



Effects of memantine on recombinant rat NMDA receptors expressed in HEK 293 cells

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1 The actions of the uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists, memantine (1-amino-3,5-dimethyladamantane) and (+)-MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, dizocilpine), on recombinant NMDA receptors has been studied by use of the whole-cell patch clamp technique.

2 Human embryonic kidney (HEK) 293 cells were transiently transfected with different NMDA receptor subunit combinations (NR1a/NR2A, NR1a/NR2B and NR1a/NR2D). A mutant form of the green fluorescent protein (GFP) was cotransfected with the NMDA receptor subunits to enable the visualization of transfected cells.

3 Memantine (0.3–30 μ M) blocked L-glutamate (100 μ M)-mediated currents in a concentration-dependent manner in NR1a/NR2A, NR1a/NR2B and NR1a/NR2D transfected cells with IC₅₀ values (at –70 mV) of 0.93 ± 0.15 μ M, 0.82 ± 0.12 μ M and 0.47 ± 0.06 μ M (mean \pm s.e. mean), respectively.

4 The memantine-induced block was strongly voltage-dependent. Alteration of the holding potential from –70 mV to +60 mV resulted in an e-fold increase in the IC₅₀ values per 30–33 mV change in membrane potential, for all 3 subunit combinations investigated.

5 The kinetics of the actions of memantine (30 μ M) were investigated for the NR1a/2A combination, in 6 cells (13–15 determinations). At –70 mV, the block and recovery from block were both best described by two exponentials with time-constants of 201 ± 23 ms ($81 \pm 2\%$) and 3.9 ± 0.6 s and 597 ± 94 ms ($18 \pm 1\%$) and 18.6 ± 2.4 s, respectively. The predominant effect of depolarization was to increase the weight of the faster recovery time-constant. Kinetic analysis suggests that these results are consistent with previously proposed Markov models.

6 (+)-MK-801 was studied briefly for comparative purposes. (+)-MK-801 (200 nM) preferentially blocked NMDA receptor currents (at –70 mV) in NR1a/NR2A and NR1a/NR2B ($82 \pm 10\%$ and $93 \pm 2\%$ depressions) compared to NR1a/NR2D ($38 \pm 7\%$) transfected cells. (+)-MK-801 appeared to be less voltage-dependent than memantine on all three receptor combinations.

7 In conclusion, memantine was a voltage-dependent antagonist of recombinant rat NMDA receptors expressed in HEK 293 cells but showed little selectivity between the subunits investigated. Its actions on these recombinant receptor combinations are similar to its actions on native NMDA receptors.

Keywords: Memantine (1-amino-3,5-dimethyladamantane); MK-801 (dizocilpine), NMDA (N-methyl-D-aspartate); patch clamp; voltage-dependency; HEK (human embryonic kidney) 293 cells

Introduction

There is considerable interest in the development of glutamate receptor antagonists because of the potential involvement of the excitotoxic properties of L-glutamate and related amino acids in acute and chronic neurological disorders, (e.g., Meldrum, 1985; Danysz *et al.*, 1995a). One possible therapeutic target at the NMDA receptor complex is the (+)-MK-801/PCP binding site within the ion channel. However, many uncompetitive antagonists such as (+)-MK-801 are known to exert effects that undermine their potential clinical use: These include stereotypy, indicative of psychotomimetic-like actions, and motor incoordination (Koek *et al.*, 1988), acute pathomorphological changes in specific neurones (Olney *et al.*, 1989), cardiovascular changes and disruption of learning and memory (e.g., Bischoff & Tiedke, 1992; Danysz *et al.*, 1995b). It has been suggested that so-called 'low-affinity' NMDA receptor antagonists, such as the 1-amino-adamantane derivative, memantine and the morphinan compounds, dextrorphan and dextromethorphan, may be more

promising as therapeutic agents because of reduced side effects (e.g., Rogawski, 1993). Indeed, memantine has been used clinically for many years in spasticity and Parkinson's disease, and more recently in dementia (Schneider *et al.*, 1984; Ditzler, 1991; Görtelmeyer & Erbler, 1992; Rabey *et al.*, 1992; Pantev *et al.*, 1993).

In previous studies of recombinant NMDA receptors (Meguro *et al.*, 1992; Monyer *et al.*, 1992; 1994), it has been shown that the antagonist, (+)-MK-801, preferentially interacts with NR1a/2A and NR1a/2B subunit combinations in binding studies using HEK 293 cells (Laurie & Seeburg, 1994) and in electrophysiological studies with oocytes (Yamakura *et al.*, 1993) compared to NR1a/2C or NR1a/2D combinations. In contrast, ketamine and SKF-10,047 block to a similar extent the mouse homologues of NR1a/2A, NR1a/2B, NR1a/2C and NR1a/2D in electrophysiological studies (Yamakura *et al.*, 1993). However, no studies of the subunit selectivity have been reported for memantine. Thus, the aim of the present study was to investigate whether memantine exhibits different pharmacological actions on distinct rat NMDA receptor subtypes, using HEK 293 cells as an expression system for different NMDA receptor subunit combinations. (+)-MK-801 was also studied for comparative purposes. Some of the results on NR1a/NR2B receptor coassemblies, have been published in abstract form (Bresink *et al.*, 1996).

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Methods

Plasmid construction

The full-length cDNAs encoding the rat NR1 (splice variant 1a, from S. Nakanishi, Institute for Immunology, Kyoto University, Japan), NR2B, NR2C and NR2D (from J. Boulter, The Salk Institute, Molecular Neurobiology Laboratory, San Diego, U.S.A.) were subcloned from the recombinant phagemid pBluescript SK(-) into the polylinker site of a mammalian expression vector containing a cytomegalovirus (CMV) promoter (pcDNA1/Amp; Invitrogen). The orientation of the cloned insert was determined by restriction mapping. The NR2A construct (inserted into a CMV promoter-containing vector RK7) and the mutant green fluorescence protein construct were obtained from P. Seeburg (University Heidelberg ZMBH, Heidelberg, Germany) and J. Pines (Wellcome/CRC Institute, Cambridge, England), respectively. The subunit-containing plasmids were amplified via growth in *E. coli* followed by a purification based on a modified alkaline lysis procedure (QIAGEN, Plasmid Mega Kit, Qiagen GmbH, Germany).

