Pharmacological Characterization of the Competitive GLU\textsubscript{K5} Receptor Antagonist Decahydroisoquinoline LY466195 in Vitro and in Vivo


Received January 13, 2006; accepted March 29, 2006

ABSTRACT

The excitatory neurotransmitter glutamate has been implicated in both migraine and persistent pain. The identification of the kainate receptor GLU\textsubscript{K5} in dorsal root ganglia, the dorsal horn, and trigeminal ganglia makes it a target of interest for these indications. We examined the in vitro and in vivo pharmacology of the competitive GLU\textsubscript{K5}-selective kainate receptor antagonist LY466195 [(3\textsubscript{S},4aR,6R,8aR)-6-[[2S]-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]decahydro-3-isoquinolinecarboxylic acid], the most potent GLU\textsubscript{K5} antagonist described to date. Comparisons were made to the competitive GLU\textsubscript{K5}/\alpha-aminoadamantane-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonist LY293558 [(3\textsubscript{S},4aR,6R,8aR)-6-[[2]-1(2)-H-tetrazole-5-yl]ethyl]-decahydroisoquinoline-3-carboxylic acid], other decahydroisoquinoline GLU\textsubscript{K5} receptor antagonists, and the noncompetitive AMPA receptor antagonist LY300168 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine]. When characterized electrophysiologically in rat dorsal root ganglion neurons, LY466195 antagonized kainate (30 \textmu M)-induced currents with an IC\textsubscript{50} value of 0.045 \pm 0.011 \textmu M. In HEK293 cells transfected with GLU\textsubscript{K5}, GLU\textsubscript{K5}/GLU\textsubscript{K5}, or GLU\textsubscript{K5}/GLU\textsubscript{K5} receptors, LY466195 produced IC\textsubscript{50} values of 0.08 \pm 0.02, 0.34 \pm 0.17, and 0.07 \pm 0.02 \textmu M, respectively. LY466195 was efficacious in a dural plasma protein extravasation (PPE) model of migraine with an ID\textsubscript{100} value of 100 \mu g/kg i.v. LY466195 was also efficacious in the c-fos migraine model, with a dose of 1 \mu g/kg i.v. significantly reducing the number of Fos-positive cells in the rat nucleus caudalis after electrical stimulation of the trigeminal ganglion. Furthermore, LY466195 showed no contractile activity in the rabbit saphenous vein in vitro. The diethyl ester prodrug of LY466195 was also efficacious in the same PPE and c-fos models after oral administration at doses of 10 and 100 \mu g/kg, respectively while having no N-methyl-D-aspartate antagonist-like behavioral effects at oral doses up to 100 mg/kg.

Glutamate is the major excitatory neurotransmitter in the central nervous system and can act at three major types of ligand-gated ion channels that are defined by the activity of the subtype-selective agonists N-methyl-D-aspartate (NMDA), kainate, and \alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Collingridge and Lester, 1989). Five kainate receptors subtypes have been cloned and classified as either high-affinity (GLU\textsubscript{K1} and GLU\textsubscript{K2}) or low-

ABBREVIATIONS: NMDA, N-methyl-D-aspartate; AMPA, \alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DRG, dorsal root ganglion; ATPA, (RS)-2-\alpha-amino-3-hydroxy-5-tert-buty1-4-isoxazolopropionic acid; PPE, plasma protein extravasation; LY344370, 4-fluoro-tert-butyl-4-isoxazolepropionic acid; LY334370, 4-fluoro-tert-butyl-4-isoxazolepropionic acid; LY293558, (3\textsubscript{S},4aR,6R,8aR)-6-[[2]-1(2)-H-tetrazole-5-yl]ethyl]-decahydroisoquinoline-3-carboxylic acid; LY382884, 3\textsubscript{S},4aR, 6S,8aR-6-(4-carboxyphenoxy)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid; LY300168, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine; GYKI 53655; HEK, human embryonic kidney; LY341495, \alpha-amino-\alpha-((1S,2S)-2-carboxycyclopropyl)-\(\alpha\)-(9H-xanthene-9-propanoic acid; FITC-BSA, fluorescein isothiocyanate-bovine serum albumin; PBS, phosphate-buffered saline; PCP, phencyclidine; LY235959, (3\textsubscript{S},4aR,6R,8aR)-6-[[2]-1(2)-H-tetrazole-5-yl]ethyl]-decahydroisoquinoline-3-carboxylic acid; MK-801, 5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine maleate); UB2936, (RS)-3-2-carboxybenzyl]in[ll]ardilnine; N35763, 5-carboxy-2,4-di-benzamido-benzoic acid; NS1209, 8-methyl-6-(4-(N,N-dimethylsulfa-moyl)phenyl)-6,7,8,9-tetrahydro-1H-pyrrolo[3,2-h]-isoquinoline-2,3-dione-3-O-(4-hydroxybutyric acid-2-yl)oxime; LY466195, (3\textsubscript{S},4aR,6R,8aR)-6-[[2S]-2-carboxy-4,4-difluoro-1-pyrrolidinyl]-methyl]decahydro-3-isoquinolinecarboxylic acid.
affinity (GLUK5, GLUK6, and GLUK7) kainate receptors. GLUK5, GLUK6, and GLUK7 receptors form functional ion channels when expressed in homomorphic configurations, whereas GLUK5 and GLUK7 receptors do not (Hollmann and Heinemann, 1994). Human GLUK5, when expressed in its homomorphic configuration, is activated by kainate and only weakly activated by AMPA (Korc zak et al., 1995), whereas GLUK6 forms a homomeric receptor channel that can be selectively activated by kainate but not by AMPA (Hoo et al., 1994).

