

Blocking kinetics of memantine on NR1a/2A receptors recorded in inside-out and outside-out patches from *Xenopus* oocytes

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Received: 18 February 2008 / Accepted: 15 June 2008 / Published online: 8 July 2008
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Abstract Previous experiments on primary cultures of hippocampal/cortical neurones revealed that the block and unblock of *N*-Methyl-D-Aspartate (NMDA) receptor channels by memantine showed double exponential kinetics and that the offset kinetics following a voltage-step were much faster than following a concentration jump. There are, however, two major problems when using such cultured primary neurones for these experiments (1) the almost certain expression of heterogeneous NMDA receptor subunits which could underlie double exponential kinetics due to different potencies at receptor subtypes and (2) slow space- and concentration-clamp due to neuronal morphology which could mask even faster kinetics. Therefore, we performed similar experiments with *Xenopus* oocytes exclusively expressing one NMDA receptor type (NR1a/2A) at high levels which allowed recordings from membrane patches with large currents. The use of inside-out patches for voltage-step and outside-out patches in combination with a piezo driven fast application system largely negated potential space- and concentration-clamp problems. Block and unblock of the NMDA receptor by memantine after both voltage jump and concentration jumps showed triple exponential kinetics. The fast onset kinetics of NMDA receptor channel block following both concentration-clamp and voltage jumps from +70 to -70 mV were similar. In contrast, offset kinetics after a voltage-step from -70 to +70 mV were much faster than following a concentration jump at the holding potential of -70 mV.

These results provide further support for the hypothesis that rapid relief of block via strong synaptic membrane depolarisation underlies the good therapeutic profile of memantine.

Keywords Memantine · *N*-Methyl-D-Aspartate (NMDA) · Patch clamp · Inside-out · Outside-out · *Xenopus* oocytes · NR1a/2A · Uncompetitive antagonist · Kinetics · Voltage-dependency

Introduction

Memantine (1-amino-3,5-dimethyladamantane) is a moderate affinity, uncompetitive, agonist activation-dependent, *N*-Methyl-D-Aspartate (NMDA) receptor open channel blocker (Chen et al. 1992; Kornhuber et al. 1991; Kornhuber et al. 1989; Parsons et al. 1993; Rogawski 1993) which is registered in both Europe and the USA for the treatment of Alzheimer's disease (Buéno et al. 2007; Danysz and Parsons 2003; Danysz et al. 2000; Doody et al. 2005; McShane et al. 2006; Parsons et al. 1999b). In this regard, it should be noted that memantine shows agonist concentration-dependency of blocking kinetics but not equilibrium block of NMDA receptors (Gilling et al. 2007).

Memantine is very well tolerated clinically (e.g., Peskind et al. 2006; Reisberg et al. 2003; Tariot et al. 2004), and this property has been attributed to its moderate affinity and associated strong voltage-dependency and rapid, open-channel unblocking kinetics (Parsons et al. 1993). These properties have been proposed to allow memantine to block the tonic, mild pathological activation of NMDA receptors in Alzheimer's disease whilst leaving their transient, strong physiological activation relatively intact (Li et al. 2007; Parsons et al. 1993, 1995, 1999b).

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Memantine is known to block and unblock open NMDA receptor channels with at least double exponential kinetics (Blanpied et al. 1997; Bresink et al. 1996; Frankiewicz et al. 1996; Sobolevsky and Koshelev 1998; Sobolevsky et al. 1998). The speed and weight of the fast component of block increases with memantine concentration. In contrast, the speed of fast unblock remains relatively constant but its weight (relative to the slow component) decreases with memantine concentration (see references above). Most importantly, the predominant effect of depolarization is to dramatically increase the weight of the faster recovery time constant (Bresink et al. 1996; Frankiewicz et al. 1996; Parsons et al. 1998). These data indicate that memantine binds to at least two sites within the channel (Sobolevsky et al. 1998).

