Antagonism of N-Methyl-D-aspartate-evoked Currents in Rat Cortical Cultures by ARL 15896AR¹


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Accepted for publication December 9, 1996

ABSTRACT

The purpose of this study was to characterize the kinetics and voltage-dependence of the block of N-methyl-D-aspartate (NMDA)-induced currents in primary cultures of rat cortical neurons by the neuroprotective, low-affinity, NMDA antagonist ARL 15896AR, using whole-cell voltage-clamp techniques. ARL 15896AR caused rapid and reversible inhibition of NMDA (50 μM)-evoked currents from neurons held at −60 mV, with an IC₅₀ of 9.8 μM. The EC₅₀ for NMDA was not significantly affected by 10 μM ARL 15896AR (P > .05), with a noncompetitive mechanism of block. ARL 15896AR antagonism was use-dependent, because application of the drug 60 sec before NMDA did not attenuate the initial NMDA-evoked current, although the block developed rapidly thereafter. Once bound, ARL 15896AR remained trapped upon removal of NMDA until subsequent NMDA re-exposure, whereupon currents recovered rapidly. The forward and reverse binding rate constants were estimated to be 2.406 × 10⁸ M⁻¹ sec⁻¹ and 0.722 sec⁻¹, respectively. Antagonism was strongly voltage-dependent; the Kᵥ values at 0 and −60 mV were 60 and 11 μM, respectively. Additionally, there was a component of the block by ARL 15896AR that was voltage-insensitive. This component of the block did not act at the ligand binding site, because it was not influenced by NMDA concentration, or at the polyamine site, because it was not affected by spermine. However, there was an interaction of ARL 15896AR with the glycine regulatory site. In contrast to many uncompetitive NMDA antagonists, like MK-801, ARL 15896AR exhibited rapid kinetics. This property may result in a large margin of safety while maintaining the efficacy associated with use-dependent NMDA antagonists, making this compound an excellent candidate for clinical trials.

Inhibition of NMDA receptors may occur by antagonist interaction at the ligand binding site, at the strychnine-insensitive glycine site, at the polyamine site or directly at the PCP site within the pore of the ion channel (Johnson and Ascher, 1987; Lodge and Johnson, 1990; Meldrum, 1991; Williams et al., 1991; Danysz et al., 1995). Uncompetitive NMDA antagonists that act at the PCP site exhibit use-dependence, resulting in block only after the channel enters its open state after activation (Huettner and Bean, 1988). Although this use-dependent antagonism would seem to be potentially advantageous in situations where excessive activation of NMDA receptors occurs, such as in the case of ischemia or epilepsy, clinical trials with several of these compounds have not been encouraging. Both MK-801 (dizocilpine) and PCP, which bind with high affinity to a site within the channel pore, have serious neurobehavioral side effects, which minimize their therapeutic potential, despite their neuroprotective effectiveness in various models of focal and global ischemia (Kemp et al., 1987; Olney et al., 1989). However, lower-affinity NMDA antagonists may have reduced toxicities because they reach a steady-state block more rapidly than the high-affinity antagonists, due to their rapid on-off kinetics (Rogawski, 1993). This difference in kinetics may be therapeutically relevant, because the slower blocking rates of MK-801 and PCP allow significant calcium entry before equilibrium block is reached. This calcium entry is only overcome at very high antagonist concentrations, which result in a long-lasting, maximal channel block (Kemp et al., 1987). The lack of serious side effects in clinical trials using the low-affinity, uncompetitive NMDA antagonists memantine, amantadine (Parsons et al., 1995) and ADCI (Rogawski et al., 1991, 1995) is likely due to their rapid kinetics and voltage-dependence of block (Chen et al., 1992; Parsons et al., 1993, 1995).