Both trigeminal neurons (Sahara et al., 1997) and dorsal root ganglion (DRG) neurons appear to express functional kainate receptors. In DRG neuron cell bodies, receptors are preferentially activated by kainate (Huettner, 1990) and (RS)-2-α-amino-3-hydroxy-5-tert-butyl-4-isoxazolopropionic acid (ATPA) (Bortolotto et al., 1999). Northern blot analysis for probes of individual AMPA and kainate receptor subunits demonstrates that GLUK5 mRNA predominates in DRG cell bodies (Partin et al., 1993). This observation, coupled with the selective modulation of kainate responses in DRG neurons by concanavalin A and not the AMPA receptor modulator cyclothiazide, suggests that GLUK5 receptors mediate the observed kainate-induced currents in DRG neurons (Huettner, 1990; Partin et al., 1993).

There is growing evidence for a role of kainate receptors in migraine pathogenesis. In addition to the demonstration that GLUK5 and GLUK6 receptors are expressed on trigeminal neurons (Sahara et al., 1997), it has been shown that kainate receptor antagonists are effective in a dural plasma protein extravasation (PPE) model of migraine in rats (Filla et al., 1998; Alt et al., 2004; Fig. 1). Several compounds that includes the competitive GLUK5/AMPA receptor antagonist LY466195 (773) have been evaluated in this model. GLUK5, GLUK5/GLUK6, or GLUK2/GLUK5 kainate receptors. 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 g for 20 min. The membrane pellets were then washed in >100% 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 of protein). For kainate receptor binding, the reaction mixture consisted of 150 µl of 50 mM Tris-HCl, pH 7.5, 25 µl of [3H]kainate (DuPont-New England Nuclear Research Products, Boston, MA), and 25 µl of an unlabeled competitor (10× 10−9 M). The final [3H]kainate concentration used in the competitive inhibition experiments was 20 nM for GLUK5–7, GLUK1, and GLUK2 receptors. For AMPA receptor binding, 20 nM [3H]AMPA (DuPont-New England Nuclear Research Products) was used for each receptor subtype and 100 mM potassium thiocyanate was added to the Tris-HCl buffer. After the 60-min incubation, the membranes were centrifuged at 50,000g for 20 min to separate bound from free ligand, and the pelleted were washed three times in cold assay buffer. Nonspecific binding was determined by incubation in the presence 10 mM glutamate. K values were estimated from 11-point competition assays from three separate preparations. All data were analyzed by GRAFIT 2.0 software. Radioligand binding studies were also performed using [3H]ATPA as a selective high-affinity GLUK5 kainate receptor ligand (Bleakman et al., 1999).

Selectivity profiling at other neurotransmitter receptors was performed according to standard procedures available in the literature. Membrane homogenates obtained from frozen rat brain tissue or commercially available transfected cell lines were used as a receptor source. Standard prototypic unlabeled ligands were used in each assay to serve as a positive control. Dimethyl sulfoxide (final concentration of ≤1% in the assay) was used to solubilize the test article. After an appropriate incubation at room temperature, assays were terminated by filtration or radioactivity bound was determined using scintillation counting. The results reported are from 11-point concentration curves run in at least two separate experiments. Metabotropic glutamate receptor studies were performed on AV12/RGT cells transfected with human metabotropic glutamate receptors 2, 3, 6, 7, and 8 using [3H]LY341485 as described previously (Johnson et al., 1999).

### Calcium Influx Measurements

Functional receptor activity of stably transfected kainate receptors was examined as described previously (Alt et al., 2004). Cells were plated on 96-well plates (Biocoat, poly-D-lysine; Becton Dickinson Labware, Franklin Lakes, NJ) containing confluent monolayers of HEK293 cells stably expressing GLUK5, GLUK5/GLUK6, or GLUK2/GLUK5 kainate receptors. Plate cells were washed three times with 100 ml of assay buffer (Hanks’ balanced salt solution; Invitrogen) with 20% 1 M HEPES and 1.48% 2.5 M CaCl2. Plates were incubated for 3 h at room temperature with 40 ml of assay buffer with 8 µM Fluo3-AM dye (Molecular Probes, Eugene, OR). Cells were washed and loaded with 100 ml of assay buffer containing 250 µg/ml concanavalin A (Sigma Chemical, St. Louis, MO) solution and incubated for 30 min at room temperature. Plates were washed with concanavalin A-containing assay buffer and fluorescence was measured using a fluorometric imaging plate

### Materials and Methods

#### Stable Transfections

Stable cell lines of human embryonic kidney (HEK) 293 cells transfected with cDNA coding for human GLUK5(Q), GLUK5(R)/GLUK5(Q), and GLUK2/GLUK5(Q) receptors were established as reported previously for human GLUK5(Q) (Korc zak et al., 1995; Clarke et al., 1997). GLUK5, GLUK5/GLUK6, and GLUK2/GLUK5 human cDNA was incorporated into the mammalian expression vector pBCEMV (Invitrogen, Carlsbad, CA) and transfected into the HEK293 cells. Transfected GLUK5, GLUK5/GLUK6, and GLUK2/GLUK5 cells were selected on the basis of G418 resistance, and mRNA was confirmed by reverse transcriptase-polymerase chain reaction. GLUK2/GLUK5 cells were also selected via blastidin resistance.

