ABSTRACT
Accumulating preclinical data suggest that compounds that block the excitatory effect of glutamate on excitatory amino acid receptors may have neuroprotective effects and utility for the treatment of neurodegeneration after brain ischemia. In the present study, the in vitro and in vivo pharmacological properties of the novel glutamate antagonist SPD 502 [8-methyl-5-(4-(N,N-dimethylsulfamoyl)phenyl)-6,7,8,9-tetrahydro-1H-pyrrolo[3,2-h]isoquinoline-2,3-dione-3-O-(4-hydroxybutyric acid-2-yloxime)] are described. In binding studies, SPD 502 was shown to display selectivity for the [3H]α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-binding site (IC50 = 0.043 M) compared with the [3H]kainate- (IC50 = 81 M), [3H]cis-4-phosphonomethyl-2-piperidine carboxylic acid- (CGS 19755), and [3H]glycine-binding sites (IC50 > 30 M) in rat cortical membranes. In an in vitro functional assay, SPD 502 blocked the AMPA-induced release of [3H]α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid from cultured mouse cortical neurons in a competitive manner with an IC50 value of 0.23 M. Furthermore, SPD 502 potently and selectively inhibited AMPA-induced currents in cortical neurons with an IC50 value of 0.15 M. In vivo electrophysiology, SPD 502 blocked AMPA-evoked spike activity in rat hippocampus after i.v. administration with an ED50 value of 6.1 mg/kg and with a duration of action of more than 1 h. Furthermore, SPD 502 increased the seizure threshold for electroshock-induced tonic seizures in mice at i.v doses of 40 mg/kg and higher. In the two-vessel occlusion model of transient forebrain ischemia in gerbils, SPD 502 (10 mg/kg bolus injection followed by a 10 mg/kg/h infusion for 2 h) resulted in a highly significant protection against the ischemia-induced damage in the hippocampal CA1 pyramidal neurons.

The role of glutamate as a potential mediator of neurodegeneration after acute cerebral ischemia is well established (Benveniste et al., 1984; Butcher et al., 1990). From in vitro and in vivo studies on neuroprotective effects of selective glutamate antagonists it is evident that both N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtypes of ionotropic glutamate receptors may be involved in the development of acute ischemic infarcts after stroke or delayed, selective neuronal degeneration after transient global cerebral ischemia (reviewed by Buchan, 1990; Gill, 1994). It appears, however, that the efficacy profile, window of opportunity, and side-effect profile may be quite different between different types of glutamate antagonists.

Although the neuroprotective effects of NMDA antagonists in animal stroke models have been remarkable (Bullock et al., 1990; Gill et al., 1991), protection generally has not been seen in global ischemia models (Buchan et al., 1991; Nellgard and Wieloch, 1992). The time window of opportunity for NMDA antagonists in animal stroke models is probably less than 90 min (Xue et al., 1994), and if this can be extrapolated to the human situation this relatively short window of opportunity will severely limit the usefulness of NMDA antagonists in the clinical stroke situation. Most classes of NMDA antagonists also have been associated with severe psychotomimetic effects (Koek et al., 1988), cardiovascular side effects (Muir et al., 1994), impairment of learning and memory (Morris et al. 1986), and neurotoxicity (Olney et al., 1991), all observed at doses well below the efficacious doses in the stroke models.

The discovery of the selective AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[quinoxaline (NBQX) (Sheardown et al., 1990) has greatly facilitated the study of this receptor subtype. In contrast to NMDA antagonists, competitive AMPA antagonists appear to provide robust neuroprotection in both global and focal cerebral ischemia models (Sheardown et al., 1990; Diemer et al., 1992;
Gill et al., 1992). The time window of opportunity appears to be rather long (more than 12 h) in global models (Sheardown et al., 1990) and more than 90 min in focal ischemia models (Xue et al., 1994; Shimizu-Sasamata et al., 1996), and AMPA antagonists do not share the psychotomimetic effects (Sang et al., 1998), cardiovascular effects, and neurotoxicity seen with NMDA receptor antagonists. Therefore, AMPA receptor antagonists should possess greater clinical potential than NMDA receptor antagonists. However, the first generation of competitive AMPA antagonists of the quinoxalinedione type had extremely poor water solubility at physiological pH, and this, combined with fast kidney excretion, led to precipitation of substance in the kidneys and nephrotoxicity after subefficacious doses in animals (Xue et al., 1994). Moreover, at higher doses respiratory depression was observed (Browne and McCulloch, 1994).

