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Study of potency, kinetics of block and toxicity of NMDA receptor antagonists using fura-2

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Abstract

NMDA receptor antagonists reduced NMDA-triggered increases in $[Ca^{2+}]_i$ measured by fura-2 and showed qualitative differences in the potency and kinetics of block. High potency antagonists produced a slow block which allowed an initial increase in $[Ca^{2+}]_i$ that was greater than the steady-state level, while compounds with moderate to low potency produced a rapid block that was at steady-state from the first measurement. The more potent antagonists showed the slowest unblocking rates. Using this simple method, novel NMDA antagonists can be screened to ascertain potency, kinetics of block and relative toxicity, prior to animal testing.

Keywords: NMDA receptor; Calcium; Neuron; Uncompetitive; Neuroprotection; MK-801

1. Introduction

High-affinity uncompetitive NMDA receptor antagonists like MK-801 bind within the activated NMDA-gated ion channel and impede the flow of Ca^{2+} into neurons. Significant neurobehavioral side effects limit the therapeutic value of these high-affinity, uncompetitive antagonists (Kemp et al., 1987). In contrast, low-affinity, uncompetitive NMDA receptor antagonists, like memantine, have reduced toxicities, possibly due to faster rates of unblocking and equilibrium block (Rogawski, 1993). Likewise, the low-affinity uncompetitive NMDA receptor antagonists, ARL 15896AR (formerly FPL 15896AR; (*S*)-α-phenyl-2-pyridine-ethanamine dihydrochloride) and the desglycinyl metabolite (ARL 12495AA; 1,2-diphenyl-2-propylamine monohydrochloride; DGR) of remacemide HCl (ARL 12924AA; 2-amino-*N*-(1-methyl-1,2-diphenylethyl)acetamide hydrochloride), block NMDA-induced toxicity in cortical cultures, and rapidly reduce NMDA-induced increases in $[Ca^{2+}]_i$ (Black et al., 1995, 1996). This rapid block of the $[Ca^{2+}]_i$ response differs from the high-affinity antagonists which exhibit a slower block of the NMDA-induced rise in $[Ca^{2+}]_i$ (Black et al., 1996). The slow blocking rate of MK-801 allows significant Ca^{2+} influx such that very high concentrations of MK-801 are required to completely block NMDA-triggered $[Ca^{2+}]_i$ responses, producing long-lasting, maximal blockade of the channel (Kemp et al., 1987).

In this report we describe a simple system to determine the kinetics of block of uncompetitive NMDA receptor antagonists to facilitate screening novel compounds. We show that there is an inverse relationship between the high- and low-affinity uncompetitive NMDA receptor antagonists and their rates of block of NMDA-induced $[Ca^{2+}]_i$ responses.

2. Materials and methods

2.1. Reagents

We purchased MK-801, ketamine, dextrorphan, dextromethorphan and NMDA from Research Biochemicals International (Natick, MA, USA), memantine from Tocris Cookson (St. Louis, MO, USA) and obtained ADCI ((±)-5-aminocarbonyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) and PCA (1-phenylcyclohexylamine) from Dr. A. Thurkauf (Neurogen, Branford, CT, USA) and desglycinyl remacemide, ARL 15896AR and ARL

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16247AA (the same chemical structure as CNS-1102; Cerestat; *N*-(1-napthyl)-*N′*-(3-ethylphenyl)-*N′*-(methylguanidine)) from Astra Arcus USA (Rochester, NY, USA).

2.2. Cortical neurons

E18 rat cortical neurons were cultured as described previously (Black et al., 1996). Experiments were performed on cultures following 12–15 days in vitro.

2.3. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was determined using the Ca^{2+}-sensitive indicator fura-2 as described previously (Black et al., 1995). $[Ca^{2+}]_i$ determinations were performed on cells grown on glass coverslips in a chamber perfused at 0.9 ml/min. The clearance time of the chamber was about 15 s. To assess the qualitative and quantitative block, and kinetics of action of uncompetitive NMDA receptor antagonists on the NMDA-triggered $[Ca^{2+}]_i$ response, cultures were exposed to NMDA (40 μM) for 60 s, then perfused with NMDA-free buffer (bath) for 3–7 min during which $[Ca^{2+}]_i$ returned to near resting levels and then reperfused with NMDA-free buffer (control; Fig. 1A) or with antagonists for 2–3 min. The cells were then reexposed to 40 μM NMDA for varying times, in the presence or absence of antagonists. In controls, the amplitude of the second NMDA-evoked rise in $[Ca^{2+}]_i$ was not significantly ($P > 0.05$) different from the first.

