ABSTRACT

Glutamic acid activates ionotropic glutamate receptors that mediate excitatory transmission in the central nervous system. The introduction of a methyl group at position 4 of glutamic acid imparts selectivity for kainate receptors, relative to other (N-methyl-D-aspartate and \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) ionotropic glutamate receptors. Among the stereoisomers of 4-methylglutamic acid, the potency of the (2S,4R)-isomer (SYM 2081) to inhibit \([\text{3H}]\text{kainic acid binding to both wild-type (rat forebrain) and recombinant (GluR6) kainate receptors (IC}_{50} \text{ values of } \sim 32 \text{ and } 19 \text{ nM, respectively}) was comparable to that of kainic acid (IC}_{50} \text{ values of } \sim 13 \text{ and } 28 \text{ nM, respectively). SYM 2081 was } \sim 800-\text{ and } 200\text{-fold less potent as an inhibitor of radioligand binding to wild-type (rat forebrain) } \alpha \text{-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and } N\text{-methyl-D-aspartate receptors, respectively. Preexposure of human embryonic kidney 293 cells stably expressing GluR6 receptors to low concentrations of SYM 2081 (30–300 nM) resulted in a reversible blockade of the rapidly desensitizing currents produced by kainate application. At higher concentrations, SYM 2081 (EC}_{50} \text{ of } \sim 1 \text{ \( \mu \)}M elicited kainate-like, rapidly desensitizing, inward currents. Pretreatment of recombinant GluR6 receptors with concanavalin A both abolished the effect of SYM 2081 to block kainate-induced currents and revealed nondesensitizing currents induced by SYM 2081 alone. The latter observations provide strong support for the hypothesis that SYM 2081 blocks kainate-induced currents through a process of agonist-induced desensitization. SYM 2081 also activated \( \alpha \text{-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor currents in primary cultures of cerebral cortex and, consistent with data obtained by radioligand binding, was } \sim 5\text{-fold less potent than kainate (EC}_{50} \text{ values of } 325 \text{ and } 70 \text{ \( \mu \)}M, respectively) in this measure. SYM 2081 is a high-affinity, selective, kainate agonist that may prove useful both as a probe to examine the physiological functions of kainate receptors and as the prototype of a novel class of therapeutic agents.

Ionotropic glutamate receptors are distributed throughout the mammalian central nervous system, where they subserve neurotransmission at the majority of excitatory synapses. Molecular cloning studies have identified more than a dozen discrete subunits that comprise this heterogeneous family of ligand-gated ion channels (Hollmann and Heinemann, 1994; Schoepfer et al., 1994). Based on pharmacological and electrophysiological criteria, the receptors have been subclassified into NMDA, AMPA and kainate receptors. Although converging lines of evidence have implicated activation of NMDA and, to a lesser extent, AMPA receptors in the neuropathologies associated with stroke, head injury and seizures (Sheardown et al., 1990; Meldrum, 1992; Collingridge and Watkins, 1994), the role of kainate receptors in both physiological and pathophysiological processes remains unclear. Five cDNAs have been cloned (GluR5-7, KA1 and KA2) (Bettler et al., 1990, 1992; Egebjerg et al., 1991; Werner et al., 1991; Herb et al., 1992; Sommer et al., 1992) that, when expressed in heterologous cells, display most of the characteristics described for native kainate receptors, including rapid desensitization by kainate. There is good evidence that receptors on neurons of sensory dorsal root ganglia 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). Despite the widespread distribution of both \([\text{3H}]\text{kainate binding sites (London and Coyle, 1979; Coyle, 1983; Honoré et al., 1986) and mRNAs encoding kainate receptors (Wisden and Seeburg, 1993) in brain, with few exceptions (Huettner, 1990; ABBREVIATIONS: AMPA, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Con A, concanavalin A; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMDA, N-methyl-d-aspartate; SYM 2081, (2S,4R)-4-methylglutamic acid.}
Paternain et al., 1995) it has been difficult to demonstrate kainate receptor-mediated currents in neurons. This may be due to the rapid desensitization produced by kainate in dorsal root ganglia and cultured hippocampal neurons (Huttner, 1990; Lerma et al., 1993; Ruano et al., 1995), the "masking" of kainate responses by larger, AMPA receptor-mediated currents (Paternain et al., 1995) and the paucity of selective, high-affinity kainate receptor ligands.

