Pergamon

PII: S0028-3908(97)00066-X

Neuropharmacology, Vol. 36, No. 6, pp. 853–863, 1997

Printed in Great Britain
0028-3908/97 $17.00 + 0.00

Desensitization of Kainate Receptors by Kainate, Glutamate and Diastereomers of 4-Methylglutamate

K. A. JONES,[1] T. J. WILDING,[2] J. E. HUETTNER[2] and A.-M. COSTA[1]

[1]*Symphony Pharmaceuticals Inc., 76 Great Valley Parkway, Malvern, PA 19355, U.S.A. and*
[2]*Washington University School of Medicine, Dept of Cell Biology and Physiology, St. Louis, MO 63110, U.S.A.*

(Accepted 29 January 1997)

Summary—The potencies of kainate, glutamate and diastereomers of 4-methylglutamate were determined for activation and steady-state desensitization of GluR6 and dorsal root ganglion-type kainate receptors using whole-cell voltage clamp. In HEK293 cells expressing GluR6, all four diastereomers induced desensitizing inward currents at relatively high concentrations (>50 μM), however, the 2S,4R diastereomer (2S,4R-4MG; SYM 2081) was approximately 100-fold more potent than the other three. The EC_{50} for receptor activation by 2S,4R-4MG (1.0 μM) was similar to that for kainic acid (1.8 μM), but 2S,4R-4MG was significantly more potent than kainate, glutamate or the other diastereomers of 4-methylglutamate at producing steady-state desensitization of GluR6 receptors. IC_{50}s for desensitization quantified using a fixed concentration of kainate as a test agonist were 7.6, 31 and 667 nM for 2S,4R-4MG, kainate and glutamate, respectively. In addition, 2S,4R-4MG fully desensitized native kainate receptors (of the GluR5 subtype) in dorsal root ganglion neurons with an IC_{50} of 11 nM, compared to 3.4 μM for glutamate. For GluR6, recovery from desensitization displayed a similar time course for kainate and glutamate (τ = 3-4 s) but was roughly 20-fold slower for 2S,4R-4MG, which suggests that the rate of recovery is not entirely dependent on the affinity of ligand for the desensitized receptor. Following exposure to concanavalin A, application of kainate, glutamate and 2S,4R-4MG evoked very similar maximal currents that showed little or no desensitization. Lectin pretreatment produced a leftward shift in the concentration–response relationship for 2S,4R-4MG with an 11-fold reduction in the EC_{50}; however, no significant change in the EC_{50} for kainate was observed. The characteristic of 2S,4R-4MG to potently and completely desensitize both recombinant GluR6 receptors and native receptors on dorsal root ganglion neurons suggests that this compound will be useful to study selective blockade of these receptors in the nervous system.

Keywords—Kainate, 4-methylglutamate, SYM 2081, kainate receptor, glutamate receptor, GluR5, GluR6, dorsal root ganglia.

Of the three subclasses of ionotropic glutamate receptors, kainate receptors are the least well studied because they are expressed at lower levels than α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or *N*-methyl-D-aspartic acid (NMDA) receptors (Paternain *et al.*, 1995; Spruston *et al.*, 1995) and because most agonists and antagonists of non-NMDA receptors show only limited selectivity between the AMPA and kainate subtypes (Wong *et al.*, 1994; Wilding and Huettner, 1996). Nevertheless, studies of native kainate receptors on peripheral neurons (Huettner, 1990; Wong and Mayer, 1993) and more recently on immature hippocampal neurons (Lerma *et al.*, 1993; Paternain *et al.*, 1995) have broadly defined the unique physiological and pharmacological signature of these receptors. This work has been greatly advanced by the cloning of five subunits, (Bettler *et al.*, 1990; Egebjerg *et al.*, 1991; Werner *et al.*, 1991; Bettler *et al.*, 1992; Herb *et al.*, 1992; Sommer *et al.*, 1992) that, when expressed in heterologous cells, display most of the characteristics described for native receptors. In particular, there is good evidence that receptors on neurons of sensory dorsal root ganglia (DRG) are composed of GluR5 and KA2 subunits (Bettler *et al.*, 1990; Herb *et al.*, 1992; Sommer *et al.*, 1992; Partin *et al.*, 1993), whereas kainate receptors in hippocampus are likely to be composed of the GluR6 subunit (Ruano *et al.*, 1995), possibly in combination with KA1 or KA2 (Wisden and Seeburg, 1993).

A prominent physiological feature of kainate receptors is their rapid desensitization in response to kainic acid and glutamate. This rapid desensitization plays a major

*To whom correspondence should be addressed at: Synaptic Pharmaceutical Corp., 215 College Rd, Paramus, NJ 07652-1431, U.S.A. Tel: 201 261-1331 ex. 176; Fax: 201 261-0623; e-mail: kjones@synapticcomp.com

role in determining the kinetics of kainate receptor channels and has been useful as a pharmacological tool to selectively block the activity of kainate receptors on neurons containing mixed populations of glutamate receptors (Lerma *et al.*, 1993). The densensitization of DRG cell kainate receptors has been studied in some detail (Huettner, 1990; Wong *et al.*, 1994), but comparable work has not yet been reported for receptors composed of the GluR6 subunit. Also, in general, the steady-state potency of the various agonists to induce desensitization of kainate receptors has not been studied at all.

The cloning of distinct AMPA-preferring and kainate-preferring glutamate receptor subunits has intensified efforts to synthesize compounds showing pharmacological specificity for either receptor subtype. Recently, two classes of heterocyclic compounds, modified 2,3 benzodiazepines (Tarnawa *et al.*, 1993) and substituted dihydrophthalazines (Pelletier *et al.*, 1996) have been shown to exhibit selective blockade of AMPA receptors (Paternain *et al.*, 1995; Wilding and Huettner, 1995; Pelletier *et al.*, 1996). The active site for these compounds is not equivalent to the ligand binding site for glutamate (Donevan and Rogawski, 1993; Zorumski *et al.*, 1993; Li *et al.*, 1995) or for positive modulators such as aniracetam and cyclothiazide (Tang *et al.*, 1991; Yamada and Tang, 1993; Kessler *et al.*, 1996; Partin and Mayer, 1996). To gain additional information concerning the ligand binding preferences for the major glutamate receptor subtypes, a series of 4-alkyl substituents of glutamate has been synthesized recently and tested for binding, activity (Gu *et al.*, 1995). From this series, the (2S,4R) diastereomer of 4-methylglutamate (2S,4R-4MG) was the most potent competitor for high affinity kainic acid binding sites. The compound also displayed a low affinity for NMDA and AMPA receptor binding sites (Gu *et al.*, 1995). A preliminary electrophysiological characterization suggested that 2S,4R-4MG activates and desensitizes GluR6 kainate receptors, but exhibits much weaker activity at AMPA receptors (Zhou *et al.*, 1997). The present study compares the potency of 2S,4R-4MG, glutamate and kainate for activation, as well as steady-state desensitization of recombinant GluR6 receptors and native kainate receptors expressed by sensory neurons.