Cell transfection

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Ltd, Paisten, England) supplemented with 10% horse serum (Sigma Chemical, Dorset, England) and 2 mM glutamine. Two days prior to transfection exponentially growing cells were plated out on poly-D-lysine-coated glass coverslips (22 mm diameter, thickness No. 0) to a density of approximately 0.18×10^6 per coverslip. The coverslips were kept in 35 mm dishes containing medium. HEK 293 cells were then transiently transfected with cDNAs encoding the NMDA receptor subunits using the calcium phosphate transfection technique (Chen & Okayama, 1987). Three hours before transfection the medium was replaced by a glutamine-free medium. Cells were then transfected with equimolar amounts of NR1a with either one of NR2A, NR2B, NR2C or NR2D. Following transfection, cells were grown in the presence of 200 μ M RS-AP5 (RS-2-amino-5-phosphonopentanoate) to prevent cell death (Anegawa *et al.*, 1995). In order to visualize transfected cells for patch clamp experiments, transfections were performed in the presence of 5 μ g DNA of mutant GFP (Heim *et al.*, 1995). The total amount of DNA added for these triple transfections was approximately 25 μ g per 35 mm dish (Marshall *et al.*, 1995). Cells were incubated for 20 h with medium containing the calcium phosphate precipitate in a humidified incubator in 5% CO₂ at 37°C. The medium was then replaced by glutamine-free medium and the cells were grown for an additional 24 h until they were used for patch clamp experiments.

Electrophysiology

Experiments were performed at room temperature (20–22°C). Cells were visualised using phase contrast optics on an inverted microscope with a 20x objective (Nikon, Japan). Epifluorescence from cells expressing mutant GFP was excited by the full output of a 75W xenon lamp using standard fluorescein filters (420–490 nm excitation filter, 510 nm dichroic mirror, 520 nm long pass emission filter (Nikon, Japan)). The transfection efficiency was approximately 20% (Figure 1a and 1b) and approximately 90% of those cells which showed a green fluorescence gave strong glutamate-mediated responses indicating that functional ion channels had been expressed.

Electrodes were pulled from thick-walled borosilicate glass (1.2 mm o.d., Clark Electromedical, U.K.) on a horizontal puller (Sutter, U.S.A.). They had resistances of 4–8 M Ω when filled with the intracellular solution, which comprised (in mM): CsMeSO₄ 130, CsCl 20, HEPES 10, EGTA 20 and NaCl 5, pH 7.3 with CsOH. The extracellular solution comprised (in

mM): NaCl 150, KCl 3, HEPES 10, D-glucose 10 and CaCl₂ 2. Calculated junction potentials of 1 mV were not corrected. Tight seal (≥ 10 G Ω) whole cell recordings (Hamill *et al.*, 1981) of membrane currents were performed using an Axoclamp 2-A in continuous voltage-clamp mode (no series resistance error compensation was applied). Signals were externally amplified (20x), filtered (2 kHz–3dB) (FLA-01, Cygnus, U.S.A.) digitized at 8–2000 Hz (Labmaster TL-125, Axon, U.S.A.), and displayed on an IBM compatible PC running pClamp (Axon, U.S.A.) or in-house software (LTP, W.W. Anderson). Access resistance (typically ≈ 30 M Ω) and membrane resistance (typically ≈ 0.9 G Ω) were monitored throughout experiments using 30 or 40 mV depolarizing steps of 60 ms duration. All drugs (L-glutamate, memantine and (+)-MK-801) were dissolved in extracellular solution and applied separately or in combination, as indicated, via an array of glass capillary tubes (0.51 mm o.d., 0.38 mm i.d., Garner Glass, U.S.A.). These were moved laterally in relation to the cell either manually, with a vernier micrometer, or automatically, with a linear stepping motor (860-A-1 HS, Newport, U.S.A.), to achieve complete and rapid solution changes. Using the stepping motor, solution changes for the cell proceeded with a time constant of under 15 ms. This speed of solution change was measured by moving from standard extracellular solution to a solution containing extracellular solution plus 25 mM K⁺. In a cell patch clamped at 0 mV, this resulted in the appearance of an inward current with a time constant of 13.5 ms.

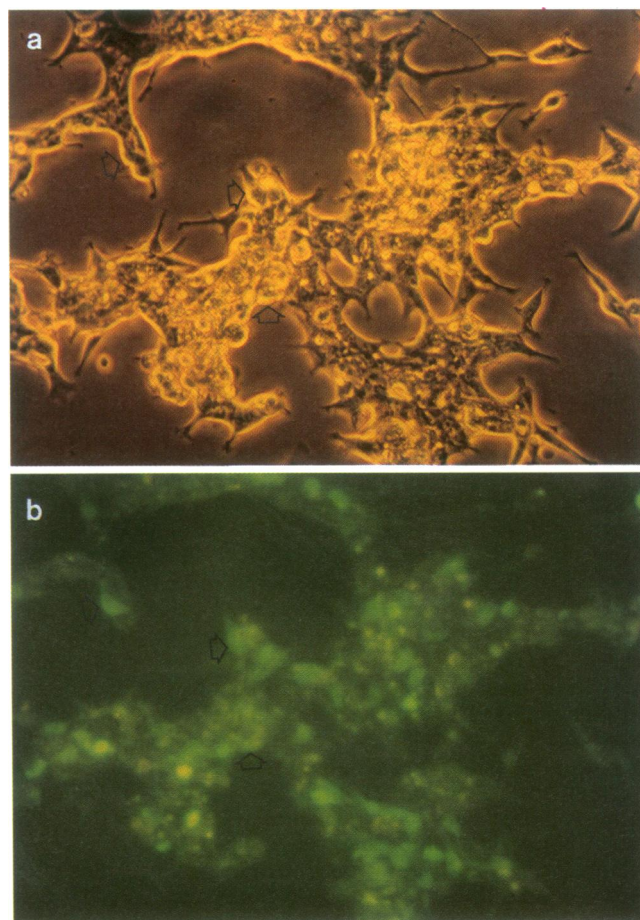


Figure 1 Mutant GFP allows rapid identification of transfected cells. HEK 293 cells were visualized with phase contrast optics (with a yellow filter) (a) and epifluorescence (b) after cotransfection of recombinant NMDA receptors and mutant GFP. Transfection efficiency was approximately 20–30%. Arrows indicate the same cell in each field.

Data analysis and statistics

To calculate the percentage block by antagonist, residual desensitization of L-glutamate-induced currents (in NR1a/2A and NR1a/2B combinations) was compensated by fitting exponentials to the pre-antagonist portion of traces. Traces were then normalised to this fit (see Figure 2). The percentage block by memantine was calculated from the final 2–5 s of a 10–20 s epoch at a given antagonist concentration, thus allowing for equilibration. Block by (+)-MK-801 was also estimated in this fashion. The extent of the block by memantine was similar whether it was applied sequentially or at a single concentration (Figure 2). IC_{50} s were obtained by fitting the data to the formula

$$\% \text{ of control} = 1/(1 + [\text{memantine}]/IC_{50}) \quad (1)$$

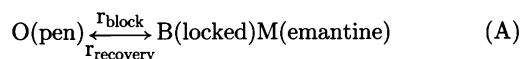
using an algorithm minimizing a X^2 criterion (Sigmaplot, Jandel Scientific, U.S.A.).