The NMDA receptor antagonist ARL 15896AR (formerly called FPL 15896AR) has been recently developed. Ligand binding studies have shown that ARL 15896AR is a low-affinity (Kᵢ = 1–2 μM in MK-801 binding studies), noncom-

ABBREVIATIONS: ADCI, 5-aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; ARL 15896AR, (S)-α-phenyl-2-pyridine-ethanamine dihydrochloride; HEPES, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid); MEM, minimal essential medium; NMDA, N-methyl-D-aspartic acid; PCP, phencyclidine.

¹This work was supported by the Canada/Astra Fight Stroke Program, funded by the National Research Council of Canada and Astra Pharma Inc. (Mississauga, Ontario, Canada).
petitive NMDA receptor antagonist (Black et al., 1995). We previously reported that ARL 15896AR blocks NMDA-induced toxicity in primary cultures of cortical neurons and that, at neuroprotective concentrations, it rapidly decreases the NMDA-induced calcium influx and subsequent rise in intracellular free calcium (Black et al., 1995). This block of the NMDA-induced calcium response occurs more rapidly than with the high-affinity antagonists MK-801 and PCP (Black et al., 1996a,b). The purpose of the present study was to characterize the kinetics and voltage-dependence of ARL 15896AR block of NMDA-induced currents, using whole-cell voltage-clamp techniques.

**Materials and Methods**

**Chemicals and reagents.** Dulbecco’s phosphate-buffered saline, HEPES, Eagle’s MEM, poly-L-lysine, tetrodotoxin and strychnine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Heat-inactivated fetal bovine serum was purchased from Gibco Laboratories (Grand Island, NY) and heat-inactivated horse serum from HyClone Laboratories Inc. (Logan, UT). NMDA and spermine were purchased from Research Biochemicals International (Natick, MA). Memantine was purchased from Tocris Cookson (St. Louis, MO). Ethylene glycol-2-bis(2-aminoethyl)-N,N,N’N’-tetraacetic acid was purchased from Fluka Biochemika (Ronkonkoma, NY).

**Cell culture.** Cortical neurons isolated from embryonic day 18 fetuses were grown in primary culture as previously described (Black et al., 1995). Briefly, timed-pregnant Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, Canada). After killing of the mother by cervical dislocation under halothane anesthesia, the fetuses were removed from the uterus on embryonic day 18, their brains were removed and placed in ice-cold phosphate-buffered saline and the cortices were isolated. The cortical neurons were dispersed by trituration with a 10-ml pipette, and the cells were centrifuged at 250 \( \times \) g for 5 min at 4°C. The cells were gently resuspended in plating medium, and viable cells (as determined by trypan blue exclusion) were counted. The cells were then plated at 105 cells/cm² on poly-L-lysine-coated, 35-mm-diameter, culture dishes (Nunc, Roskilde, Denmark) in 2 ml of plating medium at 37°C, in an atmosphere of 5% CO₂/95% air. Plating medium consisted of 80% MEM with 20 mM glucose, 10% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum and 2 mM L-glutamine.

Because the cultures contained both neurons and glial cells, they were treated with 15 mg/ml 5-fluoro-2-deoxyuridine and 35 mg/ml uridine between days 4 and 6, to minimize glial cell proliferation. On day 6, half of the medium was removed and replaced with growth serum, 10% heat-inactivated horse serum and 2 mM L-glutamine.

**Whole-cell recording.** Cortical neurons grown on 35-mm culture dishes were mounted on the stage of an inverted Zeiss microscope equipped with Hoffman Modulation optics, in a perfusion system flowing continuously at 1 ml/min at 22°C. The bathing solution contained 140 mM CsCl, 1.1 mM ethylene glycol-O,O’-bis(2-aminoethyl)-N,N,N’N’-tetraacetic acid, 10 mM HEPES and 2 mM Mg-ATP, pH 7.2.

Whole-cell currents were acquired using an Axopatch 1-D amplifier equipped with a CV-4 headstage with a 1-GΩ feedback resistor (Axon Instruments, Foster City, CA). Voltage command and current acquisition were accomplished using a personal computer equipped with a Digidata 1200 interface and pClamp 6.0 software (Axon). Data were filtered at 1 kHz and sampled at 2 kHz.