MATERIALS AND METHODS

Electrophysiological recordings

The whole-cell configuration of the patch-clamp technique was performed with glass pipettes having a resistance of 2–4 MΩ when filled with the pipette solution. Solutions used were (in mM): CsCl, 140; $MgCl_2$, 2; EGTA, 1.1; $CaCl_2$, 1.0; HEPES, 1.0; pH 7.3 for the pipette and NaCl, 150; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10 at pH 7.4 for the bath. When recording from DRG cells and cortical neurons, Na^+ currents were blocked by external TTX (300 nM). Voltage clamp recordings from HEK293 cells were made with an EPC-9 amplifier using Pulse + PulseFit software (HEKA Elektronik). Currents were low-pass filtered at 1 kHz and digitized at a rate of 5 kHz. Responses from DRG cells were recorded with an Axopatch 200 amplifier using in-house software in the Basic-Fastlab environment (Indec Systems Inc., Capitola, CA, U.S.A.). Unless otherwise noted, experiments were performed on cells voltage clamped at a holding potential of −70 mV. Rapid application of agonists was realized using a gravity-fed, perfusion system consisting of six microcapillary tubes measuring 0.32 mm diameter (J and W Scientific) placed concentrically inside a 1.7 mm O.D. glass tube. The outer tube was heated and pulled around the inner tubes to form a blunt nozzle having a final diameter of 0.3 mm O.D. Dead space between the ends of the perfusion tubes and the tip of the nozzle was 2–3 μl. Solution exchange times measured 4-6 msec (10-90% of steady-state current) at the tip of a patch pipette placed in the position of a cell. The bath was constantly perfused at a low rate with control solution.

Because some agonists when applied even for brief periods (1–2 sec) induced receptor desensitization lasting many seconds, care was taken to utilize a low frequency of agonist stimulation that would not interfere with the construction of concentration–response relationships. Therefore, for EC_{50} and IC_{50} determinations, agonist pulses were applied every 5 min, a period found to permit full recovery of maximul currents. Often this lengthy "wash" period did not allow us to apply the full range of agonist concentrations in a single cell; EC_{50}s and IC_{50}s were calculated from single curve fits constructed from the normalized response amplitudes from multiple cells. Concentration-response curves for agonists and antagonists were fitted with logistic equations of the form $I = 1/(1 + (EC_{50}/[Agonist])^n)$ for agonists and $I = 1/(1 + ([Antagonist]/IC_{50})^n)$ for antagonists, where EC_{50} is the concentration of agonist that produced half-maximal activation, IC_{50} is the concentration of antagonist that produced half-maximal inhibition and n is the Hill coefficient. Fits were made with a Marquardt-Levenberg non-linear least-squares curve fitting algorithm. All values are expressed as mean ± standard error of the mean. Statistical analysis was performed using a two-tailed paired t-test.

Cell cultures

HEK293 cells stably expressing the fully edited version (VCR) of the GluR6 subunit (Howe *et al.*, 1995), under the control of an inducible promoter (Marshall and Howe, 1994), were obtained from J. R. Howe, Yale University (New Haven, CT, U.S.A.). Cells were maintained in continuous culture in MEM-E medium (Sigma, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum, 2 μg/ml tetracycline and 0.5 mg/ml G-418. Two to four days prior to an experiment cells were trypsinized, seeded onto 35 mm tissue culture dishes and fed with medium lacking tetracycline to induce expression of the GluR6 subunit. Dorsal root

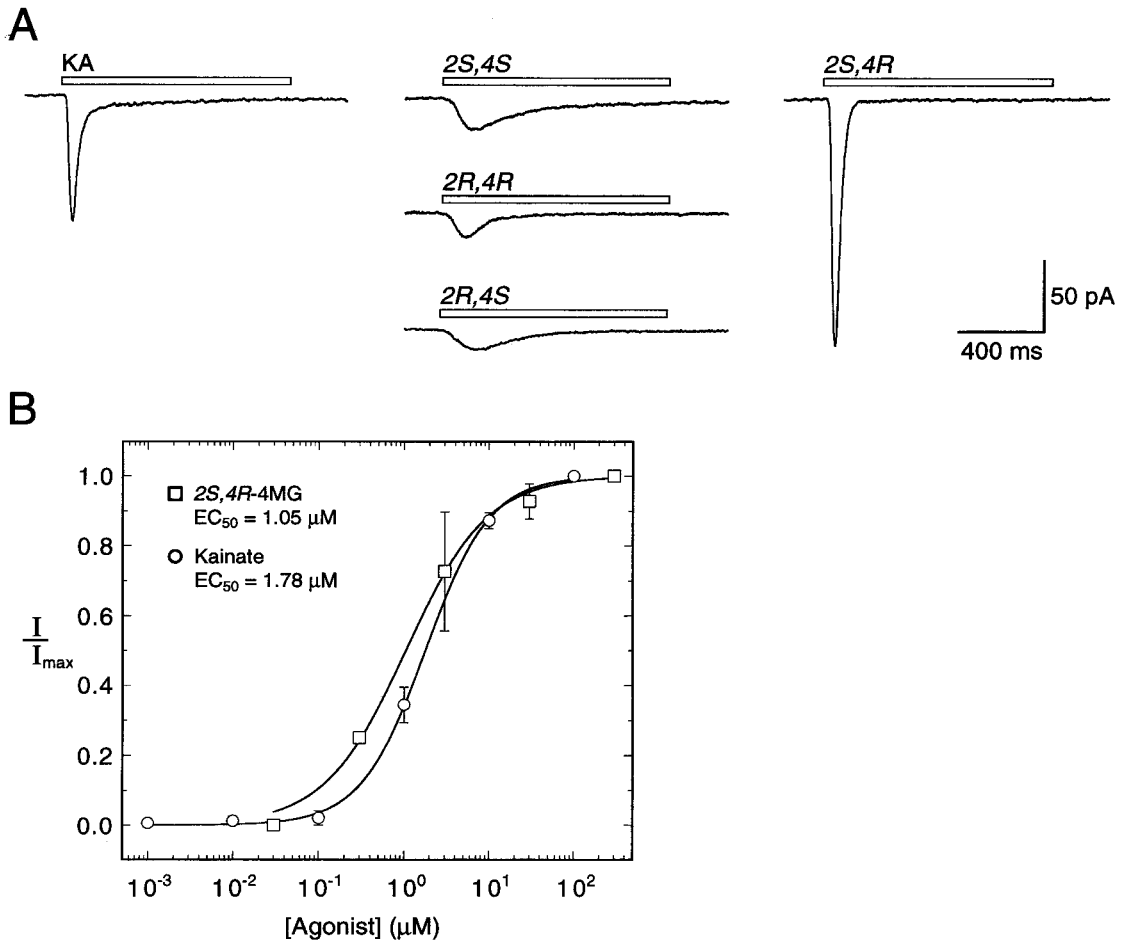

Fig. 1. Activation of currents by the four diastereomers of 4-methylglutamate and kainate (KA) in HEK293-GluR6 cells. (A) Inward current responses in a single cell voltage clamped at −70 mV. All compounds were tested at a concentration of 100 μM. "2S,4R" is 2S,4R-4-methylglutamate. In this and subsequent figures, bars indicate the duration of application of the specified drug. (B) Concentration–response profiles for 2S,4R-4MG and kainate. Each data point represents an average of responses from five cells for each concentration of kainate and three cells for each concentration of 2S,4R-4MG. Values are normalized to the current at 100 μM for kainate and 300 μM for 2S,4R-4MG. n = 0.9 for 2S,4R-4MG; n = 1.1 for kainate. Curve fits were applied as described in Materials and Methods.

ganglion (DRG) cells were isolated as described (Wilding and Huettner, 1995) from Long Evans rats 2–20 days after birth. Ganglia were incubated for 20–30 minutes with protease type XXIII (Sigma), then dissociated by trituration in buffered saline containing trypsin inhibitor.

Drugs

The four diastereomers of 4-methylglutamate were prepared using a stereo-synthetic pathway (Gu *et al.*, 1995). Purity of the enantiomers was determined by HPLC to be >99.9%. All other compounds were obtained from Sigma, unless otherwise noted.

