Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 603–608

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of a selective competitive receptor binding assay for the determination of the affinity to NR2B containing NMDA receptors

Dirk Schepmann, Bastian Frehland, Kirstin Lehmkuhl, Bastian Tewes, Bernhard Wünsch*

Fachbereich Chemie und Pharamzie, Institut für Pharmazeutische und Medizinische Chemie der Universität Münster, Hittorfstraße 58-62, D-48149 Münster, Germany

ARTICLE INFO

Article history:
Received 9 December 2009
Received in revised form 6 April 2010
Accepted 11 April 2010
Available online 18 April 2010

Keywords:
NMDA receptor
NR2B
Ifenprodil
Recombinant receptor
Binding assay

ABSTRACT

A selective, rapid and efficient competitive binding assay for the determination of the affinity of compounds towards the ifenprodil binding site of NR2B subunit containing NMDA receptors has been developed. In the assay system, [^{3}H]ifenprodil was used as radioligand and membrane homogenates from L(tk-) cells stably expressing recombinant human NR1a/NR2B receptors served as the receptor material. Sonication of the cells during work-up, performing all steps with 96-well multiplates and using a solid scintillator represent particular features of this assay. The binding kinetics was investigated by saturation and association/dissociation experiments. [^{3}H]ifenprodil bound to a single, saturable site on human recombinant NR1a/NR2B receptors, resulting in a B_{max}-value of 25.8 pmol/μg protein and K_d-value of 7.6 ± 2.3 nM (SEM). The dissociation rate constant (k_{off}) was 0.03861 min^{-1} and the association rate constant k_{on} resulted in 0.00625 nM^{-1} min^{-1}. The specificity of the assay was proved with cells not treated with dexamethasone, which has to be added to induce NMDA receptor synthesis of the cells. Additionally, the absence of α_1, σ_1 and σ_2 receptors was shown. The K_i-values of the NR2B ligands ifenprodil and eliprodil determined with the new assay are in good accordance with reported data.

1. Introduction

The amino acid (S)-glutamate is one of the most important excitatory neurotransmitters in the central nervous system (CNS), acting on both, ligand-gated ion channels (ionotropic receptors) and G-protein-coupled (metabotropic) receptors. Among the group of ionotropic glutamate receptors three different receptors, the AMPA (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid), kainate and N-methyl-D-aspartate (NMDA) receptor, have been identified [1–3]. Glutamatergic synapses typically feature both AMPA and NMDA receptors. In its resting state, the ion channel pore of the NMDA receptor is blocked by Mg^{2+}-ions. The NMDA receptor is activated by binding of two co-agonists, glutamate and glycine. Additionally, a short reduction of the postsynaptic membrane potential, typically mediated by AMPA receptors, is required to remove the voltage-dependent block of Mg^{2+}-ions. The free channel is permeable for K^+-, Na^+- and Ca^{2+}-ions [3]. The influx of cations, in particular Ca^{2+}-ions through the NMDA receptor associated ion channel mediates excitatory transmission in the CNS. Moreover, the interaction with two agonists together with the voltage gated Mg^{2+}-block plays an important role in synaptic plasticity. However, under pathophysiological conditions like cerebral ischemia, brain trauma, and inflammation, overactivation of NMDA receptors has been associated with neuronal cell death by elevating the intracellular Ca^{2+}-concentration up to cytotoxic levels [3–5].

Therefore, novel NMDA receptor antagonists represent promising drugs for the treatment of various neurodegenerative disorders like stroke, epilepsy, Parkinson's disease or Alzheimer's disease [6–8].

Functional NMDA receptors are hetero-tetrameric assemblies comprising at least one NR1 subunit and one or more NR2 subunits [9,10]. For the NR1 subunit eight splice variants (NR1a–h) and for the NR2 subunit four subtypes (NR2A–D) have been identified, which make the composition of the NMDA receptors highly variable and complex [11]. Whilst the different NR1 splice variants can be found ubiquitously in the CNS, the NR2 subunit composition and distribution differs in various regions in adult mammalian brain [12–14].