#### Ligand Binding Measurements

Ligand binding studies were performed on human recombinant AMPA and kainate receptors as described previously (Small et al., 1998). 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 g for 20 min. The membrane pellets were then washed in >100% 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 of protein). For kainate receptor binding, the reaction mixture consisted of 150 µl of 50 mM Tris-HCl, pH 7.5, 25 µl of [3H]kainate (DuPont-New England Nuclear Research Products, Boston, MA), and 25 µl of an unlabeled competitor (10× 10−9 M). The final [3H]kainate concentration used in the competitive inhibition experiments was 20 nM for GLUK5–7, GLUK1, and GLUK2 receptors. For AMPA receptor binding, 20 nM [3H]AMPA (DuPont-New England Nuclear Research Products) was used for each receptor subtype and 100 mM potassium thiocyanate was added to the Tris-HCl buffer. After the 60-min incubation, the membranes were centrifuged at 50,000g for 20 min to separate bound from free ligand, and the pellets were washed three times in cold assay buffer. Nonspecific binding was determined by incubation in the presence 10 mM glutamate. K values were estimated from 11-point competition assays from three separate preparations. All data were analyzed by GRAFIT 2.0 software. Radioligand binding studies were also performed using [3H]ATPA as a selective high-affinity GLUK5 kainate receptor ligand (Bleakman et al., 1999).

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reader (Molecular Devices, Sunnyvale, CA). Data were calculated using GraphPad Prism (GraphPad, San Diego, CA) and expressed relative to fluorescence induced by 100 μM glutamate.

Animal Use. All experiments were conducted in accordance with the National Institutes of Health regulations of animal care covered in Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985), and were approved by the Institutional Animal Care and Use Committee of Eli Lilly and Company. 

Electrophysiology. Functional GLU_Kα receptor activity was recorded in DRG neurons; NMDA and AMPA receptor activities were recorded in hippocampal neurons as described previously using whole-cell voltage clamp recordings (Bleakman et al., 1996, 1999). Cells used for recordings were chosen at random (i.e., no particular size/phenotype was selected). Curve fitting was to the following equation: percentage inhibition \( (\gamma) = 100([D]/([D] + IC_{50})) \), where \([D]\) is the antagonist concentration.

Rabbit Saphenous Vein Contractility Model. Male New Zealand White rabbits (6.6–13.2 kg) were sacrificed before removal of the saphenous vein, which was cannulated with polyethylene tubing prior to ring preparations being processed. Tissues were mounted in organ baths containing a modified Krebs’ solution maintained at 37°C and aerated with 95% O₂ and 5% CO₂. An initial optimum resting force of 4 g was applied, and isometric contractions were recorded as changes in grams of force after a 1- to 2-h equilibration period. Cumulative agonist concentration-response curves were generated. All results are expressed as a percentage of the response to 67 mM KCl (mean ± S.E.M.).

Dural Plasma Protein Extravasation Model of Migraine. Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) weighing 250 to 350 g were anesthetized with i.p. sodium pentobarbital (Nembutal; 65 mg/kg) and positioned in a stereotaxic apparatus with the incisor bar set at −2.5 mm. Core body temperature was maintained at 37°C using a rectal thermometer as an input to a proportional controller and an electric heating pad. After a midline sagittal scalp incision, two pairs of bilateral holes were drilled through the skull (3.2 mm posteriorly and 1.8 and 3.8 mm laterally with respect to bregma). Pairs of stainless-steel stimulating electrodes insulated, except at the tips, were lowered through the holes in both hemispheres to a depth of 9.2 mm below the dura. Before electrical stimulation, the femoral vein was exposed and LY466195 or saline vehicle was delivered i.v. via the femoral vein 10 min prior to trigeminal ganglion stimulation.

Two minutes before stimulation, a 20-mg/kg dose of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was injected i.v. as a marker of protein extravasation. The left trigeminal ganglion was stimulated for 5 min at a current density of 1 mA (5 Hz, 5 ms pulse duration), and the animals were euthanized by exsanguination 5 min after stimulation. Dural tissues were collected from both hemispheres, and the amount of fluorescence in each tissue due to extravasation of the FITC-BSA was determined spectrophotometrically. The extravasation ratio (ratio of the amount of extravasation in the dura from the stimulated side compared with that from the unstimulated side) was calculated. Statistical analysis was performed by comparison to the saline control group using a one-way analysis of variance and Dunnett’s method.

The diethyl ester prodrug of LY466195 was evaluated after oral dosing. Male Sprague-Dawley rats (250–350 g; Harlan Industries), fasted overnight before use, were dosed with either the diethyl ester prodrug of LY466195 or saline vehicle via oral gavage (2 ml/kg) and anesthetized with sodium pentobarbital (60 mg/kg i.p.) approximately 45 min later. Once anesthetized, the animals were placed in a stereotaxic frame and dosed with FITC-BSA. The remainder of the protocol was carried out as described above.