Rapid agonist or agonist-antagonist application was accomplished using a modified DAD-12 perfusion system (ALA Scientific Instruments, Westbury, NY). The system consisted of a custom-made manifold of eight 100-μm-diameter quartz tubes that converged into a common 100-μm tip with minimal dead volume. The tubes were fed from pressurized reservoirs equipped with miniature switching valves controlled by a computer, such that solution flowed from only a single reservoir at a time. The tip of the manifold was placed <100 μm from the patch-clamped cell under study. Solutions were degassed before use, and reservoirs were pressurized at 300 to 400 mm Hg. Switching between solutions took 12 ± 1 msec (n = 12), as determined by junction potential measurements using a 10% Cl⁻ solution in one reservoir and normal Cl⁻ in the others. In experiments designed to examine use-dependence, antagonist was preapplied to the bath using conventional perfusion (bath changeover in <5 sec), whereas agonist was rapidly applied using a U-tube (Kristal and Pidoplichko, 1980), with a switching speed comparable to that reported for the multibarrel perfusion system. All drugs were prepared fresh daily.

**Data analysis.** The fractional block of NMDA-evoked currents was calculated according to the formula

\[ B = 1 - I_b/I \]  

where \( I \) is the steady-state current evoked by NMDA and \( I_b \) is the current evoked by NMDA in the presence of a blocker.

**Concentration-effect data were fitted to the logistic equation**

\[ B = 1/(1 + (IC_{50}/[D])^n) \]

where \( IC_{50} \) is the concentration of antagonist resulting in 50% block, \( [D] \) is the concentration of the antagonist and \( n \) is the Hill coefficient.

The rate of onset of block (\( k_{on} \)) was calculated as the reciprocal of the \( \tau \) of the exponential fitting function. The mean \( k_{on} \) values were plotted as a function of antagonist concentration, and the rate constants were determined by the best fit of the data to the line generated by the equation

\[ k_{on} = k_1[D] + k_{-1} \]

where \( k_1 \) is the forward rate constant, \( k_{-1} \) is the reverse rate constant and \( [D] \) is the antagonist concentration.

The voltage-dependent binding affinity of a channel blocker can be expressed (Woodhull, 1973) as

\[ K_d(V) = K_d(0)exp(\delta zFVRT) \]

where \( K_d(0) \) is the dissociation constant of the drug binding site at a transmembrane potential of 0 mV, \( \delta \) is the fraction of the total electric field sensed at the binding site and \( z \) is the charge on the drug. Incorporating the Woodhull equation into a binding isotherm that assumes that the channel is nonconducting when occupied by a single molecule of the drug gives (Subramaniam et al., 1996)

\[ I/I_0 = (1 - \beta)(1 + [D]/K_d(0)exp(-zFVRT))^{-1} \]

where \( I \) and \( I_0 \) are the currents in the presence and absence of the drug and \( \beta \) is the fraction of the block that is voltage-independent.

Data were fitted to the curves defined by the appropriate equations indicated above, using Origin version 3.0 software (Microcal Software Inc., Northampton, MA). Data are expressed as mean ± S.E.M., where \( n \) is the number of cells. Statistical comparisons were made by analysis of variance. When significant differences were observed, Scheffe’s test was used for multiple comparisons. Statistical significance was inferred at \( P < .05 \).
Results

NMADA-evoked currents. Repetitive applications of 50 μM NMADA, 2 or 5 sec in duration at 40-sec intervals, produced rapidly activating, reproducible currents (fig. 1A). At a holding potential of −60 mV, inward currents decayed from a peak to a plateau at a rate that could be fit to a single-exponential function with a time constant (τ) of 1293 ± 149 msec (n = 38). At a holding potential of +30 mV, outward currents decayed with a τ of 2407 ± 381 msec (n = 10; data not shown). A concentration-effect curve is shown for NMADA-evoked currents from neurons clamped at −60 mV in figure 1B. The EC₅₀ for NMADA was 22.5 ± 1.3 μM (n = 10). These currents showed a strongly voltage-dependent block by the NMDA receptor antagonists Mg²⁺ and memantine (data not shown).

