Figure 2B). A particularly high affinity was seen for domoate which competed for [^{3}H]kainate with a K_i of 2.1 ± 0.8 nM (n = 3). Other K_i values in nM were 280 ± 52 (n = 3) for quisqualate, 290 ± 156 (n = 3) for glutamate and ~3000 ± 1000 (n = 3) for AMPA. The non-NMDA receptor antagonist CNQX displayed a K_i of 2 ± 0.5 μM (n = 3). This rank order of agonist potencies is largely compatible with that reported for certain high affinity kainate sites in brain (Agrawal and Evans, 1986; Hampson *et al.*, 1987). It also reflects the order of K_D values of agonists of GluR-5 channels (see below). Hence, our data are consistent with the notion that GluR-5 may be a component in kainate-sensitive channels in neuronal membranes which exhibit high affinity binding for domoate and also for kainate.

Functional characterization of GluR-5 receptor channels

Cultured 293 cells transiently expressing the GluR-5 subunit were analysed in the whole-cell configuration using patch clamp techniques for agonist-evoked currents. To facilitate the resolution of fast current components, a fast agonist application system was used and the cell was lifted from its support (Verdoorn *et al.*, 1991). In spite of these measures, no kainate- or L-glutamate-elicited currents were observed (−60 mV; six cells, three transfections) upon expressing the edited variant of GluR-5-2a [GluR-5(R)]. In contrast, inward currents were regularly evoked at −60 mV in cells expressing the non-edited variant of GluR-5-2a [GluR-5(Q)] by glutamate, kainate and domoate as well as by high concentrations of AMPA. Figure 3A illustrates the response of a lifted cell to application of different concentrations of domoate, the most potent agonist on these receptor channels, at a membrane potential of −60 mV. Responses exhibit slow desensitization in a single-exponential manner (τ = 2.23 ± 0.52 s, n = 3, for 50 μM domoate). The response to kainate consists of a fast desensitizing peak current (time constant τ = 15.3 ± 2.1 ms, n = 9, for 300 μM kainate) followed by a much slower desensitizing response (τ = 281 ± 41.8 ms, n = 9, for 300 μM kainate) (Figure 3B). This is very different from the current response to kainate in GluR-A to -D channels (Keinänen *et al.*, 1990; Sommer *et al.*, 1990). The responses to application of glutamate, desensitize completely following a dual-exponential time course (τ_1 = 8.9 ± 0.7 ms, τ_2 = 68.6 ± 7.5 ms, n = 5, for 1 mM L-glutamate) (Figure 3C). AMPA responses are qualitatively similar to those obtained with glutamate (not shown). The dose−response relations for agonist-activated peak currents at −50 mV are characterized by EC_{50} values of 1.2 μM, 33.6 μM, 631 μM and 3 mM for domoate, kainate, glutamate and AMPA, respectively (Figure 3D).

The current−voltage (I−V) relationship of the steady-state component of domoate-activated currents in normal rat Ringer extracellular solution, as measured by the ratio of