Central c-fos Expression Model of Migraine. LY466195 was dissolved in isotonic saline. Male, sodium pentobarbital-anesthetized (50 mg/kg i.p.) Sprague-Dawley rats (Harlan Industries) were placed in a stereotaxic frame with the incisor bar set at −3.5 mm. Core body temperature was maintained at 37°C using a rectal thermometer as an input to a proportional controller and an electric heating pad. A midline incision was then made in the skin covering the dorsal surface of the skull and the skin was retracted to expose the skull. Small holes were drilled in the skull to allow electrode placement. Rats were then pretreated with i.v. LY466195 or saline vehicle. Ten minutes later the left trigeminal ganglion was stimulated using a pair of stainless-steel unipolar electrodes spaced 2 mm apart to bracket the ganglion in the medial to lateral axis. The stereotactic coordinates used were: 6 mm posterior to bregma, 2 and 4 mm lateral to bregma, and 9 mm ventral from dura. The trigeminal ganglion was stimulated for 3 min at a current intensity of 10 mA (5 Hz, 4-ms duration) using a model 273 galvanostat (EG & G Princeton Applied Research, Princeton, NJ). Animals were removed from the stereotaxic apparatus and allowed to survive for 90 min before transcardiac perfusion with 120 ml of phosphate-buffered saline (PBS) (Sigma Chemical) followed by 120 ml of 4% paraformaldehyde dissolved in phosphate buffer (pH 7.4). For each animal, the brainstem and cervical spinal cord were removed and postfixed for 2 h in 4% paraformaldehyde before being placed in a 30% sucrose solution for 2 days. Coronal sections (40 μm thick) of the brainstem were cut using a cryostat set at −20°C. Free-floating sections were stained using a typical protocol. Tissue was pretreated in 10% Triton X-100 and 2% normal goat serum before being placed in a 3% hydrogen peroxide dissolved in 0.05% solution of 3,3'-diaminobenzidine tetrahydrochloride (D-5905; Sigma Chemical), 0.03% hydrogen peroxide in 0.1 M Tris-HCl, and nickel ammonium sulfate buffer (pH 7.4) for approximately 1 min. After the 3,3'-diaminobenzidine tetrahydrochloride reaction, sections were washed again with PBS, and then placed on gelatin-coated slides, air-dried, dehydrated, and cover-slipped. Quantification of cells showing Fos-like immunostaining was performed by a blinded individual using computer-assisted image analysis (Quantimet 970; Leica Microsystems, London, UK). For each animal, approximately 15 randomly selected coronal sections through the cervical spinal cord (C1) or the caudal brainstem were examined. The number of cells staining with Fos protein-like immunoreactivity in the nucleus caudalis ipsilateral and contralateral to the stimulation were quantified. A mean number of ipsilateral and contralateral cells per section were calculated for each animal. Animals were grouped by treatment and compared using a one-way analysis of variance followed by a post hoc Dunnett’s test (GraphPad Prism; GraphPad).

The ability of the diethyl ester prodrug of LY466195 to inhibit Fos protein expression in the nucleus caudalis was also evaluated after oral dosing. Male Sprague-Dawley rats (250–350 g; Harlan Industries), fasted overnight before use, were dosed with either the diethyl ester prodrug of LY466195 or saline vehicle via oral gavage (2 ml/kg) and anesthetized with sodium pentobarbital (60 mg/kg i.p.) approximately 45 min later. Once anesthetized, the animals were placed in a stereotaxic frame and the remainder of the protocol was carried out as described above.

PCP-Induced Motor Activation in Rats. Behaviors were monitored in transparent, plastic shoe box cages of dimensions 45 × 25 × 20 cm, with 1-cm depth of wood chips as bedding, and a metal grill on top of the cage. Motor monitors (Hamilton Kinder, Poway, CA) consisted of a rectangular rack of 12 photobeams arranged in an 8 × 4 formation. Shoe box cages were placed inside these racks, and the lower rack was positioned at a height of 5 cm. Male Sprague-Dawley
rats (225–275 g; Harlan Industries) were placed in the cage for an acclimation period of 30 min, then were removed, administered compound or saline (1 ml/kg) (controls), and then returned to the same cages. Activities including total ambulations, fine movements, and time at rest were continuously monitored for 4 h postdosing. The diethyl ester prodrug of LY466195 was administered orally at doses of 30, 50, and 100 mg/kg, and PCP (5 mg/kg s.c.) was used as a positive control in the same experiment. In another experiment, the potent decahydroisoquinoline competitive NMDA antagonist compound LY235959 (Ornstein et al., 1992) was tested at doses of 3, 10, and 30 mg/kg s.c. Statistical analyses of behaviors were carried out using the GraphPad Prism statistical program (GraphPad). Data were analyzed by one way analysis of variance and then post hoc comparisons for each dose group versus saline control and test compounds were made using Newman-Keuls multiple comparison test. \( P < 0.05 \) was considered significant.