During recent years a significant effort has been allocated to improve the physicochemical properties of the first generation of competitive AMPA antagonists. We report here on a potent and selective AMPA antagonist SPD 502 (8-methyl-5-(4-(N,N-dimethylsulfamoyl)phenyl)-6,7,8,9-tetrahydro-1H-pyrrolo[3,2-\text{h}]-isoquinoline-2,3-dione-3-O-(4-hydroxybutyric acid-2-yl)oxime; formerly known as NS 1209, Fig. 1). To produce this compound we have utilized an isatinoxime moiety instead of the quinoxalinedione system, known from previous AMPA antagonists, e.g., NBQX. Furthermore, attachment of an appropriate phenyl substituent combined with an optimized fusion of a piperidine part gave a potent and selective compound. Finally, water solubility was greatly improved by attachment of a selected carboxylic acid side chain. The aqueous solubility of SPD 502 (1.4 mg/ml at pH 4.6; 1.5 mg/ml at pH 6.8; 8.0 mg/ml at pH 7.3; 167 mg/ml at pH 7.8) was found to be much higher than for NBQX (<1 mg/ml at pH 4.6–8.0 (0.12 mg/ml in Britton-Robinson buffer at pH 7 as measured by Takahashi et al. (1998)]. We report here that SPD 502 has neuroprotective efficacy with a long time window of opportunity in a rodent cerebral ischemia model.

Fig. 1. Structure of SPD 502 and its (R)- and (S)-isomers.

**Experimental Procedures**

**Materials**

[3H]AMPA (41 Ci/mmol), [3H]kainic acid (58 Ci/mmol), [3H]glycine (45 Ci/mmol), and [3H]cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755; 87 Ci/mmol) were purchased from DuPont/NEN (Boston, MA). [3H]-y-Aminobutyric acid (GABA; 86 Ci/mmol) was purchased from Amersham International (Cardiff, UK).

SPD 502, the (R)- and (S)-isomers of SPD 502 (Wäätinen and Drejer, 1998), and NBQX were synthesized at NeuroSearch A/S. Fluo-3 was purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Tetrodotoxin was purchased from Alomone Laboratories (Jerusalem, Israel), and bicuculline methiodide and cyclothiazide were purchased from Research Biochemicals International (Natick, MA). All other chemicals were purchased from regular commercial sources and were of the purest grade available.

**Membrane Preparation**

Rat cerebral cortical membranes were prepared from male Wistar rats (M & B, Ry, Denmark) as described by Johansen et al. (1993). Cerebral cortices were removed rapidly after decapitation, homogenized for 5 to 10 s in 10 volumes of 30 mM Tris-HCl (pH 7.4), and centrifuged at 27,000 g for 15 min. All procedures were performed at 0–4°C unless otherwise indicated. After washing the pellet three times (resuspension in 10 volumes of ice-cold buffer and centrifugation at 27,000 g for 10 min), the pellet was homogenized in Tris-HCl buffer, incubated on a water bath (37°C) for 30 min to remove endogenous glutamate, and then centrifuged at 27,000 g for 10 min. The pellet was then homogenized in buffer and centrifuged for 10 min at 27,000 g. After one more wash, the final pellet was resuspended in 10 volumes of buffer and the preparation was frozen at −20°C. Before use, the frozen membrane preparation was thawed slowly and centrifuged at 2°C for 10 min (27,000 g). After one wash in 30 mM Tris-HCl, the final pellet was resuspended in assay buffer and used for binding experiments.

[3H]AMPA Binding. Binding conditions were as described previously (Honore and Nielsen, 1985). Samples containing 500 μl tissue suspension in assay buffer (composition: 2.5 mM CaCl₂, 100 mM KSCN, and 30 mM Tris-HCl, pH 7.4), 25 μl of [3H]AMPA (5 nM, final concentration), and 25 μl of drug at the indicated concentrations were mixed and incubated for 30 min at 2°C in triplicate. Nonspecific binding was determined in the presence of 0.6 mM l-glutamate. Binding was terminated by rapid filtration over Whatman GF/C glass fiber filters.

[3H]Kainate Binding. Binding was determined by using the method described previously by Johansen et al. (1993). In brief, the assay was performed at 2 nM [3H]kainate in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 550 μl for 60 min at 2°C in triplicate. Nonspecific binding was determined in the presence of 0.6 mM l-glutamate, and binding was terminated by rapid filtration.

[3H]CGS 19755 Binding. Binding conditions were as described previously (Murphy et al., 1988). The assay was performed at 2.5 nM [3H]CGS 19755 in 50 mM Tris-HCl (pH 7.4) buffer in a final volume of 550 μl for 20 min at 2°C in triplicate. Nonspecific binding was determined in the presence of 1 mM l-glutamate, and binding was terminated by rapid filtration.

