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In vitro characterization of NS3763, a non-competitive antagonist of GLU_{K5} receptors

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Non-standard abbreviations:

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

ATPA: α-amino-3-hydroxy-5- tertbutylisoxazole-4-propionic acid

APV: D-2-amino-5-phosphovaleric acid

HEK: human embryonic kidney

NMDA: *N*-methyl-D-aspartate

NBQX : 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline

NS1209: 8-methyl-5-(4-(*N*,*N*-dimethylsulfamoyl)phenyl)-6,7,8,9,-tetrahydro-1*H*-pyrrolo[3,2-*h*]-isoquinoline-2,3-dione-3-*O*-(4-hydroxybutyric acid-2-yl)oxime

NS3763: 5-carboxyl-2,4-di-benzamido-benzoic acid

GYKI52466: 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine

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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 NS3763 [5-carboxyl-2,4-di-benzamido-benzoic acid] are described. In functional assays in HEK293 cells expressing homomeric GLU_{K5} or GLU_{K6} receptors, NS3763 is shown to display selectivity for inhibition of domoate-induced increase in intracellular calcium mediated through the GLU_{K5} subtype (IC_{50} = 1.6 μM) of kainate receptors compared with the GLU_{K6} subtype (IC_{50} > 30 μM). NS3763 inhibits the GLU_{K5}-mediated response in a non-competitive manner and does not inhibit [^{3}H]α-amino-3-hydroxy-5-tertbutylisoxazole-4-propionic acid (ATPA)-binding to GLU_{K5} receptors. Furthermore, NS3763 selectively inhibits L-glutamate and domoate-evoked currents through GLU_{K5} receptors in HEK293 cells and does not significantly inhibit AMPA- or NMDA-induced currents in cultured mouse cortical neurons at 30 μM. This is the first report on a selective and non-competitive GLU_{K5} 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 (Schoepp et al., 1999; Collingridge and Lester, 1989). 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), non-competitive antagonists such as MK-801 (Wong et al., 1986), and the competitive AMPA/kainate receptor antagonists CNQX 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 CNQX 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 e al., 2000). The 2,3 benzodiazepine class of compounds such as GYKI52466 and GYKI53655 are non-competitive antagonists showing a relatively high degree of selectivity for AMPA receptors, and these compounds have successfully been used to isolate kainate receptors (Paternain et al., 1995). Through use of these antagonists and cloned kainate receptors it became clear that ATPA (Lauridsen et al., 1985) is a GLU_{K5}-preferring agonist potently activating homomeric

GLU_{K5} receptors and native DRG kainate receptors, but shows only weak activity at AMPA receptors, and no activity at GLU_{K6} homomers (Clarke et al., 1997; Wilding and Huettner, 2001).

Kainate receptors, as AMPA and NMDA receptors, are thought to be tetramers (reviewed by Madden, 2002) formed by homo- or heteromeric association of the kainate receptor subunits GLU_{K1}, GLU_{K2}, GLU_{K5}, GLU_{K6} and GLU_{K7}.

The first compound to be described as a competitive kainate antagonist was NS102, based on its ability to block low affinity [^{3}H]kainate binding (Johansen et al., 1993). However, functional assays yielded contradictory results since NS102 acts at GLU_{K5} and GLU_{K6} receptors and shows selectivity in some systems (Verdoorn et al., 1994; Wilding and Huettner, 1996) but poor selectivity in others (Paternain et al., 1996). Recently, LY382884 has been reported to bind specifically to GLU_{K5} but not to GLU_{K6}, GLU_{K7}, GLU_{K2} or AMPA receptor subunits (Bortolotto et al., 1999). In functional tests, LY382884 inhibits kainate-evoked currents in DRG neurons and is approximately 100 times less potent on AMPA- and NMDA-evoked responses in hippocampal neurons (Bleakman 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_{K5} 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_{K5} subtype) in pain transmission (Li et al., 1999; Procter et al., 1998), and Simmons et al. (1998) demonstrated that

the selective GLU_{K5} antagonist LY382884 was active in an animal model of persistent pain. More recently, GLU_{K5} receptors have been linked with migraine headache, and competitive GLU_{K5} antagonists have been reported to be active in animal models of acute migraine (Filla et al., 2002). Within the hippocampus, GLU_{K5} containing receptors are involved in frequency facilitation and induction of long-term potentiation, and in excitatory drives of inhibitory CA1 interneurons. In addition, GLU_{K5} antagonists have recently been reported to have anticonvulsant activity in animal models (Smolders et al., 2002).