**Results**

**Ligand-Binding Studies.** The binding affinity of LY466195 to both recombinant human kainate and AMPA receptor subtypes was evaluated. Table 1 summarizes the estimated \( K_i \) values for LY466195 at human AMPA or kainate receptors stably expressed in HEK293 cells. Comparisons with other decahydroisoquinolines are provided. The \( K_i \) value for LY466195 at GLUK5 kainate receptors was 52 ± 22 nM, measured by the displacement of \( ^{[3H]} \)kainate. LY466195 displaced \( ^{[3H]} \)ATPA from GLUK5 receptors with similar affinity (\( K_i = 128 \pm 15 \) nM). LY466195 produced no measurable inhibition of \( ^{[3H]} \)kainate binding in cells recombinantly expressing GLUK6. In addition, a greater than 100-fold selectivity for displacement at GLUK5 versus the other kainate and AMPA receptor subtypes was observed for LY466195. By comparison, LY293558 displaced binding to both AMPA and GLUK5 kainate receptors but not to other kainate receptor subtypes (Bleakman et al., 1999). LY382884 selectively displaced binding to GLUK5 receptors, but with approximately 100-fold lower affinity than LY466195 (Bortolotto et al., 1999).

LY466195 binding affinity was evaluated at a wide range of neurotransmitter receptors. Neither LY466195 nor LY466195 monohydrate demonstrated any appreciable affinity for the receptors listed in Table 2 at the concentrations shown.

**Functional Calcium Influx Activity.** The ability of LY466195 to antagonize glutamate-evoked calcium influx was measured in HEK293 cells stably transfected with kainate receptors. Cells were pretreated with concanavalin A to block agonist-induced desensitization, allowing for glutamate to produce a measurable calcium signal. LY466195 produced a concentration-dependent inhibition of glutamate (100 \( \mu M \))-evoked influx with IC\(_{50}\) values of 0.08 ± 0.02, 0.34 ± 0.17, and 0.07 ± 0.02 \( \mu M \) at GLUK5, GLUK2/GLUK5, and GLUK5/GLUK6 receptors, respectively (Fig. 2A). The \( K_i \) value for LY466195 was calculated as 0.024 ± 0.006 \( \mu M \) against glutamate-evoked calcium influx into HEK293 cells stably transfected with GLUK5. LY466195 (up to 100 \( \mu M \)) had no effect on glutamate-evoked calcium influx in cells expressing homomeric GLUK6 receptors. Table 3 shows a comparison of LY466195 antagonist activity with the antagonist activity of the decahydroisoquinolines 293558 and LY382884.

**Electrophysiological Studies.** Electrophysiology performed at rat DRG neurons demonstrated that LY466195 is a competitive antagonist at GLUK5 receptors in native tissue. Inward currents associated with activation of GLUK5 receptors were recorded in cells that had been preincubated with concanavalin A (250 \( \mu g/\)ml for 10 min). Under these conditions in rat DRG neurons, the EC\(_{50}\) value for kainate-evoked inward currents was 12 ± 1 \( \mu M \) (Alt et al., 2004). Concentration-response curves for LY466195 were determined versus 30 \( \mu M \) kainate (approximate EC\(_{50}\) value). The IC\(_{50}\) value for LY466195 was 0.045 ± 0.011 \( \mu M \), corresponding to an estimated \( K_i \) value of approximately 13 \( \mu M \) (Fig. 2B).

LY466195 was also evaluated against NMDA receptor-mediated responses in cultured hippocampal neurons. The estimated EC\(_{50}\) value for NMDA under the current experimental conditions was 7.2 ± 0.2 \( \mu M \) (Bleakman et al., 1999). For comparative purposes we have also evaluated PCP and MK-801, two noncompetitive NMDA receptor antagonists, and LY235959, a competitive NMDA receptor antagonist.

<table>
<thead>
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<th>Table 1</th>
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<td>Binding affinity of LY466195 at AMPA and kainate receptors compared with reported values for other decahydroisoquinoline kainate receptor antagonists. ( K_i ) values for LY466195 were determined at each receptor from 11-point competition assays from three separate preparations against either ( ^{[3H]} )AMPA or ( ^{[3H]} )kainate. None of the compounds in the table show measurable affinity at GLUK5 receptors.</td>
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<tr>
<td>K( _i )</td>
</tr>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>LY466195</td>
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\( ^{a} \) Reported from Filla et al. (2002).  
\( ^{b} \) Reported from Dominguez et al. (2005).  
\( ^{c} \) Reported from O’Neill et al. (1998).  
\( ^{d} \) Reported from Bortolotto et al. (1999).  
\( ^{e} \) Reported from Bleakman et al. (1999).
PCP and MK-801 antagonized inward currents activated by 10 μM NMDA in a concentration-dependent manner with estimated IC₅₀ values of 1.0 ± 0.8 and 0.3 ± 0.1 μM, respectively. As shown in Fig. 3A, LY466195, LY293558, and LY235959 produced IC₅₀ values of 2.5 ± 0.9, 12.2 ± 0.7, and 0.12 ± 0.06 μM, respectively. The NMDA receptor antagonist activity of LY466195 was competitive (data not shown).

