In Vitro Characterization of 5-Carboxyl-2,4-di-benzamidobenzoic Acid (NS3763), a Noncompetitive Antagonist of GLUK5 Receptors

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Received November 10, 2003; accepted February 24, 2004

ABSTRACT

Accumulating preclinical data suggest that compounds that block the excitatory effect of glutamate on the kainate subtype of glutamate receptors may have utility for the treatment of pain, migraine, and epilepsy. In the present study, the in vitro pharmacological properties of the novel glutamate antagonist 5-carboxyl-2,4-di-benzamido-benzoic acid (NS3763) are described. In functional assays in human embryonic kidney (HEK)293 cells expressing homomeric GLUK5 or GLUK6 receptors, NS3763 is shown to display selectivity for inhibition of domoate-induced currents in intracellular calcium mediated through the GLUK5 subtype (IC50 = 1.6 μM) of kainate receptors compared with the GLUK6 subtype (IC50 > 30 μM). NS3763 inhibits the GLUK5-mediated response in a noncompetitive manner and does not inhibit [3H]methyl-D-aspartate-5-tertbutyloxazol-4-propionic acid binding to GLUK5 receptors. Furthermore, NS3763 selectively inhibits L-glutamate- and domoate-evoked currents through GLUK5 receptors in HEK293 cells and does not significantly inhibit d-amino-3-hydroxy-5-methylisoxazole-4-propionic acid- or N-methyl-D-aspartate-induced currents in cultured mouse cortical neurons at 30 μM. This is the first report on a selective and noncompetitive GLUK5 antagonist.

Glutamate is the major excitatory neurotransmitter in the central nervous system and is involved in both physiological and pathological events in the brain through activation of G protein-coupled metabotropic receptors as well as a trio of ionotropic receptor families consisting of N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, and kainate receptors (Collingridge and Lester, 1989; Schoepf et al., 1999). The pharmacology of NMDA and AMPA/kainate receptors has been studied thoroughly. Selective competitive NMDA antagonists such as d-2-amino-5-phosphovaleric acid (APV; Davies et al., 1981), noncompetitive antagonists such as (-)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (Wong et al., 1986), and the competitive AMPA/kainate receptor antagonists 6-cyano-2,3-dihydroxy-7-nitroquinoxaline and NBQX (Honoré et al., 1988; Sheardown et al., 1990) have aided in the understanding of the physiological role of these two groups of receptors. The pharmacological isolation of kainate receptors has been very difficult because AMPA receptor activation masks the existence of kainate receptors in essentially every central neuron, and the competitive AMPA/kainate receptor antagonists 6-cyano-2,3-dihydroxy-7-nitroquinoxaline and NBQX have provided limited information because they display very poor selectivity at AMPA versus kainate receptors (Fletcher et al., 1988; Lodge et al., 1991). However, low concentrations of NBQX have been used to isolate kainate responses in hippocampal interneurons (Mulle et al., 2000). The 2,3-benzodiazepine class of compounds such as GYKI52466 and GYKI53655 are noncompetitive antagonists showing a relatively high degree of selectivity for AMPA receptors, and these compounds have successfully been used to isolate kainate receptors (Patermain et al., 1995). Through use of these antago-
nists and cloned kainate receptors, it became clear that ATPA (Lauridsen et al., 1985) is a GLU<sub>K5</sub>-preferring agonist potentially activating homomeric GLU<sub>K5</sub> receptors and native dorsal root ganglion kainate receptors, but shows only weak activity at AMPA receptors, and no activity at GLU<sub>K6</sub> homomers (Clarke et al., 1997; Wilding and Huyettner, 2001).