NMADA antagonism by ARL 15896AR. ARL 15896AR caused a rapid and reversible inhibition of 50 μM NMADA-evoked currents from neurons clamped at −60 mV, as shown in figure 2A. Fractional block (B) of NMADA-evoked currents by 0.1 to 200 μM ARL 15896AR was calculated using equation 1, where I was estimated by curve-fitting the decay of the NMADA-evoked current during the first 3 sec and extrapolating the fit to the end of the NMADA plus antagonist application. Iᵦ was the current measured at the end of NMADA plus antagonist coapplication. To verify the validity of estimating I using this technique, extrapolated current values from an NMDA/NMADA plus antagonist event were compared with an NMDA-alone event at the same time point. As can be seen in figure 2B, the values were virtually identical.

From the ARL 15896AR concentration-effect curve (fit using eq. 2) shown in figure 2C, the IC₅₀ for ARL 15896AR was estimated to be 9.8 ± 1.1 μM and the Hill coefficient was 1.4. The effect of 10 μM ARL 15896AR on the NMADA concentration-effect curve is shown in figure 1B. Compared with the EC₅₀ of 22.5 ± 1.3 μM (n = 10) for NMADA alone, ARL 15896AR did not significantly (P > .05) affect the EC₅₀ for NMADA activation (23.0 ± 2.3 μM; n = 6). The fractional block by ARL 15896AR using 10 or 100 μM NMADA was 0.47 ± 0.03 or 0.42 ± 0.02 (n = 6), respectively, and these values also were not significantly different (P > .05). These findings are consistent with a noncompetitive mechanism of block.

Use-dependence of antagonism. The application of ARL 15896AR before NMADA exposure did not affect the initial amplitude of the NMADA-evoked currents. However, these currents decayed rapidly as a block developed in the presence of NMADA. A representative example of five experiments demonstrating the use-dependence of ARL 15896AR is shown in figure 3A. A neuron clamped at −60 mV was perfused continuously for 30 sec, via a U-tube, with 50 μM NMADA. It was then perfused with normal bath solution for 1 min, followed by 50 μM ARL 15896AR for another 1 min, before a 30-sec NMADA/ARL 15896AR coapplication. The first application of NMADA produced a slowly inactivating inward current. In contrast, the current induced by the NMDA/ARL 15896AR coapplication started at the same amplitude as the first NMADA application but decayed to a plateau of about 25% of its initial amplitude within seconds.

We investigated whether ARL 15896AR, once bound to the NMDA receptor, remained trapped if the channel closed. A representative example of three experiments demonstrating trapping is shown in figure 3B. Applications of 50 μM NMADA, 5 sec in duration, were given at 40-sec intervals. During the first, third and fifth NMADA applications, ARL 15896AR was coapplied during the last 3 sec. The initial peak of the second (or fourth) NMADA-evoked current was attenuated, compared with the first (or third or fifth). However, after 2 sec of NMADA application the currents had equivalent amplitudes. This indicates that ARL 15896AR remained
trapped, as the channel closed after the first NMDA application, until the channel was reopened by the next NMDA application (35 sec later), whereupon it washed out in about 2 sec.