RESULTS

Activation of kainate receptors by diastereomers of 4-methylglutamate

Rapid delivery of micromolar concentrations of any of the four diastereomers of 4-methylglutainate produced inward currents in HEK293 cells expressing the GluR6 subunit (Fig. 1A). Untransfected HEK293 cells were unresponsive to applications of 2S,4R-4MG (60 μM), kainate (300 μM) or domoate (3 μM). The enantiomers were initially tested at 1 and 100 μM to obtain preliminary information on their potencies and general response properties. At the higher concentration, each compound elicited an inward current that subsequently decayed to baseline (Fig. 1A). Only one of the enantiomers, 2S,4R-4MG, induced substantial current at 1 μM. Comparison of peak inward currents, normalized to the peak current elicited in the same cell by a near maximal concentration of kainate, confirmed the higher potency of 2S,4R-4MG (Table 1). These results are consistent with earlier studies (Gu *et al.*, 1995) showing high affinity binding of (2S,4R)-4-methylglutamate, but not the (2R,4R), (2S,4S) or (2R,4S) stereoisomers, to

Table 1. Peak currents evoked by glutamate and the four diastereomers of 4-methylglutamate relative to kainate

	Compound				
		4-methylglutamate			
Concentration	Glutamate	2S,4R	2R,4R	2S,4S	2R,4S
1 μM	n.t.	0.42 ± 0.08 (7)	0 (2)	0 (1)	0 (5)
100 μm	0.53 ± 7 (4)	1.89 ± 0.22 (9)	0.48 (2)	0.43 ± 0.06 (3)	0.40 ± 0.06 (6)
1000 μm	1.07 ± 0.11 (4)	n.t.	n.t.	n.t.	n.t.

All values are normalized to the response to 100 μM kainate in the same cell and expressed as mean ± SEM of n cells; n.t., not tested.

high affinity kainate binding sites in brain. Therefore, 2S,4R-4MG was selected for detailed analysis.

The relative potencies of 2S,4R-4MG and kainate for activating whole-cell currents were compared by measuring peak inward currents over a range of ligand concentrations (Fig. 1B). Curve-fitting with the logistic equation yielded EC_{50} values of 1.78 ± 0.07 and 1.05 ± 0.11 μM for kainate and 2S,4R-4MG, respec-

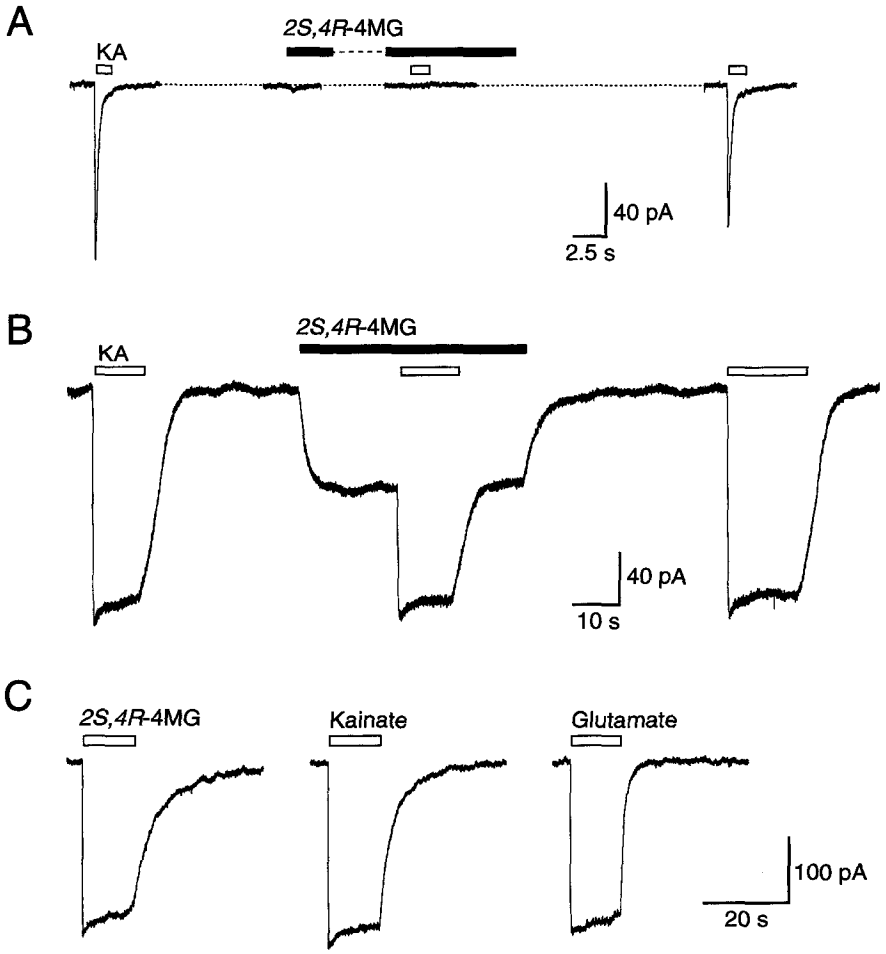

Fig. 2. Blockade of homo- and cross-desensitization by Con A. (A) Rapid application of 60 nM 2S,4R-4MG causes a small current (second trace from left) and subsequent insensitivity to kainate. Kainate responsivity returns after a 3 min wash. Open bars indicate times of kainate application (KA, 100 μM here and in (B)); solid bars indicate times of 2S,4R-4MG application; dashed line between bars represents a 1 min gap in the record. (B) Following a 10 min treatment of the cell in (A) with Con A (0.3 mg/ml), kainate generates a sustained current and 60 nM 2S,4R-4MG elicits a substantial sustained current. Coapplication of kainate induces the same amount of inward current as kainate alone. (C) In a different cell, near maximal concentrations of 2S,4R-4MG (60 μM), kainate (300 μM) and glutamate (300 μM) induce similar inward currents in the presence of Con A.

tively. Hill slopes measured 0.9–1.1 for both ligands. Full concentration–response data for glutamate was not obtained, but our data are consistent with an EC_{50} between 100 and 1000 μM; Raymond *et al.* (1993) reported an EC_{50} Value of 270 μM for glutamate at homomeric GluR6 receptors.

In measurements taken from the same cells, peak responses to 2S,4R-4MG were significantly larger ($p < 0.01$) than those stimulated by kainate (Fig. 1A); the amplitude ratio (2S,4R-4MG:kainate) was 1.89 + 0.22. The lower apparent efficacy of kainate compared to 2S,4R-4MG was not an artifact resulting from incomplete recovery from desensitization or run-down, since both were controlled for by routinely reversing the order of agonist application. Desensitization during the rising phase of the response to kainate, however, could potentially reduce the peak response, since, at least for DRG-type kainate receptors, responses to glutamate are dependent on the speed of agonist application (Huettner, 1990).

Steady-state potency in cells treated with concanavalin A

In order to study differences in the steady-state activation of GluR6 receptors, we treated cultures with concanavalin A (Con A), a lectin that is known to block desensitization of kainate receptors (Huettner, 1990; Wong and Mayer, 1993). The effects of Con A treatment on responses to 2S,4R-4MG and kainate are shown in Fig. 2. In the absence of Con A, a low concentration of 2S,4R-4MG (60 nM) evoked a threshold current and completely desensitized the receptors (Fig. 2A). In the same cell, after a 5 min treatment with Con A (0.3 mg/ml), both kainate and 2S,4R-4MG elicited sustained currents. In addition, the current evoked by co-application of kainate and 2S,4R-4MG was equal to that elicited by kainate alone, consistent with the activation of a common pool of receptors by both agonists. Therefore, Con A treatment abolished the rapid desensitization produced by high concentrations of kainate, as well as the cross desensitization of kainate by low concentrations of 2S,4R-4MG. Following exposure to Con A, near maximal concentrations of 2S,4R-4MG, kainate and glutamate evoked inward currents of similar amplitude (Fig. 2C). 2S,4R-4MG (100 μM) and glutamate (1 mM) activated 101.3 $\pm$ 1.6 ($n = 11$) and 99.2 $\pm$ 0.8 ($n = 4$) percent, respectively, of the current activated by kainate (100 μM) in the same cells.