Kainate receptors, as AMPA and NMDA receptors, are thought to be tetramers (for review, see Madden, 2002) formed by homo- or heteromeric association of the kainate receptor subunits GLU<sub>K1</sub>, GLU<sub>K2</sub>, GLU<sub>K5</sub>, GLU<sub>K6</sub>, and GLU<sub>K7</sub>. The first compound to be described as a competitive kainate antagonist was NS102, based on its ability to block low-affinity 3-Hkainate binding (Johansen et al., 1993). However, functional assays yielded contradictory results because NS102 acts at GLU<sub>K5</sub> and GLU<sub>K6</sub> receptors and shows selectivity in some systems (Verdoorn et al., 1994; Wilding and Huyettner, 1996) but poor selectivity in others (Paternain et al., 1996). Recently, LY382884 has been reported to bind specifically to GLU<sub>K5</sub> but not to GLU<sub>K6</sub>, GLU<sub>K7</sub>, GLU<sub>K2</sub>, or AMPA receptor subunits (Bortolotto et al., 1999). In functional tests, LY382884 inhibits kainate-evoked currents in dorsal root ganglion neurons and is approximately 100 times less potent on AMPA- and NMDA-evoked responses in hippocampal neurons (Beakman, et al, 2002).

Kainate receptors are believed to have diverse roles under both physiological and pathological conditions, and the novel pharmacological agents have enabled insights into the involvement of GLU<sub>K5</sub> receptors in synaptic transmission and plasticity. In the processing of nociceptive information, kainate receptors are involved at several sites, including primary afferent fibers, superficial dorsal horn neurons, and intrinsic spinal horn neurons (Ruscheweyh and Sandkühler, 2002). Several studies have implicated kainate receptors (specifically, the GLU<sub>K5</sub> subtype) in pain transmission (Procter et al., 1998; Li et al., 1999), and Simmons et al. (1998) demonstrated that the selective GLU<sub>K5</sub> antagonist LY382884 was active in an animal model of persistent pain. More recently, GLU<sub>K5</sub> receptors have been linked with migraine headache, and competitive GLU<sub>K5</sub> antagonists have been reported to be active in animal models of acute migraine (Filla et al., 2002). Within the hippocampus, GLU<sub>K5</sub>-containing receptors are involved in frequency facilitation and induction of long-term potentiation, and in excitatory drives of inhibitory CA1 interneurons. In addition, GLU<sub>K5</sub> antagonists have recently been reported to have anticonvulsant activity in animal models (Smolders et al., 2002).

Existing GLU<sub>K5</sub> antagonists show no overt behavioral side effect at doses where the beneficial effects are observed in animal models (Simmons et al., 1998; Smolders et al., 2002).

In summary, these data suggest that the GLU<sub>K5</sub> subtype of kainate receptors can be used as a target for the development of selective antagonists, which may provide a valuable approach for the future treatment of pain, migraine, and epilepsy.

In the present work, we report the in vitro pharmacology of a novel non-competitive GLU<sub>K5</sub> antagonist. In contrast to the competitive antagonist NS1209, which inhibits AMPA-induced responses in cortical neurons (Nielsen et al., 1999) and kainate-evoked responses in cells expressing GLU<sub>K5</sub> receptors equipotently (Varming et al., 2001), NS3763 did not show significant antagonistic properties on either native AMPA or native NMDA receptors.

**Materials and Methods**

**Materials and Drugs**

[1H]Kainic acid (58 Ci/mmole) and [3H]ATPA (16 Ci/mmole) were purchased from PerkinElmer Life Sciences (Boston, MA) and Amer sham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK), respectively. Fluo-4-AM (cell-permeant acetoxyethyl ester of the Ca<sup>2+</sup> indicator Fluo-4) and domoic acid was purchased from Molecular Probes Europe BV (Leiden, The Netherlands) and Tocris Cook son Inc. (Bristol, UK), respectively. GYKI52466 was purchased from Sigma/RBI (Natri, MA). NS1209 (previously known as SPD502; Nielsen et al., 1999; Varming et al., 2001) was synthesized at Neur oSearch A/S. NS3763 was identified in a compound library purchased from Chemical Diversity Labs (San Diego, CA).

Cell culture media were obtained from Invitrogen (Roskilde, Denmark). All other chemicals were purchased from regular commercial sources and were of the purest grade available.

**Cell Cultures**

GLU<sub>K5</sub>- and GLU<sub>K6</sub>-Expressing Cell Lines. HEK293 cell lines stably expressing homomeric human GLU<sub>K6Q1a</sub> and GLU<sub>K5Q1c</sub> were established as described previously (Varming et al., 2001).