**Kinetics of antagonism.** The onset and relief of block of 200 μM NMDA-evoked currents in neurons clamped to −60 mV by 5 to 150 μM ARL 15896AR (fig. 4A) could be fit by single-exponential functions. The rate of onset of block (k_{obs}) was calculated as the reciprocal of the \( t \) of the exponential fitting function. In figure 4B, the mean \( k_{obs} \) values were plotted as a function of antagonist concentration and the rate constants were determined from equation 3. Based upon this application (trace 2), acquired 40 sec after trace 1, ARL 15896AR was coapplied during the last 3 sec. When the trace 2 current during the NMDA-alone application (i.e., the first 2 sec of the trace) was curve-fit to a single-exponential function and extrapolated to the 5-sec time point (between the two arrows), it matched the actual current observed during the 5-sec NMDA-alone application (trace 1). Scale, 1.25 sec and 200 pA. C, concentration-effect plot showing the fractional block by ARL 15896AR (0.1–200 μM) of currents evoked by 50 μM NMDA at −60 mV (n = 5 for 0.1, 1.0 and 5.0 μM ARL 15896AR, n = 8 for 10, 50, 100 and 200 μM ARL 15896AR). The IC_{50} was 10 μM ARL 15896AR, and the Hill coefficient was 1.4.
analysis $k_1 = 2.406 \times 10^4 \, \text{M}^{-1} \, \text{sec}^{-1}$, $k_{-1} = 0.722 \, \text{sec}^{-1}$ and $K_0 = 30 \, \mu\text{M}$ ($K_D = k_{-1}/k_1$). The $k_{\text{obs}}$ for the relief of block was independent of antagonist concentration (fig. 4B).

Voltage-dependence of antagonism. A current-voltage relationship for currents evoked by 50 $\mu$M NMDA in the absence or presence of 10 $\mu$M ARL 15896AR, shown in figure 5A, demonstrates the voltage-dependence of antagonism. Fitting the relative current block ($I/I_0$) at membrane potentials from $-80$ to $+60$ mV to the curve defined by equation 4b (fig. 5C) gives $K_D(0 \, \text{mV}) = 60 \, \mu\text{M}$ and $z\delta = 0.73$. Incorporating these values into equation 4a gives $K_D(-60 \, \text{mV}) = 11 \, \mu\text{M}$. This compares favorably with the IC$_{50}$ of 9.9 $\mu$M estimated from the concentration-effect curve in figure 2B. The unblocking rate was also influenced by membrane potential, ranging from 1397 ± 275 msec at $-80$ mV to 286 ± 11 msec at $+60$ mV ($n = 3$) (fig. 5B). Both of these observations are consistent with an open-channel mechanism of block and support the use-dependent nature of the antagonism described earlier.

Voltage-independent fraction of antagonism. An additional component of the antagonism of NMDA-evoked currents by 10 $\mu$M ARL 15896AR was voltage-independent (fig. 5, A and C), suggesting some interaction of the antagonist at a site distinct from the channel pore or situated in the channel such that the site is shielded from the charge field. NMDA concentrations between 5 and 100 $\mu$M did not significantly ($P > .05$) influence the fractional block of ARL 15896AR at a concentration near the IC$_{50}$ (10 $\mu$M) at a holding potential of $-60$ mV (fig. 1B), suggesting that the ligand binding site was not involved. Therefore, we investigated the possibility that the voltage-independent action of ARL 15896AR was due to activity at the glycine site.

The efficacy of the fractional block of 50 $\mu$M NMDA-evoked currents by ARL 15896AR was determined with 0.1, 10 or 100 $\mu$M glycine present, to determine whether glycine could modulate ARL 15896AR. At a holding potential of $+40$ mV, where the voltage-independent portion of the block should be predominant, 10 $\mu$M ($n = 7$) or 50 $\mu$M ($n = 6$) ARL 15896AR with 10 $\mu$M glycine present caused 9 ± 2 or 31 ± 6% block, respectively (fig. 6). Increasing the glycine concentration to 100 $\mu$M did not affect the block at either concentration of antagonist, but reducing it to 0.1 $\mu$M significantly ($P < .05$) increased the block by 10 $\mu$M or 50 $\mu$M ARL 15896AR, to 47 ± 6 or 84 ± 1%, respectively. Decreasing the glycine concentration from 10 to 0.1 $\mu$M caused a 5.2-fold increase in the percent block by 10 $\mu$M ARL 15896AR but only a 2.7-fold increase in the percent block by 50 $\mu$M ARL 15896AR. At all glycine concentrations, the percent block by ARL 15896AR was significantly ($P < .05$) stronger at 50 $\mu$M than at 10 $\mu$M. Spermine, at concentrations of 10, 100 or 1000 $\mu$M, did not affect the antagonism of 50 $\mu$M NMDA-evoked currents by 10 $\mu$M ARL 15896AR at a holding potential of $+60$ mV (data not shown), suggesting that ARL 15896AR does not act at the polyamine site.