Existing GLU_{K5} 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_{K5} 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_{K5} 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_{K5} receptors equipotently (Varming et al., 2001), NS3763 did not show significant antagonistic properties on either native AMPA- or native NMDA receptors.

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Materials and Methods

Materials and drugs

[^{3}H]Kainic acid (58 Ci/mmol) and [^{3}H]ATPA (16 Ci/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA) and Amersham Pharmacia Biotech UK Limited (Little Chalfont, England), respectively. Fluo-4-AM (cell permeant acetoxymethyl ester of the Ca^{2+} indicator Fluo-4) and domoic acid was purchased from Molecular Probes Europe BV (Leiden, The Netherlands) and Tocris (Bristol, UK), respectively. GYKI52466 was purchased from Research Biochemicals International (Natrick, MA). NS1209 (previously known as SPD502; Nielsen et al., 1999; Varming et al., 2001) was synthesized at NeuroSearch. NS3763 was identified in a compound library purchased from Chemical Diversity Labs (San Diego, CA). Cell culture media were obtained from Life Technologies (Roskilde, Denmark). All other chemicals were purchased from regular commercial sources and were of the purest grade available.

Cell Cultures

GLU_{K5} and GLU_{K6} expressing cell lines. HEK293 cell lines stably expressing homomeric human $GLU_{K5Q\text{-}1a}$ and GLU_{K6IYQ} 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^2), in a humidified atmosphere of 5% CO_2 /95% air, at 37°C. Cells were cultured to 80-90% confluency before plating. The cells were rinsed with PBS and detached from the culture flask by trypsin (0.1% (w/v)) digestion, 5 min, 37°C. After addition of growth media cells were resuspended by trituration and seeded at a density of

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0.05-0.1 million cells/well in black-walled, clear bottom, 96-well plates pre-treated 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 $GLU_{K5Q\text{-}2b}$ splice variant, HEK293 cells were transiently transfected using the LipofectAMINE PLUS™ (Life Technologies, Roskilde, Denmark) transfection kit as described by the manufacturer. Cells were used the day after transfection.

For electrophysiological studies, cells were seeded on the day of experiment. Glass cover slips (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 & B, Ry, Denmark) at day 15 to 16 of gestation as described previously (Drejer et al., 1987). Briefly, dissected tissue was chopped in 0.4 mm cubes and triturated with 0.2% (w/v) trypsin and DNAse (40 µg/ml), 10 min, 37°C. The cells were suspended (1.5 million/ml) in a slightly modified DMEM (with 23 mM glucose), which contained 10% (v/v) horse serum, 7 µM *p*-amino benzoate, 0.5 mM L-glutamine, 100 mU/l insulin, 0.1 % (w/v) penicillin and 19.1 mM KCl. The cell suspension was subsequently inoculated into poly-D-lysine-coated (0.01 % w/v) 35 mm Petri dishes (2 ml/dish). Glass cover slips (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 8-13 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 buffer (10 mM HEPES, 140 mM Choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$; pH 7.4) to remove extracellular dye. The reason for using a relatively high $CaCl_2$ 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 (FLIPR, Molecular Devices, Sunnyvale, CA). Cells were preincubated for 1.5 min with test compound (50 µl) 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 DMSO, final concentration of solvent never exceeding 0.5%.