Each subunit has binding sites for different agonists and allosteric regulators. The NR1 subunit contains the glycine binding site, whereas the NR2 subunit contains the glutamate binding site and binding sites for allosteric regulators and noncompetitive antagonists such as extracellular Zn^{2+}-ions, polyamines, and ifenprodil. The variety of different binding sites provides the rationale for the development of subtype-selective therapeutic drugs. Antagonists selectively acting at the NR2B subunit are of particular interest because of their improved side effect profile in comparison to uncompetitive NMDA receptor antagonists like open channel blockers [15–18].

* Corresponding author. Tel.: +49 251 8333311; fax: +49 251 8332144.
E-mail address: wuensch@uni-muenster.de (B. Wünsch).

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doi:10.1016/j.jpba.2010.04.014

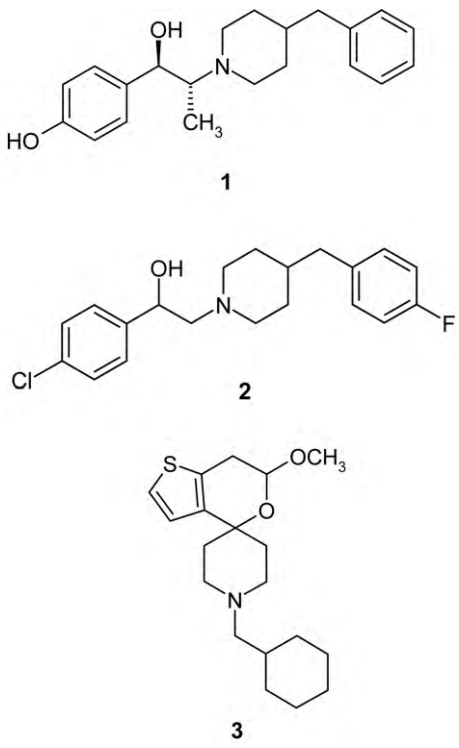

Fig. 1. Chemical structures of ifenprodil (**1**), eliprodil (**2**), and the spirocyclic σ receptor ligand **3**.

The development of selective NR2B antagonists requires selective binding assays. Assays employing preparations from rat or pig brain with [^{125}I]ifenprodil (Fig. 1) [19], [^{3}H]ifenprodil [20,21] or [^{3}H]Ro25-6981 [(1R,2S)-3-(4-benzylpiperidin-1-yl)-1-(4-hydroxphenyl)-2-methylpropan-1-ol] [22] as radioligands have been described. However the most commonly used radioligand [^{3}H]ifenprodil does not only interact with the ifenprodil binding site of NR2B subunit containing NMDA receptors, but also with α_1, σ_1 and σ_2 receptors as well as dopamine transporters [23–25]. In some assays, the selectivity for NR2B subunit containing NMDA receptors is achieved by masking the other binding sites with compounds like GBR-12909 (1-{2-[bis(4-fluorophenyl)-methoxy]ethyl}-4-(3-phenylpropyl)piperazine), GBR-12935 (1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine) and (+)-PPP [(R)-3-(1-propylpiperidin-3-yl)phenol] [20,21]. In addition to the presence of many different receptors in native material the standardization of NMDA receptor material is particular difficult, due to the variable composition of NMDA receptors in different brain regions. Therefore membrane preparations from rat brain typically show an inhomogenous distribution pattern of different NMDA receptors. A binding assay based on recombinant receptors consisting of NR1a–NR2B subunits has already been described by Grimwood et al. [26]. However, they reported that a high portion (88%) of the [^{3}H]ifenprodil binding was not related to the NMDA receptor. Therefore masking of the other specific binding sites of [^{3}H]ifenprodil with buffer additives was required. Additionally, the assay was performed at low temperature (4 °C) to reduce the binding of [^{3}H]ifenprodil to the σ_1 receptor.

Herein, we report on the development of a simple and highly selective, filtration-based receptor binding assay on 96-well multiplates for the ifenprodil binding site of NR2B subunit containing NMDA receptors using [^{3}H]ifenprodil as radioligand. The addition of buffer additives and the execution of the assay at low temperature were not necessary. Also, a solid scintillator was used to avoid the disadvantage of rather long equilibrium times of liquid scintillation cocktails before counting. Hence, the assay can be easily adopted for high-throughput screening methods.