Discussion

In this study we have used whole-cell patch-clamp and rapid perfusion techniques to characterize in detail the antagonism of NMDA-induced currents by ARL 15896AR. In line with previous reports, ARL 15896AR was shown to be an NMDA antagonist with a relatively low potency. ARL 15896AR was shown functionally to be a noncompetitive antagonist, because it did not significantly affect the EC$_{50}$ of NMDA at concentrations that blocked NMDA responses. In addition, the fractional blockade by ARL 15896AR was not overcome by increasing the concentration of NMDA. This is consistent with ARL 15896AR having affinity for the MK-801 binding site (Black et al., 1995). In addition, preapplication of ARL 15896AR before NMDA exposure did not substantially reduce the initial amplitude of the NMDA-evoked currents, indicating that antagonism by ARL 15896AR was agonist- or use-dependent. This uncompetitive mode of antagonism is characteristic of compounds acting via the MK-801 site. ARL 15896AR also exhibited a degree of trapping. Specifically, ARL 15896AR exited from the closed channel at a slower rate than from the open channel. However, if ARL 15896AR were completely unable to exit from the closed channel, trace 2 in figure 3B would have the same rate of activation as the relief of block by 100 $\mu$M ARL 15896AR in figure 4A. In fact, the relief of block in figure 4A occurred more rapidly than the activation of the second NMDA-induced current in figure 3B. Trapping has been reported with both high-affinity (MK-801)
and low-affinity (ADCI) uncompetitive NMDA antagonists (Jones and Rogawski, 1992; Rogawski, 1993).

In earlier work using intracellular calcium imaging (Black et al., 1996a), the kinetics of blockade by ARL 15896AR could not be resolved. In the present work, the rate of development of block was resolved and confirmed to be very rapid. The ability to observe the development of blockade makes it possible to strongly confirm the uncompetitive nature of antagonism by ARL 15896AR. The $z_d$ for ARL 15896AR was 0.73, similar to the values reported by Subramaniam et al. (1996) for $(R)$-$(-)$-remacemide and $(S)$-$(-)$-des-glycinyl-remacemide (0.65 and 0.82, respectively), suggesting that all three compounds act at the same site within the channel pore. The forward rate constant for ARL 15896AR ($k_{1}$) was marginally slower than that reported for $(R)$-$(-)$-remacemide or $(S)$-$(-)$-des-glycinyl-remacemide (9.7 $\times$ $10^4$ M$^{-1}$ sec$^{-1}$ and 4.5 $\times$ $10^4$ M$^{-1}$ sec$^{-1}$, respectively) (Subramaniam et al., 1996) or memantine (2.88 $\times$ $10^4$ M$^{-1}$ sec$^{-1}$) (Parsons et al., 1993). However, the reverse rate constant for ARL 15896AR ($k_{-1}$) was intermediate between those for $(R)$-$(-)$-remacemide and $(S)$-$(-)$-des-glycinyl-remacemide (2.6 sec$^{-1}$ and 0.047 sec$^{-1}$, respectively) and faster than that for memantine (0.20 sec$^{-1}$). The direct comparison of rate constants between studies may be misleading because experimental conditions, such as cultures, bath calcium and glycine concentrations and Ca$^{2+}$-buffering in the pipette, were not identical. The $K_D$ calculated from the rate constants (i.e., $K_D = k_{-1}/k_{1}$) for ARL 15896AR was 30 $\mu$M, similar to