An estimation of the voltage-dependent interaction of memantine with the NMDA receptor channel was determined using the Woodhull equation. This measure, δ , can be calculated by assuming the probability that the voltage-dependence of memantine interaction with the NMDA receptor channel follows a Boltzmann distribution (Woodhull, 1973; Mayer *et al.*, 1988)

$$K_d(V_m) = K_d(V_o) \cdot \exp[(z\delta FV_m)/RT] \quad (2)$$

where $K_d(V_m)$ is the dissociation constant for memantine binding at a membrane potential V_m , $K_d(V_o)$ is the dissociation constant at zero membrane potential, δ is the fraction of the membrane electric field that influences memantine binding, and z , F , R and T have their standard meaning. δ was calculated for each NMDA receptor subunit subtype studied using IC_{50} s as an estimate for K_d s at different V_m and fitting via an algorithm minimising a X^2 criterion (Jandel Scientific, U.S.A.). δ was also used to calculate the e-fold change of K_d per mV using $1000 \cdot RT/\delta F$.

Exponential fits of the time course of block and recovery by memantine and (+)-MK-801 were fitted with one or two exponentials (whichever provided the best fit based on a minimum X^2 criterion) using Sigmaplot or in-house software (LTPFIT, T.A. Benke). If the rates of block and recovery are reasonably well fitted by a single exponential, then the following Markov model can be assumed (Chen *et al.*, 1992) with a single conformational state (BM) in which memantine binds to the open conformation of the NMDA receptor (O):



The K_d can then be estimated according to

$$K_d = \tau_{\text{block}} \cdot [\text{drug}] / \tau_{\text{recovery}} \quad (3)$$

where, in the continuous presence of L-glutamate (100 μM), τ_{block} is the exponential describing the time course of block following the rapid addition of antagonist, $[\text{drug}]$ is the concentration of antagonist, r_{block} is $1/\tau_{\text{block}} \cdot [\text{drug}]$, τ_{recovery} is the exponential describing the time course of recovery and r_{recovery} is $1/\tau_{\text{recovery}}$. However, if the time courses of block and recovery are multiexponential, more complicated models must be assumed. The simplest, in which the NMDA receptor can exist in a desensitized state (Clements & Westbrook, 1991; Lester & Jahr, 1992), allows for a single state bound to memantine:

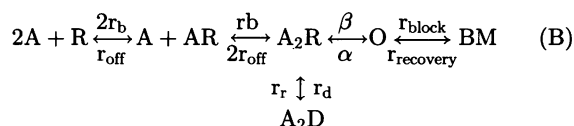
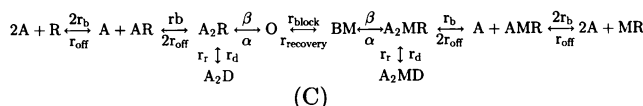


Table 1 Kinetic model rate constants

Rate constant description	Abbreviation	Value
Glutamate binding rate	r_b	$4.9 \mu\text{M}^{-1} \text{s}^{-1}$
Glutamate unbinding rate	r_u	4.9s^{-1}
Opening rate	β	100s^{-1}
Closing rate	α	322s^{-1}
Desensitization rate	r_d	4.0s^{-1}
Undesensitization rate	r_r	0.3s^{-1}
Blocking rate by memantine	r_{block}	$0.5-2 \mu\text{M} \text{s}^{-1}$
Recovery rate from memantine	r_{recovery}	$0.067-20 \text{s}^{-1}$

where A is free agonist (i.e. glutamate), R is free NMDA receptor, AR is receptor bound to one agonist molecule, A_2R is receptor bound to two agonist molecules, O is the open state, A_2D is the desensitized state and BM is the blocked state bound to memantine. Rate constants are listed in Table 1. A more complicated model allows for the receptor, once it has bound memantine, to exist in multiple states (see Huettner & Bean, 1988):



where BM is the initial state bound to memantine and A_2MR , A_2MD , AMR and MR are the other states bound to memantine which behave with rate constants equivalent to similar states unbound to memantine.

These models were tested empirically using in-house software (BLOCK, T.A. Benke) which integrated the kinetic equations describing each model via a Runge-Kutta fourth-order algorithm in order to find which model best described the data. Kinetic parameters in Table 1 were those of Clements & Westbrook (1991).

All values are given as mean \pm s.e. mean of individual cells from at least three individual transfections. Statistical analysis was made by one way analysis of variance which, if significant, was followed by the Student-Newman-Keuls test using SIGMASTAT software (Jandel Scientific).

Materials

HEK 293 cells were obtained from the European Collection of Animal Cultures (Salisbury, England). Memantine was synthesized and tested for purity at Merz & Co. (Frankfurt, Germany). (+)-MK-801 and RS-AP5 was obtained from Tocris Cookson (Bristol, England). Stock solutions were made up in distilled water and kept at -20°C until day of use. All other reagents were obtained from Sigma Chemicals (Dorset, England).

Results

Glutamate-mediated currents through recombinant NMDA receptors

L-glutamate (100 μM), applied in Mg^{2+} -free solution containing 10 μM glycine, evoked characteristic whole-cell currents at a holding potential of -70 mV in transiently transfected HEK 293 cells expressing heteromeric NMDA receptor channels consisting of NR1a in combination with NR2A, NR2B or NR2D, respectively. While specific [^3H]-MK-801 binding was detected in cell homogenates after transfection of NR1a in combination with NR2C (data not shown), we failed to detect reproducible L-glutamate-mediated currents in electrophysiological experiments using this combination of subunits. This may indicate that NR2C coassemblies are formed but not

expressed on the cell surface; special circumstances may be required for their cell surface expression (McIlhinney *et al.*, 1996).

Strong responses (approximately 1–2 nA) were obtained from NR2A-containing coassemblies, whereas currents obtained from NR2B- or NR2D-containing coassemblies were generally smaller (several hundred pA). NR1a/NR2A-mediated

responses were characterized by marked desensitization. NR1a/NR2B also exhibited desensitization, which was most pronounced when rapid solution exchange was used. In contrast, current responses from NR1a/NR2D showed no detectable desensitization. After removal of L-glutamate, deactivation of currents through the NR1a/2D combination was much slower than that with the other combinations.