The AMPA receptor antagonist activity of LY466195 was compared with the noncompetitive 2,3-benzodiazepine AMPA receptor antagonist LY300168 (GYKI 53655) and with LY293558 (Fig. 3B). The EC₅₀ value for AMPA in cultured hippocampal neurons was 4.0 ± 0.6 μM (Bleakman et al., 1999). LY300168, LY293558, and LY466195 inhibited AMPA (30 μM)-induced currents with IC₅₀ values of 1.6 ± 0.16, 8.0 ± 3.1, and 54 ± 13 μM, respectively. The low-potency AMPA antagonist activity of LY466195 was competitive in nature (data not shown).

**Dural PPE Model of Migraine.** Dural plasma protein extravasation was observed ipsilateral to the stimulated trigeminal ganglion, which allowed the unstimulated half of the dura to be used as a control. Animals dosed with vehicle alone or an ineffective dose of the test compound had an extravasation ratio of approximately 2, whereas totally effective treatments resulted in a ratio of ∼1. LY466195 significantly blocked dural extravasation after an i.v. dose of 10 and 100 μg/kg, but not 1 μg/kg in Sprague-Dawley rats (Fig. 4). The diethyl prodrug of LY466195 was also examined in this assay. Preliminary pharmacokinetic studies in rats indicated that a 30 mg/kg dose of the LY466195 prodrug had approximately 40% bioavailability after oral dosing (estimated plasma concentration = 11 μg/ml (tₘₐₓ ~ 5 min) and a half-life in plasma of 0.8 h). Like LY466195, the diethyl ester prodrug of LY466195 significantly inhibited dural extravasation after oral doses of 10 and 100 but not 1 μg/kg.

**TABLE 2**

Pharmacological selectivity profile of LY466195

<table>
<thead>
<tr>
<th>Compound</th>
<th>α₁-Adrenergic</th>
<th>α₂-Adrenergic</th>
<th>β-Adrenergic</th>
<th>D₁</th>
<th>D₂</th>
<th>5-HT₂</th>
<th>H₁</th>
<th>Muscarinic</th>
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Kᵢ at hmGlurRs Expressed in AV12/RGT Cells

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hmGlurR, human metabotropic glutamate receptor.
LY466195 (i.v.) and its prodrug ester (p.o.) did not differ significantly in either their potency or maximal effect produced in this assay.

**c-fos Expression in Rat Nucleus Caudalis.** The number of Fos-positive cells on the contralateral, nonstimulated side of the rat brain was subtracted from the number on the ipsilateral side to derive a stimulation-induced average cell number per section. Numerous cells in the ipsilateral nucleus caudalis were Fos-positive after trigeminal neuron stimulation. Consistent but significantly lower numbers of Fos-positive cells were observed in the brainstem nucleus caudalis contralateral to the stimulation. Pretreatment with LY466195 significantly attenuated the trigeminal stimulation-induced increases in the number of Fos-positive ipsilateral cells. Significant decreases in the difference scores were seen at doses of 1, 10, and 100 µg/kg but not at 0.1 µg/kg for LY466195 i.v. (Fig. 5). A maximal inhibition of approximately 50% was seen after administration of 100 µg/kg LY466195.

The diethyl ester prodrug of LY466195 also produced significant decreases in the number of Fos-positive cells after oral doses of 0.1, 1, and 10 but not 0.01 mg/kg.

**Motor Activity in Rats.** In vitro pharmacological studies with LY466195 demonstrated antagonist activity of LY466195 at NMDA receptors. Studies were then designed to examine potential NMDA receptor antagonist activity in vivo using an automated activity monitoring system in rats. Rats administered oral doses of 30, 50, or 100 mg/kg LY466195 did not show significant changes in motor activities compared with the saline control animals. In contrast, PCP administration induced rapid and prominent increases in total ambulations and fine movements and marked decreases in the animal’s rest time (seconds in a session with no new photobeams broken). These effects of PCP were rapid in onset (observed within 30 min) and significant for the entire 4-h test period. Additionally, the competitive NMDA receptor antagonist LY235959 also produced dose-related motor activation. No significant effects were observed at a dose of 3 mg/kg LY235959. However, at 10 mg/kg LY235959 produced significant effects that were somewhat delayed (significant onset of fine movements at 90 min) but prolonged (present for the 4-h duration of the experiment). The higher dose of LY235959 induced prominent increases in fine movements within 1 h, but this activity lessened at the later time periods by the prominent onset of motor ataxia in the animals. No LY235959-like motor ataxia was noted in animals receiving up to 100 mg/kg p.o. of LY466195 prodrug ester.

**Vasoconstriction in the Rabbit Saphenous Vein.** Concentration-response curves were generated for LY466195 individually, as well as in the presence of sumatriptan to assess the ability of LY466195 to significantly enhance or inhibit the contractile properties of sumatriptan. LY466195 alone was completely devoid of contractile activity in the rabbit saphenous vein preparation at concentrations up to 100 µM (Fig. 6A). In addition, LY466195 (3 µM) neither enhanced nor inhibited sumatriptan-induced contraction (Fig. 6B).

