Agonist concentration dependency of blocking kinetics but not equilibrium block of \(N\)-methyl-\(\text{D}\)-aspartate receptors by memantine

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Abstract

Memantine is an uncompetitive \(N\)-methyl-\(\text{D}\)-aspartate (NMDA) receptor antagonist which is registered in both Europe and the USA for the treatment of Alzheimer’s disease (AD). Cultured rat hippocampal neurons were used to evaluate the potency and blocking kinetics of this therapeutically very well-tolerated agent in the presence of various concentrations of the synthetic agonist NMDA and a constant, saturating concentration of the co-agonist D-serine (10 \(\text{M}\)). Whole-cell patch-clamp experiments at \(-70\) mV revealed that the degree of “equilibrium” blockade of NMDA-induced currents by memantine was largely unaffected by the concentration of the agonist NMDA. The IC\(_{50}\) values for NMDA at 300, 100, 30 and 10 \(\text{M}\) were \(0.80 \pm 0.12\), \(1.01 \pm 0.08\), \(0.92 \pm 0.13\) and \(1.31 \pm 0.09\) \(\text{M}\), respectively, giving an average IC\(_{50}\) for all agonists concentrations tested of \(1.01 \pm 0.11\) \(\text{M}\). In contrast, and as expected, the onset and offset kinetics of blockade were clearly dependent on agonist concentration. For NMDA 300, 100, 30 and 10 \(\text{M}\), \(k_{\text{on}}\) values were \(10.55 \pm 1.41\), \(8.60 \pm 0.17\), \(4.90 \pm 0.20\) and \(3.22 \pm 0.08 \times 10^8 \text{M}^{-1} \text{s}^{-1}\), respectively; \(1/t_{\text{on}}\) values at the IC\(_{50}\) concentration of memantine—i.e. 1 \(\text{M}\)—were \(0.58 \pm 0.11\), \(0.28 \pm 0.05\), \(0.15 \pm 0.02\) and \(0.11 \pm 0.03 \text{s}^{-1}\), respectively and \(k_{\text{off}}\) values were \(0.24 \pm 0.01\), \(0.19 \pm 0.01\), \(0.14 \pm 0.00\) and \(0.09 \pm 0.01 \text{s}^{-1}\), respectively. It therefore appears that the kinetics, but not the equilibrium potency, of memantine are agonist concentration-dependent. These fast agonist concentration-dependent kinetic properties, in addition to the clear voltage-dependence of memantine, are proposed to be important for the therapeutic tolerability of this compound in the treatment of AD.

Keywords: Memantine (1-Amino-3,5-dimethyladamantane); \(N\)-methyl-\(\text{D}\)-aspartate (NMDA); Patch clamp; Agonist concentration dependency; Potency; Kinetics; Hippocampal neurons

1. Introduction

Memantine (1-amino-3,5-dimethyladamantane) is a moderate affinity, uncompetitive, i.e. agonist use-dependent, \(N\)-methyl-\(\text{D}\)-aspartate (NMDA) receptor open channel blocker (Kornhuber et al., 1989; Bormann, 1989; Kornhuber et al., 1991; Chen et al., 1992; Rogawski, 1993; Parsons et al., 1993) which is registered in both Europe and the USA for the treatment of Alzheimer’s disease (AD) (Parsons et al., 1999; Danysz and Parsons, 2003; Danysz et al., 2000; Doody et al., 2005; Wenk et al., 2006; McShane et al., 2006). Memantine is very well tolerated clinically e.g. (Reisberg et al., 2003; Tariot et al., 2004; Bakchine et al., 2005; Peskind et al., 2006) 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 AD whilst leaving their transient strong physiological activation relatively intact (Parsons et al., 1993; Parsons et al., 1995, 1999). It has also been suggested that agonist concentration-dependency of memantine “equilibrium” antagonism could be an important biophysical property that is different to other NMDA receptor channel blockers and that this property contributes to the favourable therapeutic profile of memantine in the treatment of AD (Chen et al., 1992;
Lipton, 2004, 2006, 2007). However, it is possible that the initial experiments (Chen et al., 1992) discussed at length to support this theory e.g. (Lipton, 2007) were confounded by technical problems relating to lack of achievement of true equilibrium blockade at low agonist concentrations. Furthermore, these experiments were performed using cultured retinal ganglion cells and not central nervous system neurons known to be involved in the pathophysiology of AD.