2.4. Calculations

To measure the magnitude of the antagonist-mediated inhibition of the NMDA-induced $[Ca^{2+}]_i$ response, the ratios (R) of the magnitudes of the second (NMDA + drug; S_2) compared to the first (NMDA alone; S_1) NMDA-triggered $[Ca^{2+}]_i$ surges (S_2/S_1) were determined following subtraction of the baseline $[Ca^{2+}]_i$ level for S_1 and the prestimulation $[Ca^{2+}]_i$ level for S_2 (Black et al., 1995). The fractional block of each concentration of antagonist

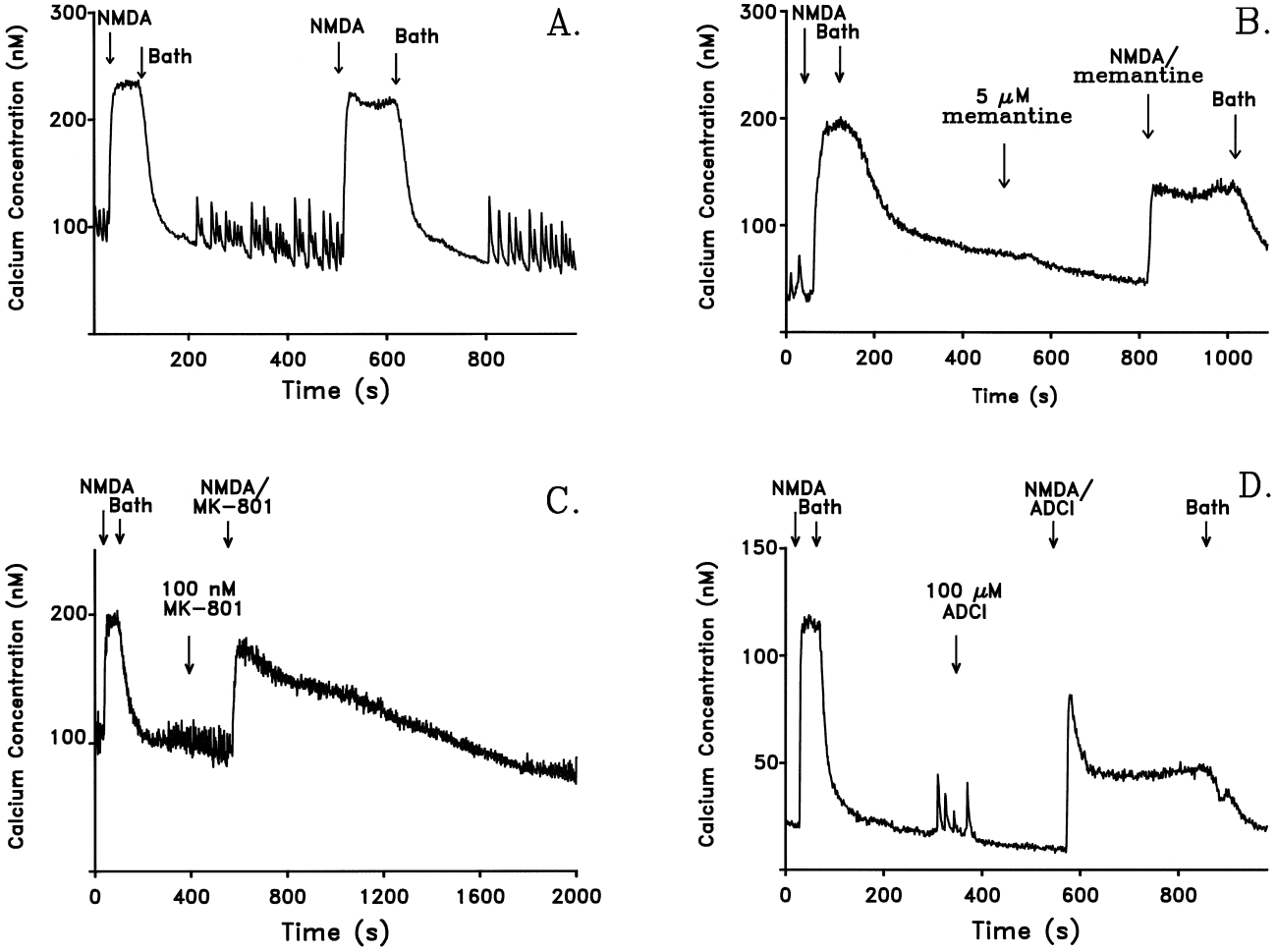

Fig. 1. Effect of NMDA receptor antagonists on NMDA-triggered $[Ca^{2+}]_i$ responses. A: Control: Neurons were stimulated with 40 μM NMDA, washed (bath), and restimulated with 40 μM NMDA. B: Neurons were treated with 40 μM NMDA, washed and then perfused with buffer containing 5 μM memantine. The cultures were then reexposed to 40 μM NMDA in the presence of memantine. C: The neurons were treated as in (B) except with 0.1 μM MK-801. D: The neurons were treated as in (B) except with 100 μM ADCI. The results are representative experiments replicated 5–10 times.