However, although double exponential kinetics might indicate two binding processes to one and the same receptor, they could also be due to different effects/potencies on two or more receptor subtypes in a heterogeneous receptor population expressed by native CNS neurons. Furthermore, it is conceivable that the blocking kinetics of memantine following activation by the full endogenous agonist L-glutamate could be different to those in the presence of the synthetic, but selective, partial agonist NMDA (Laube et al. 2004) as the kinetics of blockade and unblockade seem to depend on agonist concentrations/the level of receptor activation (Gilling et al. 2007).

In some whole cell patch clamp recordings from cultured, native CNS neurones, memantine (Parsons et al. 1998), and closely related channel blockers (Parsons et al. 1999a) have previously been reported to produce a moderate potentiation of outward currents in response to NMDA at +70 mV. Similar effects have also been reported for the physiological open channel blocker Mg^{2+} (Wang and Macdonald 1995) and also for the uncompetitive antagonist ketamine, although in this case, only in a meeting abstract form (Wang et al. 1994). The functional potentiation sometimes seen with memantine at positive potentials is very unlikely to be mediated via a channel blocking site and would therefore predict that another site for memantine might exist on the extracellular surface and/or close to the channel mouth, that is not the same as the superficial channel blocking site that could be responsible for partial untrapping blockade by memantine (Blanpied et al. 1997, 2005). Indeed, the effects of ketamine and Mg^{2+} reported by (Wang and Macdonald 1995) were attributed to an enhancement of glycine affinity, which might also indicate specific interactions of these compounds with an external binding site on NR1 receptor subunits. If this were the case, then it might be postulated that reblock from such an extracellular “holding” site following repolarization in the continuous presence of

memantine could be faster than following concentration-clamp of the same memantine concentration.

There are several caveats to this idea, such as the ability of such a higher affinity holding site to free memantine for block of a lower affinity channel blocking site with faster kinetics. Nonetheless, we wished to test this hypothesis by comparing the blocking/unblocking kinetics of memantine at NR1a/NR2A receptors expressed in *Xenopus* oocytes using concentration-clamp application (outside-out patches), and following relief of block by depolarizing steps (inside-out patches). The use of outside-out patches allowed very fast concentration-clamp estimated to be around 300 μ s tau for exchange times using the same system for outside-out patches from cultured hippocampal neurones (Rammes et al. 1998). This is considerably faster than the exchange times that can be achieved for lifted HEK-293 cells in whole cell mode which normally are not faster than 10 ms. Thus, these experiments were designed to resolve potential components of memantine open channel blocking/unblocking kinetics that might be too fast to be detected in whole cell recording mode. *Xenopus* oocytes were chosen as they allow a very high receptor expression density to increase the size of the current recorded in patches to levels which allow accurate fitting of the kinetics of blockade/unblockade.

Methods

Transfections

The cRNAs encoding the NR1a and NR2A subunits were a generous gift of Prof. J.P. Ruppertsberg, University of Tübingen, Germany. Mature female *Xenopus laevis* were anaesthetized in 0.2% tricaine on ice for 15 min prior to surgery. Oocytes were removed and incubated in 2 mg/ml collagenase (type II) in Ca^{2+} -free oocyte Ringer solution (82.5 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.5) for 30 min at room temperature and washed thoroughly with OR-2 (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.5). The remaining follicle cell layer was removed manually with fine forceps and the oocytes were kept in OR-2. The cRNA was dissolved in DEPC-treated, sterile distilled water. The NR1a subunit was mixed 1:1 with NR2A cRNA and was diluted tenfold to a final concentration of approx. 1 mg/ml. Fifty nanoliters of this cRNA mixture was injected in the oocyte's cytoplasm using a Nanoliter Injector (World Precision Instruments, Berlin, Germany). The oocytes were incubated at 19°C in OR-2 for the following 3–6 days.

One would assume with the injection of NR1a and NR2A that the receptors are tetrameric assemblies of 2*NR1a and 2*NR2A subunits. Neither subunit formed

functional receptors alone when expressed alone—see also Green et al. (2002), Schmidt et al. (2006).