[3H]Glycine Binding. Binding assays were performed by using a modification of the method of Kishimoto et al. (1981). The assay was performed at 10 nM [3H]glycine in 50 mM Tris-acetate buffer (pH 7.1) in a final volume of 550 μl for 60 min at 2°C in triplicate. Nonspecific binding was determined in the presence of 1 mM d-serine, and binding was terminated by centrifugation at 27,000 g for 5 min.

The amount of radioactivity on the filters or in the pellets was determined by conventional liquid scintillation counting using a Tri-carb liquid scintillation analyzer (model 1600CA; Packard) with a counting efficiency of 58%.
Tissue Culture

Primary cortical neuronal cultures were prepared from NMRI mice (M & B) at day 15 to 16 of gestation as described previously (Drejer et al., 1987). Dissected cortices from NMRI mouse embryos were chopped into 0.4–0.4 mm cubes by a McIlwain Tissue Chopper (Mickle Laboratory Engineering Company, Gomshall, UK). The tissue was dissociated by mild trypsinization (0.1% w/v) trypsin, 37°C, 10 min) and disaggregated by mechanical trituration through a steel needle attached to a syringe. Subsequently, the dissociated cells (4 million/ml) were plated onto poly-L-lysine-coated 30-mm Petri dishes at a density of about 8 million cells per dish in a slightly modified Dulbecco’s modified Eagle’s medium (24.5 mM KCl) supplemented with p-aminobenzoate (7 μM), insulin (100 μU/filiter), and 10% (v/v) horse serum. Cells were maintained in culture for 5 to 7 days before experiments were carried out.

For electrophysiological studies, glass coverslips (3.5 mm) were placed in the Petri dishes and 2 ml of cell suspension (1.5 million/ml) was added. After 24 h in culture, the medium was replaced by medium without serum but with 1% N2 supplement (Life Technologies, Rockville, MD). After 24 h in culture, the medium was replaced by fresh Dulbecco’s modified Eagle’s medium/N2 supplement. The cells were kept in culture for 7 to 13 days before experiments were carried out.

AMPA-Induced [3H]GABA Release

Release experiments were performed on cultured mouse cortical neurons by using the model previously described by Drejer et al. (1987). One hour before the experiment was initiated, y-aminobenzoate (100 μM) was added to the Petri dishes to prevent degradation of neuronal GABA. The cells then were preloaded for 30 min with 5 μCi [3H]GABA added to each culture. After the preloading period, the cell monolayer at the bottom of the Petri dish was covered with a piece of nylon mesh to protect the cells against mechanical damage and to facilitate dispersion of medium over the cell layer. After removal of the preloading medium, the Petri dishes were mounted in a superfusion system consisting of a peristaltic pump continuously delivering constant temperature, 37°C superfusion medium (HPS: HEPES-buffered saline (HBS): 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.6 mM MgSO4, 1.0 mM CaCl2, and 6 mM Na-glucose, pH 7.4) from a reservoir to the top of the slightly tilted Petri dish. The medium was continuously collected from the lower part of the dish and delivered to a fraction collector. Initially, the cells were superfused with HBS for 30 min (flow rate, 2 ml/min). Subsequently, the cells were stimulated for 30 s every 4 min by changing the superfusion medium from HBS to a corresponding medium containing 5 μM AMPA and test compound or vehicle. The amount of radioactivity in each fraction was determined by conventional liquid scintillation counting.

In Vitro Electrophysiology

All measurements were obtained in voltage-clamp experiments by using conventional whole-cell patch-clamp techniques (Hamill et al., 1981), and all data were obtained with an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany) run by a Power Macintosh G3 computer. Experimental conditions were set with the Pulse software accompanying the amplifier. Data was low-pass filtered and sampled directly to the hard disk. Pipettes were pulled from borosilicate glass using a horizontal electrode puller (Zeitz Instrumente, Augsburg, Germany), and the pipette resistance was 1.8 to 2.5 MΩ in the salt solutions used in the experiments.