Most agonists and antagonists of non-NMDA receptors show only limited selectivity between the AMPA and kainate receptor subtypes (Wong et al., 1994; Wilding and Huttner, 1996). For example, although kainic acid is roughly 100-fold selective for kainate receptors over AMPA receptors (Huttner, 1990; Patneau et al., 1994; Paternain et al., 1995), the agonist elicits large sustained (nondesensitizing) currents due to its activation of AMPA receptors (Paternain and Mayer, 1991) in hippocampal and neocortical neurons. This activity complicates the use of kainate as a selective compound (Paternain et al., 1995). The most selective antagonists, such as the quinoxalinedione derivative ACEA-1011 and the isatin oxime NS-102, display only 10- to 20-fold preference for kainate receptors over AMPA receptors (Verdoorn et al., 1994; Wilding and Huttner, 1996). We have recently reported the stereoselective synthesis of the four diastereomers of 4-methylglutamate (Gu et al., 1995). Among these isomers, the (2S,4R)-form (SYM 2081) displayed an affinity for kainate binding sites in brain comparable to that of kainic acid. Here, we demonstrate that SYM 2081 is a high-affinity, competitive inhibitor of [3H]kainate binding to recombinant GluR6 receptors and that SYM 2081 potently blocks kainate responses through desensitization of the ion channel. Furthermore, these effects are observed at concentrations 2 to 3 orders of magnitude lower than those required to affect ligand binding to other (NMDA and AMPA) ionotropic glutamate receptors.

Materials and Methods

Membrane preparation for [3H]kainate and [3H]AMPA binding. Cerebral cortical membranes from adult, male, Sprague-Dawley rats (175–300 g; Taconic Farms, Germantown, NY) were prepared essentially as described (London and Coyle, 1979; Honoré et al., 1986). All procedures were carried out at 0–4°C unless otherwise indicated. Tissues were disrupted (Polytron homogenizer, set-7.4) as described above. The homogenate was centrifuged at 25,000 g and recentrifuged. The resulting pellet was resuspended in 10 volumes of 5 mM HEPES/4.5 mM Tris buffer (pH 7.6) containing 0.32 M sucrose. The homogenate was diluted to 50 volumes with assay buffer (5 mM HEPES/4.5 mM Tris buffer, pH 7.6) and centrifuged at 1,000 g for 20 min. The resulting pellet was homogenized in 50 volumes of buffer and centrifuged at 8,000 g for 20 min. The supernatant and "buffy" pellet coat were collected and centrifuged at 25,000 g for 20 min. The resulting pellet was suspended in assay buffer containing 1 mM Na3EDTA, and the suspension was recentrifuged. This washing procedure was repeated four times, with EDTA being absent from the last cycle. The resulting pellet was resuspended in 5 volumes of assay buffer, frozen over solid CO2 and stored at −70°C. On the day of assay, tissues were thawed, diluted 10-fold with assay buffer and centrifuged at 25,000 g for 20 min. The resulting pellet was resuspended in 50 volumes of assay buffer for NMDA receptor binding assays.

[3H]Kainate binding. Assays were routinely performed in a total volume of 500 μl containing membrane suspension (~200 μg of protein), [3H]kainate (final concentration, 5–10 nM), test compounds (50 μM Tris-HCl buffer (pH 7.4) containing 1 mM Na4EDTA) and buffer to a final volume of 500 μl. Nonspecific binding was defined using 0.6 mM glutamate. Assays were initiated by the addition of [3H]kainate, incubated at 0–4°C for 60 min and terminated by rapid filtration (Brandel M-24R cell harvester) through Whatman GF/C glass fiber filters, followed by two 3-ml washes with ice-cold assay buffer. Saturation isotherms in HEK293-GluR6 membranes were prepared using [3H]kainate concentrations from ~2 to 150 nM.