2. Materials and methods

2.1. Preparation of TRIS/EDTA (5 mM/1 mM, pH 7.5) buffer for the binding assays

606 mg TRIS–base (Acros Organics, Geel, Belgium) and 372 mg Na–EDTA (MERCK, Darmstadt, Germany) were dissolved in approximately 900 mL of distilled water. The pH was adjusted to pH 7.5 by dropwise addition of 1 M HCl before diluting to the final volume of 1000.0 mL.

2.2. Cell culture and preparation of membrane homogenates

Mouse L(tk-) cells stably transfected with the dexamethasone-inducible eukaryotic expression vectors pMSG NR1–1a, pMSG NR2B in a 1:5 ratio were a generous gift from Prof. Dr. Dieter Steinhilber (Department of Pharmacy, University of Frankfurt, Germany) [27]. The transformed L(tk-) cells were grown in Modified Earl's Medium (MEM) containing 10% of standardized FCS (Biochrom AG, Berlin, Germany). The expression of the NMDA receptor at the cell surface was induced after the cell density of the adherently growing cells had reached approximately 90% of confluency. For the induction, the original growth medium was replaced by growth medium containing 4 μM dexamethasone and 4 μM ketamine (final concentration). After 24 h the cells were harvested by scraping and pelleted (10 min, 5000 × *g*, Hettich Rotina 35R centrifuge, Tuttlingen, Germany).

For the binding assay, the cell pellet was resuspended in PBS buffer and the number of cells was determined using an improved Neubauer's counting chamber (VWR, Darmstadt, Germany). Subsequently, the cells were lysed by sonication (4 °C, 6× 10 s cycles with breaks of 10 s, device: Soniprep 150, MSE, London, UK). The resulting cell fragments were centrifuged with a high performance cooled centrifuge (20,000 × *g*, 4 °C, Sorvall RC-5 plus, Thermo Scientific). The supernatant was discarded and the pellet resuspended in a defined volume of phosphate buffer saline (PBS) yielding cell fragments from approximately 500,000 cells/mL. The suspension of membrane homogenates was sonicated again (4 °C, 2× 10 s cycles with a break of 10 min) and stored at −80 °C. Homogenization of the cells by sonication reduced the formation of cell aggregates, led to high interday and intraday reproducibility and increased the specific binding of the radioligand considerably.

In order to get cells without NMDA receptors as negative control, the cells were harvested without the previous addition of dexamethasone and ketamine to the growing medium. This procedure inhibits the production of NMDA receptors. The membrane homogenates were prepared as described above leading to a preparation with all native receptors and proteins except NMDA receptors, which served as reference system.

2.3. Receptor binding assay

The competitive binding assay was performed with the radioligand [^{3}H]ifenprodil (60 Ci/mmol; Perkin Elmer) using standard 96-well multiplates (Diagonal, Muenster, Germany). The thawed cell membrane preparation (about 20 μg protein) was incubated with 6 different concentrations of test compounds, 5 nM [^{3}H]ifenprodil, and TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) in a total volume of 200 μL for 120 min at 37 °C. The cell membrane suspension was added last. All experiments were carried out in triplicates. The incubation was terminated by rapid filtration through filtermats using a cell harvester (MicroBeta FilterMate-96 Harvester, Perkin Elmer). Prior to harvesting, the filtermats were

presoaked in 0.5% aqueous polyethylenimine (PEI) for 2 h at room temperature. After washing each well five times with 300 μL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was placed on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at RT. The bound radioactivity trapped on the filters was counted in the scintillation analyser (Microbeta Counter, Perkin Elmer). The overall counting efficiency was 20%. The nonspecific binding was determined with 10 μM unlabelled ifenprodil.

2.4. Protein determination

The protein concentration in the membrane preparations was determined by the method of Bradford [28], modified by Stoscheck [29]. The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL EtOH (95%, v/v). 10 mL H_2O, 5 mL phosphoric acid (85%, m/v) and H_2O were added up to a total volume of 50.0 mL. Bovine serum albumine was used as reference standard. The absorption of the protein–dye complex at λ 595 nm was measured with a platereader (Tecan Genios, Tecan, Crailsheim, Germany).