Electrophysiological Studies

The electrophysiological measurements were performed in voltage clamp using conventional whole cell patch clamp techniques (Hamill et al., 1981), and all data were obtained with an EPC-9 amplifier (HEKA-electronics, Lambrect, Germany) run by a Macintosh G3 computer. Experimental conditions and data acquisition were set and obtained using 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,

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Germany), and the final pipette resistance was approximately 2 MΩ when filled with internal solution and submerged in the external solution used in the experiments. Cover slips with cultured cells were transferred to a perfusion chamber mounted on the stage of an inverted microscope supplied with Nomarski optics, and cells were continuously superfused with external solution at a rate of 2.5 ml/min. Compounds were dissolved in external solution and applied to the patched cell through double-barreled application pipettes. The application pipettes were fabricated from theta glass tubes (1.5 mm outer diameter, World Precision Instruments, Sarasota, FL). The application pipettes were mounted on a piezoelectric device (PZS-100HS, Burleigh Instruments, Quebec, Canada) connected to a piezo-driver (PZ-150M, Burleigh Instruments, Quebec, Canada) driven by TTL pulses from the EPC-9 amplifier. One minute after the onset of the gravity flow a PULSE protocol was initiated and the current was recorded 3 times separated by 30 s waiting periods. For transfected HEK293 cells the duration of the recording periods was 150 ms during which the application pipette was switched to the test solution for 100 ms. For the cortical neurons the duration of the recording period was 1.5 s and the application pipette was moved for 1 s.

GLU_{K5} or GLU_{K6} expressing cells. For recordings from GLU_{K5} or GLU_{K6} expressing cells, the pipette solution contained: 120 mM KCl, 31 mM KOH, 10 mM EGTA, 1.8 mM $MgCl_2$, 10 mM HEPES (pH 7.2). The external solution was composed of: 140 mM NaCl, 5 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4). After giga-seal formation (1-2 GΩ) and establishment of the whole cell configuration, the cells were held at a holding potential of -60 mV. For each cell a control response induced by 3 mM L-glutamate (GLU_{K5}) or 0.5 mM L-glutamate

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(GLU_{K6}) was recorded followed by recordings of the agonist-induced responses in the presence of increasing concentrations of NS3763. Because of reversibility of the effect of NS3763 and low, constant series resistance (< 5 MΩ), several concentrations could be tested on each cell. Series resistance was compensated by 80%.

Native AMPA- and NMDA receptors in cortical neurons. For recordings in native AMPA and NMDA receptors in cortical neurons, the pipette solution contained: 135 mM CsCl, 11 mM EGTA, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 4 mM ATP, 10 mM HEPES (pH 7.3). The external solution was composed of: 140 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 25 mM D-glucose, 0.5 µM tetrodotoxin, 10 mM HEPES (pH 7.4). After giga-seal formation (1-3 GΩ) and establishment of the whole cell configuration, the cells were held at a holding potential of -60 mV. For the measurement of AMPA responses, 4 mM $MgCl_2$ was added to the external solution, whereas 10 µM glycine was added for the measurement of NMDA responses. Agonists (100 µM AMPA or 100 µM NMDA) were dissolved in the extracellular solution and for each cell a control agonist response was recorded followed by recordings of the agonist-induced response in the presence of 30 µM NS3763.

For all electrophysiological measurements the current amplitudes were measured at the peak of the responses, and the effect of NS3763 was calculated as the amplitude during compound application divided by the amplitude of the agonist-induced current evoked prior to the application of compound. All experiments were performed at room temperature (20-22°C).

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 -80ºC 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 (27,000*g*). The final pellets were resuspended in Tris-HCl buffer and used for binding experiments. All procedures were performed at 0-4ºC. 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 [^{3}H]ATPA at 46-84 µg protein/assay, and GLU_{K6} receptors were labeled with 5 nM [^{3}H]kainate at 22-27 µg protein/assay. The samples were incubated in a final volume of 550 µl for 60 min at 2ºC. 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 IC_{50} values and Hill coefficients were determined based on the equation $Y = Bottom + (Top - Bottom)/(1 + (X/IC_{50})^n)$, where Y is binding/calcium increase/current in percent of total binding/calcium increase/current; X the concentration of test compound; and n is the Hill coefficient. The EC_{50} values for domoate in stimulation of intracellular calcium in HEK293 cells were determined by using the equation $Y = 100 \cdot X^n/(EC_{50}^n + X^n)$.

Estimates of IC_{50} and EC_{50} values were calculated with the nonlinear curve-fitting program GraphPad Prism™ (version 2.0; GraphPad Software, USA). K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation: K_i =

$IC_{50}/(1+(L/K_d))$. The K_d values were as follows: 2.9 nM for [^{3}H]ATPA at GLU_{K5} receptors and 5.7 nM for [^{3}H]kainate at GLU_{K6} receptors, respectively. All results are given as means ± S.E.M.