[3H]AMPA binding. [3H]AMPA binding was assayed essentially as described by Honoré et al. (1988), in 30 mM Tris-HCl buffer (pH 7.4) containing 2.5 mM CaCl2 and 100 mM KSCN. Assays contained 250 μl of membrane suspension (~200 μg of protein), 50 μl of test compounds, 50 μl of [3H]AMPA (final concentration, 10 nM) and buffer to a final volume of 500 μl. Nonspecific binding was assessed with 0.6 mM glutamate. Assays were terminated after 30 min (0–4°C) as described above.

[3H]CGP 39653 binding. Membrane suspensions (~100 μg of protein) were incubated with 5 nM [3H]CGP 39653, test compounds and assay buffer (5 mM HEPES/4.5 mM Tris, pH 7.6), in a final volume of 500 μl. Nonspecific binding was determined with 1 mM l-glutamate. After a 90-min incubation (0–4°C), assays were terminated by rapid filtration over Whatman GF/F glass filters as described above.

[3H]MK-801 binding. Assays consisted of 250 μl of membrane suspension (50–100 μg of protein), 50 μl of test compound, 50 μl of [3H]MK-801 (final concentration, 5 μM) and assay buffer (5 mM HEPES/4.5 mM Tris, pH 7.6), in a volume of 500 μl. Nonspecific binding was assessed with phencyclidine hydrochloride (100 μM). Assays were incubated at room temperature for 2 hr and terminated by rapid filtration over Whatman GF/B glass fiber filters that had been presoaked in 0.03% polyethyleneimine, as described above.
Electrophysiology. Currents generated by activation of kainate receptors were recorded from HEK293 cells expressing the GluR6 subunit, prepared as described under “Membrane Preparation in Materials and Methods.” Additional experiments were performed on 1- to 2-week-old primary cultures of rat cerebral cortical neurons prepared as previously described (Baughman et al., 1991). Patch pipettes (3–5 MΩ) contained 140 mM CsCl, 2 mM MgCl₂, 1.1 mM tetrastodium ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 1.0 mM CaCl₂ and 10 mM HEPES (pH 7.2). Cells were bathed in a solution containing 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES (pH 7.4). Currents were filtered at 0.5 to 1.0 kHz and digitized (EPC-9; HEKA Elektronik) using a sampling frequency of 1 to 2 kHz. Agonists were applied to cells using a gravity-fed perfusion system consisting of six micropipette tubes (0.32 mm o.d.; J&W Scientific) placed concentrically into the lumen of a 1.7-mm o.d. glass tube. The outer tube was pulled around the inner tubes to form a nozzle having a final o.d. of 0.3 mm. Dead space between the ends of the perfusion tubes and the tip of the nozzle was 2 to 3 μL. Solution exchange times measured 4 to 6 msec (10–90% of steady-state current) at the tip of a patch pipet placed in the position of a cell. For some experiments, a single pair of microcapillary tubes was used to deliver agonists. Results using either method were comparable and were pooled. The bath was constantly exchanged at a rate of control solution. Concentration-response curves were fitted with the logistic equation $I = I_{max} \frac{[A]^n}{[A]^n + K_d}$, where $I$ is the concentration of the drug and $n$ is the Hill coefficient. Fits were made with a Marquardt-Levenberg, nonlinear, least-squares, curve-fitting algorithm (Kaleidagraph).

Materials. [3H]Kainate (specific activity, 63 Ci/mmol), [3H]MK-801 (specific activity, 20.3 Ci/mmol) and [3H]CGP 39653 (specific activity, 42 Ci/mmol) were purchased mixture of 4-methylglutamate isomers (Olverman et al., 1988), high concentration of the individual 4-methylglutamate isomers, including SYM 2081, were low-potency NMDA receptor agonists, compared with glutamate. These isomers enhanced [3H]MK-801 binding with EC₅₀ values ranging from 14.3 ± 1.7 μM to >100 μM and inhibited the binding of [3H]CGP 39653 (a competitive glutamate antagonist) with IC₅₀ values ranging from 5.9 to 26.7 μM (table 1).