The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum, in polystyrene culture flasks (175 cm<sup>2</sup>), in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air, at 37°C. Cells were cultured to 80% confluency before plating. The cells were rinsed with phosphate-buffered saline and detached from the culture flask by trypsin (0.1% (w/v)) digestion, for 5 min at 37°C. After addition of growth media, cells were resuspended by trituration and seeded at a density of 0.05 to 0.1 million cells/well in black-walled, clear-bottom 96-well plates pretreated with 0.001% (w/v) polyethyleneimine solution (75 µl/well for 30 min). Plated cells were allowed to proliferate for 24 h before loading with dye.

For experiments with the human GLU<sub>K5Q2b</sub> splice variant, HEK293 cells were transiently transfected using the LipofectAMINE Plus (Invitrogen) transfection kit as described by the manufacturer.

Cell cultures were used the day after transfection.

For electrophysiological studies, cells were seeded on the day of experiment. Glass coverslips (3.5 mm), precoated with poly-L-ornithine [0.005% (w/v)] and laminine [0.002% (w/v)] were placed in Petri dishes, and 2.5 ml of cell suspension (0.1 million cells/ml) was added.

**Primary Cortical Neuronal Cultures.** The cultures were prepared from NMRI mice (Taconic M and B, Ry, Denmark) at day 15 to 16 of gestation as described previously (Drejer et al., 1987). Briefly, dissected tissue was chopped into 0.4-mm cubes and triturated with 0.2% (w/v) trypsin and DNase (40 µg/ml), for 10 min at 37°C. The cells were suspended (1.5 million/ml) in a slightly modified DMEM (with 23 mM glucose), which contained 7% horse serum, 0.2% (w/v) penicillin, and 19.1 mM KCl. The cell suspension was subsequently inoculated into poly-d-lysinate-coated (0.01% (w/v)) 35-mm Petri dishes (2 ml/dish). Glass coverslips (3.5 mm) were placed in the dishes before coating. After 24 h in culture, the medium was replaced by medium without serum but with 1% N2 supplement. Every 3 to 4 days, the culture medium was replaced with DMEM/N2 supplement. Cells were maintained in culture for 8 to 13 days before experiments were carried out.

**Fluorescence Measurements**

On the day of experiment, the medium was aspirated from the wells, and 50 µl of a 2 µM Fluo-4-AM loading solution was added to each well. The plates were sealed and incubated at room temperature (20–22°C) for 60 min. The loading medium was then aspirated,
and the cells were washed twice with 100 μl of buffer (10 mM HEPES, 140 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, and 2 mM CaCl₂; pH 7.4) to remove extracellular dye. The reason for using a relatively high CaCl₂ concentration was to enhance the fluorescent signal evoked secondary to activation of GLU K₅ and GLU K₆ receptors. Buffer (100 μl) was added to each well, and the fluorescence was measured at room temperature (excitation 488 nm, emission 510–570-nm band pass interference filter) using a fluorescent imaging plate reader (Molecular Devices, Sunnyvale, CA). Cells were preincubated for 1.5 min with test compound (50 μM) before addition of domoate (50 μl) to a final concentration of 2 μM (for GLU K₅) or 0.2 μM (for GLU K₆).

Stock solutions of test substances were made in ethanol or dimethyl sulfoxide, with final concentration of solvent never exceeding 0.5%.

Electrophysiological Studies

The electrophysiological measurements were performed in voltage clamps 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 Macintosh G3 computer. Experimental conditions and data acquisition were set and obtained using the PULSE-software accompanying the amplifier. Data were low pass filtered and sampled directly to the hard computer. Experimental conditions and data acquisition were set and all data were obtained with an EPC-9 amplifier clamps using conventional whole cell patch-clamp techniques (Hamill et al., 1981), and all data were obtained with an EPC-9 amplifier.