Coverslips were transferred to a 15-μl perfusion chamber mounted on the stage of an inverted microscope supplied with Nomarski optics, and cells were continuously superfused with HBS (140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 4 mM MgCl2, 10 mM HEPES, 0.3 mM tetrodotoxin, 5 μM bicuculline methiodide, 10 mM cyclothiazide, and 30 mM sucrose, pH 7.4) at a rate of 2.5 ml/min. After giga-seal formation (1–5 GΩ) and establishment of the whole-cell configuration, the cells were held at a holding voltage of ~60 mV and the current was continuously measured for 45 s to ensure a stable baseline. For the measurement of NMDA responses, 10 μM glycine was added to the extracellular solution and MgCl2 and cyclothiazide were omitted. The pipette buffer contained 120 mM CaCl2, 20 mM CsF, 4 mM MgCl2, 10 mM EGTA, 4 mM ATP, and 10 mM HEPES (pH 7.2). Agonists (30 μM AMPA or 100 μM NMDA) were dissolved in extracellular HBS and were delivered to the chamber through a custom-made, gravity-driven flowpipe, the tip of which was placed approximately 50 μm from the cell. Application was triggered when the tubing connected to the flowpipe was compressed by a valve controlled by the Pulse software. Initially, agonists were applied for 1 s every 45 s. The sample interval during application was 400 μs. After attainment of stable responses, the extracellular saline as well as the agonist containing solutions were switched to solutions containing SPD 502 and NBQX at the concentration to be tested. The compound was present until responses of a repeatable amplitude were achieved. Because of good reversibility of the effect of the compounds and low, constant series resistance (<10 MΩ), several concentrations of the compounds could be tested on each cell. Current amplitudes were measured at the peak of the responses, and the effect of a compound was calculated as the amplitude at compound equilibrium divided by the amplitude of the agonist-induced current evoked immediately before application of the compound. All experiments were performed at room temperature (20–22°C).

Animal Ethics

All procedures using animals were approved by the Danish National Committee for ethics in animal research.

In Vivo Electrophysiology

Surgical Procedure. Male Wistar rats (280–380 g; M & B) were anaesthetized with urethane (1.1 g/kg i.p.) or mebumal (50 mg/kg i.p.), and the femoral artery was catheterized to allow arterial blood pressure to be monitored throughout the experiment. The femoral vein was catheterized for i.v. injection of drugs and continuous infusion of 0.9% NaCl (0.5–1.0 ml% h) and mebumal (5–10 mg/h). Additional anesthetic was given if the rat responded to a pinch of the hind paw. The trachea was cannulated and the rats were placed in a stereotaxic frame and ventilated by a rodent ventilator (Ugo Basile, Comerio-Varese, Italy). The lateral and dorsal part of the left parietal bone was removed by craniotomy, and the dura was withdrawn, exposing the pia mater and underlying brain, which was superfused with a standard Krebs’ solution (37°C). The core body temperature of the animals was maintained at 37°C by a d.c. heating pad.

Electrophysiology. Extracellular recordings of single hippocampal neuron spikes (action potentials) were made with five-barrel glass microelectrodes (5B120F-6; World Precision Instruments Inc., Sarasota, FL) with a tip diameter of 10 to 12 μm. The individual barrels were filled with 5 M NaCl (recording), 400 mM NaCl (current balancing), and 200 mM NaCl (control current), and the last two barrels were used for solutions of AMPA (10 mM in 200 mM NaCl) and NMDA (100 mM in 100 mM NaCl). Both solutions were adjusted to pH 7.5 ± 0.1 with 1 M NaOH.

Experiments were performed on hippocampal neurons (A: anterior to interaural = 5.5–6.5 mm, L: lateral to midline = 1.5–2.0 mm, H: below the surface of the brain = 2.0–3.0 mm, according to Paxinos and Watson, 1986). Neuronal spike activity was evoked by iontophoretic application of AMPA or NMDA for 10 to 15 s with 1.5 min between each agonist stimulation. Neuronal signals were amplified 5000 times with a bandwidth of 0.3 and 3 kHz (CyberAmp 320 with an AI 402 × 50 smartprobe; Axon Instruments, Foster City, CA). On- and off-line analyses were performed by the Spike2 program with an 1401plus interface (Cambridge Electronic Design Limited, Cambridge, UK). In addition to monitoring and controlling iontophoretic application, the program recorded mean arterial blood pressure.
AMP and NMDA solutions were ejected into the hippocampus in regular cycles of 3 min. During each cycle, a saline (200 mM NaCl) ejection was performed to exclude stimulation artifacts. After establishment of stable neuronal responses (less than 10% deviation between consecutive groups of three responses) for at least 30 min and obtaining responses to AMPA or NMDA that were not more than a factor of 3 different from each other, a single dose of SPD 502 (9–36 μmol/kg corresponding to 5–20 mg/kg; dissolved in 0.9% NaCl at pH 7.3) was injected into the femoral vein. Recording of neuronal spike activity was continued for at least 45 min after drug injection, and only experiments in which AMPA and NMDA responses reversed to approximately 50% of the predrug level were accepted for analysis.