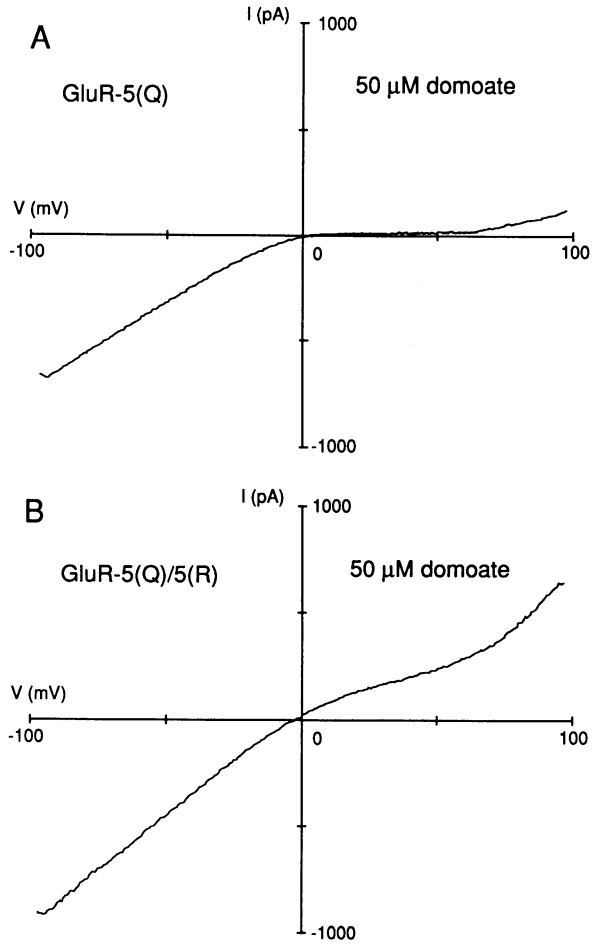

Fig. 4. Current−voltage relation of GluR-5 receptors. Ramp I−V curves constructed in a cell expressing homomeric GluR-5(Q) receptors are illustrated in (**A**). Results of a similar experiment performed on a cell expressing heteromeric edited and non-edited GluR-5 channels are shown in (**B**).

chord conductances (G_{+60}/G_{-60}), is illustrated in Figure 4. For GluR-5(Q) channels, the shape is characterized by a doubly rectifying behaviour similar to that of unedited GluR-A to -D receptor channels. The reversal potential is close to 0 mV. Following co-expression of edited and unedited versions of the GluR-5 subunit GluR-5(R) and GluR-5(Q), the shape of the I−V relationship, changed to become more linear. This change indicates that GluR-5(R) and GluR-5(Q) assemble into heteromeric receptor channels, reminiscent of the situation observed in the AMPA receptor family.

To address the question, whether GluR-5 assembles with members of the AMPA−low affinity kainate receptor family, I−V curves were taken from cells transfected with GluR-5(Q) in combination with GluR-B and GluR-5(R) in combination with GluR-D. One might expect similar changes in I−V relationship as described above, if GluR-5 assembled with GluR-B or -D. However, inwardly rectifying I−V relationships were observed upon co-expression of GluR-B with GluR-5(Q) as well as upon coexpression of GluR-5(R) with GluR-D (not shown). This indicates that GluR-5 subunits probably do not assemble with GluR-B or -D, although further measurements of other electrophysiological features may reveal emergent properties of such heteromeric channels.

Discussion

The present study by ligand binding pharmacology and electrophysiology functionally assigns the GluR-5 subunit to a class of high affinity kainate receptors, clearly different from AMPA receptors. Pharmacological studies on recombinantly expressed receptors result in a nanomolar dissociation constant for kainate, in good agreement with one of the two high affinity kainate binding sites observed in brain membranes (London and Coyle, 1979; Unnerstall and Wamsley, 1983; Hampson *et al.*, 1987). The observed rank order of potency, domoate > kainate > quisqualate > glutamate > CNQX > AMPA largely matches published affinities determined in cerebellum (Slevin *et al.*, 1983), forebrain (London and Coyle, 1979), striatum (Unnerstall and Wamsley, 1983) or total brain membranes (Hampson *et al.*, 1987), as well as dorsal root ganglia neurons of afferent C-fibres (Agrawal and Evans, 1986). The relative affinities of domoate and kainate, the most potent agonist on these binding sites, differ by a factor of two to four in the central nervous system (CNS) (Slevin *et al.*, 1983; Hampson *et al.*, 1987). On membranes of GluR-5 expressing cells, however, domoate displays >30-fold higher affinity towards recombinant receptors than kainate. This ratio between the affinities of domoate and kainate is also observed on kainate receptors of dorsal root ganglia neurons (Agrawal and Evans, 1986). In addition, the relative affinities of all agonists tested in this study match surprisingly well with relative K_D values of corresponding agonists on kainate receptors of a subpopulation of cultured dorsal root ganglia (Huettner, 1990). Pharmacological properties of the GluR-5 subunit are clearly distinct from those obtained with members of the AMPA receptor family (Keinänen *et al.*, 1990) as well as the recently published high affinity kainate receptor subunit, KA-1 (Werner *et al.*, 1991). Thus we conclude that GluR-5 represents a member of a distinct class of high affinity kainate receptors.

