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Pharmacological characterization of a GluR6 kainate receptor in cultured hippocampal neurons

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Abstract

We have examined the pharmacology of kainate receptors in cultured hippocampal neurons (6–8 days in vitro (DIV)) from embryonic rats (E17). Cultured neurons were pre-treated with concanavalin A to remove kainate receptor desensitization and whole-cell voltage clamp electrophysiology employed to record inward currents in response to glutamatergic agonists and antagonists. *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor responses were blocked using MK801 (3 μM) and the 2,3-benzodiazepine, LY300168 (GYKI53655, 50 μM), respectively. Inward currents were recorded in hippocampal neurons upon application of kainate and the 2*S*,4*R* isomer of 4-methyl glutamic acid (SYM2081) with EC_{50} values of 3.4 ± 0.4 μM and 1.6 ± 0.5 μM, respectively ($n = 6$ cells). The GluR5 selective agonists, LY339434 (100 μM) and (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butyl-4-isoxazolyl) propionic acid (ATPA) (100 μM), did not evoke detectable inward currents in any cell responding to kainate. LY293558 and the selective GluR5 antagonist, LY382884, had weak antagonist effects on responses evoked by either kainate or (2*S*,4*R*)-4-methyl glutamate (IC_{50} > 300 μM). The quinoxalinedione, 2,3-dihyro-6-nitro-7-sulfamoyl-benzo(*f*)quinoxaline (NBQX), blocked both kainate and (2*S*,4*R*)-4-methyl glutamate-activated currents at much lower concentrations (IC_{50} approximately 10 μM). These results provide pharmacological evidence that ion channels comprised of GluR6 kainate receptor subunits mediate kainate receptor responses in hippocampal neurons cultured 6–8 DIV.

Keywords: GluR5 antagonist; (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butyl-4-isoxazolyl) propionic acid (ATPA); Quinoxalinedione

1. Introduction

Glutamate is the primary amino acid transmitter in the central nervous system (Collingridge and Lester, 1989). Functional studies using selective agonists and, more recently, molecular biological studies have resulted in a subtype classification of ionotropic glutamate of NMDA, AMPA and kainate (KA) receptor classes (Hollmann and Heinemann, 1994; Fletcher and Lodge, 1996; Bleakman and Lodge, 1998). For kainate receptors, there appear to be three subunits, GluR5, GluR6 and GluR7 that form functional ion channels (Bettler et al., 1990; Egebjerg et al., 1991; Sommer et al., 1992; Schiffer et al., 1997). There are also KA1 and KA2 subunits that appear to modify both the pharmacological and biophysical properties of the other kainate receptor ion channels when co-expressed in recombinant systems, or when present in neurons (Hollmann and Heinemann, 1994; Sahara et al., 1997).

Several agents have recently been used to investigate the pharmacology of non-NMDA receptor responses (Bleakman and Lodge, 1998). The quinoxalinedione series of compounds (e.g., CNQX, NBQX) have been useful for competitive antagonism of non-NMDA responses (Honoré et al., 1988; Sheardown et al., 1993). The 2,3-benziodiazepine class of non-competitive AMPA receptor antagonists including LY300168 (GYKI53655) has allowed pharmacological isolation of kainate receptor-mediated responses from those activated by kainate at AMPA receptors (Paternain et al., 1995; Bleakman et al., 1996). In addition, several recent reports have identified ligands that appear to show selectivity for the GluR5 kainate receptor. LY293558, LY294486 and LY382884 from the decahydroisoquinoline series of compounds have been shown to be GluR5 receptor antagonists. These compounds have varying degrees of AMPA antagonist activity (LY293558 > LY294486 > LY382884) whilst maintaining activity at the

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GluR5 kainate receptor subunit and having no significant activity at GluR6 kainate receptors (Bleakman et al., 1996; Clarke et al., 1997; Simmons et al., 1998). For kainate selective agonists there are GluR5 preferring compounds such as the *tert*-butyl substituted AMPA analogue, ATPA (Clarke et al., 1997), the napthyl, γ-substituted glutamate analogue, LY339434 (Small et al., 1997) and the GluR5/GluR6 agonist (2*S*,4*R*)-4-methylglutamic acid (Gu et al., 1995; Small et al., 1997).