Characteristics of both wild-type (Huettner, 1990; Lerma et al., 1993; Patneau et al., 1994) and recombinant (Herb et al., 1992; Sommer et al., 1992; Raymond et al., 1993; Verdoorn et al., 1994) kainate receptors, fast application of kainic acid (100 μM) to HEK293 cells expressing GluR6 elicited rapidly desensitizing inward currents (fig. 2A, left). Sixty-second pre-applications of SYM 2081 using either fast perfusion or slow exchange in the bath reversibly blocked kainate currents (fig. 2A, middle and right). Inhibition measured 72 ± 8% (n = 5) at a concentration of 30 nM and 94 ± 4% (n = 5) at a concentration of 300 nM SYM 2081. Fast applications of

### Results

The effects of the four isomers of 4-methylglutamate were initially examined with wild-type glutamate receptors prepared from rat forebrain (table 1). Although all isomers exhibited selectivity for kainate receptors, compared with glutamate, the potency of SYM 2081 to inhibit [3H]kainate binding (IC₅₀, 32 ± 3 nM) was comparable to that of kainic acid (IC₅₀, 13 ± 2 nM). [3H]Kainate labels at least two populations of binding sites in rat brain (Honore et al., 1986; Johansen et al., 1993). Because the initial assay conditions used primarily label a “high-affinity” [3H]kainate binding site, the potency of SYM 2081 was also determined in the presence of 20 mM Ca²⁺, which optimizes radioligand binding to a population of “low-affinity” kainate binding sites (Honore et al., 1986; Johansen et al., 1993). Under these conditions, the IC₅₀ for SYM 2081 was 212 ± 16 nM (n = 4), compared with 62 ± 2.3 nM (n = 4) for kainic acid. Additional radioligand binding studies were performed in HEK293 cells that stably express the GluR6 isoform of the kainate receptor. SYM 2081 inhibited [3H]kainate binding to GluR6 receptors with a Kᵢ of 9.8 ± 3.5 nM (n = 3), compared with 14.3 ± 3.8 nM for kainic acid (n = 4) (fig. 1). Consistent with a competitive mode of action at GluR6, SYM 2081 (15 nM) increased the Kᵢ of [3H]kainic acid (from 6.6 ± 0.5 nM to 25.7 ± 1.8 nM) without significantly increasing the Bₘₐₓ (187.6 ± 31.7 vs. 192.6 ± 9.3 fmol/mg of protein).

Compared with glutamate, the 4-methylglutamate analogs were ±50-fold less potent as inhibitors of [3H]AMPA binding to rat brain membranes (table 1). SYM 2081 was ±5-fold less potent than kainate as an AMPA receptor ligand (table 1). Consistent with previous results obtained using the unresolved mixture of 4-methylglutamate isomers (Olverman et al., 1988), high concentrations of the individual 4-methylglutamate isomers, including SYM 2081, were low-potency NMDA receptor agonists, compared with glutamate. These isomers enhanced [3H]MK-801 binding with EC₅₀ values ranging from 424 Zhou et al.
higher concentrations of SYM 2081 (EC$_{50}$ 1.0 ± 0.1 μM; n = 3) produced rapidly desensitizing inward currents (fig. 2B) that resembled those elicited by kainate (fig. 2A). At these higher concentrations, complete desensitization was observed within 1 sec over the entire concentration range of SYM 2081 examined (0.3–30 μM).

In another series of experiments, cells were pretreated with Con A (0.3 mg/ml, for 5 min) to block agonist-induced desensitization (Huettner, 1990; Partin et al., 1993). In Con A-pretreated cells, kainate (100 μM) elicited large nondesensitizing currents that were unaffected by SYM 2081 (fig. 2C). Kainate-evoked currents in the presence of SYM 2081 (30 nM) were 98 ± 4% (n = 4) of control kainate responses in the absence of SYM 2081. In contrast, the same concentration of SYM 2081 reduced kainate-evoked currents to 28 ± 8% (n = 5) of control values in cells that were not treated with Con A.