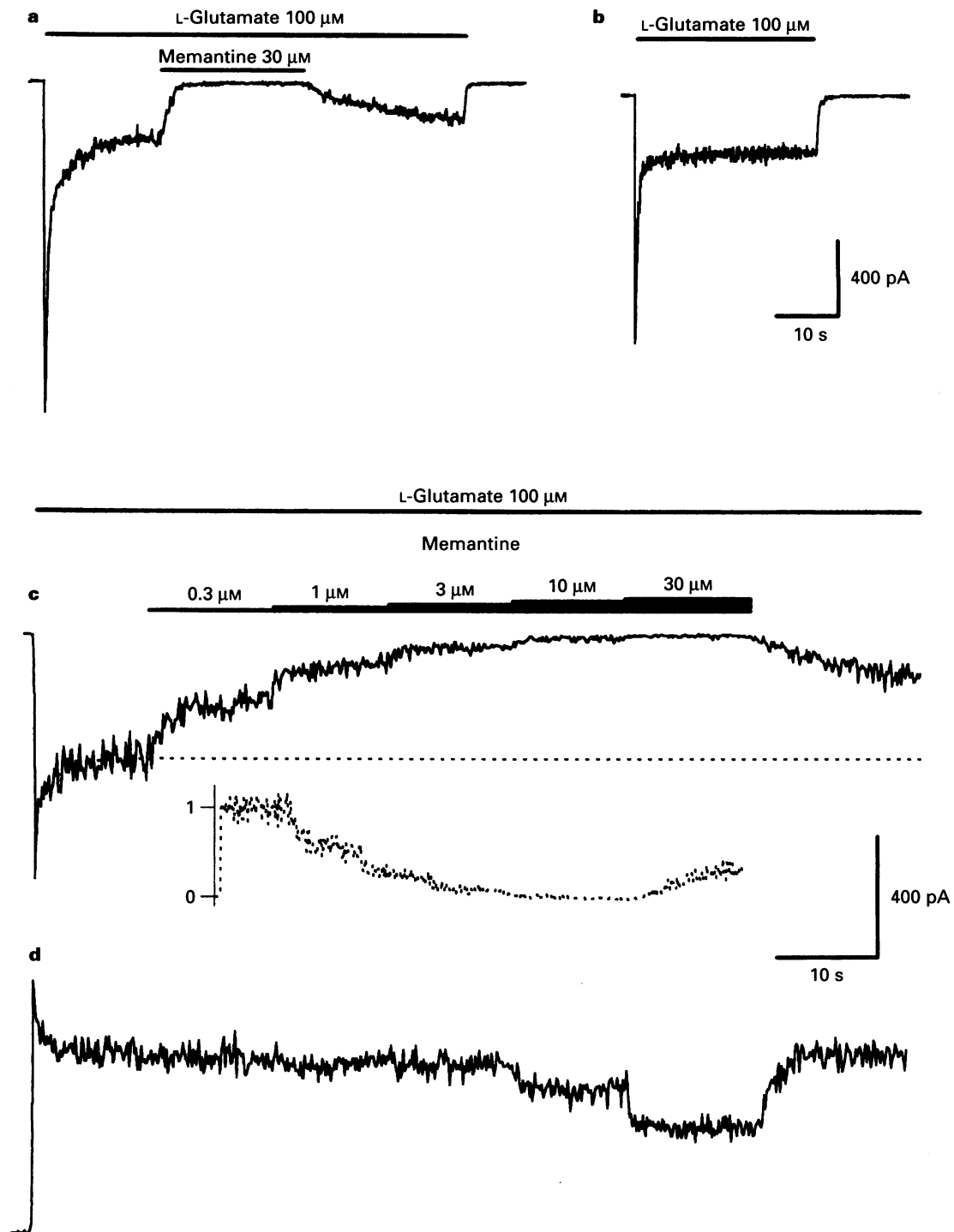


Figure 2 Memantine block of recombinant NMDA receptor currents is concentration- and voltage-dependent. (a) Representative block of 100 μM L-glutamate-mediated current by the application of 30 μM memantine on recombinant NR1a/NR2A receptors expressed in HEK 293 cells at a holding potential of -70 mV. Wash-out of memantine was not complete in this trace; however, repeated applications of L-glutamate restored responses to that of control (b). Peak responses appear different because of the low sampling rate. (c) Representative block of 100 μM L-glutamate-mediated current by the application of increasing memantine concentrations (0.3–30 μM) on recombinant NR1a/NR2B receptors expressed in HEK 293 cells at a holding potential of -70 mV. The inset shows this trace normalised to the fit of two exponentials to the pre-memantine portion of the trace; the time base was compressed to 60%. The IC_{50} for memantine in this cell was calculated to be 0.34 μM. (d) Representative block of L-glutamate mediated current by memantine in the same cell at a holding potential of $+60$ mV. The IC_{50} was 40.8 μM.

Antagonism by memantine

Memantine (0.3–30 μM) antagonized L-glutamate-mediated responses in a concentration-dependent manner (Figure 2) with IC_{50} values (equation 1) of $0.93 \pm 0.15 \mu\text{M}$, $0.82 \pm 0.12 \mu\text{M}$ and $0.47 \pm 0.06 \mu\text{M}$ (at -70 mV), for NR1a/NR2A, NR1a/NR2B, and NR1a/NR2D combinations, respectively (Figure 3).

The effect of memantine was highly voltage-dependent. Increasing the holding potential from -70 mV to $+60 \text{ mV}$ resulted in a shift of the IC_{50} towards higher values on all of the subunit combinations investigated (Figure 3a,b,c). The affinity decreased e-fold per 33 mV, 33 mV and 30 mV in NR1a/2A, NR1a/2B and NR1a/2D coassemblies, respectively (r^2 range of fits: 0.969–0.997) (Figure 3d). Using equation (2), this estimates that memantine experiences 76–83% of the membrane field when it exerts its blocking action.

A statistical comparison of the IC_{50} s of memantine on the different NMDA receptor ion channels revealed that memantine was significantly more potent on NR2D containing

coassemblies at a holding potential of -70 mV as compared to NR1a/2A combinations ($P < 0.05$, one-way ANOVA). At holding potentials of -30 and $+30 \text{ mV}$ memantine blocked L-glutamate-mediated currents to a significantly less extent on NR1a/2A containing receptors as compared to the two other subunit combinations ($P < 0.05$, one-way ANOVA).