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Results

Calcium measurements.

Domoate concentration-dependently increased intracellular calcium in GLU_{K5} and GLU_{K6} expressing cells with EC_{50} values of 1.5 ± 0.2 μM 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 IC_{50} 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 an IC_{50} value of 0.21 ± 0.03 μM. No inhibition of GLU_{K6}-mediated responses was seen at concentrations up to 300 μM (n = 3; Table 1).

The competitive AMPA/kainate antagonist NS1209 potently inhibited domoate-induced calcium increase mediated by GLU_{K5} receptors with an IC_{50} value of 0.63 ± 0.09 μM (n=4). GLU_{K6} mediated responses were inhibited with an IC_{50} value of 65 ± 4 μM by NS1209 (n = 4; Table 1; Fig. 2).

Electrophysiology on GLU_{K5} and GLU_{K6} receptors

Both L-glutamate and domoate evoked concentration-dependent inward currents in cells stably expressing GLU_{K5} and GLU_{K6} receptors. The estimated EC_{50} values for L-glutamate were 3.6 mM and 1.0 mM for GLU_{K5} and GLU_{K6} (data not shown), respectively, and for the inhibition studies 3 mM and 0.5 mM L-glutamate were chosen as agonist concentration for GLU_{K5} and GLU_{K6}, respectively. The peak inward current responses to 3 mM L-glutamate in GLU_{K5} expressing cells ranged

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from 122 to 1147 pA (n=31). The L-glutamate-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. 3A 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, τ_{fast}, 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, τ_{slow}, 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).

Domoate was a more potent agonist than L-glutamate in evoking currents in GLU_{K5} and GLU_{K6} expressing cells, and the estimated EC_{50} values were 7.5 μM and 0.4 μM, respectively (data not shown). The peak inward current responses obtained at the estimated EC_{50} 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

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IC_{50} 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 $GLU_{K5\text{-}1a}$ isoform, which contains 15 extra amino acids in the NH_2-terminal domain and has a shorter COOH-terminal domain than the $GLU_{K5\text{-}2b}$ isoform reported for rat (Sommer et al., 1992) and for human by Korczak et al. (1995). In order to investigate whether NS3763 had different modulatory effect at $GLU_{K5\text{-}1a}$ and $GLU_{K5\text{-}2b}$ receptors, HEK293 cells transiently expressing the $GLU_{K5\text{-}2b}$ isoform were used. In these cells an EC_{50} 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 $GLU_{K5\text{-}2b}$ (IC_{50} = 0.38 μM; n=3) than the $GLU_{K5\text{-}1a}$ isoform (IC_{50} = 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. 6, the maximal increase in intracellular calcium occurred at 30 μM domoate, and 3 μM NS3763 caused a reduction of the maximal response. However, the EC_{50} value for domoate in the presence of NS3763 (1.7 ± 0.2 μM; n=3) was similar to the control 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

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change in the maximal response (Fig. 6A); the EC_{50} 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 non-competitive mechanism.

The non-competitive action of NS3763 was supported by radioligand binding studies, since NS3763 did not inhibit [^{3}H]ATPA binding to GLU_{K5} receptors (Table1). In contrast, NS1209 inhibited ligand binding to GLU_{K5} and GLU_{K6} (Varming et al., 2001), whereas ATPA only displaced binding to GLU_{K5}.

The non-competitive mechanism of action for NS3763 was confirmed in electrophysiological studies (Fig. 6B); the EC_{50} 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).

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 non-competitive 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).

Discussion

The present study shows that NS3763 selectively and non-competitively inhibits homomeric GLU_{K5} receptor mediated responses. This is the first demonstration of a non-competitive kainate antagonist.

