Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neurochemistry International 46 (2005) 369–379
NEUROCHEMISTRY International
www.elsevier.com/locate/neuint

Inducible expression and pharmacological characterization of recombinant rat NR1a/NR2A NMDA receptors

Dalma Kurkó *, Péter Dezső, András Boros, Sándor Kolok, László Fodor, József Nagy, Zsolt Szombathelyi

Pharmacological and Drug Safety Research, Gedeon Richter Ltd., Budapest, Hungary

Received 11 October 2004; received in revised form 7 December 2004; accepted 9 December 2004
Available online 2 February 2005

Abstract

In this study, we have established a non-neuronal cell line stably and inducibly expressing recombinant NMDA receptors (NRs) composed of rat NR1a/NR2A subunits.

EcR-293 cells were transfected with rat NR1a and NR2A cDNAs using the inducible mammalian expression vector pIND. Cell colonies resistant for the selecting agents were picked and tested for NR2A mRNA as well as protein expression using quantitative RT-PCR and flow cytometry based immunocytochemistry. Clonal cells expressing functional NMDA receptors were identified by measuring NMDA-evoked ion currents, and NMDA-induced increase in cytosolic free calcium concentration in whole-cell patch-clamp and fluorimetric calcium measurements, respectively. One clone named D5/H3, which exhibited the highest response to NMDA, was chosen to examine inducibility of the expression and for pharmacological profiling of recombinant NR1a/NR2A NMDA receptors.

To check inducibility, NR2A subunit expression in D5/H3 cells treated with the inducing agent muristerone A (MuA) was compared with that in non-induced cells. Both NR2A mRNA and protein expression was several folds higher in cells treated with the inducing agent. As part of the pharmacological characterization, we examined the activation of the expressed NR1a/NR2A receptors as a function of increasing concentration of NMDA. NMDA-evoked concentration-dependent increases in cytosolic [Ca^{2+}] with an EC_{50} value of 41 ± 1 μM. In addition, whereas the NMDA response was concentration-dependently inhibited by the channel blocker MK-801 ($IC_{50} = 58 \pm 6$ nM), NR2B subunit selective NMDA receptor antagonists were ineffective. Thus, this cell line, which stably and inducibly expresses recombinant NR1a/NR2A NMDA receptors, can be a useful tool for testing NMDA receptor antagonists and studying their subunit selectivity.

Keywords: NMDA receptor; Rat NR2A subunit; EcR-293 cell line; Inducible expression

1. Introduction

NMDA receptors (NRs) are ligand-gated ion channels that constitute a distinct class within the family of ionotropic glutamate receptors. They are considered to play a central role in the formation of neural networks during development as well as in neuronal plasticity underlying memory and learning (Tang et al., 1999, 2001; Reed and Zukin, 2002). Besides these physiological roles of NRs, they are also implicated in a variety of neuropathological processes (Ozawa et al., 1998; Hynd et al., 2004), therefore, NRs are considered as relevant molecular targets for therapeutic agents in the treatment of stroke, epilepsy, head trauma, pain and alcohol dependence (Cull-Candy et al., 2001; Nagy et al., 2003b, 2004). Several lines of evidence indicate that both $GABA_A$ receptor subtype-selective ligands (Sinkkonen et al., 2004), and NMDA receptor antagonists (Chojnacka-Wójcik et al., 2001) modulate anxiety in rodents and are widely tested in animal models detecting anxiolytic activity of these drugs.

The involvement of NRs in diverse processes reflects their unique features, which include voltage-sensitive blockage by extracellular Mg^{2+} ions and a high permeability to Ca^{2+} ions. Furthermore, besides binding of both L-glutamate and the co-agonist glycine (Meguro et al., 1992),

* Corresponding author. Fax: +36 1 2605000.
E-mail address: d.kurko@richter.hu (D. Kurkó).

0197-0186/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2004.12.002

depolarization of the plasma membrane resulting in displacement of the Mg^{2+} ion blockade is also required for activation of these channels.

Functional NRs are heteromeric complexes comprising multiple copies of NR1 and NR2 (NR2A, NR2B, NR2C or NR2D) subunits (Mori and Mishina, 1995) in yet unknown stoichiometry (Chazot et al., 1994; Blahos and Wenthold, 1996; Luo et al., 1997; Bigge, 1999; Pina-Crespo and Gibb, 2002). Whereas NR1, which has eight alternative splice variants (NR1a–h), serves as a fundamental subunit to form heteromeric NRs, the NR2A–D subunits are regarded as modulatory ones (Monyer et al., 1992; Sheng et al., 1994; Mori and Mishina, 1995). Two recently described members of a third type of NMDA receptor subunits, NR3A and NR3B (Goebel and Poosch, 1999; Chatterton et al., 2002), do not form functional receptors alone, but can co-assemble with NR1/NR2 complexes (Perez-Otano et al., 2001).