Receptor Binding

GLU K₅- and GLU K₆-expressing cells were harvested and washed once with 50 mM Tris-HCl (pH 7.1) and stored at −80°C until the day of experiment. The thawed membrane pellets were resuspended in >100 volumes of ice-cold Tris-HCl buffer and centrifuged for 10 min (27,000g). The final pellets were resuspended in Tris-HCl buffer and used for binding experiments. All procedures were performed at 0 to 4°C.

Binding conditions for GLU K₅ and GLU K₆ were as described previously (Varming et al., 2001). Briefly, binding to GLU K₅ receptors was performed using 3 nM [³H]ATPA at 46 to 84 μg of protein/assay, and GLU K₆ receptors were labeled with 5 nM [³H]kainate at 22 to 27 μg of protein/assay. The samples were incubated in a final volume of 550 μl for 60 min at 2°C. Nonspecific binding was determined in the presence of 0.6 mM L-glutamate, and binding was terminated by rapid filtration. Radioactivity was determined by conventional liquid scintillation counting.

Data Analysis

In binding and functional studies, compounds were tested over a wide range of concentrations, and IC₅₀ values and Hill coefficients were determined based on the equation Y = Bottom + (Top − Bottom)/(1 + (X/IC₅₀)^n)), where Y is binding/calcium increase/current in percentage of total binding/calcium increase/current; X the concentration of test compound; and n is the Hill coefficient. The IC₅₀ values for domoate in stimulation of intracellular calcium in HEK293 cells were determined by using the equation Y = 100 - X^n/(E_C₅₀^n + X^n).

Estimates of IC₅₀ and EC₅₀ values were calculated with the non-linear curve-fitting program GraphPad Prism (version 2.0; GraphPad Software Inc., San Diego, CA). Kᵥ values were calculated from IC₅₀ values using the Cheng and Prusoff equation: Kᵥ = IC₅₀/1 + (L/Kᵣ). The Kᵥ values were as follows: 2.9 nM for [³H]ATPA at GLU K₅ receptors and 5.7 nM for [³H]kainate at GLU K₆ receptors, respectively. All results are given as means ± S.E.M.

Results

Calcium Measurements. Domoate concentration dependently increased intracellular calcium in GLU K₅- and GLU K₆-expressing cells with EC₅₀ values of 1.5 ± 0.2 and 0.13 ± 0.03 μM, respectively (data not shown). Based on these potencies, the following inhibition studies were conducted at 2 μM domoate for GLU K₅ and 0.2 μM for GLU K₆ cells. NS3763 (Fig. 1) inhibited GLU K₅-mediated responses with an IC₅₀ value of 1.6 ± 0.2 μM, whereas no inhibition of GLU K₆-mediated response was seen at concentrations up to 30 μM (n = 4, Table 1; Fig. 2). ATPA did not induce agonist responses at concentrations ranging from 0.01 to 3 μM, but the compound potently and selectively inhibited domoate-induced increase in intracellular calcium in GLU K₅ cells with...
described in “S.E.M. of three separate experiments. Curve fitting was performed as described under Materials and Methods. Results are means ± S.E.M. from at least three separate determinations.

**TABLE 1**

Selectivity profile of GLU K5 ligands

<table>
<thead>
<tr>
<th>Compound</th>
<th>GLU K5 IC₅₀</th>
<th>GLU K6 IC₅₀</th>
<th>[³H]ATPA Kᵢ</th>
<th>[³H]Kainate Kᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPA</td>
<td>0.21 ± 0.03</td>
<td>&gt;300</td>
<td>0.0094 ± 0.0010</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NS1209</td>
<td>0.63 ± 0.09</td>
<td>65 ± 4</td>
<td>0.62 ± 0.11</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>NS3763</td>
<td>1.6 ± 0.2</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

Functional selectivity at recombinant homomeric GLU K5 and homomeric GLU K6 receptors expressed in HEK293 cells, determined by measurements of intracellular calcium. IC₅₀ values were calculated from percentage of inhibition of domoate-induced calcium influx.

Selectivity in ligand binding assays to homomeric GLU K5 and homomeric GLU K6 receptors expressed in HEK293 cells. Kᵢ values for NS1209 are from Varming et al. (2001).