The aim of the present study was to test whether the true equilibrium blocking potency of memantine is indeed increased in the presence of higher compared to lower agonist concentrations — in this case we also used the synthetic, selective agonist NMDA. Longer application times were used to try to ensure equilibrium block at low agonist/antagonist concentrations in order to adjust for (1) reduced open probability of NMDA receptors activated by lower concentrations of agonist and (2) slower open channel block at low antagonist concentrations due to the law of mass action. In addition, rat cultured hippocampal neurons were used. We also directly tested the hypothesis that the kinetics of uncompetitive channel block/unblock should indeed be dependent on agonist concentration as there seems to be little doubt in the literature that memantine is indeed an uncompetitive NMDA receptor antagonist—see (Parsons et al., 1999).

2. Methods

2.1. Preparation of hippocampal cultures

Commercially available cryopreserved rat (E18/19) hippocampal neurons (order code R-HI-501, Cambrex Biosciences, Belgium) were used in this investigation. Cells were defrosted according to the suppliers directions, and plated at a density of approximately 2.5 x 10^4 cells cm^-2 onto poly-DL-ornithine/laminin precoated plastic Petri dishes. We wished to culture the cells in the same medium which we use for classically prepared primary hippocampal cultures, so that the medium could not affect comparisons between data. However, the cells could not be initially seeded in this medium after defrosting, so the culturing steps were performed in such a way that the medium was gradually switched. The cells were initially nourished with 2% B27 Neurobasal medium which we use for classically prepared primary hippocampal cultures and was adjusted to pH 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), CaCl2 (0.2), glucose (10), ATP (2), cAMP (0.25), and was adjusted to pH 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), CaCl2 (0.2), glucose (10), HEPES (10), sucrose (4.5) and was adjusted to pH 7.35 using NaOH or HCl. Test substances were added to this basic solution at the concentrations detailed in results. D-serine (10 μM) was present in all solutions. Only results from stable cells were included in the final analysis i.e. those showing at least 75% recovery of the responses to NMDA following removal of memantine. Despite this, the recovery from drug actions was almost never 100% due to receptor rundown. This was compensated by basing the % of antagonism at each concentration on both control and recovery and assuming a linear time course for this rundown. All compounds were obtained from Sigma unless otherwise noted. Memantine was synthesised by Merz Pharmaceuticals GmbH.

2.3. Analysis and statistics

IC₅₀ values were calculated according to the four parameter logistic equation using the GraFit computer program (Erithacus Software Ltd., UK). Results are expressed as mean ± S.E.M.

Exponential fits were made using the program TIDA for windows. Most responses were much better fit by a double exponential. However, it should be stressed that such double exponential fits were sometimes not clearly better than single exponential fits, in particular for lower concentrations of agonist/antagonist. As such, we undertook the following normalizing procedure in order to be able to compare kinetic data at different agonist/antagonist concentrations. All “superior” double exponential fits were integrated to single exponentials according to the following relationship \[ ((t_{fast} \times weight_{fast}) + (t_{slow} \times weight_{slow})) / (weight_{fast} + weight_{slow}) \].

3. Results

Memantine was applied between 0.3 and 30 μM to antagonize currents evoked by 300, 100, 30, and 10 μM NMDA (Figs. 1 and 2). The IC₅₀ value of blockade was calculated for each agonist concentration (Fig. 2, Table 1) and it is clear from these data that there was no real trend towards memantine having higher equilibrium potency when higher concentrations of NMDA were used, unlike reported in a previous study (Chen et al., 1992). The IC₅₀ values for 300, 100, 30 and 10 μM NMDA were 0.80 ± 0.12, 1.01 ± 0.08, 0.92 ± 0.13 and 1.31 ± 0.09 μM, respectively, giving an average IC₅₀ for all agonists concentrations tested of 1.01 ± 0.11 μM (n = 25). It should be stressed that this concentration is the therapeutically relevant level of memantine achieved in the treatment of AD.

For comparison, the data in Fig. 3 have also been presented in a similar manner to those of the previous study from (Chen et al., 1992) to reveal any agonist concentration-dependency at different concentrations of memantine. The data for 10 μM memantine are at the concentration closest to that used by (Chen et al., 1992)—who tested 6 μM. Nonetheless, all memantine concentrations tested are also illustrated to...
highest concentration of NMDA tested, i.e. 300 µM. All attempts were made to allow full recovery after each memantine application. Panel (A) shows the effects of memantine in the presence of the lowest concentration of NMDA and a saturating concentration of the co-agonist D-serine (10 µM). Control currents for each agonist concentration, but different memantine concentrations, were normalized to give a better impression of memantine concentration-dependence on both “equilibrium” blockade and kinetics. This measure was necessary, as some degree of receptor rundown must be accepted during such long patch clamp experiments. The onset kinetics at therapeutically relevant IC₅₀ concentrations of memantine i.e. 1/τ_on at 1 µM memantine, was chosen as a useful parameter as this gives more information on how quickly memantine could gain access to the activated NMDA receptor channel under therapeutically relevant conditions (Parsons et al., 1995). Moreover, this parameter has previously been shown to correlate with antagonist potency per se, i.e. onset of block at IC₅₀ concentrations of antagonist is slower for more potent compounds. The same was true for k_off and τ_off whereas k_on showed little dependence on antagonist affinity (Parsons et al., 1995).