Patch clamp

Outside-out and inside-out patch clamp recordings were made from transfected oocytes with polished quartz glass electrodes (0.5–1 m Ω) at room temperature (20–22°C) and a holding potential of –70 mV using an EPC-9 amplifier and the Macintosh program Pulse v 7.21. Fast capacitive currents were corrected for by the EPC-9 upon formation of the gigaseal, and membrane patch capacitance and series resistance correction was performed after the inside- or outside-out patches were obtained. These procedures were performed semi-automatically using the EPC-9 amplifier software. The current signal was filtered by the EPC-9 amplifier using the 3-pole pre-filter with Bessel 10 kHz bandwidth and the 4-pole filter set to 2.9 kHz with Bessel characteristic. Current measurements were acquired at a rate of 10 kHz to avoid potential problems of aliasing.

For outside-out recordings, the intracellular pipette solution was as follows: 100 mM KCl; 10 mM HEPES; 10 mM BAPTA, pH 7.2. The extracellular “bath” solution was as follows: 100 mM NaCl, 2 mM KCl, 5 mM HEPES, 2 mM CaCl₂, pH 7.35. Rapid concentration-clamp was achieved using a custom designed piezo application system which completely switched channels within 100 μ s, assessed measuring junction currents of open recording pipettes switching between 100 and 340 Osm solutions. Application times were somewhat slower for outside-out patches (onset 250–300 μ s offset 150–200 μ s), see Rammes et al. (1998). Modification of this system allowed the contents of each side of the application pipette to be completely exchanged within 2–5 s depending on the speed of flow of solutions. The dead space of the application pipette was minimized by sliding polyethylene tubing (PE-10, 15 cm, Clay Adams) to the tip and then filling with molten dental wax almost to the tip. The dead space of the custom designed manifold connecting different solutions to the PE10 tubing was also minimized such that the total dead space of the system was 15–30 μ l. Both the piezo applicator and valves switching solutions (LFAA1201718H, Lee, Westbrook, CT, USA) into the theta glass pipette were controlled automatically by the Macintosh program Pulse v. 7.21 (Heka, Lambrecht, Germany).

For inside-out recordings, the “extracellular” pipette solution was as follows: 100 mM NaCl, 2 mM KCl, 5 mM HEPES, 2 mM CaCl₂, 100 μ M glutamate, 10 μ M glycine and memantine 10 μ M, pH 7.35. Additionally, this solution contained 10 μ M nifedipine, 1 μ M ω -conotoxin GVIA and 1 μ M tetrodotoxin to block voltage-gated calcium and sodium channels. Control experiments

confirmed that such channel activation did not contaminate currents under the recording conditions used. The intracellular “bath” solution was as follows: 100 mM KCl, 10 mM HEPES, 10 mM BAPTA, pH 7.2. NB: NMDA receptors in these inside-out patches were continuously stimulated by glutamate (100 μ M) but currents at the resting membrane potential of –70 mV were nearly completely blocked by memantine (10 μ M) in the extracellular that is patch pipette solution. Relief from memantine blockade at –70 mV was achieved by clamping the patch to +70 mV for 4 s of every 30 s. During such depolarizations, a very fast portion of the current relaxation should be due to the rapid change in driving force. The amplitude should be about twice the residual NMDA receptor current at –70 mV (since a 140 mV jump was used), which was probably very small, but could still have accounted for a relevant fraction of the fastest component. This component of the relaxation, which is not due to memantine unblock, was taken into consideration during the fitting process by assuming that memantine 10 μ M blocked only to $\leq 5\%$ of control currents in the inside-out patch experiments and not including this portion of the trace to generate the exponential fit.