Because radioligand binding studies demonstrated a lower affinity of SYM 2081 for AMPA receptors, compared with kainate (table 1), parallel concentration-response experiments were performed in rat neocortical neurons. Others have shown that, when stimulated by kainate, these neurons exhibit large sustained currents due to the activation of AMPA receptors (Wilding and Huettner, 1995); currents induced by stimulation of kainate receptors have not been reported for these cells (but see Paternain et al., 1995, for hippocampal neurons). High concentrations of SYM 2081 (>10 μM) produced sustained (nondesensitizing) currents similar in waveform to those evoked by kainate (fig. 3). However, SYM 2081 was ~4.6-fold less potent than kainate (EC$_{50}$ of 325 ± 23 μM vs. 70 ± 6 μM, n = 4).

**Discussion**

The identification of cDNAs encoding a family of kainate receptors (GluR5-7, KA1 and KA2) has resulted in significant insight into their structure and organization (Hollmann and Heinemann, 1994; Schoepfer et al., 1994; Bettler and Mulle, 1995). Nonetheless, the physiological functions of kainate receptors remain obscure, due, in part, to a lack of selective, high-affinity ligands. We demonstrate here that introduction of a methyl group at the 4-position of glutamic acid results in a marked increase in potency and selectivity for kainate receptors. Among the four stereoisomers, SYM 2081 exhibited the greatest selectivity for kainate receptors, inhibiting [3H]kainate binding to both high- and low-affinity sites in rat brain with a potency comparable to that of kainic acid.

**Fig. 1.** Left, inhibition of [3H]kainate ([3H]KA) binding to HEK293-GluR6 membranes by kainate ([ ]) and SYM 2081 ([ ]). In this experiment, the IC$_{50}$ values for kainate and SYM 2081 were 24.3 and 19.7 nM, respectively (see table 1 for details). Right, [3H]kainate binding to HEK293-GluR6 membranes and effect of SYM 2081, shown in a representative saturation isotherm and Scatchard plot (inset) of [3H]kainate binding in the absence ([ ]) and presence ([ ]) of SYM 2081 (15 nM). In this experiment, the K$_D$ of [3H]kainate was 7.1 and 29.3 nM in the absence and presence of SYM 2081, respectively; the corresponding B$_{max}$ values were 187 and 196 fmol/mg of protein.

**Fig. 2.** Electrophysiological effects of SYM 2081 in HEK293 cells expressing recombinant kainate (GluR6) receptors. A, left, application of kainic acid (KA) (100 μM) (open bar) results in rapidly desensitizing current. Middle, a 60-sec preapplication of SYM 2081 (30 nM) blocks kainate-induced currents. Right, kainate responses are fully recovered after a 4-min wash. B, high concentrations of SYM 2081 (3 μM) elicit kainate-like rapidly desensitizing inward currents. An EC$_{50}$ of 1.0 ± 0.1 μM was determined from three cells, each tested at five concentrations of SYM 2081 ranging from 0.03 to 300 μM (data not shown). C, Con A abolishes both kainate-induced desensitization and SYM 2081 inhibition of kainate currents. Left, kainic acid (100 μM) (open bars) induces a nondesensitizing current after Con A treatment (0.3 mg/ml for 5 min) of cells. Right, subsequent application of SYM 2081 (30 nM) (open bar) elicits a small, slowly activating current. After a 10-sec application of SYM 2081, coapplication of kainate induces a current equal in magnitude to that obtained in the absence of SYM 2081.
a potent effect on GluR6 receptors. Preapplication of this compound at a concentration of 30 nM induced a steady-state desensitization that abolished responses to kainate in a reversible manner. At much higher concentrations, SYM 2081 (1 μM) elicited currents that rapidly desensitized, resembling those evoked by kainate. These findings, taken together with the failure of SYM 2081 to block kainate currents in Con A-treated cells, support the hypotheses that SYM 2081 1) acts at the same binding site as kainic acid and 2) inhibits kainate currents via an agonist-induced desensitization. In toto, these findings lead us to conclude that SYM 2081 potentially and effectively desensitizes kainate receptors.