**Discussion**

Ligand-binding studies demonstrate that the decahydroisoquinoline LY466195 is a potent antagonist of GLUK5 -
LY466195 is the most potent GLU\textsubscript{K5} antagonist described to date. Interestingly, LY466195 also shows low micromolar affinity for GLU\textsubscript{K7} receptors, although the functional significance of this activity is not known. The binding affinity of ethyl (3S,4aR,6S,8aR)-6-(4-ethoxy carbonyl)imidazol-1-yl methyl)decahydroisoquinoline-3-carboxylic acid (compound 3 from Filla et al., 2002) is approximately 3-fold less than that of LY466195 at GLU\textsubscript{K5}, and 5-fold less at GLU\textsubscript{K7}.

Functional competitive antagonism by LY466195 was examined using agonist-dependent calcium influx at recombinant GLU\textsubscript{K5}-containing receptors. For heteroreceptor combinations of GLU\textsubscript{K5} receptors with other GLU\textsubscript{K5} or GLU\textsubscript{K6} subunits, LY466195 exhibited functional antagonism of glutamate-induced calcium influx. As previously reported for LY293558 and LY382884 (Alt et al., 2004; Christensen et al., 2004a), functional antagonists of heteroreceptor receptors appear to be determined by the presence of a GLU\textsubscript{K5} subunit.

We also demonstrated that LY466195 acted as a competitive antagonist at rat GLU\textsubscript{K5}-containing receptors in dorsal root ganglion neurons. In agreement with the results using recombinant human receptors, LY466195 was more potent at rat GLU\textsubscript{K5} receptors in DRG neurons than either LY293558 (Bleakman et al., 1996) or LY382884 (Smolders et al., 2002) (approximately 45- and 20-fold, respectively). LY466195 also demonstrated marked selectivity when examined against NMDA and AMPA-mediated responses in cultured hippocampal neurons.

It is interesting to note that although LY466195 shows only a 3-fold greater binding affinity to recombinant human GLU\textsubscript{K5} receptors than ethyl (3S,4aR,6S,8aR)-6-(4-ethoxy carbonyl)imidazol-1-yl methyl)decahydroisoquinoline-3-carboxylic acid (compound 3 from Filla et al., 2002), this difference in potency widens to 10-fold for functional antagonism of recombinant human GLU\textsubscript{K5} receptor activity. In addition, this difference further widens to 20-fold for functional antagonism of native GLU\textsubscript{K5}-containing receptors in DRG neurons (using kainate as the agonist in both the binding experiments and to activate currents in DRG neurons). Whether this reflects the difference in species (human versus rat), experimental conditions, or subunit composition of native versus recombinantly expressed receptors has yet to be determined.

The in vitro selectivity and potency of LY466195 compare favorably to other known antagonists of GLU\textsubscript{K5}, kainate receptors. For example, the willardiine derivative UBP296 inhibits both homeric and heteromeric GLU\textsubscript{K5}-containing receptors with approximate \( K_h \) values of 0.6 ± 0.1, 0.8 ± 0.1, and 1.0 ± 0.4 \( \mu M \) at GLU\textsubscript{K5}, GLU\textsubscript{K5}/GLU\textsubscript{K6}, and GLU\textsubscript{K2}/GLU\textsubscript{K5} receptors, respectively (More et al., 2004). UBP296 did not, however, have any appreciable activity at NMDA receptors (More et al., 2004). The antagonist derivative of ATPA, (S)-2-amino-3-[5-tert-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid, has been reported to be an antagonist at both GLU\textsubscript{K5} and AMPA receptors, with a \( K_h \) value of 23 \( \mu M \). (S)-2-Amino-3-[5-tert-butyl-3-(phosphonomethoxy)-4-isoxazolyl]propionic acid was also reported to not significantly attenuate NMDA-mediated currents in the rat cortical wedge model (Møller et al., 1999). Another recently reported AMPA and kainate receptor antagonist, a 3-(5-tetrazolylmethoxy) analog of AMPA, inhibits GLU\textsubscript{K5} receptors (\( IC_{50} = 131 ± 4 \mu M \)), but is 7-fold more potent at the GLU\textsubscript{K2}/GLU\textsubscript{K6} receptor (\( IC_{50} = 19 ± 1 \mu M \)), while also inhibiting AMPA receptors with \( IC_{50} \) values ranging from 48 to 161 \( \mu M \) (Fro-
The analogs tested by Frolund et al. (2005) showed very low-potency antagonist effects on NMDA-mediated currents in the rat cortical wedge preparation. Kainate receptor antagonists that are noncompetitive in nature have also been reported. Christensen et al. (2004b) reported that the noncompetitive kainate receptor antagonists NS3763 and NS1209 inhibited domoic acid-induced calcium influx at GLU$_{K5}$ with IC$_{50}$ values of 1.6 ± 0.2 and 0.63 ± 0.09 μM, respectively. Both NS3763 and NS1209 showed at least 30-fold selectivity for GLU$_{K5}$ versus GLU$_{K6}$ homomeric receptors. NS3763 was further shown to be selective for homomeric GLU$_{K5}$ receptors versus heteromeric conformations containing GLU$_{K2}$ or GLU$_{K6}$ subunits (Christensen et al., 2004a). Other reported antagonists of GLU$_{K5}$ kainate receptors are nonselective and/or low-potency molecules such as 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzol[f]quinoxaline-7-sulfonamide, 6-cyano-7-nitroquinoxaline-2,3-dione, kynurenic acid, and γ-D-glutamylaminomethylsulfonic acid that act at multiple kainate and AMPA receptors (Wilding and Huettner, 1996; Alt et al., 2004).