For both inside-out and outside-out recordings, to subtract any residual capacitive currents despite EPC-9 compensation, mirror voltage-clamp (P5) protocols with smaller (20%) voltage-steps in the opposite direction were run between agonist/antagonist applications or voltage-step relief of antagonist blockade for example, the equivalent for a step from –70 to +70 mV (difference of +140 mV) was –70 to –98 mV (difference of –28 mV). These “opposing” capacitive currents were then multiplied by a factor of 5 and then “added” to the recorded receptor-mediated currents before fitting the data. A necessary assumption for this compensation procedure was that any receptor-mediated current at –70 mV in the inside-out patch experiments was nearly completely blocked by memantine. Such 100% block was almost certainly not really achieved, but the purpose of these experiments was to determine the kinetics of block/unblock, not the magnitude of the memantine blockade.

Results

In the present study, the use of inside-out and outside-out patches from *Xenopus* oocytes expressing NR1a/2A receptors and a fast piezo application system revealed that memantine has triple exponential blocking and unblocking kinetics (Figs. 1 and 2, Table 1). Visual inspection, comparison of the SDs of individual fits and *F*-statistics indicated that triple exponentials were always superior to double exponentials.

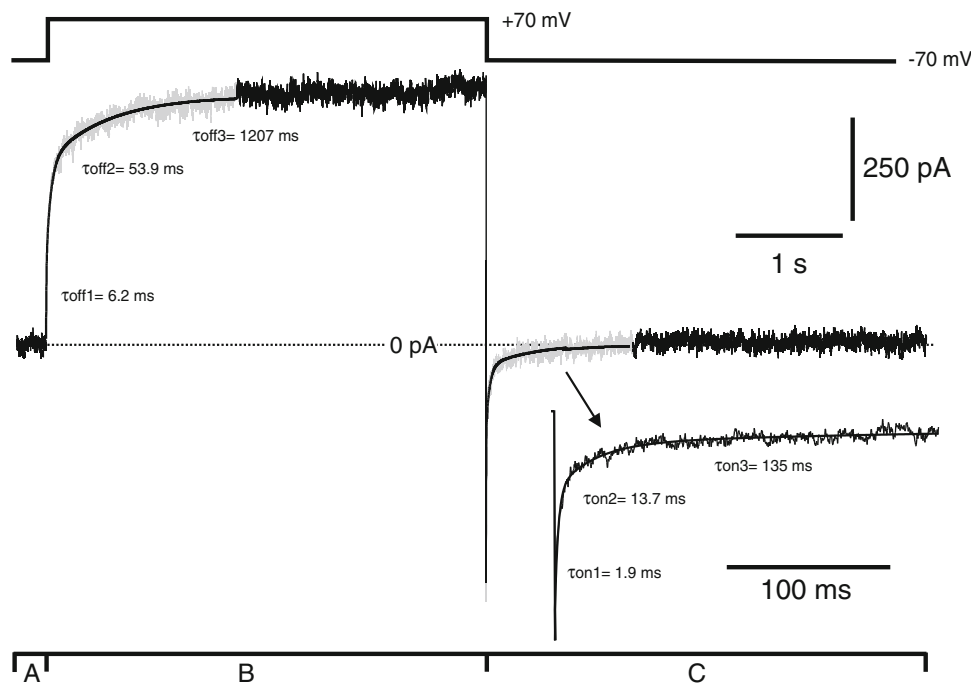


Fig. 1 Kinetics of unblockade/blockade of memantine following voltage jumps using inside-out patches from *Xenopus* oocytes expressing NR1a/2A receptors. NMDA receptors in these inside-out patches were continuously stimulated by glutamate (100 μ M) but currents at the resting membrane potential of -70 mV were nearly completely blocked by memantine (10 μ M) in the extracellular that is, patch pipette solution. Relief from memantine blockade was achieved by clamping the patch to $+70$ mV for 4 s of every 30 s. The trace illustrated is an average of ten such recordings from the same patch. Grey areas represent the portion of the trace used for triple exponential fits, which are shown overlaid in black. Reblock following the return to -70 mV was so fast that the relevant fitted portion of the trace has been expanded in the insert-note different time