Electroshock-Induced Seizures in Mice

To test the effect of SPD 502 and NBQX on electroshock-induced seizures, compound solutions or vehicle (5.5% glucose) was administered i.v. to female NMRI mice (20–25 g; M & B) 5 or 30 min before testing. In time-course studies, compounds were administered relative to the time of testing. The mice were stimulated for 0.3 s by corneal electrodes connected to a rodent shocker (Hugo Sachs Elektronik, March, Germany). The electroshock seizure threshold was determined as the current (in mA) necessary to induce tonic extension of the hindlegs in 50% of the mice by the up-and-down method described by Dixon and Mood (1948). At each dose of the test compound the initial current was selected by the experimenter based on expectations. According to the response obtained, the next mouse got a 1-mA-lower current (if the previous mouse experienced tonic seizures) or a 1-mA-increased current (if the previous mouse did not experience a tonic seizure). This schedule was continued until at least 10 mice had been tested using a current close to the threshold value, which was calculated as the average of the current used in the last 10 mice tested. The maximal current used was 150 mA.

Transient Forebrain Ischemia in Mongolian Gerbils Induced by Bilateral Occlusion of the Carotid Arteries (BCAO)

Mongolian gerbils (57–68 g; NeuroSearch, Glostrup, Denmark) were anesthetized with halothane (2% halothane in 30% O2/70% N2O), and the left jugular vein was catheterized for i.v. injection and infusion. Subsequently, both carotid arteries were exposed and occluded for 4 min using artery clips. The core temperature of the animals was controlled before and after surgery and maintained at ~37°C by using heating lamps. During surgery the gerbils were placed on heating pads, and body temperature was controlled and maintained at 37 ± 0.5°C. In one study, temperature-sensitive transmitters (Data Sciences International, St. Paul, MN) were implanted in the peritoneum of the gerbil, and the body temperature was monitored every fifth min by telemetry. A feedback system switched a heating lamp on if body temperature dropped below 37.0°C and off when the temperature was higher than 37.5°C.

SPD 502 was dissolved in 5.5% glucose, and vehicle or drug solution was administered to the gerbils as a bolus injection (10 mg/kg) immediately after reperfusion followed by infusion of 10 mg/kg/h for 2 h.

Four days after surgery the animals were sacrificed and the brains were removed and frozen on dry ice. The brains were cut in 20-μm coronal sections on a cryostat (CM 3000; Leica, Nussloch, Germany), and five to seven sections with hippocampal tissue were selected and stained with H&E. Based on the degree of hippocampal damage in the CA1 pyramidal neurons, an ischemic score of 2 to 8 was determined for each animal, as described by Jensen and Møller (1992). Briefly, the following scale was used: 1) no loss of CA1 neurons, 2) CA1 layer partly damaged, 3) total loss of CA1 neurons, and 4) total loss of CA1 neurons, expanding into other hippocampal subregions. The score for each animal was the sum of the score from the two hippocampi. All assessments of histological sections were made by an observer who was unaware of the drug treatment for each gerbil.

Data Analysis

In in vitro studies, compounds that displaced radioligand binding or inhibited neurotransmitter release were tested over a wide range of concentrations, and IC50 values and Hill coefficients were determined based on the equation B = 100 − [100 · D/(IC50 + Dn)], where B is the binding/release in percentage of total specific binding/release, D is the concentration of test compound, and n is the Hill coefficient. The EC50 values for AMPA in neurotransmitter release were determined by using the equation B = 100 · Dn/(EC50 + Dn). Estimates of binding parameters were calculated with the nonlinear curve-fitting program GraFit (Leatherbarrow, 1992). All results are given as mean ± S.E.M.

In in vitro electrophysiology, the IC50 values and Hill coefficients were determined from data points fitted to the equation y = 100 · Dn/(IC50 + Dn). Results are given as mean ± S.D.

In in vivo electrophysiology, evoked neuronal spike activity was analyzed on-line by a computer, saving single spikes and time of event. Neuronal spike activity (number of action potentials per s) was monitored on a pulse-rate histogram together with indicators for tonic extension and seizure activity. This schedule was continued until at least 10 mice had been tested using a current close to the threshold value, which was calculated as the average of the current used in the last 10 mice tested. The maximal current used was 150 mA.