LY466195 had significant inhibitory effects in two preclinical models of migraine. In particular, i.v. administered LY466195 was able to block PPE induced by electrical stimulation of the trigeminal ganglion in the dural membranes of rats. The diethyl ester prodrug of LY466195 was also active in a dural PPE model after oral dosing. LY466195 dosed i.v. and p.o. showed significant effects in reducing extravasation in the dural membranes.

Fig. 4. Dose-response curve of LY466195 and LY466195 diethyl ester prodrug in the PPE model after oral administration. Values are the mean ratios of extravasation in the stimulated side of the dura to that in the unstimulated side ± S.E.M. (n = 3). A compound was considered fully efficacious at a ratio of approximately 1 and ineffective at a ratio of approximately 2.

Fig. 5. Suppression of Fos expression by LY466195 in the rat nucleus caudalis ipsilateral to trigeminal ganglion stimulation. A, animals pretreated with LY466195 monohydrate. B, animals pretreated with LY466195 prodrug ester. For each animal, 10 to 15 sections were analyzed to provide an average number of stained cells per section. These averages were combined for vehicle- and compound-pretreated animals. Asterisks denote a significant difference from the vehicle group (P < 0.05), and vertical bars represent the S.E.M. from four to nine animals.

Fig. 6. A, contractile concentration-response curves for LY466195 and sumatriptan in the rabbit saphenous vein. B, contractile concentration-response curves for sumatriptan and sumatriptan in the presence of LY466195 (3 μM) in the rabbit saphenous vein. Points are mean values, and vertical bars represent the S.E.M. from three to four experiments.
and its prodrug ester administered orally both had an ID\textsubscript{100} of approximately 100 \mu{g}/kg, with significant reductions in extravasation observed at 10 \mu{g}/kg. At 10 \mu{g}/kg, it appeared that the prodrug of LY466195 (p.o.) was slightly more potent than LY466195 itself (i.v.). However, the different pretreatment times used (1 h p.o. versus 10 min i.v.) may preclude direct comparisons of potencies.

LY466195 and the diethyl ester prodrug of LY466195 also decreased the number of Fos-positive cells in the trigeminal nucleus caudalis after electrical stimulation of the trigeminal ganglion. Significant reductions in the number of Fos-positive cells were achieved with LY466195 at doses of 1 to 100 \mu{g}/kg i.v. and for the prodrug of LY466195 starting at 100 \mu{g}/kg. LY466195 (i.v.) appeared to exhibit greater potency than its prodrug (p.o.) in its ability to inhibit Fos expression, whereas both compounds were approximately equipotent in their inhibition of dural extravasation. Although the pretreatment times for each compound (10 min i.v. for LY466195 and 1 h p.o. for the prodrug) were the same in both assays, differences in the duration and intensity of the electrical stimulation used or in the time after stimulation at which the effects were measured may account for the difference in relative potency observed between the two assays.

Another decahydroisoquinoline GLUK\textsubscript{5} antagonist prodrug had previously been shown to be active in both the c-fos migraine model and a slightly different version of the PPE model (Filla et al., 2002). In addition, LY293558, a decahydroisoquinoline GLUK\textsubscript{5}/AMPA receptor antagonist, has been shown to be efficacious in the clinical treatment of acute migraine (Sang et al., 2004). However, the affinity of both of these compounds for AMPA receptors (Filla et al., 2002; Alt et al., 2006) makes it difficult to conclusively ascribe their effects in these models to antagonism of GLUK\textsubscript{5} receptors. Although LY466195 also displays some affinity for AMPA receptors (Table 1), its improved potency at GLUK\textsubscript{5} receptors results in a greater selectivity for GLUK\textsubscript{5} versus AMPA receptors than is displayed by either of these other described compounds. A comparison of the relative affinity of several decahydroisoquinoline GLUK\textsubscript{5} antagonists for kainate and AMPA receptors is provided in Table 1. In addition to its improved selectivity for kainate versus AMPA receptors, LY466195 was found to have no measurable affinity for any of a wide range of other receptor types tested (Table 2).

Because of the modest antagonist effect observed with LY466195 at NMDA receptors when evaluated in an in vitro assay, the diethyl ester prodrug of LY466195 was evaluated in a behavioral test paradigm designed to detect NMDA antagonist activity in vivo. Two well characterized NMDA antagonists, PCP and LY235959, were also evaluated as positive controls. PCP and LY235959 induced significant dose-related motor activation as expected. In contrast, the positive controls, PCP and LY235959 induced significant antagonistic activity in vivo. Two well characterized NMDA antagonist activity in vivo. The relative roles of AMPA and kainate receptors in the initiation and maintenance of migraine are unknown. However, the current data suggest a potential role of GLUK\textsubscript{5} receptors in migraine as inferred from the prevention of electrically stimulated dural extravasation and Fos expression in the current study. It will be of great interest to determine whether a GLUK\textsubscript{5} mechanism is associated with the establishment of migraine in humans.

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Characterization of Competitive GLU₅₀ Antagonist LY466195

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