![Fig. 5. A, inset, currents evoked by 50 $\mu$M NMDA at holding potentials from -80 to +60 mV. After an initial 3-sec NMDA application, 10 $\mu$M ARL 15896AR was coapplied for 4 sec, followed by a subsequent 4-sec NMDA application. The protocol was repeated every 40 sec, and the holding potential was increased in 10-mV steps. Scale, 3 sec and 300 pA. A, current-voltage relationship. NMDA-evoked currents measured after 4 sec of ARL 15896AR application (arrow 2, inset) were used to construct a current-voltage plot (C). Currents measured in the presence of NMDA alone were fit to single-exponential curves and extrapolated from arrow 1 to arrow 2, and the extrapolated values at these points were plotted (B). B, voltage-dependence of relief of block of NMDA (50 $\mu$M)-evoked currents by 10 $\mu$M ARL 15896AR (n = 3). C, predominant voltage-dependence of ARL 15896AR antagonism of currents evoked by 50 $\mu$M NMDA. Fitting the relative current block ($I/I_0$) at membrane potentials from -80 to +60 mV to the curve defined by equation 4b, with estimated $\beta = 0.25$, gives $K_D(0 \text{ mV}) = 60 \mu$M and $z_D = 0.73$ (n = 3).]
that for ADCI (Jones and Rogawski, 1992). This is 3-fold higher than the \( K_D \) calculated from the Woodhull equation or the IC\(_{50}\) measured from the concentration-effect curve. However, a similar order of discrepancy was reported for ADCI (Jones and Rogawski, 1992). The source of this discrepancy remains to be determined.

The antagonism of NMDA-evoked currents by ARL 15896AR was strongly voltage-dependent. Fitting the relative current block at membrane potentials between −80 and +60 mV gave \( K_D \) values at 0 and −60 mV of 60 and 11 \( \mu \)M, respectively. For a 1:1 binding reaction, the \( K_D \) should equal the IC\(_{50}\) and, in fact, the \( K_D \) for ARL 15896AR at −60 mV compares well with the IC\(_{50}\) of 10 \( \mu \)M determined from the concentration-effect curve measured at −60 mV. Similar agreement between \( K_D \) (−60 mV) and IC\(_{50}\) was observed with (S)-(−)-des-glycinyl-remacemide but not with (R)-(−)-remacemide (Subramaniam et al., 1996); these are two NMDA antagonists structurally similar to ARL 15896AR. In the latter case, it was speculated that the \( K_D \) (−60 mV) was greater because part of the block occurred at a voltage-independent site that had a higher affinity than the voltage-dependent site. In fact, those authors showed that remacemide had a significant voltage-independent component to its antagonism, whereas des-glycinyl-remacemide did not exhibit a voltage-independent component of blockade. ARL 15896AR also has a voltage-independent component of blockade. However, at −60 mV the voltage-independent component is small, compared with the voltage-dependent component, and may not significantly affect the overall potency of ARL 15896AR. It is intriguing that the small structural differences between these compounds produce large changes in antagonist character. The unblocking rate of ARL 15896AR was also influenced by membrane potential, becoming more rapid at depolarized potentials. Both this and the voltage-dependence of ARL 15896AR block are consistent with an open-channel mechanism of block and support the use-dependent nature of the antagonism described earlier.

From the concentration-effect curve, the Hill coefficient was 1.4, suggesting that there is more than one binding site for ARL 15896AR. Indeed, a fraction of the block was voltage-independent, indicating that this portion of the block occurred at a site outside, shielded from or insensitive to the transmembrane electric field. It is unlikely that the voltage-independent block by ARL 15896AR is due to an interaction at the ligand binding site, because NMDA concentration (5−100 \( \mu \)M) did not influence the fractional block with the antagonist at its IC\(_{50}\) concentration. It is also unlikely that ARL 15896AR acts at the polyamine site of the NMDA receptor, because spermine did not influence antagonism. However, decreasing the glycine concentration from 10 to 0.1 \( \mu \)M caused a 5.2-fold increase in the percent block by 10 \( \mu \)M ARL 15896AR, suggesting an interaction between ARL 15896AR and the glycine modulatory site. The nature of this interaction will require further study. A competitive interaction may be suggested by the fact that reduction of glycine produced only a 2.7-fold increase in the percent block by 50 \( \mu \)M ARL 15896AR. On the other hand, glycine (10 \( \mu \)M) did not affect the displacement of \(^{[3]H}\)MK-801 by ARL 15896AR in a binding assay (Black et al., 1995). Given that the physiological interaction with glycine was evident at 10 \( \mu \)M, which approximates the concentration in cerebrospinal fluid, the glycine interaction, although only a small portion of the block at physiological membrane potentials, could be a relevant characteristic of ARL 15896 antagonism, especially in pathological situations like cortical spreading depression.