In studies measuring intracellular calcium, NS3763 was shown to inhibit domoate-induced responses in GLU_{K5} receptor-expressing HEK293 cells (IC_{50} value of 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 GLU_{K5} expressing cells with IC_{50} 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 GLU_{K5} receptors (Lerma et al., 1993; Lerma et al., 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 domoate concentration response curves to the right in a parallel fashion indicative of competitive antagonism, whereas the effect of NS3763 was non-competitive. The non-competitive mechanism of action of NS3763 was further supported by the fact that NS3763, in contrast to ATPA and NS1209, did not inhibit [^{3}H]ATPA binding to GLU_{K5} receptors.

In electrophysiological studies, NS3763 inhibited domoate-evoked currents in GLU_{K5} expressing cells (IC_{50} value of 1.3 μM), with a potency similar to that determined using intracellular calcium measurements. The inhibition of agonist-evoked currents by NS3763 was, as expected from its non-competitive mechanism of action,

independent of the concentration of agonist used. Currents evoked by 30 μM domoate were inhibited by 10 μM NS3763 to the same extend (58 ± 5%) as seen at 8 μM 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 micro molar range by NS3763 with a maximal inhibitory effect varying from 30 to 50% (Fig. 5 and 6B), and the non-competitive 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 μM. NS3763 is somewhat less potent than NS1209 (IC_{50} = 0.075 μM; 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 μM. NS3763 thus shows selectivity for GLU_{K5} receptors and displays a selectivity profile different from NS1209. These data are the first describing a selective non-competitive antagonist of GLU_{K5} receptors.

Several compounds interacting non-competitively 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 (Bleakman et al., 1996). The known positive allosteric modulators are benzothiadiazines (e.g. cyclothiazide) and benzoylpiperidines (e.g. aniracetam, 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; Rammes 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 since 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 appeared to distinguish between two isoforms of GLU_{K5}, being 10-fold more potent in inhibiting L-glutamate-evoked currents in the $GLU_{K5\text{-}2b}$ than in the $GLU_{K5\text{-}1a}$ 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 appears 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 μM) at pH 7.4). However, it cannot be excluded that NS3763 is unable to inhibit the functional responses completely due to its non-competitive interaction with the GLU_{K5} receptor.

ATPA has been shown to be not completely selective for homomeric GLU_{K5} receptors since it also activates heteromeric GLU_{K5} receptors containing GLU_{K2} or GLU_{K6} subunits and heteromers consisting of $GLU_{K6/K2}$ 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.

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Footnote

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Figure 1

Chemical structure of NS3763.

Figure 2

Inhibition of domoate-induced increase in intracellular calcium in HEK293 cells expressing homomeric GLU_{K5} or GLU_{K6} receptors by NS3763 (●, ○) and NS1209 (■, □). Closed symbols represent GLU_{K5}, open symbols GLU_{K6}. Each point represents the mean ± S.E.M. of three separate experiments. Curve fitting was performed as described in Data Analysis. The IC_{50} values are given in Table 1.

Figure 3

NS3763 selectively blocks L-glutamate-evoked currents in HEK293 cells expressing homomeric GLU_{K5} receptors. A, inhibition of L-glutamate-evoked current in a GLU_{K5} 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 GLU_{K6} 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.

Figure 4

NS3763 blocks domoate-evoked currents in HEK293 cells expressing homomeric GLU_{K5} 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.

Figure 5

Effect of NS3763 on L-glutamate-induced currents in HEK293 cells expressing the homomeric GLU_{K5} receptor isoform GLU_{K5-1a} (●) and GLU_{K5-2b} (▲). The cells were voltage clamped at -60 mV. The current amplitudes were expressed as percentage of the current induced by 3 mM L-glutamate. Each data point represents the mean ± S.E.M. of three to four separate experiments. Curve fitting was performed as described in Data Analysis.

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Figure 6

NS3763 interacts non-competitively with homomeric GLU_{K5} receptors.

A, comparison of the effects of NS3763 and NS1209 on domoate concentration-response curves in HEK293 cells expressing homomeric GLU_{K5} 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 GLU_{K5} 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.

Figure 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 Mg^{2+}-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.

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Table 1

Selectivity profile of GLU_{K5} ligands

(a) Functional selectivity at recombinant homomeric GLU_{K5} and homomeric GLU_{K6} receptors expressed in HEK293 cells, determined by measurements of intracellular calcium. IC_{50} values were calculated from percent inhibition of domoate-induced calcium influx.