2.5. Reduction of nonspecific binding

The filtermats were presoaked with 0.1, 0.2, 0.5, 0.7 or 1% aqueous polyethylenimine (PEI) solution for 2 h at RT. Subsequently, 200 μL of a solution containing 5 nM [^{3}H]ifenprodil in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) in 96-well multiplates was filtered through the filtermats using the cell harvester and washed five times with water. The same procedure was performed in the presence of 20 μg membrane receptor protein to determine the ratio between nonspecific binding and total binding to the receptor protein.

2.6. Determination of binding kinetics

To determine the optimum incubation time and the association rate constant (k_{on}), 5 nM [^{3}H]ifenprodil was incubated together with 20 μg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) in a total volume of 200 μL for 5, 15, 30, 45, 60, 120, 180 and 240 min at 37 °C, respectively. The incubation was terminated by filtration and analysed as described in Section 2.3. The observed rate constant (k_{ob}) and association constant (k_{on}) were calculated as described in Section 2.9.

For the determination of the dissociation rate constant (k_{off}), 5 nM [^{3}H]ifenprodil was incubated together with 20 μg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) for 2 h at 37 °C to reach equilibrium in the formation of the receptor–radioligand complex. To initiate the dissociation of the radioligand, a large excess (10 μM) of non-labelled ifenprodil was added and the mixture was incubated for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 120, 180 and 240 min, respectively. The incubation was terminated and analysed using the same procedure as described in Section 2.3. The dissociation constant (k_{off}) was calculated as described in Section 2.9.

2.7. Saturation experiment

The saturation analysis was performed by incubating increasing concentrations of [^{3}H]ifenprodil (0.5, 1, 2.5, 5, 10, 15, 20, 25 and 50 nM) together with 20 μg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) for 2 h at 37 °C. For each concentration, the nonspecific binding was determined with an excess of non-labelled ifenprodil (10 μM). K_d and B_{max} were calculated as described in Section 2.9.

2.8. Selectivity towards α_1, σ_1 and σ_2 and receptors

Competition experiments with prazosine, (+)-pentazocine and rimcazol were performed as described in Section 2.3. The residual binding of the radioligand [^{3}H]ifenprodil at a concentration of the test compound of 1 μM was calculated using the formula:

$$\text{Residual binding}\,(\%) = \frac{\text{measured binding} - \text{nonspecific binding}}{\text{total binding} - \text{nonspecific binding}} \times 100\%$$

Definitions of the terms

measured binding: counts per minute measured in the presence of a specified concentration of test compound; nonspecific binding: counts per minute measured in the presence of a large excess of ifenprodil; total binding: counts per minute measured after incubation of [^{3}H]ifenprodil without inhibitor or test compound.

From the residual binding, the inhibition of the radioligand was calculated with the following formula:

$$\text{Inhibition}\,(\%) = 100\% - \text{Residual binding}\,(\%)$$

2.9. Data analysis

Data analysis was performed with Graph Pad Prism® Software, Version 3.0 (Graph Pad Software Inc., San Diego, CA, USA). The observed rate constant (k_{ob}) of the association experiment was calculated by nonlinear regression using the "one phase exponential association" equation. The dissociation rate constant (k_{off}) was calculated by nonlinear regression using the exponential decay calculation method.

Subsequently, the association rate constant (k_{on}) was calculated with the following equation:

$$k_{on} = \frac{k_{ob} - k_{off}}{\text{Radioligand}} \quad (1)$$

The association rate constant and dissociation constant were used to estimate the K_d-value using the formula:

$$K_d = \frac{k_{off}}{k_{on}} \quad (2)$$

Saturation analyses were made by nonlinear regression using the "one-site-saturation" calculation method. The Scatchard (Rosenthal) plot was generated by linear regression using the least squares method. The IC_{50}-values of the reference compounds used in the competitive binding experiments were determined by nonlinear regression using the "one-site-competition" calculation method. Subsequently, the K_i-values of the reference compounds were calculated according to the equation of Cheng and Prusoff [30]. The K_i-values are given as mean values from three independent experiments ± Standard Error of the Mean (SEM).