At the IC\(_{50}\) concentration, ARL 15896AR failed to protect cultured cortical neurons from glutamate toxicity (Black et al., 1995), although increasing the dose to 25 \( \mu \)M afforded maximal protection. At this protective concentration, we would predict that some channel activity would remain, based on our concentration-effect curve and assuming that neurons were at a membrane potential of −60 mV. It is likely, however, that the neurons in the glutamate toxicity study were depolarized to some degree by glutamate before a block could develop. Because the block by ARL 15896AR is strongly voltage-dependent, more drug would be required to achieve a block in depolarized cells. This suggests that complete block of NMDA-mediated synaptic transmission is not required for neuroprotection by ARL 15896AR. The rapid kinetics of block by ARL 15896AR (due to its low affinity and therefore relatively high requisite concentrations) would be especially effective in achieving this protective level of block within physiologically relevant time frames, without having to produce significant tonic blockade of NMDA-mediated synaptic transmission.

ARL 15896AR is structurally similar to the anticonvulsant and antiparkinsonian agent remacemide hydrochloride (Palmer et al., 1992). However, there are a number of significant differences between these compounds. The characteristics of block of NMDA-induced currents by remacemide and its des-glycinyl metabolite have recently been reported (Subramaniam et al., 1996). Although generally similar, remacemide exhibits a voltage-independent blockade that the des-glycinyl metabolite does not. This could result in remacemide itself mediating more of the NMDA antagonism in pathological depolarized states. However, although remacemide and des-glycinyl-remacemide are both NMDA antagonists, remacemide is significantly less potent and probably not responsible for most of the in vivo NMDA antagonist activity at therapeutic concentrations, due to its rapid metabolism to des-glycinyl-remacemide. The antagonism of NMDA-evoked currents by ARL 15896AR is more similar to that by remacemide, in that it contains a voltage-independent component of block. ARL 15896AR, however, is significantly more potent than remacemide. In addition to NMDA blockade, both remacemide and des-glycinyl-remacemide have sodium channel-blocking activity (Wamil et al., 1996), which is not found in ARL 15896AR (Palmer et al., 1996). In view of these differences in action, ARL 15896AR provides a useful alternative to remacemide for testing in various pathological conditions, such as stroke.

ARL 15896AR blocks NMDA-induced toxicity in cultured rat cortical neurons and, at neuroprotective concentrations, rapidly decreases the NMDA-induced calcium influx and subsequent rise in intracellular free calcium (Black et al., 1995). It has been shown to be neuroprotective in models of acute neuronal damage (Palmer et al., 1996). In the present study, it has been shown to have relatively low potency, so it exhibits a fast development of block at therapeutic concentrations. In addition, its blockade is not overcome by increasing concentrations of glutamate, is use-dependent and contains a voltage-independent component of blockade that may be important in pathological depolarized states. These mechanistic attributes, coupled with its lack of side effects (Palmer et al., 1996), make ARL 15896AR a promising agent for further investigation.
et al., 1996), make ARL 15896AR an excellent candidate to be a clinically effective neuroprotective agent.

Acknowledgments

ARL 15896AR was provided by Astra Arcus USA (formerly Fisons Pharmaceuticals, Rochester, NY). Thanks go to Dr. M. Poulter for advice in designing the U-tube perfusion system.

References


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