Domoate was a more potent agonist than L-glutamate in evoking currents in GLU K5- and GLU K6-expressing cells, and the estimated EC₅₀ values were 7.5 and 0.4 μM, respectively (data not shown). The peak inward current responses obtained at the estimated EC₅₀ value for domoate in GLU K5 cells ranged from 41 to 1474 pA (n = 9), and the amplitude of the response evoked by 8 μM domoate was inhibited by 63 ± 10% in the presence of 10 μM NS3763 (n = 5; Fig. 4A). NS3763 (0.01–30 μM) caused a concentration-dependent inhibition of GLU K5 responses to 8 μM domoate with an IC₅₀ value of 1.3 μM, and a maximal inhibitory effect of approximately 60% (n = 5; Fig. 4B). Currents evoked by 30 μM domoate were inhibited 58 ± 5% (n = 6) in the presence of 10 μM NS3763.

All data reported above were obtained using the GLU K5-1a isoform, which contains 15 extra amino acids in the NH₂-terminal domain and has a shorter COOH-terminal domain than the GLU K5-2b isoform reported for rat (Sommer et al., 1992) and for human by Korczak et al. (1995). To investigate whether NS3763 had different modulatory effects at GLU K5-1a and GLU K5-2b receptors, HEK293 cells transiently expressing the GLU K5-2b isoform were used. In these cells an EC₅₀ value of 1.2 mM was obtained for L-glutamate (data not shown).

As illustrated in Fig. 5, NS3763 caused a concentration-dependent inhibition of L-glutamate responses in both isoforms; however, the drug was approximately 10-fold more potent at the GLU K5-2b (IC₅₀ = 0.38 μM; n = 3) than the GLU K5-1a isoform (IC₅₀ = 5.7 μM; n = 4).

In electrophysiological studies, as well as in assays measuring intracellular calcium (Fig. 2), only a partial inhibition of the responses could be obtained.

**Mechanism of Action of NS3763 on GLU K5 Receptors.** To investigate the mechanism of inhibition by NS3763, its effect on the concentration-response relationship for domoate was characterized. As illustrated in Fig. 6A, the maximal increase in intracellular calcium occurred at 30 μM domoate, and 3 μM NS3763 caused a reduction of the maximal response. However, the EC₅₀ value for domoate in the presence of NS3763 (1.7 ± 0.2 μM; n = 3) was similar to the control evoked currents were considerably larger in GLU K6-expressing cells ranging from 1.1 to 11.8 nA (n = 23) at 0.5 mM L-glutamate.

In the presence of 10 μM NS3763, the amplitude of the L-glutamate-evoked response in GLU K5 cells was inhibited by 44 ± 10% (n = 4), whereas no effect was seen on GLU K6-mediated responses up to 30 μM (n = 3, Fig. 3, A and B).

It was examined whether the effect of NS3763 was due to changes in the desensitization kinetics of GLU K5 receptors. For selected pairs of current responses evoked by 3 mM L-glutamate in the presence and absence of 30 μM NS3763, the decay phase of the peak-current was fitted to a double-exponential function and compared by paired t test. The decay time constant of the fast component, τₑₚₑₜ, in the presence of NS3763 (1.75 ± 0.08 ms; n = 7) was not significantly different from the control value of 1.62 ± 0.10 ms (n = 7). Similarly, the slow component, τₛₒₜ, was not different in the presence (8.87 ± 1.81 ms; n = 7) and absence (7.20 ± 0.69 ms; n = 7) of NS3763. The ratio of the two components was also unaffected by the application of the compound, the fast component accounting for 0.78 ± 0.03 (control) and 0.81 ± 0.04 (30 μM NS3763).
value of 1.4 ± 0.1 μM (n = 3). In contrast, 3 μM NS1209 caused a concentration-dependent rightward shift in the concentration-response curve with no change in the maximal response (Fig. 6A); the EC₅₀ value for domoate in the presence of 3 μM NS1209 was 7.1 ± 1.3 μM (n = 3). Thus, unlike NS1209, which interacts competitively at the domoate binding site, NS3763 inhibits domoate responses by a noncompetitive mechanism.

