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Comparative pharmacology of the human NMDA-receptor subtypes R1-2A, R1-2B, R1-2C and R1-2D using an inducible expression system

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ABSTRACT

Pharmacological characterization of *N*-methyl-D-aspartate (NMDA) receptors has been hampered by the difficulty to outwit cytotoxicity after functional expression in recombinant systems. In this study a muristerone-inducible expression system for the NNMDA-R1 subunit was used. This was combined with constitutive expression of NMDA-R2A, 2B, 2C and 2D in different cell clones. After establishment of the cell lines, quantitative RT-PCR demonstrated the inducibility of the NNMDA-R1 subunit, and verified the expression of the NMDA-R2 subunits in the different cell clones. Functional responses were characterized using calcium influx through the ion channel as a robust assay system. Stimulation of the NMDA-receptor subtypes in the different cell lines led to calcium transients which were rising gradually, peaked after 30–160 s and declined thereafter very slowly. The expression of the four different NMDA-receptor subtypes in the same cellular background allowed a direct pharmacological comparison of the different receptors. Glutamate showed the highest potency at the NMDA-R1-2D. NMDA displayed at all subtypes a lower potency compared to glutamate and was a partial agonist except at the NMDA-R1-2D. 20 antagonists were tested in this study and the pharmacological characterization of the inhibition of glutamate-evoked elevation of intracellular free Ca^{2+} revealed a distinct rank order of antagonist potency for each receptor subtype. These data illustrate that assessment of calcium transients upon receptor stimulation in the same cellular background is a powerful tool to compare the functional effects of compounds acting at the different NMDA-R2 receptors.

1. Introduction

Glutamatergic synapses mediate most of the excitatory neurotransmission in mammalian brains. Glutamate released from presynaptic terminals activates several types of ligand gated ion channels, including *N*-methyl-D-aspartate (NMDA) receptors. To date seven genes have been identified that encode NMDA-receptor subunits, namely one NMDA-R1 subunit, four NMDA-R2 subunits (2A-D), and two NR3 subunits (NR3A and B) (Dingledine et al., 1999). Eight splice variants exist for the NMDA-R1 subunit (Waxman and Lynch, 2005), that differentially influence NMDA-receptor mediated gene expression (Bradley et al., 2006). NMDA-receptor subunits have specific regional and temporal expression patterns. Adult rodent cortex expresses mainly NMDA-R1, NMDA-R2A, and NMDA-R2B subunits. NMDA-R2C expression is restricted to the granule cell layer I in the cerebellum, hippocampal interneurons, glial cells in cortex, the corpus callosum and the pineal gland (Monyer et al., 1994). NMDA-R2D expression is even more restricted to midline thalamic nuclei, hypothalamus, superior colliculus and the substantia nigra.

Functional NMDA receptors form hetero-tetrameric complexes of two NR1 subunits and two NMDA-R2 subunits (Bigge, 1999). The NMDA-NR1 subunit binds glycine and the NMDA-R2 subunits harbor a glutamate binding site. The glycine site must be occupied before the glutamate site, however it seems that the glycine site is occupied most of the time (Johnson and Ascher, 1987). In addition, magnesium acts as a voltage-dependent antagonist at resting membrane potential while membrane depolarization relieves the block. This magnesium block is much weaker for the NMDA-R1-2C and NMDA-R1-2D receptors (Ishii et al., 1993) and the conductances are smaller compared to the other NMDA-receptor subunits (Farrant et al., 1994). Activation of NMDA-receptor requires binding of both glutamate and glycine, as well as a simultaneous depolarization of the plasma membrane. Signaling by NMDA receptors involves the influx of extracellular calcium and is crucial for inducing synaptic plasticity processes like LTD or LTP. NMDA receptors activate intracellular signaling pathways like MAPK and the transcription factor Cyclic-AMP Response Element Binding protein (CREB) (Papadia et al., 2005; Ivanov et al., 2006). NMDA receptors are involved in the

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pathophysiology of a range of diseases like brain injury, epilepsy, Alzheimer's disease and schizophrenia.