scales. Triple exponential kinetics is documented next to these fits. The time segments detailed in the lower bar can be described as follows: **a** memantine steady-state block; **b** memantine unblock following the voltage-step to $+70$ mV; **c** memantine reblock following the voltage-step back to -70 mV. The dotted line represents the 0 pA current level and it is clear that (1) the memantine resting block level was close to this and (2) the maximal unblocked current at $+70$ mV was of very similar magnitude to the peak current measured upon rapidly clamping back to -70 mV, albeit of the opposite polarity. NB: it was not the purpose of these experiments to determine the magnitude of steady-state memantine blockade, but rather the kinetics. For averaged kinetic analysis see Table 1

Inside-out patches

For inside-out patches in the continuous presence of memantine (10 μ M) voltage-steps from -70 mV to $+70$ mV for 4 s. caused rapid relief from blockade with triple exponential kinetics: $\tau_{\text{off}1} = 6.9$ (weight 28.7%), $\tau_{\text{off}2} = 84.8$ (weight 34.6%), $\tau_{\text{off}3} = 1,623$ ms (weight 36.7%). Reblock by memantine was even more rapid following return of the membrane potential to -70 mV and the fastest component had the largest weight: $\tau_{\text{on}1} = 2.8$ (weight 45.6%), $\tau_{\text{on}2} = 25.6$ (weight 36.4%), $\tau_{\text{on}3} = 998$ ms (weight 18.0%) (Fig. 1, Table 1).

Outside-out patches

In recordings from outside-out patches, memantine (10 μ M) applied for 4 s at a holding potential of -70 mV also blocked glutamate-induced currents with triple exponential kinetics and the fastest component again had the largest weight: $\tau_{\text{on}1} = 3.5$ (weight 55.8%), $\tau_{\text{on}2} = 52.8$ (weight 19.5%), $\tau_{\text{on}3} =$

1032 ms (weight 24.7%). Relief of block following removal of memantine again showed triple exponential kinetics, but in this case the slowest component had the largest weight $\tau_{\text{off}1} = 36.1$ (weight 12.3%), $\tau_{\text{off}2} = 348.3$ (weight 30.3%), $\tau_{\text{off}3} = 1343$ ms (weight 57.4%) (Fig. 2, Table 1).

It should be noted, that the relative lack of residual capacitive artefacts following voltage-steps from -70 mV to 0 mV following wash-out of memantine in the outside-out patch clamp experiments (Fig. 2) seems to confirm that the measures taken to block voltage-activated channels and compensate for possible capacitive artefacts were sufficient.

Comparison

For onset kinetics, the magnitude of the fastest component was the largest for both concentration-clamp (Fig. 1) and following return to -70 mV from a depolarizing pulse (Fig. 2) and the very fast time constants detected would most likely not have been resolvable with previous concentration-clamp experiments in the whole cell mode.