3. Results and discussion

3.1. Reduction of nonspecific binding

The nonspecific binding of the radioligand to filter material is a frequently observed problem in radioligand binding assays [31,32]. Since the radioligand ([^{3}H]ifenprodil) used for the development of the assay system has a basic amino moiety, which is at least partly protonated at pH 7.5, the cationic polymer polyethylenimine was used for the reduction of the nonspecific binding [33]. The nonspecific binding of [^{3}H]ifenprodil was reduced by almost 50% after pretreatment of the filtermats with 0.5% PEI for 2 h. Under these conditions, at a concentration of 5 nM [^{3}H]ifenprodil 10.6% of total binding was nonspecific. Higher concentrations of

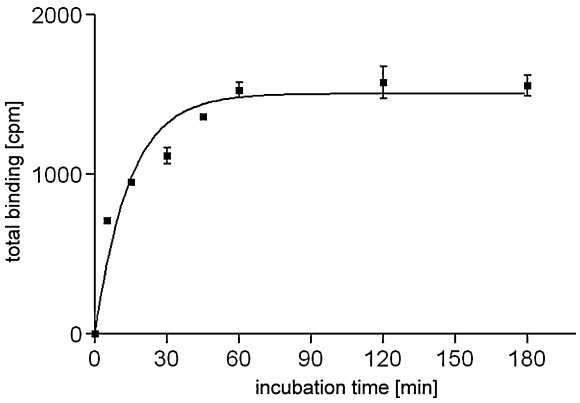

Fig. 2. Association experiment. 5 nM [^{3}H]ifenprodil was incubated together with 20 μg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) for 0–180 min at 37 °C. Results from one experiment in triplicates.

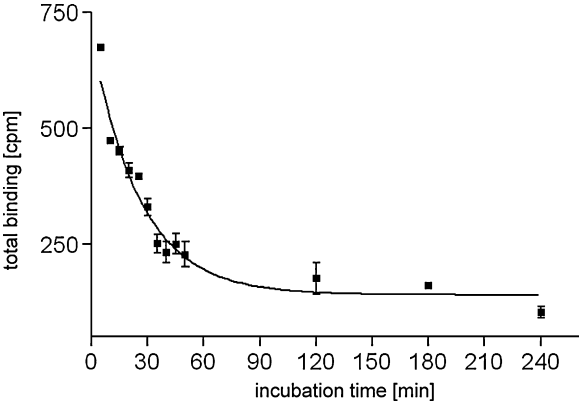

Fig. 3. Dissociation experiment. 5 nM [^{3}H]ifenprodil was incubated together with 20 μg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) for 2 h at 37 °C. Dissociation was initiated by addition of 10 μM non-labelled ifenprodil. Results from one experiment in triplicates.

PEI (0.75 and 1%) did not lead to a further decrease of the non-specific binding. Concentrations equal or higher than 1% of PEI caused a strong swelling of the filtermats rendering the filtration difficult.

3.2. Determination of binding kinetics

The minimal incubation time required to reach equilibrium was determined by incubation of the receptor protein with the radioligand for different periods of time at 37 °C. At a concentration of 5 nM [^{3}H]ifenprodil, the equilibrium was achieved after approximately 60 min (Fig. 2) and the total binding started to decrease after 240 min. Therefore, all further experiments were carried out with an incubation period of time of 120 min.

The same experiment was used to determine the observed rate constant (k_{ob}). The nonlinear regression data analysis resulted in a k_{ob} of 0.06986 min^{-1}.

The off-rate constant for the radioligand dissociating from the receptor (k_{off}) was determined by performing a dissociation experiment in which the radioligand and the receptor protein were incubated until reaching equilibrium. Subsequently, the dissociation was started by adding a high concentration (10 μM) of non-labelled ifenprodil as inhibitor and measuring the reduction of the radioligand binding over a time period of 4 h (Fig. 3). The calculation resulted in a k_{off} of 0.03861 min^{-1}. After determination of k_{ob} and k_{off}, the association constant k_{on} was calculated as described in Section 2.9 and resulted in 0.00625 nM^{-1}· min^{-1}.