(b) Selectivity in ligand binding assays to homomeric GLU_{K5} and homomeric GLU_{K6} receptors expressed in HEK293 cells. K_i values for NS1209 are from Varming et al., 2001.

Functional and receptor binding assays were performed as described under *Materials and Methods.* Results are means ± S.E.M from at least three separate determinations.

Compound	**GLU_{K5}**	**GLU_{K6}**	**[^{3}H]ATPA**	**[^{3}H]Kainate**
	(a) IC_{50} (μM)		(b) K_i (μM)	
ATPA	0.21 ± 0.03	>300	0.0094 ± 0.0010	>100
NS1209	0.63 ± 0.09	65 ± 4	0.62 ± 0.11	13 ± 4.0
NS3763	1.6 ± 0.2	>30	>30	>30

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Fig. 1

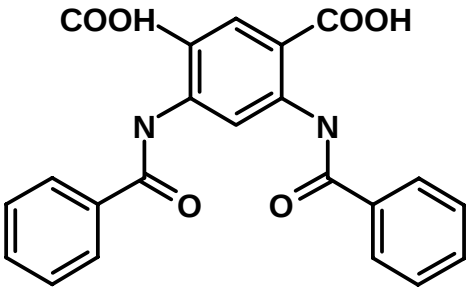

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Fig. 2

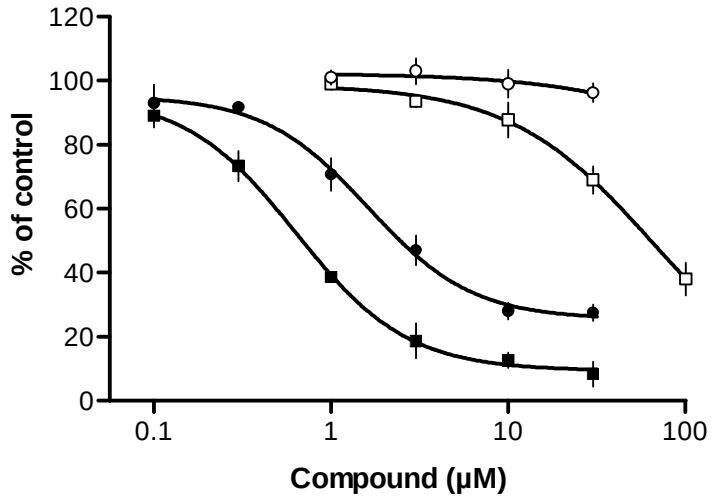

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Fig. 3