Kainate receptors have been studied in hippocampal neurons in vitro, both in slices and in dissociated cell culture. In hippocampal slices, both GluR5 and GluR6 receptor-mediated responses have been described on both excitatory glutamatergic neurons and gamma-aminobutyric acid-ergic (GABA-ergic) inhibitory neurons (Clarke et al., 1997; Vignes et al., 1997; Mulle et al., 1998; Vignes et al., 1998). In hippocampal neuronal cultures from embryonic rats, there is evidence from physiological properties of currents and mRNA analysis that the majority of neurons have kainate receptors comprised of GluR6 receptor subunits (Ruano et al., 1995). However, kainate receptor responses in dissociated cell cultures of hippocampal neurons from neonatal rats have been studied and display properties distinct from those expected from GluR6 kainate receptors (Wilding and Huettner, 1997). In the light of these studies, we have employed a pharmacological approach to investigate the kainate receptor subtypes in cultured hippocampal neurons from embryonic rats. A preliminary report of this work has appeared (Ogden et al., 1998)

2. Methods

2.1. Transfected cell lines

Stable cell lines of human embryonic kidney 293 (HEK293) cells transfected with cDNA coding for the human GluR1–4 (flip), GluR5–7, KA2 and GluR6 + KA2 receptors were established as reported previously (Hoo et al., 1994; Fletcher et al., 1995; Korczak et al., 1995; Clarke et al., 1997). Stable cell lines were established by transfection of HEK293 cells with the mammalian expression vector pRc/CMV (Invitrogen) incorporating the cDNA for human GluRs. Transfected cells were selected on the basis of G418 resistance, and mRNA confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR).

2.2. Ligand binding studies

Cell membranes were prepared from frozen HEK293 cells expressing either recombinant AMPA or kainate receptors by resuspending the cells in ice-cold distilled water, sonicating and centrifuging at $50{,}000 \times g$ for 20 min. The membrane pellets were then washed in $> 100 \times$ volumes of 50 mM Tris–HCl buffer, pH 7.5 and centrifuged to remove endogenous glutamate. Binding reactions were performed at 4°C for 60 min in a total volume of 250 μl containing 50 μl of membrane suspension (100–150 μg protein). For kainate receptor binding, the reaction mixture consisted of 150 μl 50 mM Tris–HCl, pH 7.5, 25 μl [^{3}H]kainate (NEN, Dupont) and 25 μl unlabelled competitor (10^{-11}–10^{-3} M). The final [^{3}H]kainate concentrations used in the competitive inhibition experiments was 20 nM. For AMPA receptor binding, 20 nM [^{3}H]AMPA (NEN, Dupont) was used for each receptor subtype and 100 mM KSCN was added to the Tris–HCl buffer. Following the 60 min incubation, the membranes were centrifuged at $50{,}000 \times g$ for 20 min to separate bound from free ligand and the pellets were washed three times in cold assay buffer. Non-specific binding was determined by incubation in the presence 10 mM glutamate. All data were analyzed by GRAFIT 2.0 software.

2.3. Primary culture of hippocampal pyramidal neurons

Hippocampal pyramidal neurons were cultured via modification of the method of Brewer et al. (1993) from E17 Sprague–Dawley rat fetuses. Neurons were mechanically dispersed in 1 ml HBSS without Mg^{2+} and Ca^{2+} (Gibco) supplemented with 1 mM sodium pyruvate and 5 mM HEPES utilizing a fire polished glass Pasteur pipette. The suspension was then diluted with HBSS with Mg^{2+} and Ca^{2+} (Gibco) supplemented as above and non-dispersed tissue was allowed to settle for 3 min. The supernatant was transferred to a 15 ml conical tube and centrifuged for 1 min at $200 \times g$. The pellet was resuspended in Neurobasal™ Media (Gibco) supplemented with B27 (Gibco), 0.5 mM glutamine, and 25 μM glutamic acid. Neurons were plated at 2×10^5 per collagen/laminin (Sigma, St. Louis, MO) coated glass coverslips (15 mm). Cultures were maintained in a humidified 5% CO_2 incubator at 37°C. On day 4 and every seventh day after, media were half exchanged with plating media lacking glutamic acid.