Receptor Binding

SPD 502 displaced [3H]AMPA binding in rat cortical membranes in a concentration-dependent manner with an IC50 value of 0.043 ± 0.007 μM (n = 3; Fig. 2 and Table 1) and a Hill coefficient value of 0.94 ± 0.10 (data not shown). NBQX displaced binding with an IC50 value of 0.083 ± 0.018 μM (n = 3; Fig. 2 and Table 1). In contrast to its affinity for the [3H]AMPA-binding sites, SPD 502 was more than 1000-fold less potent (IC50 value of 81 ± 12 μM, n = 3) in displacing [3H]kainate binding from kainate-bind-
ing sites present in rat brain (Table 1). No displacement of [3H]CGS 19755 and [3H]glycine binding in rat brain was seen at 30 μM SPD 502 (Table 1). NS 1219 and NS 1220, the optical (R)- and (S)-isomers of the racemic SPD 502 (Fig. 1), both showed similar affinity for the [3H]AMPA-binding sites (IC50 values of 0.070 ± 0.012 μM and 0.063 ± 0.018 μM, respectively), NBQX and SPD 502 were equipotent in displacing [3H]AMPA binding, butNBQX showed less selectivity for the AMPA receptor, as it displaced [3H]kainate binding with an IC50 value of 5.4 ± 1.5 μM (n = 3; Table 1). However, NBQX, like SPD 502, showed no affinity for the glutamate and glycine sites on the NMDA receptor at 30 μM (Table 1), as determined by [3H]CGS 19755 and [3H]glycine binding, respectively.

SPD 502 was also examined in 57 other receptor-binding and enzyme activity assays at MDS Panlabs (Bothell, WA) (Table 2) and showed little affinity (IC50 > 10 μM) for GABA receptors as well as other members of the ligand-gated ion-channel superfamily, including nicotinic and or (5-HT or serotonin) type-α members of the G protein-coupled receptor superfamily, including adenosine, adrenaline, angiotensin, bradykinin, cholecystokinin, dopamine, endothelin, galanin, histamine, leukotriene, muscarinic, neuropeptide Y, opiate, platelet-activating factor, serotonin, tachykinin, and vasoactive intestinal peptide; 1-type calcium, chloride, and sodium channel proteins; receptors for estrogen, insulin, phosphol ester, progesterone, testosterone, thromboxane, and thyrotropin releasing hormone, and did not inhibit the activity of calpain, nitric oxide (NO) synthase, tyrosine kinase, protein kinase C, and calcineurin phosphatase.

Neurotransmitter Release. The antagonistic properties of SPD 502 in vitro was evaluated in cultured mouse cortical neurons. Concentration-response curves for SPD 502 in AMPA-induced release of [3H]GABA from these neurons showed that the compound inhibited AMPA responses with an IC50 value of 0.23 ± 0.07 μM (n = 4). Comparable antagonist potency was observed for NBQX (IC50 value of 0.19 ± 0.04 μM, n = 3; Fig. 3A and Table 3). Application of 0.01 to 1 μM SPD 502 alone did not affect the spontaneous release of [3H]GABA, indicating that it acts as a pure antagonist (data not shown). Again, no stereoselectivity was observed for the (R)- and (S)-isomers (Table 3). AMPA induced a concentration-dependent increase in release of [3H]GABA with maximal effect at 30 μM and an EC50 value of 3.0 ± 0.3 μM (n = 4; Fig. 3B). Inclusion of 0.25 μM SPD 502 or 0.5 μM NBQX resulted in a right-hand shift in the dose-response curve for AMPA and an increase in the EC50 value for AMPA to 10 ± 1.9 μM (n = 4) and 15.6 ± 2.7 μM (n = 3; Fig. 3B), respectively.

In Vitro Electrophysiology. Brief applications of 30 μM AMPA to voltage-clamped cultured mouse cortical neurons resulted in inward currents that varied considerably with respect to desensitization. With 10 μM cyclothiazide present in the extracellular solution to block desensitization, the amplitude of control responses was 1865 ± 963 pA (six cells, data not shown). In the presence of 0.3 μM SPD 502, the amplitude of the agonist response was inhibited by approximately 60%, and the rise time of the response was significantly slower (Fig. 4). To obtain current plateau, it was necessary to extend the AMPA pulse from 1 to 2 s, when SPD
502 was present. The inhibition by SPD 502 of AMPA-induced responses was concentration-dependent with an IC<sub>50</sub> value of 0.15 ± 0.05 μM (n = 3; Fig. 5). For comparison, the IC<sub>50</sub> value for the antagonist NBQX was 0.10 ± 0.06 μM (n = 3; Fig. 5).

Cultured mouse cortical neurons invariably responded to 100 μM NMDA, and the inward currents varied from cell to cell with respect to desensitization kinetics, with control responses of 630 ± 164 pA (three cells, data not shown). In the presence of 100 μM SPD 502, the NMDA responses were inhibited by 9.3 ± 2.3% (Fig. 6).