GluR-5 is capable of forming homomeric channels that can be gated by domoate, kainate, L-glutamate and AMPA. It was previously described that L-glutamate is the only ligand to be active on homomeric GluR-5 receptors (Bettler *et al.*, 1990). This discrepancy with our study might be explained by the limitations of the oocyte recording system used by Bettler *et al.* (1990), which does not allow for fast drug application in the whole-cell configuration. Therefore fast desensitizing L-glutamate-evoked currents are easily missed. The application of kainate and domoate, the agonist evoking the slowest desensitizing currents, were not described by Bettler *et al.* (1990).

When compared to the AMPA−low affinity kainate receptor family, several differences in gating properties can be observed. Glutamate-evoked currents on AMPA receptors can be separated in a fast desensitizing peak component and a non-desensitizing steady state component, whereas currents elicited by L-glutamate on GluR-5 receptor channels desensitize completely, following a dual-exponential time course. Kainate application leads to non-desensitizing steady state currents in AMPA−low affinity kainate receptors (Sommer *et al.*, 1990). However, on GluR-5 receptor channels, kainate-induced currents desensitize in a dual-exponential fashion. These properties of GluR-5 are similar to those observed for GluR-6, another recombinant glutamate receptor subunit, with which it displays ~80% sequence identity. GluR-6, however, cannot be gated by AMPA up to concentrations of 10 mM (Egebjerg *et al.*, 1991), a result not anticipated from the close sequence similarity of these subunits.

Although several reports describe increased excitability of neurons in the CNS following application of nanomolar amounts of kainate (Robinson and Deadwyler, 1981; Westbrook and Lothman, 1983), the only apparently pure population of high affinity kainate receptor channels *in vivo* has been described in the peripheral nervous system in the dorsal root ganglia neurons (Agrawal and Evans, 1986). This receptor population shows a desensitization behaviour towards kainate application reminiscent of that observed on recombinant GluR-6 (Egebjerg *et al.*, 1991) and GluR-5 (this study) receptor channels. When compared with the most extensive study to date on kainate receptors of peripheral dorsal root ganglia (DRG) in culture (Huettner, 1990), it becomes apparent that recombinant GluR-5 receptor channels best resemble these receptors. K_D values for kainate and domoate on recombinant GluR-5 receptor channels match those of kainate receptors of DRG neurons determined by dose−response analysis (Huettner, 1990). Desensitization patterns of kainate receptors on DRG neurons after agonist application (Huettner, 1990) also match those determined on recombinant GluR-5 receptor channels. In contrast to the closely related GluR-6 subunit, GluR-5 receptor channels can be gated by AMPA, a feature also described for the receptors of cultured DRG neurons (Huettner, 1990). I−V relations reported by Huettner (1990) resemble those of heteromeric edited and non-edited GluR-5 receptor channel complexes, suggesting that these channels may be composed of GluR-5(R) and GluR-5(Q). In this respect the arginine residue in the channel pore has a similar influence on high affinity kainate receptors and AMPA−low affinity kainate receptors (Verdoorn *et al.*, 1991).

Since high levels of GluR-5 mRNA have been observed in DRG neurons (Bettler *et al.*, 1990) and there is a close correspondence in pharmacological profile as well as electrophysiological characteristics between recombinant GluR-5 receptors and native high affinity kainate receptors found in DRG neurons of primary afferent C-fibre, we conclude that GluR-5 is a major component of these receptors.