SYM 2081 was ~5-fold less potent than kainate both as an inhibitor of radioligand binding to AMPA receptors in rat brain membranes and as an activator of AMPA receptors in primary cultures of cerebral cortex. The lower affinity of SYM 2081 at AMPA receptors, relative to kainate receptors, may in part explain the absence of seizures in mice after parenteral administration of up to 512 mg/kg (data not shown), whereas far smaller doses of kainate are convulsant (Olney et al., 1974; Coyle, 1983; Sperk et al., 1985). Although it could be argued that SYM 2081 does not cross the blood-brain barrier, steady-state brain concentrations are ~6 μM after parenteral administration of 200 mg/kg (data not shown). The low affinity of SYM 2081 for AMPA receptors may increase its usefulness in future functional studies of the behavioral consequences of in vivo blockade of kainate receptors.

To date, the most compelling evidence linking activation of glutamate receptors to the neuropathologies associated with stroke, head injury and seizures stems from studies using selective, high-affinity compounds that act at the multiple allosteric regulatory sites on NMDA receptors (Collingridge and Watkins, 1994). In contrast, the involvement of non-NMDA subtypes of glutamate receptors in these and other pathophysiological processes is far less compelling, which may be attributed, at least in part, to the paucity of high-affinity, selective AMPA and kainate receptor ligands. SYM 2081 has significantly higher affinity and selectivity for kainate receptors than do previously described competitive antagonists such as NS-102 and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzoylquinoxaline (Honore et al., 1988; Sheardown et al., 1990; Verdoorn et al., 1994). SYM 2081 represents a prototypical, high-potency, kainate-selective ligand that may be useful both for the elucidation of the physiological roles of kainate receptors and for the development of therapeutic agents.

References


---

**Fig. 3.** Concentration-response relationships for SYM 2081 and kainate at AMPA receptors on rat neocortical neurons. A, trace showing inward currents recorded from a neuron voltage-clamped at −60 mV, resulting from applications of SYM 2081 at concentrations ranging from 30 to 3000 μM. B, currents from the same neuron in response to 10 to 1000 μM kainate. C, plot showing averaged data normalized to the maximal responses from four cells each for SYM 2081 and kainate. EC_{50} values are 325 ± 23 μM (Hill coefficient = 1.21) for SYM 2081 and 70 ± 6 μM (Hill coefficient = 1.16) for kainate.

Because native kainate receptors are heterogeneous (Honore et al., 1986; Johansen et al., 1993), the properties of SYM 2081 were also examined with recombinant GluR6 receptors. These receptors exhibit saturable [3H]kainate binding (Lomeli et al., 1992; Tygesen et al., 1994; Verdoorn et al., 1994) and produce rapidly desensitizing currents in response to kainate and glutamate (Herb et al., 1992; Raymond et al., 1993; Verdoorn et al., 1994), characteristic of native kainate receptors (Huettnner, 1990; Lerma et al., 1993; Patneau et al., 1994; Ruano et al., 1995). The K_{d} of [3H]kainate at GluR6 has been previously reported to range between 12.9 and 95 nM (Bettler et al., 1992; Tygesen et al., 1994; Verdoorn et al., 1994), and the K_{d} obtained in this study (6.6 nM) is consistent with the former value. The potency of SYM 2081 to inhibit [3H]kainate binding to GluR6 was somewhat higher than that of kainate itself, and the increase in K_{d} of [3H]kainic acid (without a change in B_{max}) observed in the presence of SYM 2081 is consistent with a competitive mode of action.

Patch-clamp measurements confirmed that SYM 2081 has


Send reprint requests to: Dr. Kenneth Jones, Synaptic Pharmaceutical Corp., 215 College Rd., Paramus, NJ 07652.