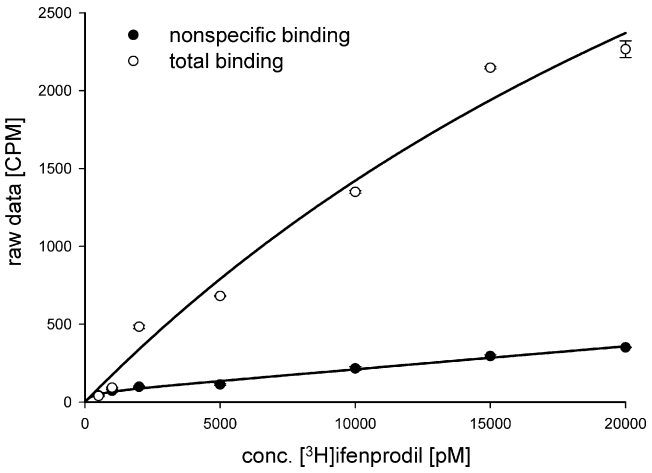

Fig. 4. Saturation experiment. Increasing concentrations of [^{3}H]ifenprodil were incubated together with 20 μg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) for 2 h at 37 °C. Nonspecific binding was determined with 10 μM of non-labelled ifenprodil. Results from three experiments in triplicates.

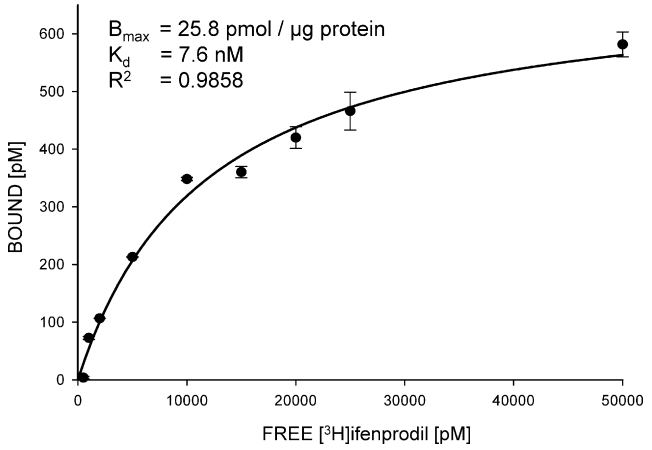

Fig. 5. Saturation plot. The saturation analysis was made by nonlinear regression using the "one-site-saturation" calculation method (Graph Pad Prism® Software 3.0). Results from three experiments in triplicates.

The K_d-value of [^{3}H]ifenprodil was calculated according to Eq. (2) from k_{off} and k_{on} and resulted in 6.1 nM.

To obtain a more precise K_d-value, a saturation experiment was performed (Fig. 4). B_{max} and K_d-values were determined by nonlinear regression using the one-site-saturation calculation method (Fig. 5). For a better visualization and to demonstrate that only one binding site exists for the radioligand [^{3}H]ifenprodil in the investigated concentration range, a Scatchard (Rosenthal) plot was generated (Fig. 6). The nonlinear regression analysis and the Scatchard plot led to very similar K_d-values of 7.6 ± 2.3 and 7.4 nM, respectively. Since the data transformation used in Scatchard plots violates the assumptions of linear regression, the data obtained by nonlinear regression are generally considered to be more accurate [32]. Therefore, the K_d-value calculated by nonlinear regression (7.6 nM) was used for all further calculations.

3.3. Specificity of the assay

The specificity of the assay system was determined with the control cell membrane preparation, which was prepared starting with the same mouse L(tk-) cells used in the normal assay. In contrast to the normal cells dexamethasone had not been added to the growing medium of the control cells, though the cells were not stimulated

Table 1
Measured K_i-values and Hill slopes compared to reported IC_{50}-values of ifenprodil and eliprodil.

Compound	$K_i \pm$ SEM [nM] ($n = 3$)	Hill slope	IC_{50}-value [ref.] [nM]
ifenprodil (**1**)	10 ± 0.7	−1.14 ± 0.31	13.3 [40]
eliprodil (**2**)	13 ± 2.5	−0.91 ± 0.08	82 ± 10 [41]