The recombinant expression of NMDA receptor in a defined cellular background allows the efficient characterization of the subtype selectivity and activity of agonists, antagonists or allosteric modulators. Several groups have reported on the characterization of human, mouse and rat NMDA receptors in recombinant systems (Priestley et al., 1995; Grimwood et al., 1996; Varney et al., 1996; Uchino et al., 2001; Steinmetz et al., 2002; Nagy et al., 2003; Kurko et al., 2005; Hansen et al., 2008). However, none of these studies has compared more than two NMDA-receptor ligands at all 4 NMDA-R2 receptor subtypes. In this report an inducible expression system is used for the NR1 subunit, in combination with stable expression of either the NMDA-R2A, 2B, 2C or the 2D subunit. Using influx of extracellular Ca^{2+} as read-out, the pharmacological characteristics of the distinct NMDA-receptor subtypes in a single cellular background were established with the aid of pharmacological tool compounds.

2. Materials and methods

2.1. DNA constructs

The NMDA-receptor expressing cell lines were designed such that the NR1 subunit is inducible upon muristerone induction, whereas the different NR2 subunits are expressed constitutively. hNMDA-R1 cDNA was cloned into the NheI/NotI site of the pIND/Hygro vector (Invitrogen, Groningen, The Netherlands). The hNMDA-R2A cDNA was inserted into the EcoRI/NotI site and the hNMDA-R2D cDNA into the EcoRV/EcoRI site of the pCDNA3.1neo(−) vector (Invitrogen, Groningen, The Netherlands). The hNMDA-R2C cDNA was cloned into the BamHI/EcoRI site of the pCDNA3.1neo(+) vector (Invitrogen, Groningen, The Netherlands), whereas the hNMDA-R2B cDNA was cloned into the EcoRI/EcoRV site of the pCMV-T7 vector (Invitrogen, Groningen, The Netherlands).

2.2. Cell culturing and generation of stable cell lines

293EcR cells (Invitrogen, Groningen, The Netherlands) were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM, Seromed, Biochrom, Berlin, Germany; 3.7 g/l $NaHCO_3$; 1.0 g/l sucrose; with stable glutamine) and Ham's F-12 Nutrient Mixture (Seromed; 1.176 g/l $NaHCO_3$; with stable glutamine) supplemented with 10% (v/v) dialyzed fetal bovine serum (FBS; Gibco BRL) and 0.2 mg/ml zeocine at 37 °C, 5% CO_2 and 95% relative humidity. For passaging, the cells were detached from the cell culture flask by washing with phosphate-buffered saline (PBS) and brief incubation with trypsine (0.5 mg/ml)/EDTA (0.2 mg/ml) (Gibco BRL). The cells were passaged every 3 days.

293EcR cells were transfected using the SuperFect kit (Quiagen, Hilden, Germany) according to the manufacturer's instruction with both the hNMDA-R1 and the hNMDA-R2 cDNA's simultaneously. For each combination a separate transfection was carried out. In case of the hNMDA-R2B receptor the plasmid was transfected together with 1/10 of the pCDNA3.1 vector since the hNMDA-R2B was cloned in a plasmid which did not contain a neomycin resistance gene. Neomycin and hygromycin B selection was initiated 48 h after transfection with 0.2 mg/ml neomycin and 0.2 mg/ml hygromycin B. After individual colonies were visible about 200 cell clones were picked using cloning cylinders and expanded for further analysis.