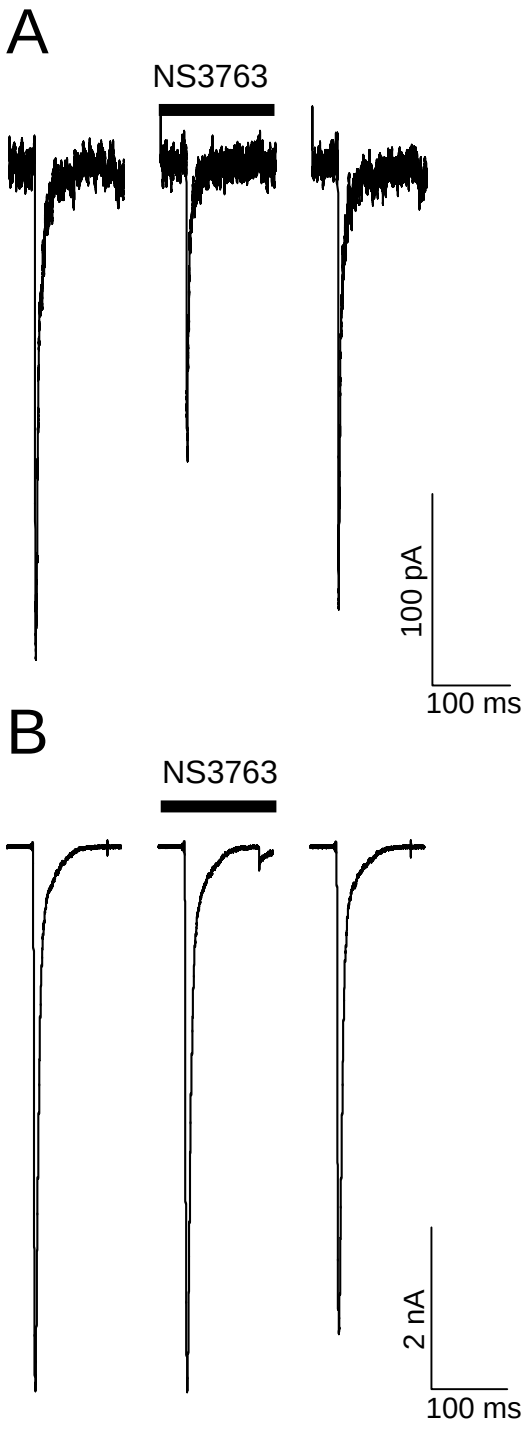

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Fig. 4

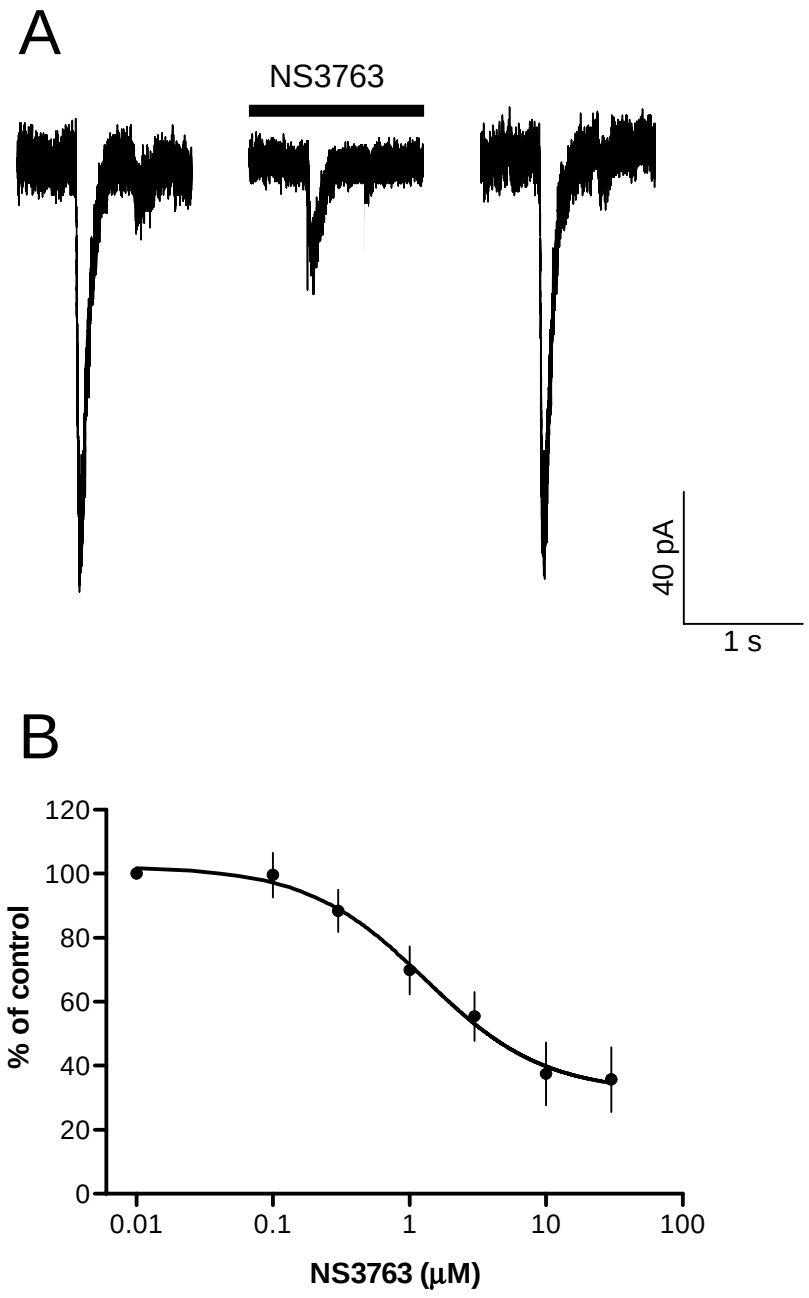

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Fig. 5

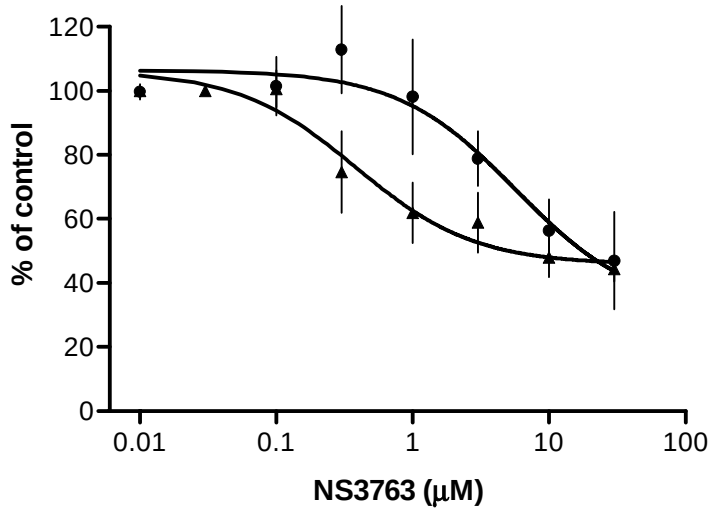

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Fig. 6

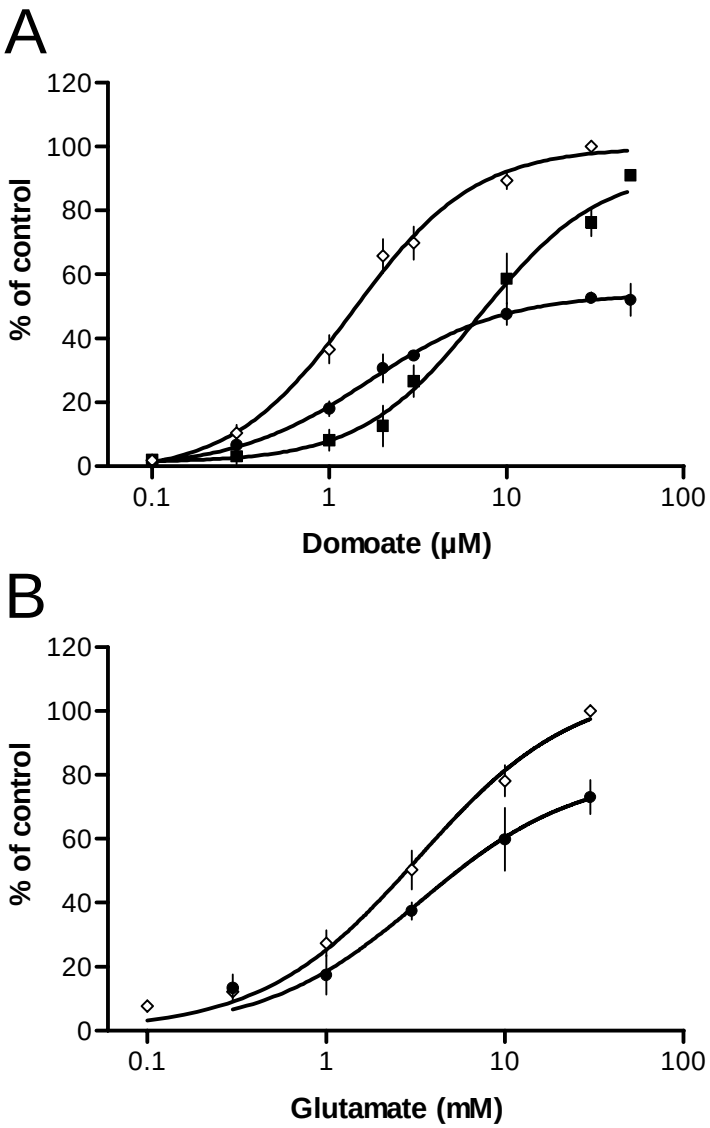

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Fig. 7