The observation that threshold concentrations of 2S,4R-4MG were able to elicit substantial currents in Con A-treated cells (Fig. 2B) suggested that lectin treatment had significantly altered the concentration-response function for this compound (see Discussion). Therefore, we repeated concentration–response experiments for 2S,4R-4MG and kainate in cells that had been pretreated with Con A. For 2S,4R-4MG the EC_{50} decreased 11-fold from 1050 nM (in the absence of Con A) to 91 nM, whereas for kainate the EC_{50} changed only slightly, decreasing from 1780 to 980 nM after treatment with Con A (Fig. 3).

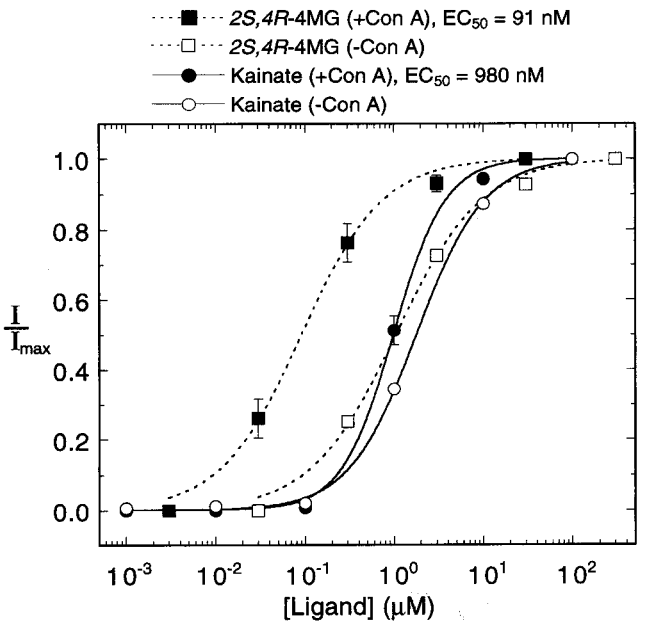

Fig. 3. Concentration–response relationships for 2S,4R-4MG and kainate in the presence of Con A. ■ (2S,4R-4MG) and ● (kainate) indicate averaged responses to three cells pretreated with Con A. $n = 1.0$ for 2S,4R-4MG; $n = 1.5$ for kainate. Concentration–response data in the absence of Con A from (Fig. 1B) is shown for comparison (○, □).

Potencies for desensitization by 2S,4R-4MG, kainate and glutamate

In cells that had not been exposed to Con A, both 2S,4R-4MG and kainate caused rapid and complete desensitization of GluR6 receptors at all concentrations that elicited substantial currents. To determine whether desensitization was produced by concentrations of 2S,4R-4MG near the threshold for current activation, we applied 1 min prepulses of the drug at 100 nM and tested for inhibition of the current evoked by a much higher concentration (30 μM). Although the low concentration of 2S,4R-4MG stimulated very little inward current, it completely eliminated the response to a saturating dose of the compound (Fig. 4A). One minute preapplications of 2S,4R-4MG caused a similar reduction of currents stimulated by a near saturating concentration of kainate (Fig. 4B) or glutamate (data not shown). We also observed cross desensitization of 2S,4R-4MG responsivity by pre-application of 1 μM kainic acid (data not shown).

The potencies of 2S,4R-4MG, kainate and glutamate for inducing steady-state desensitization of GluR6 receptors were determined using the same procedure as outlined above. Peak currents in response to kainate (100 μM) were measured before and immediately following a 1 min pre-application of one of the three

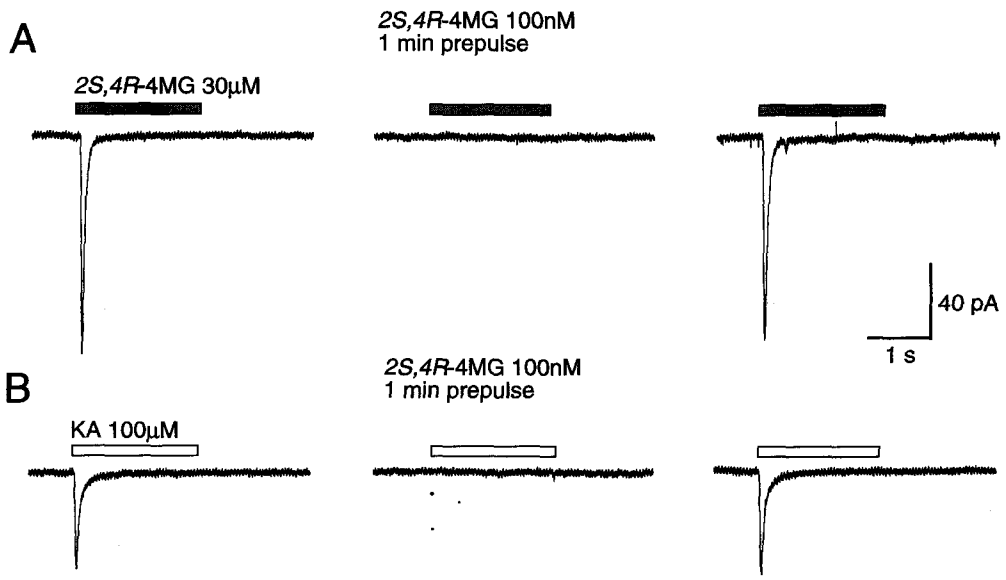

Fig. 4. Inhibition of currents generated by 2S,4R-4MG and kainate by low concentrations of 2S,4R-4MG. (A) One minute preapplication of 100 nM 2S,4R-4MG completely blocks (center trace) currents produced by a near maximal concentration of 2S,4R-4MG. (B) A similar preapplication of 2S,4R-4MG abolishes current induced by a near saturating concentration of kainate. Responses in (A) and (B) are from the same cell.

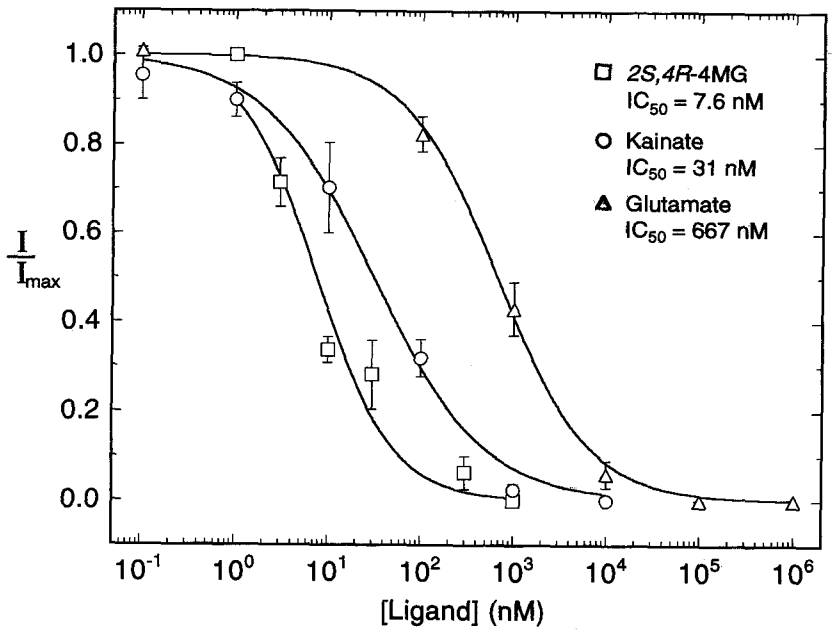

Fig. 5. Concentration–inhibition curves for 2S,4R-4MG, kainate and glutamate. Each compound was tested for its ability to inhibit the response to a 100 μM application of kainate. Compounds were applied for 1 min before application of kainate. Data points represent the average of responses from 5–12 cells for 2S,4R-4MG, 4–10 cells for kainate and three to five cells for glutamate. Hill slope n = 1.1 for 2S,4R-4MG, 0.7 for kainate and 0.9 for glutamate.

ligands. As shown in Fig. 5, 2S,4R-4MG desensitized GluR6 receptors with an IC_{50} of 7.6 nM, whereas kainate and glutamate were significantly less potent, displaying IC_{50}s of 31 and 667 nM, respectively. For all three agonists, desensitization was complete at the highest concentrations tested. Preliminary experiments revealed that the three low potency diastereomers of 4-methylglutamate also produced approximately half-maximal steady-state desensitization at a concentration of 1 μM (data not shown).