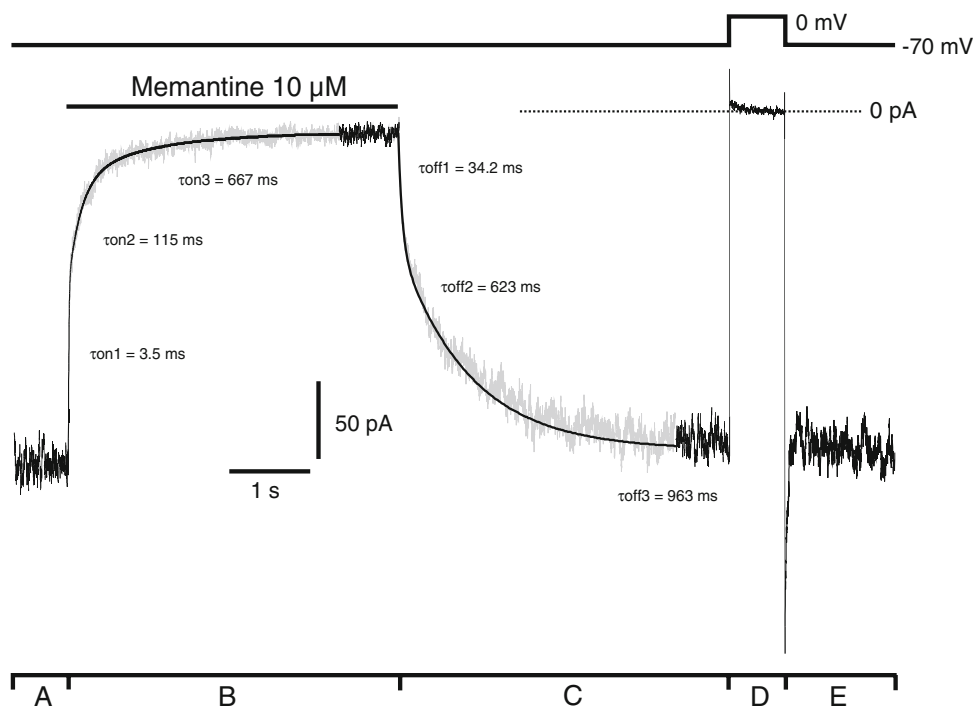


Fig. 2 Kinetics of blockade/unblockade of memantine following concentration jumps using outside-out patches from *Xenopus* oocytes expressing NR1a/2A receptors. Glutamate (100 μM) was applied for 12 s of every 60 s at -70 mV in the continuous presence of glycine (10 μM). Memantine (10 μM) was applied for 4 s, as indicated by the bar. Four seconds following the removal of memantine, patches were clamped to 0 mV for 750 ms in the continuing presence glutamate to facilitate complete recovery from antagonism. The trace illustrated is an average of ten such recordings from the same patch. Grey areas represent the portion of the trace used for triple exponential fits,

which are shown overlaid in *black*. Triple exponential kinetics is documented next to these fits. The time segments detailed in the lower bar can be described as follows: **a** maximal L-glutamate induced control current; **b** memantine block; **c** memantine unblock; **d** voltage-step to 0 mV to facilitate complete recovery from antagonism; **e** maximal L-glutamate induced recovery current. The dotted line represents the 0 pA current level and it is clear that the memantine resting block level was close to this. For averaged kinetic analysis see Table 1

Table 1 Comparison of the kinetics of blockade by memantine in concentration-clamp and voltage-step experiments in membrane patches from *Xenopus* oocytes. These results are the average from 10 inside-out and 12 outside-out patches. Conc. refers to results from concentration-clamp experiments in outside-out patches. Volt. refers to results from voltage-step experiments in inside-out patches. Tau 1, 2 and 3 refer to the fastest, middle and slowest components of triple exponential fits respectively

Kinetic	Conc. τ_{on}		Weight (%)	Volt τ_{on}		Weight (%)
	Mean	SEM		Mean	SEM	
Tau 1 (ms)	3.5	0.3	55.8	2.8	0.5	45.6
Tau 2 (ms)	52.8	9.8	19.5	25.6	6.4	36.4
Tau 3 (ms)	1032.0	293.0	24.7	998.0	506.0	18.0
Kinetic	Conc. τ_{off}		Weight (%)	Volt τ_{off}		Weight (%)
	Mean	SEM		Mean	SEM	
Tau 1 (ms)	36.1	8.0	12.3	6.9	1.1	28.7
Tau 2 (ms)	348.3	78.6	30.3	84.8	22.8	34.6
Tau 3 (ms)	1343.0	472.0	57.4	1623.0	380.0	36.7

The onset of blockade by memantine following concentration jumps whilst recording from outside-out patches was similar to that following voltage jumps giving no support for the existence of an extracellular “holding” site for memantine (Figs. 1 and 2, Table 1).