2.4. Electrophysiological recordings and drug application

Whole-cell voltage clamp recordings (Vh = −70 mV) were made from single cells with use of the tight seal whole cell configuration of the patch-clamp technique (Hamill et al., 1981). Glass fragments of coverslips with adherent cells were placed in a perfusion chamber and rinsed with buffer of composition: 138 mM NaCl, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$,10 mM HEPES and 10 mM glucose, pH 7.5 with NaOH (osmolality: 315 mosM/kg). Pipette solutions contained 140 mM CsCl, 1 mM $MgC1_2$, 14 mM diTris creatine phosphate, 50 U/ml

creatine phosphokinase, 14 mM MgATP, 10 mM HEPES and 15 mM BAPTA, pH of 7.2 with CsOH (osmolality: 295 mosM/kg). Experiments were performed at room temperature (20–22°C) and recorded on an Axopatch 200A amplifier using pClamp6 software (Axon Instruments). Pipette resistances were typically 1.5–2.5 MΩ. Drug application was via bath perfusion and occurred within approximately 5 s. Curve fitting to data points was based upon the equation $y = 100\ (D^n/D^n + \mathrm{IC}_{50}^n)$, where the slope of the line n was fixed to a value of 1, D is the agonist concentration. Estimates of EC_{50} values were made from curves generated using data from a minimum of three separate cells. Electrophysiological recording were made from neurons up to 15 days in vitro (DIV) for AMPA and NMDA currents and the effects of LY300168 and MK801, respectively. TTX (1 μM) was also included in all recording solution to prevent synaptic activity in these cultures. For kainate-induced currents in neurons, we used cultures that had been grown in culture for 6–8 days.

2.5. Chemicals

GYKI 53655 (LY300168), LY339434, ATPA, LY293558, LY382884 and (2*S*,4*R*)-4-methylglutamic acid were synthesized at Eli Lilly and Company. NBQX, AMPA, NMDA and MK-801 were obtained from Tocris Cookson (Bristol, UK). All other reagents were obtained from Sigma.

3. Results

3.1. Pharmacological isolation of kainate receptor-mediated currents

Steady-state inward currents were evoked in whole-cell voltage clamped hippocampal neurons (Vh = −70 mV) by AMPA in a concentration-dependent manner. An estimated EC_{50} value of 4.0 ± 0.6 μM was obtained for AMPA (Fig. 1A). Inward currents evoked by AMPA (30 μM) were completely inhibited by 50 μM of the non-competitive AMPA receptor antagonist LY300168 (GYKI53655). We also demonstrated that activation of NMDA receptors (in the presence of 10 μM added glycine and Mg^{2+} free solutions, EC_{50} approximately 7.2 ± 0.2 μM, Fig. 1B) was prevented by the NMDA receptor antagonist MK801 (3 μM). LY300168 (50 μM) and MK801 (3 μM) were subsequently included in experiments in order to prevent activation of AMPA receptors by kainate and NMDA receptors by LY339434 and (2*S*,4*R*)-4-methylglutamic acid (Small et al., 1997).

3.2. Ligand binding profile of LY293558 and NBQX at recombinant non-NMDA receptors expressed in HEK293 cells

Cell membranes expressing glutamate receptors GluR1–4 were radiolabelled with [^{3}H]AMPA and [^{3}H]kainate for GluR5–7, GluR6 + KA2 or KA2 as described in Section 2. K_i determinations performed for LY293558 and NBQX at these glutamate receptor subunits are shown in Table 1. LY293558 shows affinity for AMPA receptors (K_i values rank order GluR2 > GluR1 > GluR3 > GluR4), and also affinity for the GluR5 kainate receptor but not other kainate receptor subtypes. NBQX had approximately 1 μM K_i values across the GluR1–4 AMPA receptors and approximately 10-fold lower affinity at GluR5–7. NBQX also showed submicromolar K_i values for the GluR6/KA2 heteromeric with > 100 μM affinity for KA2 alone. We have previously demonstrated that the binding profile shown in Table 1 agrees well with functional data for these compounds in neuronal and recombinant preparations (Bleakman et al., 1996). LY382884 is a compound that has previously been demonstrated to show selective binding affinity for GluR5 kainate receptors (Simmons et al., 1998). Based upon the ability of these compounds to display affinity for GluR5, and, in the case of LY293558 and LY382884, show selectivity for GluR5 over other kainate receptors, we utilized these compound as agents to probe kainate receptors in cultured hippocampal neurons in vitro.