Kinetics of onset and recovery from desensitization

To determine if the larger transient currents observed for 2S,4R-4MG compared to kainate were due to

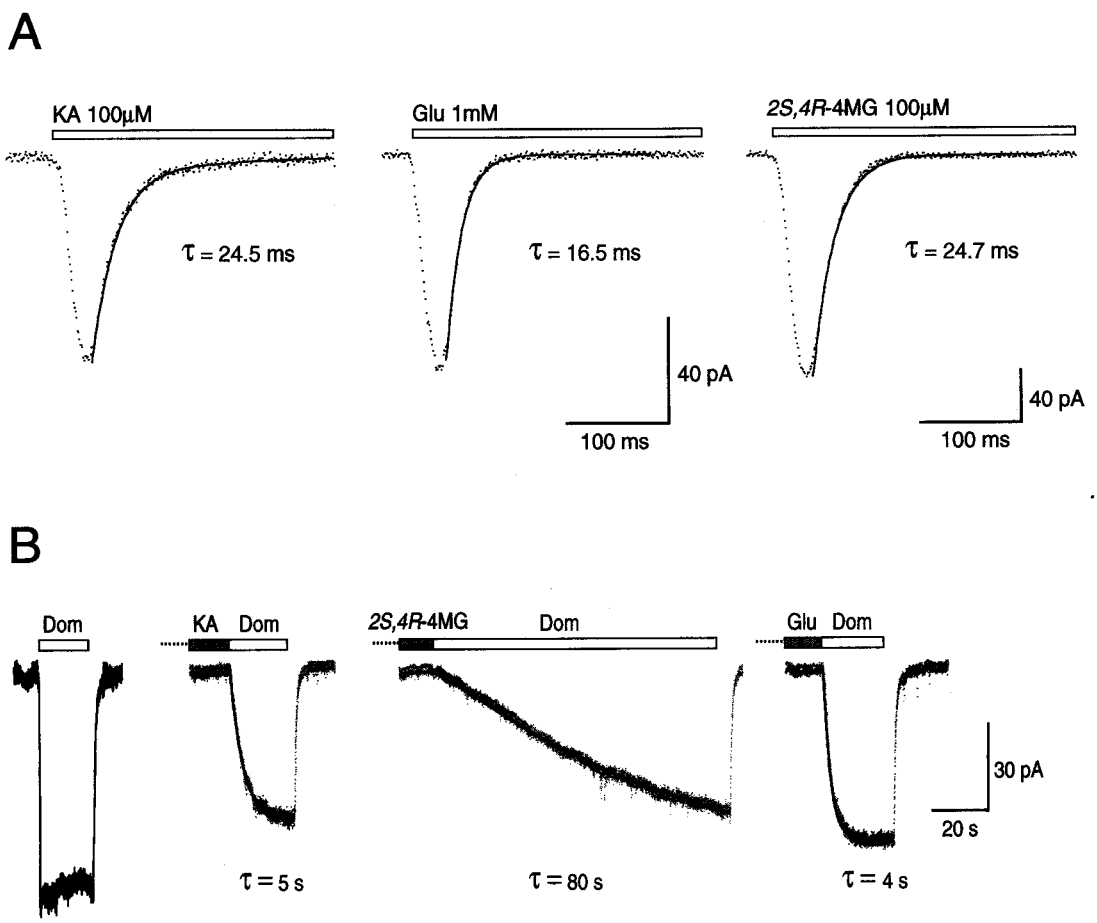

Fig. 6. Kinetics of entry and recovery from desensitization in HEK293-GluR6 cells. (A) Rate constants of entry into desensitization by kainate, glutamate and 2S,4R-4MG. Inward currents were elicited by the concentration of compounds as shown. The rate of current decay was measured by fitting a portion of the curve using the exponential curve-fitting routine in Pulse + PulseFit (HEKA Elektronik). Rate constants for these examples are as shown. Note that the trace for 2S,4R-4MG is scaled to match the amplitude of the other traces. All responses are from the same cell. (B) Rates of recovery from desensitization by kainate, 2S,4R-4MG and glutamate. A pulse of domoic acid (Dom; 2 μM) elicits a non-desensitizing current in an HEK293-GluR6 cell. Subsequently, kainate (3 μM) is applied for 1 min (indicated by dotted line) to induce desensitization. The solution is then rapidly switched from kainate to domoic acid (2 μM). Single exponential fit of the data (continuous dark curve) yields a rate constant of 5 sec. The same protocol was used to measure the rate of recovery from desensitization induced by 2S,4R-4MG (1 μM) and glutamate (100 μM). All traces are from the same cell. The concentration of domoate is 2 μM throughout.

differences in desensitization kinetics, the decaying phases of whole-cell currents were fitted with exponential functions. In general, the time courses of desensitization elicited by all three agonists were well fit by single exponential functions (Fig. 6A). In the few cases where time courses were better fit by double exponential functions, the slower time constant accounted for less than 15% of the total amplitude of the decay and, therefore, only the faster time constants were included in the data analyzes. Exponential decay times were similar for 2S,4R-4MG, kainate and glutamate, having mean values of 26 $\pm$ 5 (n = 5), 31 $\pm$ 5 (n = 5) and 23 $\pm$ 8 msec (n = 4), respectively.

In early experiments, we noticed that currents took longer to recover from pre-exposure to 2S,4R-4MG than they did to either kainate or glutamate. We quantified rates of recovery from desensitization using domoic acid, an agonist that stimulates a large steady-state current in cells expressing the GluR6 receptor (Köhler *et al.*, 1993). In control cells, the rising phase of current evoked by 2 μM domoic acid was well fit by a single exponential having a time constant of 182 $\pm$ 42 msec (n = 4; data not shown). After a 1 min application of a maximal concentration of kainate, glutamate or 2S,4R-4MG, however, the onset of current elicited by domoic acid was slowed considerably (20–400-fold), reflecting the slow kinetics of recovery from desensitization (Fig. 6B). For each of the compounds, recoveries from desensitization were well fit by single exponentials, but for 2S,4R-4MG the rate constant for recovery (83.0 $\pm$ 3.1 sec) was

20-fold slower than for either kainate (4.0 ± 0.4 sec) or glutamate (3.2 ± 0.4 sec).