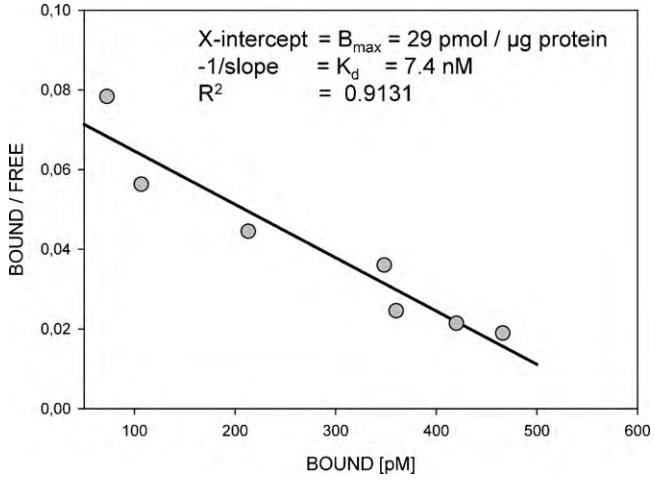

Fig. 6. Scatchard (Rosenthal) plot of the saturation experiment. Data analysis was performed by linear regression with the method of least squares. Results from one representative experiment in triplicates.

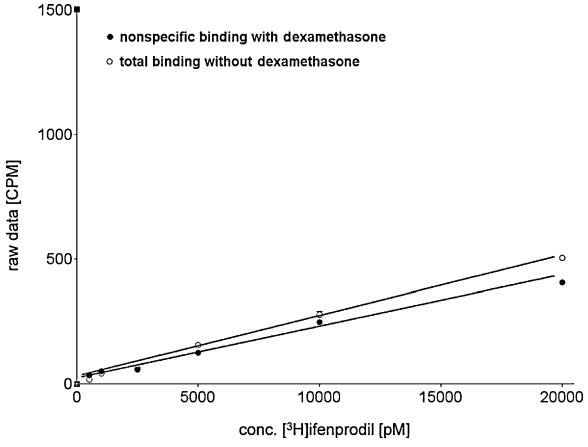

Fig. 7. Increasing concentrations of [^{3}H]ifenprodil were incubated with the control membrane preparation obtained from L(tk-)cells, which were not stimulated with dexamethasone. The total binding of the radioligand is compared with the nonspecific binding of the radioligand employing membrane preparations from dexamethasone induced cells. Results from one experiment in triplicates.

to produce NMDA receptors. This membrane preparation without NMDA receptors was incubated with increasing concentrations of [^{3}H]ifenprodil. In Fig. 7 the resulting binding of the radioligand is compared with the nonspecific binding of cell membrane preparations induced with dexamethasone. Fig. 7 clearly demonstrates that the total binding of the non-NMDA containing control cells is almost identical with the nonspecific binding of cells containing NMDA receptors. This experiment nicely proves the specificity of the assay, as the cells according to these results do not produce any proteins or other components, which selectively bind the radioligand [^{3}H]ifenprodil.

3.4. Selectivity towards α_1, σ_1 and σ_2 receptors [34–37]

In addition to these control experiments, the absence of σ_1 and σ_2 receptors as well as α_1 receptors in the cell membrane preparation was investigated, since the radioligand [^{3}H]ifenprodil interacts with high affinity with these receptors.

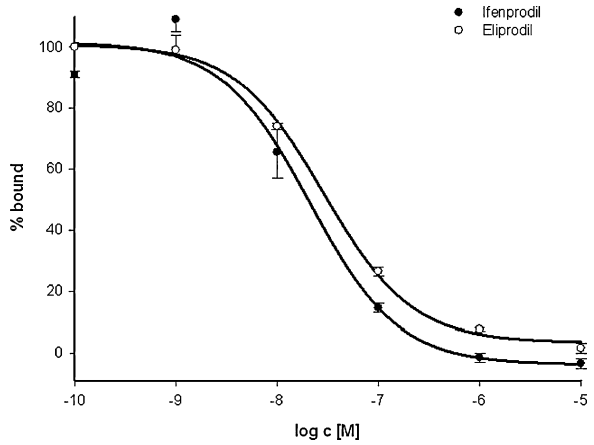

Fig. 8. Competition curves of ifenprodil and eliprodil. Different concentrations of the reference compounds were incubated together with 5 nM [^{3}H]ifenprodil and 20 µg receptor protein in TRIS/EDTA-buffer (5 mM/1 mM, pH 7.5) for 120 min at 37 °C. Results from three experiments in triplicates.