Since the action of memantine on each of the subunit coassemblies investigated was similar, a kinetic investigation was performed on just one of the combinations, NR1a/2A. At all holding potentials tested, the time-course of block and recovery was best described by two exponentials. At -70 mV , memantine (30 μM) blocked L-glutamate (100 μM)-mediated currents with time constants of $201 \pm 23 \text{ ms}$ ($81 \pm 2\%$) and $3.9 \pm 0.6 \text{ s}$ (15 determinations in 5 cells). Recovery from block proceeded with time constants of $597 \pm 94 \text{ ms}$ ($18 \pm 1\%$) and $18.6 \pm 2.4 \text{ s}$ (13 determinations in 5 cells) (see Figure 4a,c). Lumping the time constants by their weights into a single exponential, equation (3) predicts $1.76 \mu\text{M}$ as an estimate of the K_d , compared to the measured IC_{50} of $0.93 \pm 0.15 \mu\text{M}$ at

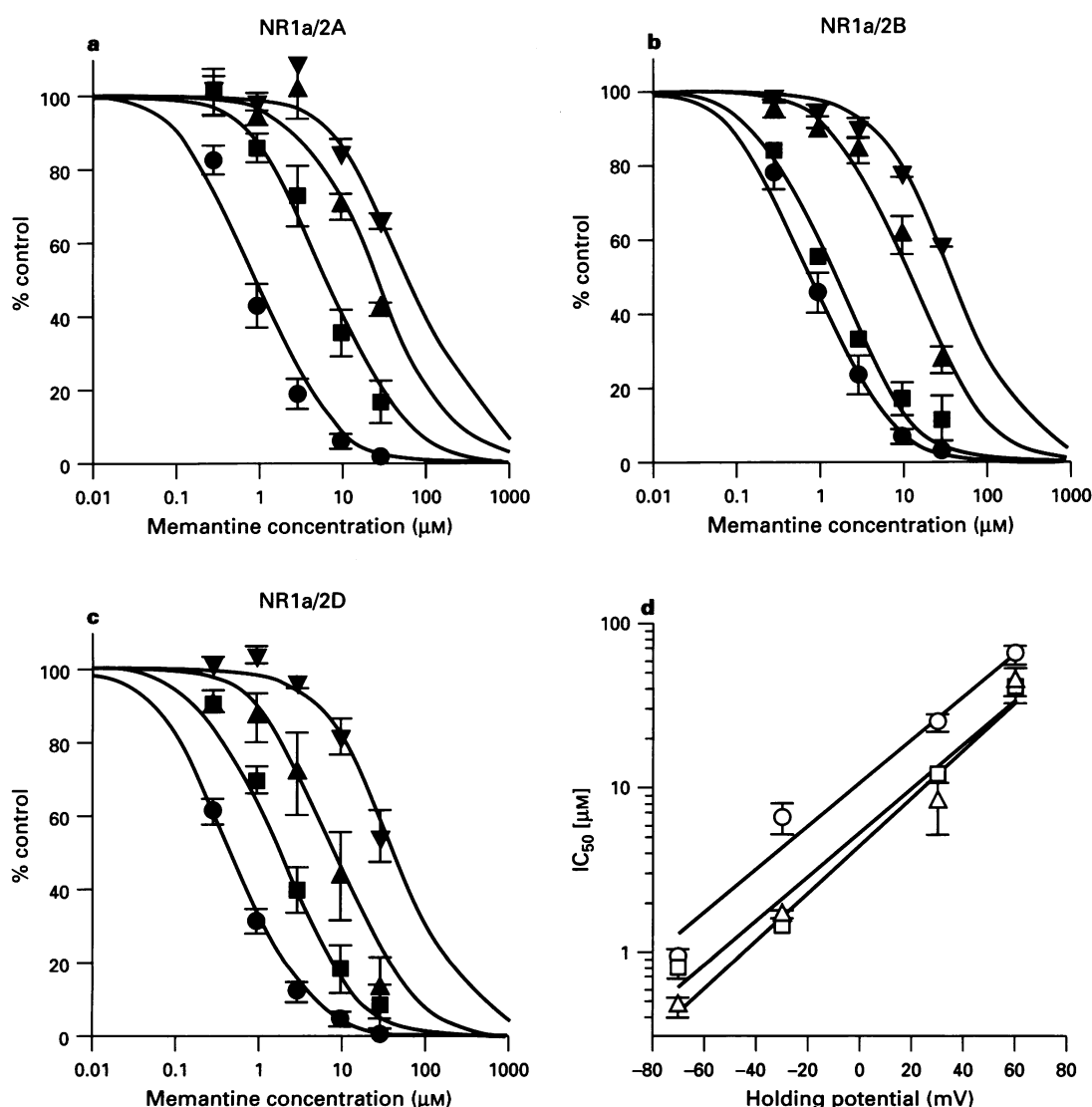


Figure 3 Memantine blocks different subunit coassemblies in a similar concentration- and voltage-dependent manner. Data obtained as in Figure 2 were pooled for each subunit combination at different holding potentials and fitted according to equation (1). Increasing the holding potential resulted in a decrease in affinity of memantine in HEK cells expressing NR1a/2A (a), NR1a/2B (b) or NR1a/2D (c) combinations. Data depict means \pm s.e. mean of at least 4 individual experiments at different holding potentials (\bullet -70 mV ; \blacksquare -30 mV ; \blacktriangle $+30 \text{ mV}$; \blacktriangledown $+60 \text{ mV}$). The respective IC_{50} values (μM) were 0.93 ± 0.15 , 6.7 ± 1.4 , 25.2 ± 3.9 and 64.8 ± 9.4 for NR1a/2A; 0.90 ± 0.11 , 1.5 ± 0.14 , 12.1 ± 1.4 and 40.1 ± 3.3 for NR1a/2B; and 0.47 ± 0.06 , 1.7 ± 0.1 , 8.1 ± 2.9 and 42.4 ± 10.2 for NR1a/2D. The voltage-dependent block of recombinant NMDA receptor by memantine follows a Boltzmann distribution (d). Pooled IC_{50} s \pm s.e. mean are plotted versus holding potential for NR1a/2A (\circ), NR1a/2B (\square), and NR1a/2D (\triangle) coassemblies. Fits of the data to equation (2) imply that memantine interacts with each recombinant channel in a similar way, i.e. at distances 75–82% into the membrane field, but with different apparent affinity, i.e. NR1a/2A < NR1a/2B \approx NR1a/2D.