Activation and desensitization of kainate receptors in DRG cells by 2S,4R-4MG

In contrast to the large transient currents elicited by 2S,4R-4MG at GluR6 receptors, rapid applications of this compound to freshly dissociated DRG cells evoked much smaller peak currents than kainate or glutamate (Fig. 7A). Compared to 300 μM kainate, the amplitudes of currents evoked by 4 and 16 μM 2S,4R-4MG were 20 ± 1% and 45 ± 6%, respectively ($n = 4$ cells). Higher concentrations of 2S,4R-4MG did not evoke larger currents (data not shown). Similar to the GluR6 receptors, however, pre-exposure to 2S,4R-4MG caused complete desensitization of kainate currents in the DRG cells (Fig. 7B). The IC_{50} for steady-state desensitization produced by a 10 sec prepulse of 2S,4R-4MG or glutamate was determined from the reduction in peak current elicited by 300 μM kainate. As shown in Fig. 7C, 2S,4R-4MG desensitized DRG kainate receptors with an IC_{50} of 11 nM, whereas glutamate was significantly less potent ($IC_{50} = 3.4$ μM). Full recovery from desensitization to 2S,4R-4MG took at least 5–7 min, even when drug concentrations of 0.25–1 μM were applied for only 1–10 sec.

As previously described (Huettner, 1990; Wong and Mayer, 1993), brief exposure of DRG cells to Con A eliminated kainate receptor desensitization. As in the HEK293 cells expressing the GluR6 subunit, 2S,4R-4MG, kainate and glutamate activated sustained currents with very similar maximal amplitudes following treatment with Con A. The EC_{50} for activation of whole-cell current by 2S,4R-4MG was 163 nM (Fig. 7D) in Con A treated cells, a concentration that did not produce any discernible current prior to incubation with the lectin. Coapplication of the competitive antagonists ACEA-1011 (30 μM) and NS-102 (10 μM) produced 93 ± 2% and 46 ± 5% inhibition of currents activated by 250 nM 2S,4R-4MG, respectively, which is consistent with the potency of these drugs against DRG kainate receptors (Wilding and Huettner, 1996).

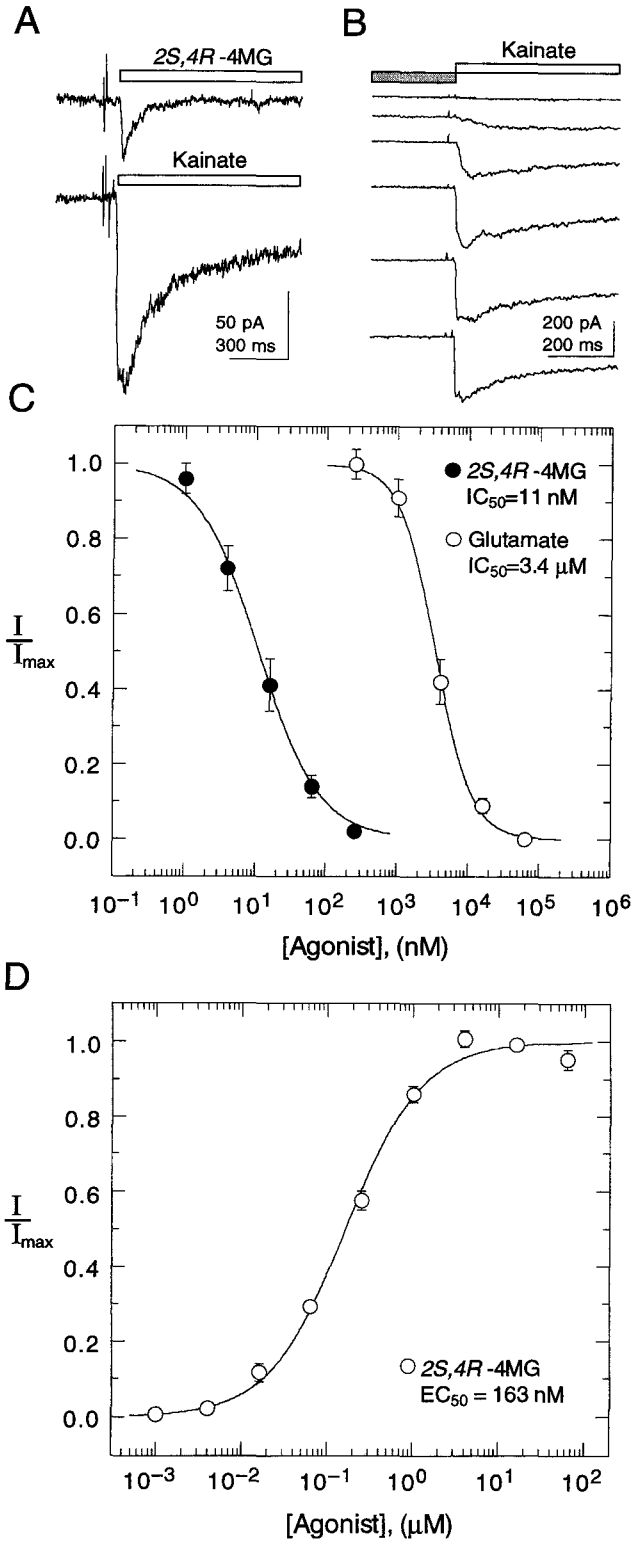

Fig. 7. Activation and desensitization of kainate receptors, by 2S,4R-4MG in DRG neurons. (A) Whole cell currents evoked by 300 μM kainate and 16 μM 2S,4R-4MG in a single DRG cell held at −80 mV. (B) Desensitization induced by a 10 sec prepulse of 2S,4R-4MG. Traces show the effect of (top to bottom) 250, 63, 16, 4, 1 and 0 nM 2S,4R-4MG (shaded bar) on current evoked by 300 μM kainate (open bar). (C) Desensitization induced by 2S,4R-4MG (●; six to eight cells/point) or glutamate (○; three to five cells/point). Points show the mean ± SEM of peak current as a fraction of the maximal response to kainate alone. Curves were fit as described in Materials and Methods. For 2S,4R-4MG, $IC_{50} = 11$ nM, $n = 1.0$; glutamate $IC_{50} = 3.4$ μM, $n = 1.7$. (D) Concentration–response relation for 2S,4R-4MG in cells exposed to Con A (mean ± SEM for 5–14 cells/data point). $EC_{50} = 163$ nM, $n = 0.98$.

DISCUSSION

This study has demonstrated the potency of kainate, 4-methylglutamate and glutamate to induce steady-state desensitization of cloned and native kainate receptors. One of the 4-methylglutamate diastereomers, 2S,4R-4MG, was the most potent of all tested compounds,

producing half maximal desensitization at 7.6 nM for GluR6 receptors and 11 nM for native receptors on DRG neurons. Kainate and glutamate produced steady-state desensitization of GluR6 receptors with IC_{50} values of 31 nM and 667 nM, respectively. The three other diastereomers of 2S,4R-4MG had potencies roughly equal to glutamate. At DRG neurons the IC_{50} for glutamate was 3.4 μN; kainate produced only incomplete desensitization in these cells (Huettner, 1990). Receptors that include the GluR5 subunit, found in DRG neurons (Bettler *et al.*, 1990; Herb *et al.*, 1992; Sommer *et al.*, 1992; Partin *et al.*, 1993), are known to show less complete desensitization than those containing GluR6 (Huettner, 1990; Herb *et al.*, 1992; Sommer *et al.*, 1992; Patneau *et al.*, 1994). The present study highlights the fact that glutamate, and close glutamate analogs such as 2S,4R-4MG, induce rapid and complete desensitization of both GluR6 and GluR5 (DRG) subtypes of kainate receptors.

The activity of 2S,4R-4MG was highly stereoselective, confirming an earlier binding study (Gu *et al.*, 1995). All four of the 4-methylglutamate diastereomers stimulated inward currents at high concentrations, but only the 2S,4R diastereomer evoked current at micromolar concentrations. In addition, only 2S,4R-4MG produced desensitization at nanomolar concentrations.