**In Vivo Electrophysiology.** Fig. 7 shows an example of selective inhibition of AMPA-evoked spike activity in the hippocampus after i.v. administration of 20 mg/kg SPD 502. The duration of the block of AMPA responses was about 2 h. SPD 502 dose-dependently inhibited AMPA-evoked spike activity with significant effects at 10 and 20 mg/kg, and with a maximal inhibition of 86% and an ED<sub>50</sub> value of 6.1 mg/kg.
NMDA were performed using Mg2+-free solutions, as indicated by the black, horizontal bar. The experiments with M.

ments.

m (only two responses shown), SPD 502 (100 μM) was included in the solutions, as indicated by the black, horizontal bar. The experiments with NMDA were performed using Mg2+-free solutions with 10 μM glycine. The recordings are representative of findings in three separate experiments.

(Fig. 8). However, NMDA-evoked spike activity was only inhibited significantly at 10 mg/kg (Fig. 8). The duration of action of SPD 502 on AMPA-evoked spike activity in hippocampus increased in a dose-dependent manner: 25 to 50 min, 80 to 180 min, and >180 min after administration of 5, 10, and 20 mg/kg SPD 502, respectively.

Electroshock-Induced Seizures. SPD 502 increased the seizure threshold for electroshock-induced tonic seizures in mice at i.v. doses of 50 mg/kg and higher, when administered 5 min before testing (Fig. 9A). Similar protection was obtained 30 min after administration (Fig. 9B). For NBQX, full protection was seen after a single i.v. dose of 50 mg/kg and 100 mg/kg when administered 5 and 30 min before testing, respectively (Fig. 9, A and B). A single i.v. injection of 60 mg/kg SPD 502 resulted in total protection against electroshock-induced seizures in a time interval between 15 and 90 min after administration (Fig. 9C). However, after dosing of 60 mg/kg i.v. of NBQX, full protection was observed 5 min after administration, whereas no protection could be obtained when testing was performed 30 min after dosing (Fig. 9C). Thirty minutes after i.v. administration of the (R)- and (S)-isomers, NS 1219 and NS 1220, an increase in seizure threshold for electroshock-induced tonic seizures similar to that seen after administration of SPD 502 was observed (data not shown).

Transient Forebrain Ischemia in Mongolian Gerbils. The neuroprotective effect of SPD 502 was evaluated in the model of global cerebral ischemia in gerbils. A highly significant (p < .001) protection against hippocampal CA1 cell loss was seen in the group-administered SPD 502 immediately after reperfusion as a bolus injection (10 mg/kg) followed by infusion (10 mg/kg/h) for 2 h (Fig. 10A). Neuropeptidase also was obtained when treatment was delayed up to 2 h postocclusion (p < .05; Fig. 10B). In one study, body temperature was regulated to normal for 24 h (adjusted every 5th min), and the neuroprotective effect was maintained (p < .05; data not shown), indicating that the neuroprotective effect is not due to a drug-induced decrease in body temperature. No respiratory depression was observed in the gerbils at the dose tested.

Kidney Toxicology. The possible effects of SPD 502 on the kidney were determined in a pilot study in which SPD 502 was administered to groups of three male Wistar rats (200 g) as an infusion of 10, 30, or 60 mg/kg/h or vehicle for 2 h. The rat was chosen instead of the gerbil because NBQX has been reported to deposit in rat kidneys (Xue et al., 1994). The rats were decapitated 48 h after infusion, and histological slices of the kidneys were examined under the light microscope (Scantox, Lille Skensved, Denmark). No microscopic changes in the kidneys were observed 48 h after the 2-h infusions of SPD 502. Rats administered NBQX either as a single i.v. injection (10 mg/kg) or as three successive i.p. injections (30 mg/kg, T = 30, 60, and 90 min) were run in parallel. At both dose regimens microscopic changes in the kidneys were observed as intraluminal crystals, necrosis, and inflammation 48 h after administration of the compound.

Discussion

The present study describes the in vitro and in vivo pharmacological properties of a novel AMPA antagonist, SPD 502. In radioligand-binding studies, SPD 502 was shown to display selectivity for the [3H]AMPA-binding sites (IC50 = 0.043 μM) present in rat cortical membranes relative to the kainate and NMDA sites. The (R)- and (S)-isomers of SPD 502, NS 1219, and NS 1220, respectively, both showed affinities similar to that of SPD 502 in [3H]AMPA-binding studies, indicating no stereoselectivity. In general, potent AMPA antagonism can be achieved without the introduction of chirality. That (R)- and (S)-SPD 502 are equipotent indicates that this part of the receptor, when occupied by an antagonist, is nonstereoselective. In neurotransmitter-release studies SPD 502 was shown to potently inhibit AMPA-induced [3H]GABA release from cultured cortical neurons. The observed increase in the EC50 value for AMPA in stimulation of [3H]GABA release in the presence of SPD 502 or NBQX indicates that the two compounds are competitive AMPA antagonists. The affinity of SPD 502 in [3H]AMPA-binding studies was very similar to that of NBQX, but the former showed approximately 30 times greater selectivity for the [3H]AMPA-binding site compared with the [3H]kainate-binding site. However, NBQX, like SPD 502, showed no affinity for the glutamate- and glycine-binding sites on the NMDA receptor at 30 μM. Further ligand-binding studies showed that SPD 502 at 10 μM was without affinity at a broad range of receptors.