Memantine is known to block and unblock open NMDA receptor channels with double exponential kinetics and this phenomenon was also confirmed in the present experiments. The speed and weight of the fast component of block increases with memantine concentration. In contrast, the speed of fast unblock remains constant but its weight (relative to the slow component) decreases with memantine concentration (Bresink et al., 1996; Frankiewicz et al., 1996; Blanpied et al., 1997; Sobolevsky and Koshelev, 1998; Sobolevsky et al., 1998). Moreover, 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 and Koshelev, 1998; Sobolevsky et al., 1998).

In the present experiments, increasing the concentration of agonist had the following effects on double exponential
Table 1
Summary of the potency and kinetics of memantine determined at different concentrations of NMDA

<table>
<thead>
<tr>
<th>Concentration NMDA (µM)</th>
<th>IC50 (µM)</th>
<th>S.E.M.</th>
<th>Hill</th>
<th>S.E.M.</th>
<th>t/τ on at IC50 i.e. 1 µM</th>
<th>S.E.M.</th>
<th>k on (×10^6 M^-1 s^-1)</th>
<th>S.E.M.</th>
<th>k off (s^-1)</th>
<th>S.E.M.</th>
<th>Kd (µM)</th>
<th>S.E.M.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>0.80</td>
<td>0.12</td>
<td>0.93</td>
<td>0.09</td>
<td>0.58</td>
<td>0.11</td>
<td>10.55</td>
<td>1.41</td>
<td>0.24</td>
<td>0.01</td>
<td>2.27</td>
<td>0.45</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>1.01</td>
<td>0.08</td>
<td>1.07</td>
<td>0.07</td>
<td>0.28</td>
<td>0.05</td>
<td>8.60</td>
<td>0.17</td>
<td>0.19</td>
<td>0.01</td>
<td>2.21</td>
<td>0.18</td>
<td>7</td>
</tr>
<tr>
<td>30</td>
<td>0.92</td>
<td>0.13</td>
<td>1.04</td>
<td>0.15</td>
<td>0.18</td>
<td>0.02</td>
<td>4.90</td>
<td>0.20</td>
<td>0.14</td>
<td>0.00</td>
<td>2.86</td>
<td>0.12</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>1.31</td>
<td>0.09</td>
<td>1.20</td>
<td>0.09</td>
<td>0.48</td>
<td>0.01</td>
<td>3.22</td>
<td>0.08</td>
<td>0.09</td>
<td>0.01</td>
<td>2.80</td>
<td>0.38</td>
<td>5</td>
</tr>
</tbody>
</table>

The mean k on was calculated from the kinetic experiments as the ratio Kd = k off/k on. Whilst these values are somewhat higher than the equilibrium IC50, it is clear that the magnitude of the NMDA concentration dependence of both onset and offset kinetics was similar and therefore not associated with a change in potency.

Again, the speed and weight of the fast component of block increased with agonist concentration e.g. for 10 µM memantine, the values for the fitted parameters for 30 µM NMDA were: τ off(1) 0.49 ± 0.23 s, weight(1) 45.5%, τ off(2) 3.18 ± 0.42 s, weight(2) 54.5%. With 300 µM NMDA these values changed to: τ off(1) 0.22 ± 0.02 s, weight(1) 63.0%, τ off(2) 2.04 ± 0.28 s, weight(2) 37.0%. In the case of onset kinetics, increasing agonist concentration increased the speed of both the faster and slower components, but there was less effect on their weighting. e.g. for 10 µM memantine, the values for the fitted parameters for 30 µM NMDA were: τ on(1) 1.87 ± 1.00 s, weight(1) 16.2%, τ on(2) 16.75 ± 5.8 s, weight(2) 83.8%. With 300 µM NMDA these values changed to: τ on(1) 0.65 ± 0.13 s, weight(1) 22.2%, τ on(2) 5.93 ± 0.46 s, weight(2) 77.8%.