The selectivity against the σ_1 receptor was investigated using (+)-pentazocine, which features a high affinity and selectivity towards the σ_1 receptor (K_i = 4.2 nM [37], 2.2 nM [35] and 2.1 nM [34]). Even a concentration of 1 µM of (+)-pentazocine did not lead to a considerable displacement of the radioligand [^{3}H]ifenprodil (1% inhibition at a concentration of 1 µM). A similar result was obtained with the σ_2 receptor ligand rimcazol [38] which caused an inhibition of 16% at a concentration of 1 µM. The thienopyran **3**, which shows very high affinities to both σ receptor subtypes (σ_1: K_i = 0.3 nM; σ_2: K_i = 25.4 nM) [39], revealed a 31% inhibition of [^{3}H]ifenprodil binding at a concentration of 1 µM. The IC_{50}-value is greater than 1 µM and therefore these experiments prove that σ_1 and σ_2 receptors do not contribute to the specific binding of [^{3}H]ifenprodil in this assay system.

The selectivity against α_1 receptors was investigated with the α_1 receptor antagonist prazosine [36]. In the described assay system, a K_i-value of 480 nM for prazosine was determined using the radioligand [^{3}H]ifenprodil. Considering the high affinity of prazosine at α_1 receptors (K_i = 0.1 nM), it is possible that this value is caused by a small amount of α_1 receptors expressed on the surface of the cells, which have been labelled by the radioligand [^{3}H]ifenprodil. However, due to the basic piperazinylquinazoline structure of prazosine we assume that the observed K_i-value is the result of a low specific interaction of prazosine with the ifenprodil binding site of the NR2B subunit, instead of displacement of [^{3}H]ifenprodil from competing α_1 receptors. Nevertheless, this interaction will not disturb the NR2B assay.

3.5. Competition experiments

For the validation of the assay, the binding affinities of the reference compounds ifenprodil (**1**) and eliprodil (**2**) were determined in homologous competition experiments as described in Section 2.3. The IC_{50}-values were determined by nonlinear regression analysis and transformed into the corresponding K_i-values using the Cheng–Prusoff equation [30]. Typical competition curves are pre-

sented in Fig. 8. The K_i-values determined in the new assay are in good accordance with the binding affinities reported in the literature. The Hill slopes of both competition curves are close to 1.0 indicating that only one binding site is involved in this competition process. The data are summarized in Table 1.

4. Conclusion

A selective and sensitive receptor binding assay for measuring the interaction of ligands with NR2B subunit containing NMDA receptors using 96-well multiplates and homogenates of cells stably expressing recombinant human NR1a/NR2B receptors has been developed. Sonication of the cells during the membrane preparation led to high specific binding of the radioligand [^{3}H]ifenprodil. All crucial steps of the assay (incubation, filtration and washing) were carried out using microtiter plates and, therefore, only low amounts of receptor material and radioligand were required and low amounts of waste were produced. The bound radioactivity on the filter was measured with a solid scintillator, avoiding the disadvantages of conventional liquid scintillation cocktails. After careful optimization of the assay conditions, only 10.6% of total binding was nonspecific. The binding kinetics was fully characterized by association and dissociation experiments and, moreover, a saturation experiment. These experiments led to a B_{max}-value of 25.8 pmol/μg protein, a K_d-value of 7.6 nM and a K_i-value of 10.4 nM for ifenprodil. The specificity of the assay was proved with mouse L(tk-) cells, which were not treated with dexamethasone. These experiments showed that the specific binding of the radioligand [^{3}H]ifenprodil to the NR2B receptor binding sites was remarkably high, other specific binding sites (e.g. receptors or other proteins) did not contribute to the specific binding of [^{3}H]ifenprodil. Therefore, in contrast to the reported assay systems (see Section 1), additives for masking of competing receptors or proteins were not necessary. Altogether the assay represents a valuable tool for the development of novel NMDA receptor antagonists, which selectively interact with the ifenprodil binding site on the NR2B subunit. Moreover, automation for high-throughput screening should be possible.

Acknowledgement

We wish to thank Prof. Dr. D. Steinhilber, Department of Pharmacy, University of Frankfurt, for donation of stably transfected mouse L(tk-) cells.

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