2.3. Measurement of intracellular Ca^{2+}

1.5×10^4 cells per well were seeded 48 h prior to the experiment on black poly-L-lysine coated 96-well plates (Costar, New York, USA) in normal growth medium and incubated at 37 °C in a humidified atmosphere (5% CO_2/95% air). 16 h before the experiment medium was changed to 1% BSA in HEPES-buffered salt solution (HBSS, in mM: NaCl 130, KCl 5.4, $MgSO_4$ 4, NaH_2PO_4 0.9, glucose 25, $CaCl_2$ 1.8 mM, ketamine 0.1, muristerone 0.001, Hepes 20, pH 7.4). On the day of the experiment medium was replaced with 100 µl HBSS/1%BSA containing 2 µM Fluo-4, (Molecular Probes) in the presence of 0.1 mM probenecid (Sigma). The cells were incubated at 37 °C in a humidified atmosphere (5% CO_2/95% air) for 30 min. Plates were flicked to remove excess of Fluo-4, washed twice with HBSS/1%BSA and refilled with 100 µl of Mg-free HBSS containing 25 µM glycine, 100 µM probenecid, 5 mM $CaCl_2$, 20 mM HEPES and antagonists when appropriate. The incubation in the presence of the antagonist lasted 5 min. Plates were then placed in the cell plate stage of the FLIPR (Molecular Devices, Sunnyvale, CA, USA). A baseline consisting in 5 measurements of 0.4 s each (laser: excitation 488 nm at 1 W, CCD camera opening of 0.4 s) was recorded. Agonists (50 µl) were added to the cells using the FLIPR 96-tip pipettor simultaneously to fluorescence recordings for 3 min. To assess the activity of antagonists 10 µM glutamate was used for NMDA-R1-2A and -2B, 30 µM glutamate for the NMDA-R1-2C and 3 µM for the NMDA-R1-2D. Calcium kinetic data were normalized to the maximal fitted response induced by glutamate which was included in each experiment. Four parameter Hill equations were fitted to the concentration–response data (GraphPad Prism 3.0). Values of E_{max} (maximal effect), EC_{50} (concentration producing half the maximal effect) and IC_{50} were derived from this fit. Each graph is a representative plot from at least three determinations. All measurements were performed in triplicates and error bars in the graphs are standard deviation (S.D.).

2.4. Relative quantification of NMDA-receptor transcripts

Total RNA was isolated from cell lines using the S.N.A.P.™ Total RNA Isolation Kit (Invitrogen) according to the manufacturer's instructions, except that the DNAse I treatment was done twice to remove all traces of genomic DNA. The amount of total RNA was determined by staining the RNA with RiboGreen (Molecular Probes). The concentration of the RNA stained with RiboGreen was determined using a fluorescence microplate reader (Fluorskan II; BioConcept). For the reverse transcription, 2 µg of total RNA was transcribed into cDNA in 20 µl of buffer containing 1.5 µg random hexamer primers, 10 mM DTT, 0.4 mM dNTPs, 50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$. The reaction mix without the reverse transcriptase was incubated at 95° C for 10 min, followed by 70° C for 10 min and then at 37° C for 5 min. 200 U of M-MLV reverse transcriptase (GIBCO/BRL) were added and the reaction was incubated at 37° C for 60 min. The reaction was stopped by heating up to 95° C for 10 min. After completion of the reaction, the cDNA was diluted with H_2O to a final concentration of 10 ng/µl. For the TaqMan assays the following thermal cycling profile was used: 50° C/2 min, 95° C/10 min followed by 40 cycles of 95° C/15 s and 60° C/1 min. The forward and reverse primers and the TaqMan probe were designed by using the Primer Express software Version 1.0 (PE Applied Biosystems). Specificity of the primer and probes was checked by running BLAST searches against the GeneBank/EMBL databases. The forward and reverse primers as well as the TaqMan probe were synthesized by Microsynth AG (Balgach, Switzerland). The probes were labelled at the 5′ end with FAM and at the 3′ end with TAMRA (see Table 1). For the TaqMan "Real Time PCR" approach, the TaqMan Universal PCR master mix (Perkin-Elmer) was used. The mix is supplied as a 2× concentrated solution containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, ROX and the buffer components. For all TaqMan assays, the primers and the probe were used at concentrations of 300 nM and 175 nM, respectively. The expression levels of the NMDA-receptor subunits were analyzed by determining the C_T (threshold cycle; cycle at which a statistically significant increase in fluorescence is first detected) values.

2.5. Compounds

The substances were obtained from Tocris (Anawa Trading SA, Wangen, Switzerland) or were synthesized at Novartis Pharma AG,