In the present experiments, for the sake of clarity, such double exponential fits were integrated to single exponentials (see Section 2) to facilitate a simpler representation of the agonist concentration-dependency of blocking/unblocking kinetics. This measure was viewed to be particularly important at low agonist/antagonist concentrations because clear double exponential behaviour could sometimes not be resolved at these concentrations for technical reasons. In addition, this procedure allows the calculation of simple Kd values on the basis of k off/k on to verify with this kinetic calculation that memantine equilibrium potency is unchanged by agonist concentration (see Table 1). The hastening of both onset and offset kinetics at higher concentrations of agonist also had no net effect on the calculated equilibrium potency of memantine i.e. both onset and offset kinetics were influenced to a similar degree by agonist concentration.

It should be noted that kinetics were, per se, slightly faster than in some previous studies from our group—this most likely reflects the use of slightly different hippocampal culturing methods (frozen compared to classically prepared freshly isolated cells) and underlies the need to perform such comparative analyses under identical conditions, as performed in this study.

4. Discussion

The primary aim of this study was to challenge the hypothesis that memantine antagonizes NMDA receptor-mediated currents with greater potency when this agonist is present at higher concentrations e.g. (Chen et al., 1992; Lipton, 2004, 2006, 2007). The results shown here indicate that the degree of “true” equilibrium blockade of NMDA-induced currents by memantine was not affected by the concentration of this agonist, although the onset and offset kinetics clearly were. It is therefore also proposed that the degree of steady-state “resting” blockade by memantine under therapeutic conditions will not be dependent upon the concentration of the in vivo agonist, glutamate, which was not used here due to its agonism of AMPA and kainate receptors expressed by hippocampal neurons.

Although, there might seem to be a slight difference between memantine’s apparent potency with 300 and 10 µM NMDA, this was most probably due to problems in achieving “true” equilibrium blockade with the lowest agonist/antagonist concentrations tested, even when we did our best to
address this issue with very long agonist/antagonist application times at the lower concentrations of agonist tested. It should be clear from the kinetic analysis that, even under the stringent conditions used, “true” equilibrium blockade by memantine at the lowest concentrations of agonist/memantine tested had probably still not been fully achieved. This factor alone is probably responsible for any apparent differences in “true” equilibrium concentration-dependent blockade of NMDA receptors by memantine. This should also be clear from the Hill coefficients of memantine blockade, which were apparently higher for lower concentrations of agonist (see Table 1), i.e. the lowest concentrations of memantine tested at low NMDA concentrations had probably not really been achieved equilibrium blockade, even under the stringent conditions used.

To support the relevance of this basic pharmacological principle for memantine, we calculated rough estimates of the degree of equilibrium blockade achieved in the present experiments on the basis of the fitted, and then normalized to single exponential, onset kinetics at different concentrations of antagonist/agonist, taking into account the different antagonist application times used. With the lowest concentration of memantine tested (0.3 μM) 97.4%, 99.2%, 99.8% and 99.99% of the achievable equilibrium blockade would have been predicted for 10, 30, 100 and 300 μM NMDA, respectively. Had we used more “conventional”, shorter 10 s antagonist application times as in the study of (Chen et al., 1992), these values would only have been 70.5%, 74.5%, 87.8% and 99.5%, respectively. It should be stressed that the use of such normalized single exponential kinetics to predict the degree of blockade achieved can only give a very rough estimate of these values. Nonetheless, equilibrium blockade would clearly not have been achieved with lower concentrations of agonist/antagonist had we only applied antagonist for 10 s as in previous studies (Chen et al., 1992). In summary, in the present study, near equilibrium blockade was probably achieved at all NMDA concentrations tested (all greater than 99.7% of the achievable blockade) with memantine (at 10 or 30 μM) and it should be clear from Figs. 2 and 3 that there was absolutely no NMDA concentration dependency of memantine block at these memantine concentrations.

The results of the present study do not support the theory that the equilibrium blocking potency of memantine is dependent upon agonist concentration, nor that this factor contributes to the good in vivo/proven clinical therapeutic profile of memantine (Chen et al., 1992; Lipton, 2004, 2006, 2007). These results seem rather to confirm the hypothesis that other characteristics are much more important in this regard, such as strong voltage dependence combined with swift, agonist concentration-dependent unblocking kinetics, as has previously been suggested (Parsons et al., 1993, 1995, 1999). When the concentrations of agonist are transiently high and associated with strong synaptic depolarization, such as during physiological synaptic activation, the voltage-dependent relief of memantine blockade would be very fast, thus allowing neuronal transmission. However, unblock would be slower when lower, pathological concentrations of agonist are present and membrane potentials are closer to resting levels.

References