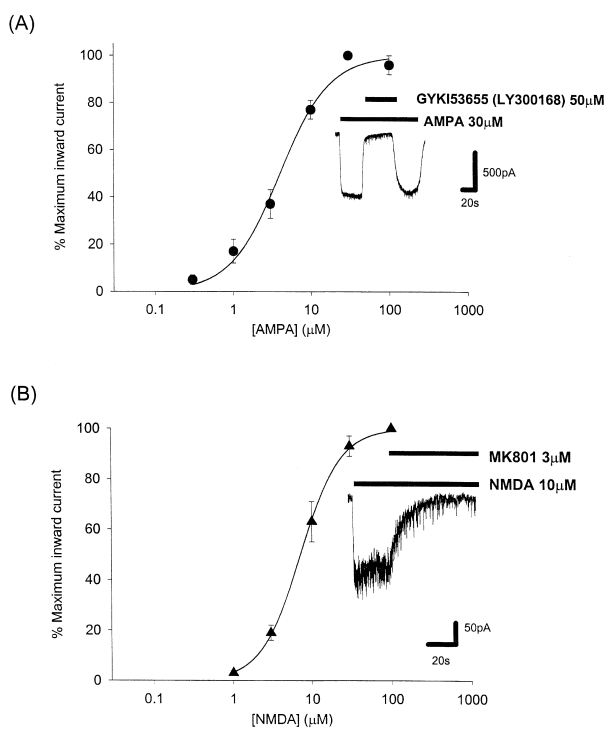

Fig. 1. Agonist response curves in cultured hippocampal neurons voltage clamped at −70 mV. (A) AMPA concentration–response curve and in the inset the effect of the non-competitive AMPA receptor antagonist LY300168 on inward currents activated by 30 μM AMPA. (B) Concentration–response curves generated using NMDA as the agonist. The inset shows the inhibition of NMDA (10 μM) receptor-mediated responses by MK801 (3 μM). The scale bars represent time (s), horizontal and current (pA), vertical.

Table 1
Ligand binding profiles of LY293558 and NBQX at recombinant GluR receptors expressed in HEK293 cell membranes. [^{3}H]AMPA and [^{3}H]kainate were used as radioligands for AMPA and kainate receptors, respectively as described in Section 2. Data represents mean K_i values for three separate determinations performed in triplicate using 11 point dose–response relationships

GluR subunit	LY293558 (K_i, μM)	NBQX (K_i, μM)
GluR1 ([^{3}H]AMPA)	9.2 ± 3.9	1.6 ± 0.9
GluR2 ([^{3}H]AMPA)	3.2 ± 0.3	0.26 ± 0.04
GluR3 ([^{3}H]AMPA)	32.0 ± 5.2	0.9 ± 0.3
GluR4 ([^{3}H]AMPA)	50.5 ± 12.7	0.6 ± 0.3
GluR5 ([^{3}H]kainate)	4.2 ± 0.3	11.6 ± 4.4
GluR6 ([^{3}H]kainate)	> 100	13.2 ± 1.9
GluR6 + KA2 ([^{3}H]kainate)	> 100	0.6 ± 0.1
GluR7 ([^{3}H]kainate)	> 100	23.9 ± 6.9
KA2 ([^{3}H]kainate)	> 100	> 100

3.3. Neuronal responses to kainate and (2S,4R)-4-methyl glutamate

Of the 44 neurons (6–8 DIV) examined, 35 neurons responded to kainate (10 μM) with an inward current > 20 pA under conditions that pharmacologically isolated kainate receptors (pre-treatment of cultures with 250 μg/ml concanavalin A for 10 min, recorded in the presence of 50 μM LY300168 and 3 μM MK801). Estimated EC_{50} values for kainate and (2*S*,4*R*)-4-methyl glutamate were calculated from concentration–response curves shown in Fig. 2 and were 3.4 ± 0.1 μM and 1.6 ± 0.5 μM, respectively. We also examined the effects of ATPA and LY339434 on voltage clamped hippocampal neurons. These agents have previously been shown to be potent and selective GluR5 kainate receptor agonists (Clarke et al., 1997; Small et al., 1997). In the present study we were unable to detect any inward currents following exposure of cells to ATPA or LY339434 (100 or 300 μM).