Activity of SPD 502 at the AMPA receptor was assessed further using electrophysiological recordings from single-mouse cortical neurons in culture. Stimulation of cortical neurons by AMPA resulted in an inward current, which was strongly potentiated by cyclothiazide because of prevention of rapid AMPA receptor desensitization (Wong and Mayer, 1993). In the presence of 10 μM cyclothiazide, excitations produced by 30 μM AMPA were inhibited by SPD 502 with an estimated IC50 value of 0.15 μM, a potency close to that...
The inhibition of AMPA-evoked spike activity lasts only 30 to 40 min and 10 to 20 min, respectively (Mathiesen et al., 1998).

Blockade of electroshock-induced tonic seizures in mice was used as the first measure of in vivo behavioral AMPA antagonistic activity. Total protection against electroshock-induced tonic seizures was seen at doses of 50 mg/kg i.v. and 60 mg/kg i.v. for SPD 502 and NBQX, respectively, when measured 5 min after administration. However, when the anticonvulsant effect of the compounds was measured 30 min after administration, 100 mg/kg i.v. of NBQX was needed to obtain full protection. The duration of anticonvulsant effect of SPD 502 was at least 90 min at 60 mg/kg i.v. compared with less than 30 min for NBQX, indicating that the half-life for SPD 502 is longer than for NBQX. Another AMPA receptor antagonist, 1,4,7,8,9,10-hexahydro-9-methyl-nitropyrido[3,4-f]-quinoxaline-2,3-dione (PNQX), recently has been desribed (Bigge et al., 1995). This compound is more potent than NBQX against maximal electroshock seizures, probably because of an additional antagonist action at the glycine site of the NMDA receptor, but the duration of action for PNQX was as short as that observed for NBQX (Bigge et al., 1995).

Bilateral occlusion of the carotid arteries of the gerbil for 5 min produces a selective loss of CA1 pyramidal cells in the hippocampus a few days after the ischemic insult (Kirino 1982; Crain et al., 1988). This delayed degeneration of neurons is regarded as a model for the brain damage induced by transient cardiac arrest. SPD 502 protected hippocampal neurons from ischemic damage after complete forebrain ischemia not only when administered immediately after the onset of cerebral ischemia (i.v. bolus followed by infusion for 2 h), but also when administration first was initiated 2 h after the ischemic insult. Furthermore, the neuroprotective effect of SPD 502 was maintained in gerbils where core body temperature was controlled for 24 h after surgery, suggesting that the neuroprotective effect of SPD 502 is not exerted via a hypothermic action. A similar long therapeutic time window in global ischemia in gerbils has been reported for NBQX (Bigge et al., 1995; Buchan et al., 1991; Lin et al., 1996), suggesting that AMPA receptors play an important role in delayed neuronal death of CA1 pyramidal cells. The neuroprotective effect of NMDA antagonists in models of global ischemia is, however, controversial under conditions of controlled core temperature (Buchan et al., 1991; Lin et al., 1993), and they fail to prevent degeneration when administered more than 1 h after ischemia (Sheardown et al., 1993). The clinical development of NBQX was hampered because of solubility problems, and the compound was found to de-
posit in kidneys, resulting in nephrotoxicity (Xue et al., 1994). SPD 502, however, is devoid of these solubility problems and does not precipitate into the renal tubules after i.v. dosing. In conclusion, SPD 502 is a selective, potent, and competitive AMPA antagonist with an in vitro potency comparable to that of NBQX, but with a duration of action substantially longer than that of NBQX. We have demonstrated that SPD 502 significantly reduces hippocampal damage after global cerebral ischemia in gerbils when administered up to 2 h after the ischemic insult. In addition, SPD 502, with its improved water solubility, does not produce precipitates in the kidneys, in contrast to NBQX. On the basis of these results, SPD 502 has been selected for development as a therapeutic agent for the prevention of neurodegeneration after acute cerebral ischemia.

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References


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