The noncompetitive action of NS3763 was supported by radioligand binding studies, because NS3763 did not inhibit [³H]ATPA binding to GLU₉₅ receptors (Table 1). In contrast, NS1209 inhibited ligand binding to GLU₉₅ and GLU₁₀₅ (Varming et al., 2001), whereas ATPA only displaced binding to GLU₉₅.

The noncompetitive mechanism of action for NS3763 was confirmed in electrophysiological studies (Fig. 6B); the EC₅₀ value for l-glutamate in the presence of 10 μM NS3763 (3.28 mM; n = 5–9) was similar to the control value of 3.35 mM (n = 3–7; p > 0.05; paired t test).

**Discussion**

The present study shows that NS3763 selectively and noncompetitively inhibits homomeric GLU₉₅ receptor-mediated noncompetitive GLU₉₅ Antagonist 1007

![Fig. 3.](image1.png)

Fig. 3. NS3763 selectively blocks l-glutamate-evoked currents in HEK293 cells expressing homomeric GLU₉₅ receptors. A, inhibition of l-glutamate-evoked current in a GLU₉₅-expressing cell by 10 μM NS3763. The cell was voltage clamped at −60 mV in the whole cell configuration and exposed to a 100-ms pulse of 3 mM l-glutamate. After attainment of responses of a repeatable amplitude (only one response shown), 10 μM NS3763 was included in the solution as indicated by the black horizontal bar. B, effect of 30 μM NS3763 on l-glutamate-evoked current in a GLU₉₅-expressing cell. The experiment was performed as described above but using 0.5 mM l-glutamate. The interval between stimulations was 30 s. The recordings are representative of findings in three to four separate experiments.

![Fig. 4.](image2.png)

Fig. 4. NS3763 blocks domoate-evoked currents in HEK293 cells expressing homomeric GLU₉₅ receptors. A, inhibition of domoate-evoked current by 10 μM NS3763. The cell was voltage clamped at −60 mV in the whole cell configuration and exposed to a 100-ms pulse of 8 μM domoate. After attainment of responses of a repeatable amplitude (only one response shown), 10 μM NS3763 was included in the solution as indicated by the black horizontal bar. B, concentration-dependent inhibition of domoate-induced currents by NS3763. The experiment was performed as described above. The current amplitudes were expressed as percentage of the current induced by 8 μM domoate. Each data point represents the mean ± S.E.M. of five separate experiments. Curve fitting was performed as described in “Data Analysis”.

**Native AMPA and NMDA Receptors.** The possible antagonistic activity of NS3763 on native AMPA- and NMDA receptors was investigated in cultured mouse cortical neurons. The peak inward current responses to 100 μM AMPA or NMDA ranged from 104 to 244 pA (n = 4) and from 525 to 1237 pA (n = 4), respectively. In the presence of 30 μM NS3763, the amplitude of the responses to 100 μM AMPA was inhibited by 0.6 ± 5.4% (n = 4; Fig. 7A). In contrast, the AMPA-induced current was blocked by 89 ± 2% in the presence of 30 μM GYKI52466, a noncompetitive AMPA antagonist (n = 4; Fig. 7A). The response to 100 μM NMDA was blocked by 10 ± 4% in the presence of 30 μM NS3763 and by 79 ± 11% by 50 μM APV (n = 4; Fig. 7B). The inhibition of AMPA- and NMDA-induced currents by NS3763 was not significant (p > 0.05; paired t test).
responses. This is the first demonstration of a noncompetitive kainate antagonist. In studies measuring intracellular calcium, NS3763 was shown to inhibit domoate-induced responses in GLUK5 receptor-expressing HEK293 cells (IC50 = 1.6 μM). ATPA and the mixed AMPA/kainate receptor antagonist NS1209 (Nielsen et al., 1999; Varming et al., 2001) also inhibited domoate-induced calcium responses in GLUK5-expressing cells with IC50 values of 0.21 and 0.63 μM, respectively. The lack of agonist response observed for ATPA at concentrations up to 3 μM and its inhibitory action on domoate-induced responses is most likely due to rapid desensitization of homomeric GLUK5 receptors (Lerma et al., 1993, 2001). Similarly, no agonist response could be obtained for L-glutamate (data not shown).