3.4. Antagonist activities of NBQX, LY293558 and LY382884

The effects of NBQX and LY293558 were examined on inward currents elicited by either kainate or (2*S*,4*R*)-4-methyl glutamate (10 μM). NBQX inhibited responses evoked by kainate and (2*S*,4*R*)-4-methyl glutamate with approximate IC_{50} values of 10 μM. LY293558 was much

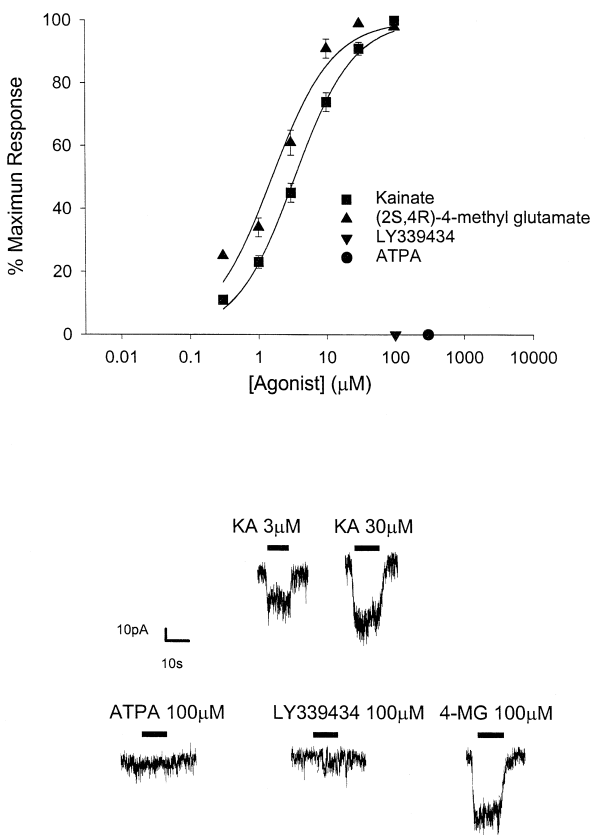

Fig. 2. Agonist concentration response curves for kainate (■), (2S,4R)-4-methyl glutamate (▲) in cultured rat hippocampal neurons (6–8 DIV). Also shown are data points for ATPA (●) and LY339434 (▼). Current traces shown are for each of the four agonist recorded in the same cell. The scale bar denotes time (s), horizontal and current (pA), vertical.

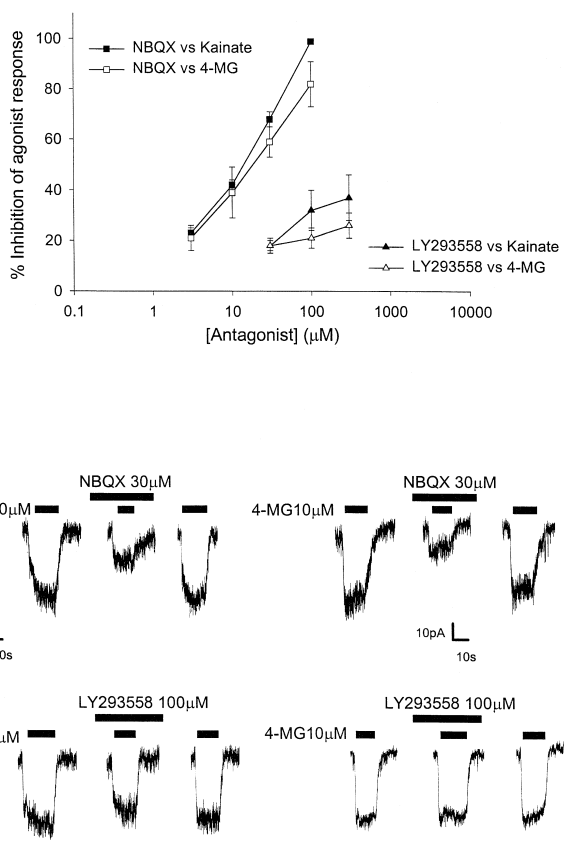

Fig. 3. Effects of GluR5/6 (NBQX) and GluR5 (LY293558) antagonists on agonist response evoked in hippocampal neurons by kainate or (2*S*,4*R*)-4-methyl glutamate (4-MG). The current traces show individual currents evoked by either kainate (10 μM) or 4-MG (10 μM) and the effects of NBQX or LY293558. The scale bar denotes time (s), horizontal and current (pA), vertical.