–70 mV. Similarly, K_d s of 2.36 and 20.0 μM were predicted at –30 mV and +30 mV (compared to 6.7 ± 1.42 and $25.25 \pm 3.92 \mu\text{M}$, respectively). However, this does not account

for the multiexponential nature observed. Using model (B) which accounts for NMDA receptor desensitization, the bi-exponential nature is reproduced with a single state blocked by

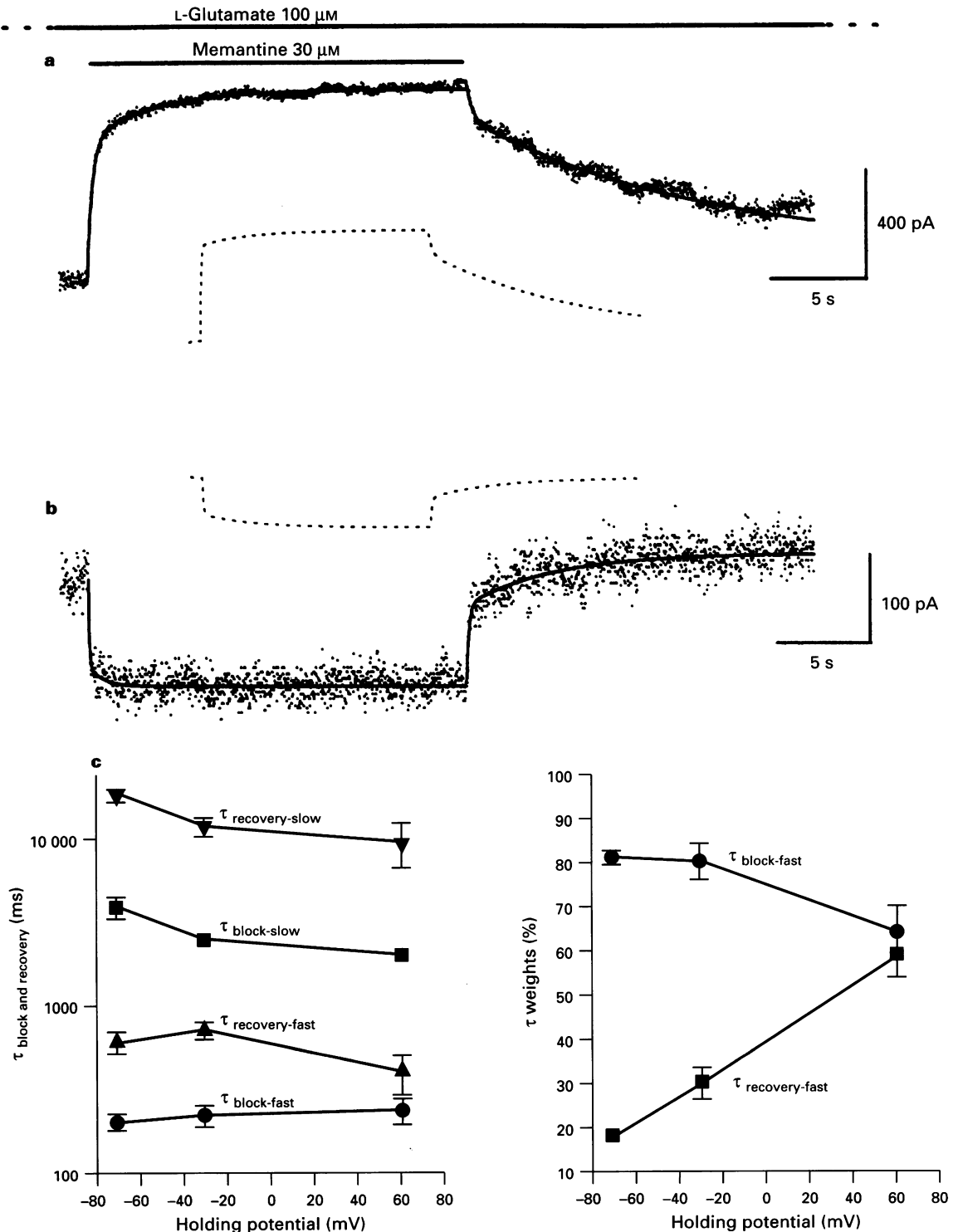


Figure 4 Time course of block by and recovery from memantine. Experiments were performed using the NR1a/2A combination. In (a), NMDA receptor-mediated currents at a holding potential of –70 mV induced by 100 μM L-glutamate are blocked by 30 μM memantine and recover upon its removal. Exponential fits of the time course of block and recovery are superimposed on the currents. The time course of block in this cell was fitted by time constants of 269 ms (72%) and 3.5 s. The time course of recovery was fitted by time constants of 309 ms (24%) and 24.0 s. The dotted trace inset shows results of kinetic model (B) with a blocking rate of $2 \text{ s}^{-1} \mu\text{M}^{-1}$ and a recovery rate of 0.067 s^{-1} . In (b), exponential fits of the time-course of block and recovery are superimposed on the currents recorded at +60 mV. The time course of block in this cell was fitted by time constants of 55 ms (76%) and 779 ms. The time course of recovery in this cell was fitted by time constants of 128 ms (61%) and 4.6 s. Dotted trace inset shows results of kinetic model (C) with a blocking rate of $0.5 \text{ s}^{-1} \mu\text{M}^{-1}$ and a recovery rate of 20 s^{-1} . In (c), pooled data (\pm s.e. mean) from 6 cells (minimum of 4 determinations per point) demonstrates the voltage-dependence of the time course of block and recovery and the relative weights of each exponential.