was calculated according to the formula $B = (R_0 - R_D)/R_0$ where R_O and R_D are the NMDA-induced $[Ca^{2+}]_i$ increase in the absence and presence of drug, respectively. Concentration-response curves were fit to a sigmoid curve determined by the following equation: $y = 100/(1 + (x/c)^b$, where b is the slope parameter, c is the inflexion point, y is the percent block and x is the concentration of drug tested.

2.5. Kinetic analysis

To determine antagonists' on- and off-rates, the decrease in $[Ca^{2+}]_i$ following exposure to NMDA in the presence of the antagonist was fit to a single exponential based on the following equation: $f = a \exp(-bx)$, where a is the response amplitude at time = 0 (i.e., the peak of the initial NMDA-evoked rise in $[Ca^{2+}]_i$), and $[Ca^{2+}]_i$ decreases with a time constant, $\tau = 1/b$. To determine the on- (k_1) and off-rates (k_{-1}) of these compounds, τ was plotted vs. concentration. The on-rate was estimated from the slope and the off-rate was taken as the y-axis intercept. The $time_{on}$ (s) of these compounds at the IC_{50} was determined from the equation: $time_{on} = [k_{-1} + (k_1 \times IC_{50})]^{-1}$ as described by Jones and Rogawski (1992).

3. Results

3.1. Block of NMDA-triggered $[Ca^{2+}]_i$ responses

Memantine, a low-affinity antagonist, reduced the peak NMDA-induced $[Ca^{2+}]_i$ to a suprabasal plateau level for as long as NMDA and the antagonist were perfused (Fig. 1B) as did the low-affinity antagonists ARL 15896AR (Black et al., 1995) and desglycinyl remacemide (Black et al., 1996). A second pattern of blockade was observed with the high-affinity antagonists, MK-801 (0.1 μM; Fig. 1C), PCP (1–10 μM; data not shown) and ARL 16247AA (1 and 5 μM; data not shown). All allowed an initial $[Ca^{2+}]_i$ spike followed by a drop to, or near, baseline levels. The peak initial NMDA-evoked rise in $[Ca^{2+}]_i$ and the rate of decrease of the amplitude of the NMDA-triggered $[Ca^{2+}]_i$ response were dependent on antagonist concentration. A third pattern, produced by the high-, intermediate- and low-affinity NMDA receptor antagonists ADCI (100 μM; Fig. 1D), PCA (10 and 50 μM; data not shown), ketamine (1–100 μM; data not shown), dextrorphan (1–10 μM; data not shown) and dextromethorphan (1–100 μM; data not shown), consisted of two phases of block. There was an initial $[Ca^{2+}]_i$ spike that rapidly dropped to a lower, but still suprabasal plateau level, the rate of which was dependent on the concentration of the antagonist. The amplitude of the plateau $[Ca^{2+}]_i$ level was also dependent on antagonist concentration.

3.2. Potency of block

The IC_{50} for each NMDA receptor antagonist was determined from the S_2/S_1 ratios. For low-affinity antagonists, S_2 was calculated at the plateau of the response (at 100–300 s). For high-affinity antagonists, where a plateau was not observed, S_2 was calculated at 300 s after the second NMDA application, such that the potency of these

Table 1
Experimental and published IC_{50} values and values of binding affinities of NMDA antagonists

Compound	IC_{50} (μM) peak	IC_{50} (μM) plateau	IC_{50} (peak/plateau)	Literature values	
				IC_{50} (μM)	Binding affinities (μM)
MK-801	3.25	0.11	29.5	0.021 [b], 0.14 [i]	0.003 [a,i], 0.010 [f]
ARL 16247AA	5.33	0.17	31.4	0.028 [c]	0.037 [a]
PCP	25.8	0.76	34.0	1.0 [i]	0.063 [e], 0.5 [i]
Dextrorphan	6.93	0.75	9.2	1.3 [i]	0.17 [a]
PCA	3.99	1.5	2.7	2.0 [e]	0.53 [b,e]
Ketamine	5.22	3.0	1.7	1.6 [i], 9 [f]	0.2 [i], 1.0 [f]
Dextromethorphan	10.6	6.4	1.7	6 [g], 6.1 [i]	1.5 [a], 1.3 [g]
ADCI	134.2	33.7	4.0	14 [b]	9.3 [a]
DGR	8.1	8.1	1.0	0.7 [d], 0.48 [a]	1.1 [d]
Memantine	4.1	4.1	1.0	1.2 [h], 2.3 [i]	0.7 [i], 0.54 [a]
ARL 15896AR	52.5	52.5	1.0	NR	NR