The effects of the two antagonists NS3763 and NS1209 on domoate responses, however, were different. NS1209 shifted

Fig. 6. NS3763 interacts noncompetitively with homomeric GLUK5 receptors. A, comparison of the effects of NS3763 and NS1209 on domoate concentration-response curves in HEK293 cells expressing homomeric GLUK5 receptors. Concentration-response relationships for domoate (0.1–50 μM) under control conditions (○) and in the presence 3 μM NS3763 (●) or 3 μM NS1209 (■) were obtained by measurements of intracellular calcium. Values were normalized to maximum calcium increase (30 μM domoate; 100%) and represent the mean ± S.E.M. of three separate experiments. B, effect of NS3763 on L-glutamate-induced currents in HEK293 cells expressing homomeric GLUK5 receptors. Concentration-response relationships for L-glutamate (0.1–30 mM) under control conditions (○) and in the presence 10 μM NS3763 (●) were obtained in electrophysiological studies. The cell was voltage clamped at −60 mV in the whole cell configuration and exposed to a 100-ms pulse of L-glutamate. For each cell, the data were normalized to the current evoked by 30 mM L-glutamate. Points indicate the mean ± S.E.M. of the peak current amplitude values for three to nine cells separate experiments. Curve fitting was performed as described in "Data Analysis".

Fig. 7. NS3763 has no effect on AMPA- and NMDA-induced responses in cultured mouse cortical neurons. A, effect of 30 μM NS3763 on AMPA-induced current in a cortical neuron. The cell was voltage clamped at −60 mV in the whole cell configuration and exposed to 1-s pulses of 100 μM AMPA. After attainment of responses of a repeatable amplitude, 30 μM NS3763 was included in the solution as indicated by the black horizontal bar. Finally, the AMPA-induced responses were inhibited by 30 μM GYKI52466 ("GYKI"). The interval between stimulations was 30 s. B, effect of 30 μM NS3763 on NMDA-induced current in a cortical neuron. The cell was voltage clamped at −60 mV in the whole cell configuration and exposed to 1-s pulses of 100 μM NMDA. The experiments were performed using Mg2+-free solutions containing 10 μM glycine. After attainment of responses of a repeatable amplitude, 30 μM NS3763 was included in the solution as indicated by the black horizontal bar. The NMDA-induced current was blocked by 50 μM APV (APV). The interval between stimulations was 30 s. The recordings are representative of findings in three separate experiments.
dominate concentration-response curves to the right in a parallel manner indicative of competitive antagonism, whereas the effect of NS3763 was noncompetitive. The noncompetitive mechanism of action of NS3763 was further supported by the fact that NS3763, in contrast to ATPA and NS1209, did not inhibit \(^3\)HATPA binding to GLU\(_{K5}\) receptors.

In electrophysiological studies, NS3763 inhibited domoate-evoked currents in GLU\(_{K5}\)-expressing cells (IC\(_{50}\) = 1.3 \(\mu\)M), with a potency similar to that determined using intracellular calcium measurements. The inhibition of agonist-evoked currents by NS3763 was, as expected from its noncompetitive mechanism of action, independent of the concentration of agonist used. Currents evoked by 30 \(\mu\)M domoate were inhibited by 10 \(\mu\)M NS3763 to the same extend (58 \pm 5\%) as seen at 8 \(\mu\)M of the agonist (Fig. 4B). Despite the different functional endpoints (current versus cytosolic calcium) in electrophysiological and imaging studies, the potency and maximal inhibitory effect (60–70\%) of NS3763 against domoate-induced responses were very similar.

Currents evoked by the endogenous ligand L-glutamate were also inhibited in the low micromolar range by NS3763 with a maximal inhibitory effect varying from 30 to 50\% (Figs. 5 and 6B), and the noncompetitive mechanism of action of the compound was confirmed.