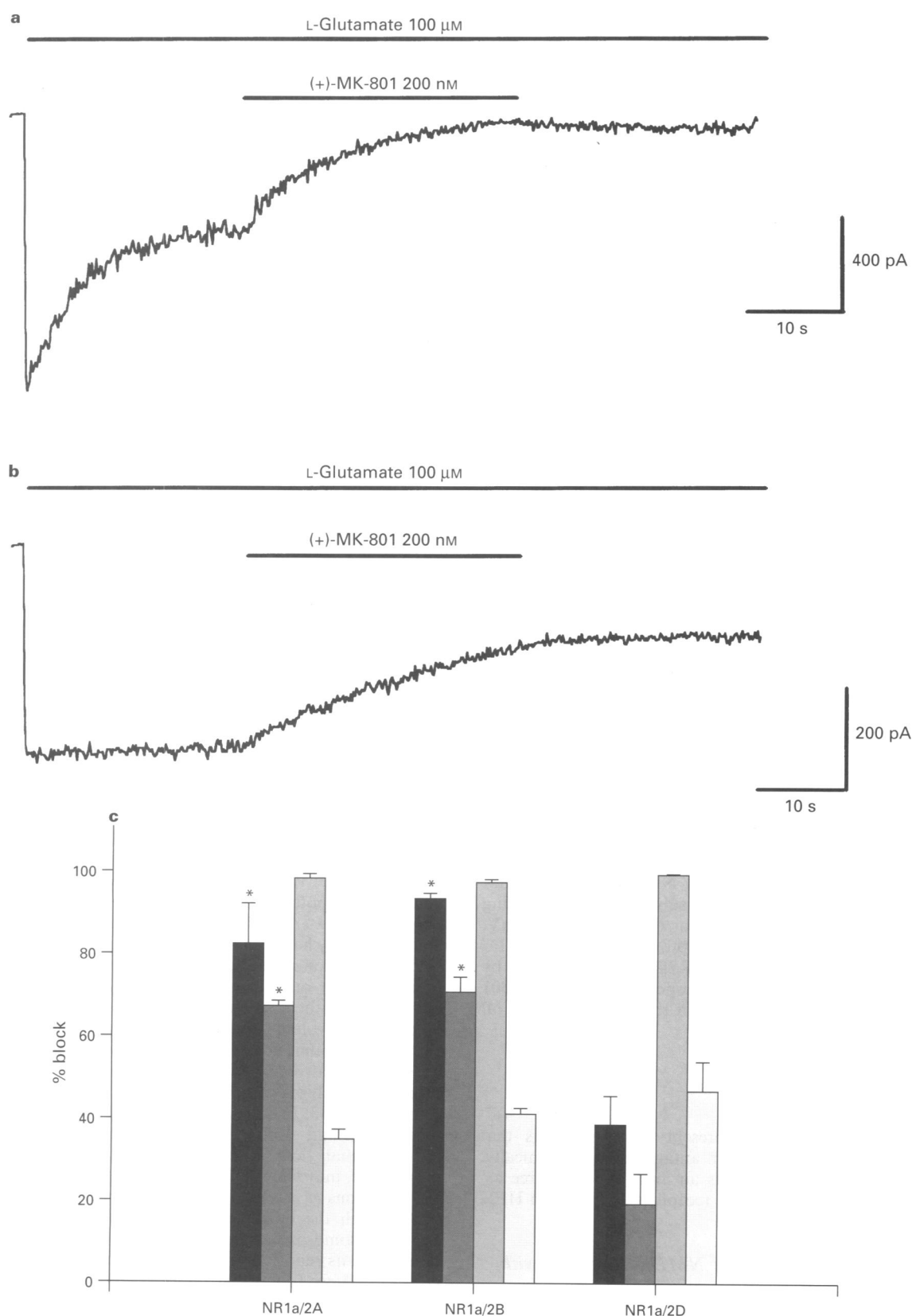


Figure 5 Differences between (+)-MK-801 and memantine. Representative block of 100 μ M L-glutamate-mediated current by the application of 200 nM (+)-MK-801 on (a) NR1a/NR2B and (b) NR1a/NR2D transfected cells. The percentage of block of 200 nM (+)-MK-801 was calculated to be 92% for NR1a/NR2B and 55% for NR1a/NR2D for these respective cells. The currents show the slow on- and offset kinetics characteristic of (+)-MK-801. (c) Block of 100 μ M L-glutamate-mediated whole-cell currents by 200 nM (+)-MK-801 is compared to that for 30 μ M memantine at -70 mV and at +60 mV. (columns, from left to right, (+)-MK-801 at -70 mV; (+)-MK-801 at +60 mV; memantine at -70 mV; memantine at +60 mV). Note that MK-801 is considerably more potent than memantine, it shows less voltage-dependence and greater subunit selectivity. (+)-MK-801 block is significantly stronger on NR2B- and NR2A-containing receptors (* P < 0.05 with respect to NR1a/NR2D transfected cells at the same holding potential). The data shown are the mean \pm s.e. mean, n = 3–5.

memantine (Figure 4a, inset). Equation (3) predicted the K_d to be $1.0 \mu\text{M}$ using the estimated rates into ($r_{\text{block}} = 2 \text{ s}^{-1} \mu\text{M}^{-1}$) and out of ($r_{\text{recovery}} = 0.067 \text{ s}^{-1}$) this state.

At $+60 \text{ mV}$, memantine ($30 \mu\text{M}$) blocked L-glutamate ($100 \mu\text{M}$)-mediated currents with time constants of $235 \pm 42 \text{ ms}$ ($64 \pm 6\%$) and $2.0 \pm 0.2 \text{ s}$ (8 determinations in 5 cells). Recovery from block proceeded with time constants of $399 \pm 108 \text{ ms}$ ($59 \pm 5\%$) and $9.6 \pm 2.9 \text{ s}$ (7 determinations in 5 cells) (see Figure 4b,c). An estimate of the K_d using equation (3) with lumped time constants predicted a low value ($6.23 \mu\text{M}$) compared to the measured IC_{50} ($65 \mu\text{M}$) as a result of the multiexponential time course of block and recovery by memantine observed at this holding potential (similar to what has been reported for the action of ketamine on native NMDA receptors at positive potentials; Mayer *et al.*, 1988). This behaviour could not be predicted by model (B) (data not shown) but could be predicted by model (C) (Figure 4b, inset) in which the NMDA receptor, once it has bound memantine, is allowed to exist in 'closed', 'desensitized' and even 'unbound' states (in which glutamate has unbound), as suggested for MK-801 (Huettnner & Bean, 1988) and ketamine (Mayer *et al.*, 1988). In this model, memantine was allowed to block at a slower rate ($r_{\text{block}} = 0.5 \text{ s}^{-1} \mu\text{M}^{-1}$) but recovered at a rate consistent with diffusion of memantine away from the channel pore ($r_{\text{recovery}} = 20 \text{ s}^{-1}$). Similar rapid rates of recovery from block have been observed for memantine in cultured cortical neurones (Frankiewicz *et al.*, 1996). Model (C) also reasonably predicted the memantine block at -70 mV using a blocking rate of $2 \text{ s}^{-1} \mu\text{M}$ and a recovery rate of 2 s^{-1} (data not shown).