As has been suggested for AMPA receptors (Patneau and Mayer, 1991; Raman and Trussell, 1992), our IC_{50} determinations are likely to reflect the affinities of each ligand for the binding site of the kainate receptor in the desensitized conformation. A comparison of our IC_{50} values with those obtained from equilibrium measurements of [^{3}H]kainate displacement suggests that this relationship is true for the GluR6 kainate receptor. Previous studies using [^{3}H]kainate have reported IC_{50}s for 2S,4R-4MG, kainate and glutamate of 19, 28 and 540 nM, respectively (Verdoorn *et al.*, 1994; Zhou *et al.*, 1997).

Strong evidence that the mechanism of inhibition of GluR6 and DRG receptors by 2S,4R-4MG involves desensitization came from the cross-desensitization observed between kainate and 2S,4R-4MG, and from the currents recorded following exposure to Con A. Previous work has shown that Con A selectively prevents the desensitization of native and recombinant kainate receptors by kainate, glutamate and other agonists (Huettner, 1990; Partin *et al.*, 1993; Wang *et al.*, 1993; Wong and Mayer, 1993). In the present study, Con A pretreatment abolished the steady state inhibition produced by low concentrations (nM) of 2S,4R-4MG. Furthermore, the currents evoked by near saturating concentrations of kainate, glutamate and 2S,4R-4MG were of comparable amplitudes and were maintained for many seconds in the continued presence of agonist following exposure to the lectin.

We compared agonist EC_{50}s of kainate and 2S,4R-4MG using both steady-state (Con A pretreatment) and non-steady-state conditions. Because of limitations on the exchange of extracellular solutions during whole-cell recordings, our steady-state EC_{50} values are inherently more reliable than those derived from peak current amplitudes. Nevertheless, the values we obtained for kainate were virtually identical before and after exposure to Con A (EC_{50}s of 1.8 and 0.98 μM with Con A; see also Huettner (1990) and Wong *et al.* (1994)) and were comparable to those reported by others for the GluR6 receptor (0.8 μM; Howe *et al.*, 1995; 1.0 μM with Con A; Egebjerg *et al.*, 1991). In contrast, EC_{50} determinations for 2S,4R-4MG in the presence and absence of the lectin differed approximately 11-fold (1050 nM versus 91 nM with Con A). This shift in the apparent affinity for 2S,4R-4MG, but not kainate, may have resulted from desensitization during the onset of agonist exposure, which would be most pronounced for a high affinity agonist at low agonist concentrations. On the other hand, we can not rule out the possibility that exposure to Con A may have produced a genuine change in receptor activation by 2S,4R-4MG. For example, previous work on recombinant AMPA receptors has shown that blockade of desensitization by cyclothiazide is associated with an increase in agonist potency for specific subunit splice variants (Partin *et al.*, 1994). Further work using techniques that allow for more rapid solution exchange, such as recordings from outside-out patches (Trussell and Fischbach, 1989; Patneau *et al.*, 1993), will be necessary to resolve these possibilities.

The characteristic of kainate receptors to desensitize at agonist concentrations far below those required to elicit detectable currents appears to be an intrinsic property of the receptor. All three agonists, kainate, glutamate and 2S,4R-4MG, displayed a difference between IC_{50}s for desensitization and EC_{50}s for activation of 1.8–2.1 log units. This difference in apparent affinity for activation versus desensitization is similar to that described for AMPA receptors (Kiskin *et al.*, 1986; Trussell and Fischbach, 1989) where additional whole-cell and single channel data support the interpretation that channels may enter a desensitized state before going into a measurable open conformation.

In earlier work on a series of halogenated willardiines, Wong *et al.* (1994) observed that for short agonist pulses (2 sec), the rate of recovery from desensitization in DRG neurons was inversely dependent on agonist potency. This result led them to propose that the time course of recovery was governed mainly by the rate of agonist unbinding from desensitized receptors. In the present study of GluR6 receptors, which employed desensitizing prepulses of longer duration (1 min), 2S,4R-4MG appeared to fit with this pattern, because it was the most potent desensitizing agent and also showed the slowest rate of recovery. Comparison of kainate and glutamate, however, revealed very little correlation between potency and recovery rate. Therefore, recovery from desensitization proceeded at very similar rates for kainate and glutamate (Huettner, 1990), despite more than a 20-fold difference in their potency for inducing steady-state

desensitization. These results suggest that, at least for long prepulses, the time course of recovery from desensitization is not strictly linked to agonist potency. Further work will be needed to determine whether the same holds true for shorter agonist applications or whether there exist multiple, time-dependent desensitized states for kainate receptors (Wong *et al.*, 1994).

Desensitization plays an important role in the physiological and pathological regulation of glutamate receptors (Zorumski and Mennerick, 1994). The present study suggests that for homomeric GluR6 and DRG-type kainate receptors, concentrations of glutamate of approximately 1 μM produce considerable desensitization. Although it is difficult to estimate the concentration of glutamate in synaptic spaces *in vivo*, micro-dialysis of extracellular fluid suggests that tonic levels of glutamate may be as high as 2–4 μM (Jacobson *et al.*, 1985; Lerma *et al.*, 1986); much higher concentrations are expected when ischemic or traumatic injury occurs (Benveniste *et al.*, 1984; Rothman and Olney, 1986; Choi, 1988). Recent work suggests that one role of kainate receptors may be to regulate presynaptic release of glutamate (Chittajallu *et al.*, 1996). Pharmacological control of their desensitization by drugs such as 2S,4R-4MG may offer a new route to the study of kainate receptor function in normal and pathological processes. 2S,4R-4MG displays a high potency for kainate receptors relative to AMPA receptors (EC_{50} 325 μM on rat cortical neurons; Zhou *et al.*, 1997) therefore, its subtype selectivity profile may be useful for distinguishing kainate from AMPA receptor mediated responses at synapses with mixed populations of receptors.

Acknowledgements—This work was supported by NIH grant NS30888 (JEH) and by the McDonnell Center for Cellular and Molecular Neurobiology (JEH).

REFERENCES

Benveniste H., Drejer J., Schousboe A. and Diemer N. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *Journal of Neurochemistry* **43:** 1369–1374.

Bettler B., Boulter J., Hermans-Borgmeyer I., O'Shea-Greenfield A., Deneris E. S., Moll C., Borgmeyer U., Hollmann M. and Heinemann S. (1990) Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. *Neuron* **5:** 583–595.

Bettler B., Egebjerg J., Sharma G., Pecht G., Hermans-Borgmeyer I., Moll C., Stevens C. F. and Heinemann S. (1992) Cloning of a putative glutamate receptor: a low affinity kainate-binding subunit. *Neuron* **8:** 257–265.

Chittajallu R., Vignes M., Dev K. K., Barnes J. M., Collingridge G. L. and Henley J. M. (1996) Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. *Nature* **379:** 78–81.

Choi D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1:** 623634.

Donevan S. D. and Rogawski M. A. (1993) GYKI 52466, a 2,3-benzodiazepine, is a highly selective noncompetitive antagonist of AMPA/kainate receptor responses. *Neuron* **10:** 51–59.

Egebjerg J., Bettler B., Hermans-Borgmeyer I. and Heinemann S. (1991) Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. *Nature* **351:** 745–748.

Gu Z.-Q., Hesson D., Pelletier J., Maccecchini M.-L., Zhou L.-M. and Skolnick P. (1995) Synthesis, resolution, and biological evaluation of the four stereoisomers of 4-methylglutamic acid: selective probes of kainate receptors. *Journal of Medical Chemistry* **38:** 2518–2520.

Herb A., Burnashev N., Werner P., Sakmann B., Wisden W. and Seeburg P. (1992) The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron* **8:** 775–785.

Howe J. R., Skryabin B. V., Belcher S. M., Zerillo C. A. and Schmauss C. (1995) The responsiveness of a tetracycline-sensitive expression system differs in different cell lines. *Journal of Biological Chemistry* **270:** 14168–14174.

Huettner J. E. (1990) Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. *Neuron* **5:** 255–266.