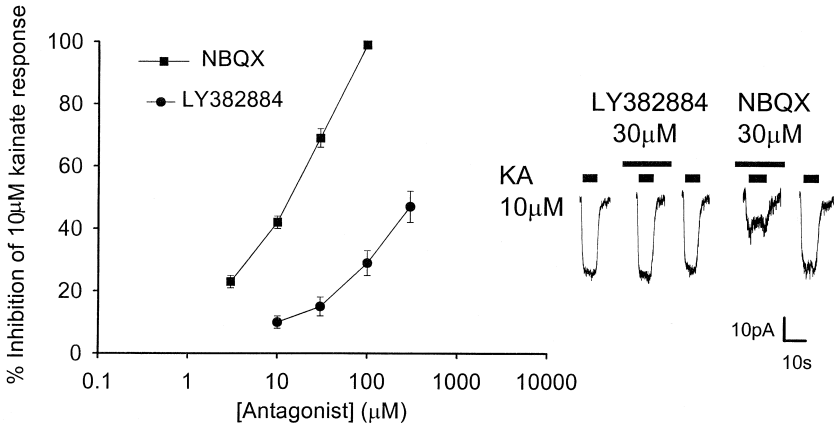

Fig. 4. Effects of GluR5/6 (NBQX, ■) and GluR5 (LY382884, ●) antagonists on agonist response evoked in hippocampal neurons by kainate (10 μM). The current traces show individual currents evoked by either kainate (10 μM) and the effects of NBQX or LY382884. The scale bar denotes time (s), horizontal and current (pA), vertical.

weaker in its ability to antagonize these agonist responses, with less than a 30% inhibition of currents at concentrations of LY293558 exceeding 100 μM (Fig. 3). The effect of the GluR5 selective antagonist, LY382884 was also examined on currents induced by kainate. As with LY293558 there was only weak inhibition of kainate-induced currents by LY382884 (< 50% inhibition at 300 μM, Fig. 4).

4. Discussion

The present studies have examined the subtypes of kainate receptors present in young cultures of hippocampal neurons (6–8 DIV). Our initial studies have identified conditions that have allowed the isolation of AMPA and NMDA receptor-mediated responses from those mediated by kainate receptors. Thus, recordings were made in the presence of the non-competitive AMPA receptor antagonist, LY300168 (50 μM) and the NMDA receptor antagonist, MK801 (3 μM).

We have used agonists that activate both GluR5 and GluR6 kainate receptors (kainate and (2*S*,4*R*)-4-methyl glutamate) and also GluR5 selective agonists (ATPA and LY339434) to investigate the agonist pharmacology of kainate receptors in these cultures. In the present study, kainate induced steady state currents recorded and an approximate EC_{50} value of 3 μM. The experiments were performed after pre-treatment of cultures with concanavalin A to remove receptor desensitization (Huettner, 1990; Wong and Mayer, 1993). In the majority of studies published, concanavalin A appears to have little effect on the EC_{50} values for kainate (Huettner, 1990; Wong et al., 1994; D. Bleakman, unpublished observations) although small increases in agonist potency have been observed (Wilding and Huettner, 1997; Jones et al., 1997). Kainate has previously been shown to evoke responses in embryonic cultures of hippocampal neurons that have resulted in an EC_{50} value of 22 μM (Lerma et al., 1993) and 23 μM in rat hippocampal dissociated cultures from neonatal animals (Wilding and Huettner, 1997). In dorsal root ganglion (DRG) neurons, neurons thought to express functional GluR5 kainate receptors subunits (Huettner, 1990; Partin et al., 1993; Bleakman et al., 1996), EC_{50} values range between 6–15 μM (Huettner, 1990; Wong et al., 1994). In recombinant systems expressing kainate receptors, EC_{50} values have been determined for kainate at homomeric GluR5 of 34 μM (Sommer et al., 1992) and 1–2 μM for homomeric GluR6 (Sommer et al., 1992; Jones et al., 1997; Small et al., 1997). Given the overlapping range of kainate EC_{50} values reported in both neuronal and recombinant systems it appears unlikely kainate EC_{50} values would be useful in defining the subtype of kainate receptors in these cultures.