Furthermore, the data indicates that NS3763 is selective for homomeric GLU\(_{K5}\) over homomeric GLU\(_{K6}\) receptors at concentrations up to 30 \(\mu\)M. NS3763 is somewhat less potent than NS1209 (IC\(_{50}\) = 0.075 \(\mu\)M; Varming et al., 2001) in inhibiting GLU\(_{K5}\) receptor-mediated currents, but whereas NS1209 displays equipotent inhibition of AMPA-induced responses in cortical neurons (Nielsen et al., 1999) and kainate-evoked responses in cells expressing GLU\(_{K5}\) receptors (Varming et al., 2001), NS3763 did not show significant antagonistic properties on either native AMPA or native NMDA receptors at concentrations up to 30 \(\mu\)M. NS3763 thus shows selectivity for GLU\(_{K5}\) receptors and displays a selectivity profile different from NS1209. These data are the first describing a selective noncompetitive antagonist of GLU\(_{K5}\) receptors.

Several compounds interacting noncompetitively with AMPA receptors are known, and it is well described that AMPA receptor-mediated responses can be either diminished or enhanced by drugs acting at allosteric sites. The 2,3-benzodiazepines such as GYKI52466 and GYKI53655 are well characterized as negative allosteric modulators (Bleckman et al., 1996). The known positive allosteric modulators are benzothiadiazines (e.g., cyclothiazide) and benzoylpiperidines (e.g., aniracetam and CX516). The precise mechanism by which 2,3-benzodiazepines act as negative modulators is not known; they do not affect deactivation or desensitization and are not open channel blockers (Donevan and Rogawski, 1993; Rammers et al., 1998).

Rapid desensitization is one of the most characteristic features of kainate receptors, and modification of this feature is one of the means by which kainate receptor responses can be altered by pharmacological agents. The lectin concanavalin A has long been known to markedly reduce kainate receptor desensitization by an allosteric mechanism (Huettner, 1990), and thereby potentiates current responses of native and recombinant receptors (Huettner, 1990; Partin et al., 1993). The inhibition of L-glutamate-evoked currents by NS3763 is apparently not due to an increase in kainate receptor desensitization because the drug had no effect on the rate of L-glutamate current desensitization.

The binding site for NS3763 at homomeric GLU\(_{K5}\) receptors is not known. However, the compound seemed to distinguish between two isoforms of GLU\(_{K5}\), being 10-fold more potent in inhibiting L-glutamate-evoked currents in the GLU\(_{K5}\)-containing GLU\(_{K5K6}\) than in the GLU\(_{K5}\)-containing GLU\(_{K5\alpha}\) isoform, which has 15 additional amino acids present in the NH\(_2\) terminal (Sommer et al., 1992). The reason for these potency differences is currently being studied.

The potencies of NS3763 in inhibiting agonist-induced responses in assays measuring intracellular calcium and in electrophysiological studies were very similar, but it seems that only a partial inhibition of the responses can be obtained. The reason for this is not known, but it should be noted that the compound has limited water solubility (<0.05 mg/ml (<125 \(\mu\)M) at pH 7.4). However, it cannot be excluded that NS3763 is unable to inhibit the functional responses completely due to its noncompetitive interaction with the GLU\(_{K5}\) receptor.

ATPA has been shown to be not completely selective for homomeric GLU\(_{K5}\) receptors because it also activates heteromeric GLU\(_{K5}\) receptors containing GLU\(_{K2}\) or GLU\(_{K6}\) subunits and heteromers consisting of GLU\(_{K6K6}\) or GLU\(_{K2K6}\) receptor subunits (Paternain et al., 2000). LY382884 has recently been reported to also be effective against heteromeric assemblies of GLU\(_{K5}\) and GLU\(_{K6}\) subunits (Smolders et al., 2002), so there is still a need for new pharmacological tools. The activity of NS3763 on heteromeric kainate receptors and the potential analgesic effect of the drug in animal models of pain are currently being studied.

Acknowledgments

We thank Anne B. Fisher, Kirsten V. Haugegaard, Kristina Christensen, and Aino Munch (Departments of Biochemical Screening and Receptor Biochemistry) for skillful technical assistance.

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