[a] Rogawski (1993), NMDA-induced current. [b] Jones and Rogawski (1992), NMDA-induced current. [c] Gamzu (1994). [d] Subramaniam et al. (1996), NMDA-induced current. [e] Rogawski et al. (1992), NMDA-induced current. [f] MacDonald et al. (1987), NMDA-induced current. [g] Church et al. (1994), NMDA-induced current and NMDA-induced increase in $[Ca^{2+}]_i$. [h] Chen et al. (1992), NMDA-induced current. [i] Parsons et al. (1995), NMDA-induced current. NR = not reported.

IC_{50} values for the various NMDA antagonists were calculated for the peak and plateau phases of block of NMDA-induced $[Ca^{2+}]_i$ responses. Published IC_{50} and binding affinity values are also given for each NMDA antagonist. The literature values for binding affinities are only roughly comparable since experimental conditions differ.

Table 2
Experimental and published values for the kinetics of block by NMDA receptor antagonists.

Compound	k_1 ($\times 10^6$/Ms^{-1})	k_{-1} (s^{-1})	k_d (k_{-1}/k_1)	Time$_{on}$ at IC$_{50}$ (s)	Literature values			
					k_1 ($\times 10^6$/Ms^{-1})	k_{-1} (s^{-1})	k_d (k_{-1}/k_1)	Time$_{on}$ at IC$_{50}$ (s)
MK-801	0.0039	0.0081	2.08	117.8	0.027 [c,e], 0.04 [a]	0.005 [a,d], 0.003 [e]	0.125 [a]	86 [c], 34 [a,c]
ARL 16247AA	0.0105	0.0034	0.32	191.3	NR	NR	NR	NR
PCP	0.0069	0.0046	0.67	94.3	0.014 [a], 0.035 [e]	0.037 [e], 0.05 [a]	3.6 [a], 1.1 [e]	13.8 [a]
Dextrorphan	0.0040	0.043	10.75	21.6	0.035 [a]	0.075 [a]	2.14 [a]	4.8 [a]
PCA	0.0029	0.063	21.72	14.7	0.023 [b]	0.06 [b]	2.60 [b]	9.7 [b]
Ketamine	0.0025	0.080	32.00	11.4	0.09 [a]	0.075 [a]	0.8 [a]	1.02 [a]
Dextromethorphan	0.0061	0.041	6.72	16.6	0.03 [a]	0.18 [a]	6.0 [a]	2.13 [a]
ADCI	0.0001	0.033	330.0	27.6	0.00056 [c]	0.017 [c]	30.36 [c]	40 [c]

[a] Parsons et al. (1995). [b] Rogawski et al. (1992). [c] Jones and Rogawski (1992). [d] Subramaniam et al. (1996). [e] MacDonald et al. (1991). NR = not reported.

compounds would be underestimated. For NMDA receptor antagonists which displayed two phases of block, IC_{50} values were calculated for each phase of the block. All antagonists blocked NMDA-triggered $[Ca^{2+}]_i$ responses in a concentration-dependent manner. The calculated IC_{50} values are summarized in Table 1. The rank order of potency, MK-801 > ARL 16247AA > PCP = dextrorphan > PCA ≥ ketamine ≥ memantine > dextromethorphan > desglycinyl remacemide > ADCI > ARL 15896AR was similar to the reported rank order of binding affinities, MK-801 > ARL 16247AA > PCP > dextrorphan > PCA ≥ desglycinyl remacemide ≥ memantine > ketamine ≥ dextromethorphan > ADCI (for References, see Table 1).

IC_{50} values for the initial $[Ca^{2+}]_i$ response were also calculated for the NMDA receptor antagonists which showed a rapid block of the initial $[Ca^{2+}]_i$ spike. The percent block of the $[Ca^{2+}]_i$ response was calculated at the peak response, following application of NMDA in the presence of the antagonist. The ratio of the IC_{50} values (peak/plateau) was much less for the low-affinity antagonists ADCI, PCA, ketamine and dextromethorphan than for the high-affinity antagonists MK-801, PCP and ARL 16247AA. Since desglycinyl remacemide, ARL 15896AR and memantine produced steady-state blockade from the onset of the $[Ca^{2+}]_i$ response, their IC_{50} ratios are 1.