Materials and methods

Isolation and expression of GluR-5 clones

For PCR (Mullis and Falooona, 1987), oligonucleotide primers and reaction conditions were as described (Keinänen *et al.*, 1990). Rat forebrain cDNA was used as a template. The PCR product (~500 bp) was gel-purified, cleaved with *Eco*RI and *Kpn*I, subcloned into doubly cut M13mp19 RF-DNA (Yanisch-Perron *et al.*, 1985) and sequenced (Sanger *et al.*, 1977). A PCR-generated DNA fragment of a GluR-5 specific M13 subclone was internally labelled with [^{3}P]dCTP to a specific activity of $2 \times 10^9/\mu g$ and used as a probe to screen a rat forebrain cDNA library constructed in λ-Zap (Stratagene). Five independent cDNAs from these screens were subcloned into pBluescript SK plasmid vectors (Stratagene, CA) or M13mp18 and mp19 RF-DNA (Yanisch-Perron *et al.*, 1985) and these subclones were used for sequence analysis (Sanger *et al.*, 1977). Expression constructs of GluR-5-2 specifying different C-termini were engineered by subcloning 3′ *Bam*HI−*Xho*I or *Bam*HI−*Eco*RI fragments encoding C-termini of GluR-5-2a or -2-c, respectively, and a 5′ *Hin*dIII−*Bam*HI fragment directly into a mammalian expression vector with a CMV promoter (Gorman *et al.*, 1990). The heterogeneity concerning glutamine and arginine codons in TM2 was generated by exchanging a 400 bp *Bsp*EI−*Msc*I fragment covering TM regions I to III of edited and non-edited GluR-5 cDNA clones. The constructs

were used to transfect human embryonic kidney cells 293 (ATCC CRL 1573) as described (Chen and Okayama, 1987; Pritchell *et al.*, 1989). These were subjected to binding studies and electrophysiology.

Binding studies

Cells were harvested 48 h after transfection by washing twice with phosphate buffered saline (PBS) prewarmed to 30°C and rinsed off the plate support with ice-cold PBS. Cell pellets were resuspended in binding buffer (50 mM Tris−citrate, pH 7.0) and homogenized in an ultraturrax homogenizer. Membranes were spun at 15 000 × *g* for 10 min. Homogenization and pelleting was repeated three times and finally membrane pellets were resuspended in binding buffer to obtain the membrane equivalent of 2×10^6 cells per 0.4 ml. Binding with [^{3}H]kainate (58 Ci/mmol, NEN) was performed in a total volume of 0.5 ml for 60 min at 0°C with nonspecific binding defined in the presence of 1 mM L-glutamate (Werner *et al.*, 1991). Competition studies were carried out in the presence of 50 nM [^{3}H]kainate. Following incubation, the reactions were quenched by addition of 5 ml ice-cold binding buffer and subsequently filtered through glass fibre filters (Schleicher and Schuell, No. 34). Filters were washed twice with 5 ml ice-cold binding buffer and filter-bound radioactivity was determined by liquid scintillation counting. Saturation isotherms and competition curves were determined by the non-linear regression function of the GraphPad program package.

Electrophysiology

48 h after transfection, agonist-activated currents were measured using standard patch clamp techniques in the whole-cell configuration (Hamill *et al.*, 1981) essentially as described (Keinänen *et al.*, 1990; Sommer *et al.*, 1990; Verdoorn *et al.*, 1991). The internal solution consisted of 140 mM CsCl, 1 mM $MgCl_2$, 11 mM EGTA and 10 mM HEPES pH 7.3. Normal rat Ringer, used as extracellular solution, contained: 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES pH 7.2. Agonists were dissolved in extracellular solution and applied rapidly by means of a piezo driven theta tubing pipette, (Keinänen *et al.*, 1990; Sommer *et al.*, 1990). Currents were filtered at 1 or 2 kHz (−3 dB, eight pole Bessel), digitized at 4−10 kHz and stored directly on-line to a VME bus computer system. I−V curves were constructed by the Ramp method. The command voltage was ramped from 0 mV to 100 mV over 2 s. Currents recorded in the absence of agonists were digitally substracted from those recorded during application of agonist to produce the agonist-activated I−V relation. Three to five substrated I−V curves were averaged under each condition. Domoate desensitized the receptors to a lesser extent and was thus the preferred agonist for the production of I−V curves. The shape of the agonist-activated I−V curves and the reversal potentials were determined by averaging the digitized current records over 4 mV and fitting the points to polynomial equations. The inwardly rectifying curves were usually fit to 8th order of polynomials whereas 4th order polynomials adequately described the more linear I−V relations. The resulting equations were used to calculate the reversal potential and chord conductances at selected voltages.

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