All subunits have distinct distributions (Watanabe et al., 1993). In situ hybridization studies revealed that NR1 subunit mRNAs are expressed throughout the whole rat CNS (Buller et al., 1994; Paupard et al., 1997; Prybylowski and Wolfe, 2000; Prybylowski et al., 2001), whereas expression of the different types of NR2 subunits are both developmentally and anatomically regulated (Buller et al., 1994; Monyer et al., 1994; Laurie et al., 1997; Wenzel et al., 1997; Goebel and Poosch, 1999). The distinct distribution of NR subunits implies that NMDA receptor complexes are heterogeneous in their pharmacological and physiological properties.

The molecular heterogeneity of NMDA receptor ion channels made it possible to develop subunit and site-specific agents for selective therapeutic interventions. Since heteromeric recombinant NRs display electrophysiological (Stern et al., 1994) as well as pharmacological (Ishii et al., 1993) properties similar to those of native NMDA receptors, in vitro cellular model systems expressing recombinant receptors with defined subunit combinations are convenient tools to identify and characterize subtype selective modulators of NMDA receptors.

The human embryonic kidney cell line HEK-293 was proved to be suitable for both transient and stable expression of ligand-gated ion channels. Since the transfection rate of transient expression systems is varying, stably expressing non-neuronal cell lines are usually preferable (Grimwood et al., 1996; Varney et al., 1996; Uchino et al., 1997). However, a major obstacle for producing such cell lines is the inherent cellular toxicity caused by the Ca^{2+} ion permeability of this ligand-gated ion channel (Cik et al., 1993; Anegawa et al., 1995; Boeckman and Aizenman, 1996). In order to overcome this problem, many investigators try to regulate the level of expression. For this purpose a dexamethasone-, or a heat shock-, a tetracycline-, as well as an ecdysone-inducible mammalian expressional system (Priestley et al., 1995; Uchino et al., 1997; Renard et al., 1999; Nagy et al., 2003a) have been developed.

In this study, we have established a non-neuronal, HEK-293 based cell line, which stably expresses recombinant rat NR1a/NR2A NMDA receptors, using an ecdysone-inducible mammalian expression system. According to its pharmacological profile, the generated cell line can be used in functional assays for testing several NMDA antagonists with or without subtype specificity.

2. Experimental procedures

2.1. Materials

Dulbecco's modified Eagle's medium (D-MEM), and fetal bovine serum were obtained from GIBCO BRL Life Technologies (Gaithersburg, MD, USA). The EcR-293 cell line, pIND(SP1)Hygro and pIND(SP1)Neo vectors as well as lipofectamine, muristerone A (MuA), G418 and hygromycin were purchased from Invitrogen (Groningen, The Netherlands). The High Pure RNA Isolation Kit, the LightCycler RNA Master SYBR Green I kit and the h-PBGD housekeeping gene set were from Hoffmann-La Roche (Basel, Switzerland). Primers were produced by TIB MOLBIOL (Berlin, Germany). NR2A subunit specific polyclonal antibody generated in goat was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fluo-4/AM was from Molecular Probes (Eugene, OR, USA). DL-2-Amino-5-phosphonopentanoic acid (DL-AP5) was obtained from Tocris Cookson Ltd. (Avonmouth, UK). NR2B subunit selective NMDA antagonists (erythro-ifenprodil, CP-101, 606 and CI-1041) were synthesised by G. Richter Ltd. (Budapest, Hungary). The isotype specific control (goat) antibody, secondary antibodies and all other chemicals were purchased from Sigma.

2.2. Generation of stably transfected cell lines expressing rat NR1a/NR2A recombinant NMDA receptors

The cDNAs, encoding rat NR1a and NR2A subunits were obtained from Prof. P. Seeburg (Monyer et al., 1992, 1994), and were subcloned into the ecdysone-inducible mammalian expression vectors pIND(SP1)Hygro and pIND(SP1)Neo, respectively. For transfection EcR-293 cells (HEK-293 cells stably expressing the ecdysone and the retinoid X receptors) were grown to ~50% confluency in Dulbecco's modified Eagle's medium (D-MEM), containing 10% fetal bovine serum, 2.5 μg/ml amphotericin B, 100 U/ml penicillin G, 100 μg/ml streptomycin. Cells were incubated with NR1a and NR2A subunits containing vectors (ratio 1:1) for 4 h in serum-free D-MEM in the presence of lipofectamine in a humidified atmosphere with 5% CO_2 at 37 °C. Following the 4 h transfection period cells were placed in serum containing D-MEM and next day they were exposed to selection medium, containing hygromycin (100 μg/ml) and G418 (600 μg/ml). The medium was replaced every two or three days for two weeks. One of the hygromycin and G418