3.3. Kinetics of block

For ARL 15896AR, desglycinyl remacemide and memantine, steady-state block was reached more rapidly than the temporal resolution limit of these experiments. NMDA receptor antagonists which displayed slower kinetics reduced the NMDA-induced rise in $[Ca^{2+}]_i$ at a rate which could be fit with a single exponential. The values for the on- and off-rates of these antagonists are summarized in Table 2. The rates of block obtained in this study are compared to published values from electrophysiological studies measuring the potency and rates of block on NMDA-evoked currents. The rank order of k_{-1} rates is similar to that of time$_{on}$ rates, while k_1 rates do not correlate with time$_{on}$ rates (Table 2).

4. Discussion

In this report, we compare the kinetics of block and potency of uncompetitive NMDA antagonists in blocking NMDA-triggered increaes in $[Ca^{2+}]_i$ in rat cortical neurons. Our data suggest that the effects of antagonists at the NMDA receptor ion channel can be evaluated qualitatively and quantitatively by this method. The IC_{50} values (plateau) for the block of NMDA-induced $[Ca^{2+}]_i$ responses by the NMDA receptor antagonists compare favourably to values reported previously (Rogawski, 1993). Furthermore, the rank order of potency of the antagonists was similar to the reported rank order of affinities of these compounds. Moreover, we show the kinetics of block of the NMDA-induced increase in $[Ca^{2+}]_i$ by several antagonists was similar (within one order of magnitude) to the kinetics of block of NMDA-evoked currents. Therefore, the action of these antagonists in blocking the NMDA-induced rise in $[Ca^{2+}]_i$ can provide an approximation of the events occurring at the level of the membrane. Some of the differences in potency may be attributed to the voltage dependence of compounds and whether they were determined under voltage-clamp conditions. In this study, membrane potential was not controlled.

The rates of block and unblock by NMDA receptor antagonists are a function of their binding affinities (Rogawski, 1993). Antagonists with the highest affinities exhibited the slowest rate of block. The time$_{on}$ at the IC_{50} for these antagonists was 7 times slower than for the low-affinity antagonists, ADCI, ketamine, PCA, dextromethorphan and dextrorphan. The time$_{on}$ is a reflection of both the blocking (k_1) and unblocking (k_{-1}) rate constants. Like others (MacDonald et al., 1991; Parsons et al., 1995), we found that differences in reverse rate constants appear to account for differences in the potency of the

NMDA antagonists. Those antagonists which had k_{-1} values in the range of 0.003–0.008 s^{-1}, i.e., MK-801, ARL 16247AA and PCP, exhibited the slowest block of the NMDA-induced $[Ca^{2+}]_i$ rise. Kinetics could not be resolved for the low-affinity antagonists, ARL 15895AR, desglycinyl remacemide and memantine, because they were beyond the limits of temporal resolution. The rapid block by memantine is consistent with its rapid kinetics in whole-cell and single-channel recordings (Chen et al., 1992; Parsons et al., 1993, 1995).

The high-affinity antagonists, MK-801, ARL 16247AA and PCP, displayed the slowest and most potent block (with IC_{50} values at the plateau < 1 μM). They exhibited a narrow range of concentrations where they could modulate, rather than completely block, the NMDA channel. At concentrations near their IC_{50} values, they blocked the NMDA-induced rise in $[Ca^{2+}]_i$ slowly, allowing a significant $[Ca^{2+}]_i$ increase while at concentrations 2–3-fold above their IC_{50} values, they completely blocked the response to NMDA. In addition, even at concentrations that produced complete blockade, an initial $[Ca^{2+}]_i$ increase occurred which lasted many seconds. In order to reduce this initial spike, very high concentrations were used (the IC_{50} values at the peak were about 30-fold greater than the IC_{50} values at the plateau). By contrast, the effective range of block by low-affinity antagonists was broader; the concentration of antagonist that reduced the NMDA-evoked $[Ca^{2+}]_i$ response to near basal levels was approximately 10-fold greater than their IC_{50} values (plateau). Moreover, the difference between the IC_{50} values at the initial peak and the plateau was smaller for the low-affinity than the high-affinity antagonists.

The present report demonstrates that uncompetitive NMDA receptor antagonists can be easily screened by measuring their kinetics of block of NMDA-induced increases in $[Ca^{2+}]_i$. Although a limitation of this method is that kinetics of block for low-affinity antagonists showing a rapid rate of block cannot be determined, we demonstrate that high-affinity antagonists blocked the NMDA-induced $[Ca^{2+}]_i$ increase more slowly, and exhibited a narrower range of effective block than low- and intermediate-affinity antagonists.

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