In contrast, offset kinetics following washout of memantine at -70 mV were much slower than following voltage jumps to +70 mV in the continuous presence of memantine (Figs. 1 and 2, Table 1). Moreover, depolarization increased the weight of the fastest component of unblockade. Thus, kinetics of unblock by memantine are faster under conditions more relevant for the therapeutic situation following synaptic depolarization in the continuous presence of memantine (Figs. 1 and 2, Table 1).

Discussion

In the present study, the use of inside-out and outside-out patches from *Xenopus* oocytes expressing NR1a/2A receptors and a very fast piezo application system revealed

that memantine has triple exponential blocking and unblocking kinetics. Regarding the onset kinetics, the magnitude of the fastest component was the largest and the very fast time constants detected following concentration-clamp (fastest $\tau_{\text{on}} = 3.5$ ms, weight 55.8%) would not have been resolvable with previous experiments in whole cell mode. For the offset kinetics, the magnitude of the fastest component following concentration-clamp was the smallest and the fastest time constants determined should also have been resolvable in previous experiments in whole cell mode—the fastest τ_{off} following concentration-clamp was 36.1 ms. Following voltage-steps, this value was faster i.e., 6.9 ms, but this should also not represent a real technical limitation in whole cell mode from well clamped cells.

In general, the pattern of changes in kinetics was very similar in the present and previous experiments using similar concentrations of memantine (Blanpied et al. 1997; Bresink et al. 1996; Frankiewicz et al. 1996; Sobolevsky and Koshelev 1998; Sobolevsky et al. 1998). For example, offset kinetics following washout of memantine at -70 mV were slower than those following the voltage jumps to $+70$ mV in the continuous presence of memantine (see reference above). In agreement with the literature, depolarization increased the weight of the fastest component of unblockade. Thus, the kinetics of unblock by memantine are faster under conditions more relevant for the therapeutic situation i.e., following synaptic depolarization in the continuous presence of memantine (Parsons et al. 1993). These observations agree with those previously published for whole cell recordings for both native receptors in cultured hippocampal neurons and NR1a/2A receptors expressed in HEK-293 cells (Blanpied et al. 1997; Bresink et al. 1996; Frankiewicz et al. 1996; Sobolevsky and Koshelev 1998; Sobolevsky et al. 1998).

The voltage-dependent, rapid unblocking kinetics of memantine have previously been proposed to allow it to block the tonic, mild pathological activation of NMDA receptors in Alzheimer's disease whilst leaving their transient strong physiological activation relatively intact. These properties, in turn, have been proposed to account for the good tolerability (Parsons et al. 1993, 1995, 1999b), and both the symptomatic and neuroprotective effects of memantine in the treatment of Alzheimer's disease (Danysz and Parsons 2003; Li et al. 2007; Parsons et al. 1999b).

An additional aspect of the present experiments was to assess the possibility of the existence of an extracellular "holding" site for memantine (Parsons et al. 1998, 1999a; Wang and Macdonald 1995; Wang et al. 1994). The hypothesis was that, if such an external holding site exists, it might offer the opportunity to develop antagonists with high affinity for this site but moderate affinity for the channel site. The logic behind these experiments was that

reblock following repolarization in the continuous presence of memantine could be faster than following concentration-clamp of the same memantine concentration if there is an external site "holding" memantine near to the channel blocking domain. However, this did not seem to be the case. There was very little difference in the triple exponential onset kinetics under the two experimental conditions and the fastest and largest component of onset blocking kinetics was almost identical. As such, if there is an extracellular binding site for memantine on the NMDA receptor, then this does not serve as an extracellular "holding" site with characteristics that could be exploited to design drugs with even better channel blocking properties. At least, this seems to be the case for NR1a/2A receptors heterologously expressed in *Xenopus* oocytes, but different effects on NR1a/2B receptors cannot be excluded and the previous observations of the potentiation of outside-out currents were made in cultures of native cortical/hippocampal neurones that also express NR2B subunits (Parsons et al. 1998, 1999a).

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