We also examined the effect of the GluR5/6 agonist (2*S*,4*R*)-4-methyl glutamate in hippocampal neurons. (2*S*,4*R*)-4-methyl glutamic acid produced an EC_{50} value of approximately 1.6 μM. The effects of (2*S*,4*R*)-4-methylglutamic acid have previously been examined in neurons and recombinant kainate expressing cells. (2*S*,4*R*)-4-methylglutamic acid evokes non-desensitizing currents following concanavalin A treatment in rat DRG neurons with an EC_{50} value of 0.1–0.3 μM (Jones et al., 1997; Small et al., 1997). In recombinant human and rat GluR6 expressing cells, (2*S*,4*R*)-4-methyl glutamic acid produced EC_{50} values of approximately 0.7 μM and 0.1 μM following concanavalin A treatment, respectively (Small et al., 1997; Jones et al., 1997). As with kainate, (2*S*,4*R*)-4-methyl glutamic acid agonist potencies appear of limited use in the discrimination of GluR5 and GluR6 containing kainate receptors under the present recording conditions. The compounds ATPA and LY339434 have previously been demonstrated to show agonist selectivity for GluR5 kainate receptors both in ligand binding studies and in functional studies in neurons and recombinant kainate receptors (EC_{50} values of approximately 1 μM; Small et al., 1997; Clarke et al., 1997). Interestingly, in the present studies, we were

unable to evoke inward currents in hippocampal neuronal cultures using these agents. Thus, based upon the agonist pharmacology in these cells it would appear that the level of functional GluR5 receptor expression in young hippocampal neurons is low.

In order to investigate further the potential absence of a GluR5 receptor-mediated response, the effects of the GluR5/6 antagonist NBQX and the GluR5 selective antagonists, LY293558 and LY382884 were examined. In DRG neurons and at recombinant human GluR5 kainate receptors, both NBQX and LY293558 inhibit currents activated by kainate with low micromolar potencies (pK_b for LY293558 in DRG neurons and human recombinant GluR5 of 6.24 ± 0.07 and 6.24 ± 0.06, respectively, Bleakman and Lodge, 1998). In human GluR6 expressing cells, currents are inhibited by NBQX (IC_{50} of 2.76 ± 0.35 vs. kainate at 1 μM) but unaffected by LY293558 at concentrations up to 100 μM. Thus, LY293558 and LY382884 are compounds with GluR5 kainate receptor selectivity whereas NBQX is an antagonist at both GluR5 and GluR6 kainate receptors. For both kainate and (2*S*,4*R*)-4-methyl glutamic acid, we chose approximate EC_{80} values of each agonist to examine the effects of the described antagonists. Neither LY293558 or LY382884, the GluR5 selective antagonists, were able to inhibit responses in hippocampal neurons to either kainate or (2*S*,4*R*)-4-methyl glutamic acid at concentrations likely to completely block GluR5 receptor-mediated responses. However, NBQX, a compound with antagonist activity at GluR6 receptors, produced a concentration-dependent reduction in inward currents associated with application of both kainate and (2*S*,4*R*)-4-methyl glutamic acid. NBQX also had affinity at GluR7 receptors and the block of currents in hippocampal neurons would not necessarily preclude an agonist action at GluR7 kainate receptors. However, activation of recombinant GluR7 receptors requires high concentrations of kainate (> 1 mM) (Schiffer et al., 1997) whereas in the present study kainate evoked currents with an EC_{50} of approximately 3 μM.

The present study provides pharmacological evidence that in young cultures (6–8 DIV) of rat embryonic hippocampal neurons GluR5 kainate receptors appear to be absent. Whether this is the case for older neurons in culture or from tissue isolated from neonatal or adult animals remains to be established. It is also possible that the present culture conditions define the developmental expression of neuronal kainate receptor subtypes. The present system, however, should allow an investigation of the properties and functional role of GluR6 expressing neurons in hippocampal neurons in vitro.

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