Jacobson I., Sandberg M. and Hamberger A. (1985) Mass transfer in brain dialysis devices: a new method for the estimation of extracellular amino acids concentration. *Journal of Neuroscience Methods* **15:** 263–268.

Kessler M., Arai A., Quan A. and Lynch G. (1996) Effect of cyclothiazide on binding properties of AMPA-type glutamate receptors: lack of competition between cyclothiazide and GYKI 52466. *Molecular Pharmacology* **49:** 123–131.

Kiskin N. I., Krishtal O. A. and Tsyndrenko A. Y. (1986) Excitatory amino acid receptors in hippocampal neurons: kainate fails to desensitize them. *Neuroscience Letters* **63:** 225–230.

Köhler M., Burnashev N., Sakmann B. and Seeburg P. (1993) Determinants of Ca^{++} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. *Neuron* **10:** 491–500.

Lerma J., Herranz A., Herreras O., V V. A. and Rio R. D. (1986) *In vivo* determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. *Brain Research* **384:** 145–155.

Lerma J., Paternain A., Naranjo J. and Mellström B. (1993) Functional kainate-selective glutamate receptors in cultured hippocampal neurons. *Proceedings of the National Academy of Science U.S.A.* **90:** 11688–11692.

Li F., Owens N. and Verdoorn T. A. (1995) Functional effects of mutations in the putative agonist binding region of recombinant α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Molecular Pharmacology* **47:** 148–154.

Marshall J. and Howe J. R. (1994) Stable expression of GluR6(R) in HEK293 cells using a tetracycline inducible promoter. *Social Neuroscience Abstracts* **20:** 493.

Partin K. and Mayer M. (1996) Negative allosteric modulation of wild-type and mutant AMPA receptors by GYKI 53655. *Molecular Pharmacology* **49:** 142–148.

Partin K., Patneau D. and Mayer M. (1994) Cyclothiazide differentially modulates desensitization of α-amino-3-hy-

droxy-5-methyl-4-isoxazolepropionic acid receptor splice variants. *Molecular Pharmacology* **46:** 129–138.

Partin K. M., Patneau D. K., Winters C. A., Mayer M. L. and Buonanno A. (1993) Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavalin A. *Neuron* **11:** 1069–1082.

Paternain A. V., Morales M. and Lerma J. (1995) Selective antagonism of AMPA receptors unmasks kainate receptor-mediated responses in hippocampal neurons. *Neuron* **14:** 185–189.

Patneau D. and Mayer M. (1991) Kinetic analysis of interactions between kainate and AMPA: evidence for activation of a single receptor in mouse hippocampal neurons. *Neuron* **6:** 785–798.

Patneau D., Vyklicky L. and Mayer M. (1993) Hippocampal neurons exhibit cyclothiazide-sensitive rapidly desensitizing responses to kainate. *Journal of Neuroscience* **13:** 3496–3509.

Patneau D. K., Wright P. W., Winters C., Mayer M. L. and Gallo V. (1994) Glial cells of the oligodendrocyte lineage express both kainate- and AMPA-preferring subtypes of glutamate receptors. *Neuron* **12:** 357–371.

Pelletier J., Hesson D., Jones K. A. and Costa A.-M. (1996) Substituted dihydrophthalazines: novel allosteric inhibitors of the AMPA subtype of the glutamate receptor. *Journal of Medical Chemistry* **39:** 343–346.

Raman I. M. and Trussell L. O. (1992) The kinetics of the response to glutamate and kainate in neurons of the avian cochlear nucleus. *Neuron* **9:** 173–186.

Raymond L. A., Blackstone C. D. and Huganir R. L. (1993) Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. *Nature* **361:** 637–641.

Rothman S. M. and Olney J. W. (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Annals of Neurology* **19:** 105–111.

Ruano D., Lambolez B., Rossier J., Paternain A. and Lerma J. (1995) Kainate receptor subunits expressed in single cultured hippocampal neurons: molecular and functional variants by RNA editing. *Neuron* **14:** 1009–1117.

Sommer B., Burnashev N., Verdoorn T. A., Keinänen K., Sakmann B. and Seeburg P. H. (1992) A glutamate receptor channel with high affinity for domoate and kainate. *EMBO Journal* **11:** 1651–1656.

Spruston N., Jonas P. and Sakniann B. (1995) Dendritic glutamate receptor channels in rat hippocampal CA3 and CA1 pyramidal neurons. *Journal of Physiology* **482:** 325–352.

Tang C.-M., Shi Q.-Y., Katchman A. and Lynch G. (1991) Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. *Science* **254:** 288–290.

Tarnawa I., Berzenyi P., Andrasi F., Botca R., Hamori T., Ling I. and Korosi J. (1993) Structure-activity relationships among 2,3-benzodiazepine compounds with AMPA antagonistic action. *Bioorganic Medical Chemistry Letters* **3:** 99–104.

Trussell L. O. and Fischbach G. D. (1989) Glutamate receptor desensitization and its role in synaptic transmission. *Neuron* **3:** 209–218.

Verdoorn T., Johansen T., Drejer J. and Nielsen E. (1994) Selective block of recombinant glur6 receptors by NS-102, novel non-NMDA receptor antagonist. *European Journal of Pharmacology* **269:** 43–49.

Wang L.-Y., Taverna F., Huang X.-P., MacDonald J. and Hampson D. (1993) Phosphorylation and modulation of a kainate receptor (GluR6) by cAMP-dependent protein kinase. *Science* **259:** 1173–1175.

Werner P., Voight M., Keinänen K., Wisden W. and Seeburg P. H. (1991) Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. *Nature* **351:** 742–744.

Wilding T. J. and Huettner J. E. (1995) Differential antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-preferring and kainate-preferring receptors by 2,3-benzodiazepines. *Molecular Pharmacology* **47:** 582–587.

Wilding T. J. and Huettner J. E. (1996) Antagonist pharmacology of kainate- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-preferring receptors. *Molecular Pharmacology* **49:** 540546.

Wisden W. and Seeburg P. H. (1993) Mammalian ionotropic glutamate receptors. *Current Opinions in Neurobiology* **3:** 291–298.

Wong L. A. and Mayer M. L. (1993) Differential modulation by cyclothiazide and concanavalin A of desensitization at native α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- and kainate-preferring glutamate receptors. *Molecular Pharmacology* **44:** 504–510.

Wong L. A., Mayer M. L., Jane D. E. and Watkins J. C. (1994) Willardiines differentiate agonist binding sites for kainate- versus AMPA-preferring glutamate receptors in DRG and hippocampal neurons. *Journal of Neuroscience* **14:** 3881–3897.

Yamada K. and Tang C. -M. (1993) Benzothiadiazides inhibit rapid glutamate receptor desensitization and enhance glutamatergic synaptic currents. *Journal of Neuroscience* **13:** 3904–3915.

Zhou, L.-M., Gu, Z.-Q., Costa, A. M., Yamada, K. A., Mansson, P. E., Giordano, T., Skolnick, P. and Jones K. A. (1997) (2S,4R)-4-Methylglutamic acid (SYM 2081): A selective, high affinity ligand for kainate receptors. *Journal of Pharmacology and Experimental Therapeutics* **280:** 422–427.

Zorumski, C. F. and Mennerick, S. J. (1994) Desensitization and the short-term modulation of AMPA/kainate receptors in vertebrate neurons. In: *Direct and Allosteric Control of Glutamate Receptors* (M. G. Palfreyman, I. J. Reynolds and P. Skolnick, Eds), pp. 57-75. CRC Press, Boca Raton.

Zorumski C. F., Yamada K. A., Price M. T. and Olney J. W. (1993) A benzodiazepine recognition site associated with the nonNMDA glutamate receptor. *Neuron* **10:** 61–67.