Antagonism by (+)-MK-801

For purposes of comparison to memantine, the channel blocker (+)-MK-801 was investigated with respect to its blockade of L-glutamate-mediated currents on recombinant NR1a/NR2A, NR1a/NR2B and NR1a/NR2D receptors at holding potentials of -70 mV and $+60 \text{ mV}$ (Figure 5). However, since inhibition by (+)-MK-801 reached steady state and recovered very slowly (see below) it was not possible to determine the IC_{50} by serial application of this drug. The experiments were therefore restricted to a fixed (+)-MK-801 concentration of 200 nM . (+)-MK-801 preferentially blocked NMDA receptor currents containing NR2A and NR2B subunits compared to NR2D containing subunits, at both holding potentials investigated (Figure 5). For example, at -70 mV , (+)-MK-801 blocked NMDA receptor currents in NR1a/NR2A, NR1a/NR2B and NR1a/NR2D transfected cells by $82 \pm 10\%$, $93 \pm 2\%$ and $38 \pm 7\%$, respectively. (+)-MK-801 appeared to be less voltage-dependent than memantine on all three receptor combinations.

Discussion

The principal finding of the present investigation is that memantine, an NMDA receptor antagonist used clinically, shows little subunit preference as an antagonist of three assemblies of recombinant NMDA receptors expressed in HEK 293 cells.

Cotransfection of recombinant NMDA receptors with mutant GFP

As has been recently reported by Marshall and colleagues (1995), GFP provides a useful tool for the visualization of transiently transfected HEK 293 cells for the investigation of recombinant NMDA receptors using patch clamp techniques. We have used a modified GFP which is more easily visualized with standard fluorescein epifluorescent optics and is resistant to photobleaching. Membrane currents through the different NMDA receptor subunit coassemblies were very similar to those reported previously using rat cDNAs in HEK 293 cells with regard to time course of desensitization and deactivation

(Monyer *et al.*, 1992; 1994; Köhr *et al.*, 1994). In addition, these membrane currents were similar to those described for human cDNAs in a permanently transfected cell line (Priestley *et al.*, 1995).

Antagonism by memantine

The experimental conditions for testing the effects of memantine on recombinant receptors were chosen for several reasons. The extracellular concentration of calcium was kept at 2 mM in order to mimic physiological conditions. The concentration of glycine was kept at a saturating level of $10 \mu\text{M}$ in order to maximize responses. Similarly, no external magnesium was added to allow maximal responses at negative membrane potentials and to avoid any possible interaction between memantine and magnesium (Chen *et al.*, 1992). These conditions also allow for the direct comparison of the effects of memantine (and (+)-MK-801) on recombinant NMDA receptors with those previously reported for native NMDA receptors.

Whilst the affinity of memantine for the different subunit coassemblies was statistically different at some membrane potentials, the magnitude of this effect was very small. In this respect, memantine is similar to ketamine and SKF-10,047 which showed little selectivity amongst the mouse equivalents of NR1/2A–2D (Yamakura *et al.*, 1993). These findings are in good agreement with receptor binding and autoradiographic studies where memantine was found to inhibit [^3H]-MK-801 binding with similar affinities in distinct brain regions which are enriched in different subtype assemblies (e.g. NR1a and NR2A, ubiquitous; NR2B, hippocampus and cortex; NR2D, cerebellum (Monyer *et al.*, 1992; Bresink *et al.*, 1995; Porter & Greenamyre, 1995).

We were not successful in our attempts to obtain functional NR1a/2C combinations. Although it is possible to generate functional responses with this combination (Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993; Wafford *et al.*, 1993) others have also reported difficulties with these subunits or have presented results which indicate possible lack of cell surface expression of this combination (Chazot *et al.*, 1994; Anegawa *et al.*, 1995).

We chose to use NR1a since it is the most predominant NR1 isoform across several brain regions (Laurie *et al.*, 1995). However, splice variants of NR1 (Sugihara *et al.*, 1992) may influence the potency of uncompetitive NMDA antagonists (Rodriguez-Paz *et al.*, 1995; see also Hollmann *et al.*, 1993). Since our study has been limited to three of numerous possible NMDA receptor subunit combinations, we cannot exclude the possibility that other combinations may display pronounced differences in their sensitivity to memantine. However, the potency and voltage-dependence of memantine for the recombinant subunit coassemblies tested was similar to that reported for native receptors in retinal ganglion, superior collicular and hippocampal neurones (Chen *et al.*, 1992; Parsons *et al.*, 1993; Frankiewicz *et al.*, 1996). In contrast, recent biochemical and electrophysiological studies in striatal tissue indicates that 'low affinity' NMDA antagonists, such as memantine, may show some subtype selectivity (Nankai *et al.*, 1995; Parsons *et al.*, 1996).

Although the blocking and recovery rates of memantine could, at some membrane potentials, be fitted by single exponentials, as reported previously (Chen *et al.*, 1992; Parsons *et al.*, 1993), we found that the rates were better described by two exponentials. We suspect that this is due to the complex gating properties of NMDA receptor channels (Clements & Westbrook, 1991; Lester & Jahr, 1992). Indeed, the blocking action of memantine was best accounted for by a kinetic model which took this into account. Thus, when memantine is removed most NMDA channels will be closed, with memantine trapped. At very positive potentials, memantine is likely to diffuse away quickly from the channel when it next opens. However, at negative potentials it has less tendency to leave and may become trapped again when the channel closes. Therefore the most pronounced effect of depolarization is to

increase the likelihood that memantine will leave during the next channel opening. These data are similar to those of Frankiewicz *et al.* (1996) where the block and relief of blockade of native NMDA receptors, by the application of voltage steps in the continuous presence of memantine, also followed a double exponential time-course.

Comparison to (+)-MK-801

Although we found little difference with memantine, our system was able to detect the subunit selectivity previously reported for (+)-MK-801 in studies using recombinant NMDA receptors in *Xenopus* oocytes (Yamakura *et al.*, 1993) and using [³H]-MK-801 binding in HEK 293 cells (Laurie & Seeburg, 1994). Consistent with this difference, homogenate and autoradiographical binding studies have found that (+)-MK-801 and PCP, unlike memantine, have lower affinities in the cerebellum compared to the cortex (Ebert *et al.*, 1991; Chazot *et al.*, 1994; Bresink *et al.*, 1995; Porter & Greenamyre, 1995). The slow kinetics of block and apparent relatively weak voltage-dependence of MK-801, relative to memantine, are consistent with previous studies (Huettner & Bean, 1988; Parsons *et al.*, 1993; Frankiewicz *et al.*, 1996).

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Clinical implications

It is possible that different selectivity for various NMDA receptor subunit combinations can contribute to the clinical utility of uncompetitive antagonists. In this context, compounds such as (+)-MK-801, that are not well tolerated in man, are more potent on receptor combinations that contain subunits which are enriched in cortex. However, a more important factor in the clinical tolerability of memantine is likely to be its relatively low affinity, rapid kinetics and strong voltage-dependency (Chen *et al.*, 1992; Parsons *et al.*, 1993; Frankiewicz *et al.*, 1996).

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