

Orphenadrine is an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist: binding and patch clamp studies

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Summary. Orphenadrine has been used as an antiparkinsonian, antispastic and analgesic drug for many years. Here we show that orphenadrine inhibits [³H]MK-801 binding to the phencyclidine (PCP) binding site of the N-methyl-D-aspartate (NMDA)-receptor in homogenates of postmortem human frontal cortex with a K_i -value of $6.0 \pm 0.7 \mu\text{M}$. The NMDA receptor antagonistic effects of orphenadrine were assessed using concentration- and patch-clamp techniques on cultured superior colliculus neurones. Orphenadrine blocked open NMDA receptor channels with fast kinetics and in a strongly voltage-dependent manner. The IC_{50} -value against steady state currents at -70mV was $16.2 \pm 1.6 \mu\text{M}$ ($n = 6$). Orphenadrine exhibited relatively fast, concentration-dependent open channel blocking kinetics ($K_{\text{on}} 0.013 \pm 0.002 10^6 \text{M}^{-1} \text{S}^{-1}$) whereas the offset rate was concentration-independent ($K_{\text{off}} 0.230 \pm 0.004 \text{S}^{-1}$). Calculation of the ratio $K_{\text{off}}/K_{\text{on}}$ revealed an apparent K_d -value of $17.2 \mu\text{M}$ which is nearly identical to the IC_{50} calculated at equilibrium.

Keywords: Uncompetitive N-methyl-D-aspartate receptor antagonist, NMDA, orphenadrine, patch clamp, superior colliculus culture, [³H]MK-801 binding, cortical membranes, glutamate.

Introduction

The development of neuroprotective agents for the prevention of neuronal loss in acute conditions like stroke and epilepsy or chronic neurodegenerative disorders including Alzheimer's disease and Huntington's chorea is currently focusing, among others, on drugs that inhibit excitatory amino acid neurotransmission. Unfortunately, potent antagonists at the N-methyl-D-aspartate (NMDA) type glutamate receptor, e.g., MK-801 or phencyclidine (PCP), share a high probability of inducing psychotomimetic side effects (Kornhuber and Weller, 1995). Further, these drugs have been associated with acute neurotoxicity in vitro and in vivo, precluding their clinical use.

Orphenadrine [N,N-dimethyl-2(o-methyl- α -phenylbenzyloxy)ethylamine] has been widely used for the control of Parkinson's disease and acute phases of drug-induced Parkinsonism (Mindham et al., 1972; Bassi et al., 1986). Furthermore, orphenadrine has muscle relaxant and analgesic properties (Hunnskaar and Donnell, 1991). While the antiparkinsonian effects have been explained by its anticholinergic properties (Syvälahti et al., 1988), the mechanisms of the analgesic effects of orphenadrine remained elusive. Two other antiparkinsonian agents, namely amantadine and budipine, may mediate their symptomatological effects at least partially via open channel blockade of NMDA receptors (Kornhuber et al., 1991, 1995; Klockgether et al., 1993; Danysz et al., 1994, 1995; Jackisch et al., 1994; Porter and Greenamyre, 1995; Parsons et al., 1995a,b). This raises the possibility that at least part of the antiparkinsonian effects of orphenadrine are also mediated by blockade of hyperactive glutamatergic pathways in the basal ganglia (see Carlsson and Carlsson, 1990; Schmidt et al., 1992; Riederer et al., 1992; Klockgether and Turski, 1993; Danysz et al., 1994, 1995).

The good clinical tolerability of amantadine has been attributed, at least in part, to its low potency and associated fast open channel kinetics and strong voltage-dependency. These properties may allow it to block prolonged pathological low level activation of NMDA receptors whilst leaving their transient physiological activation relatively intact (Parsons et al., 1995b). The aim of the present study was to assess whether orphenadrine is an NMDA receptor antagonist using displacement of [3 H]MK-801 binding to cortical membranes and patch clamp recordings from cultured superior colliculus neurones.

Methods

Tissue from the frontal cortex was taken at autopsy from 3 subjects with no apparent history of neurological or psychiatric disorders. There were 2 males and 1 female aged 60, 68 and 70 years, respectively. The postmortem interval was between 4 and 44 hours. The samples were placed in a freezer at -80°C until analysis. All details of the experimental procedures were identical to those previously described (Kornhuber et al., 1991). In brief, membrane homogenates of brain tissue were prepared and binding experiments were carried out at $+21^{\circ}\text{C}$ in plastic microtitre plates in a total volume of 200 μl . The incubation medium consisted of 5 mM Tris-HCl (pH 7.4) containing 3 nM [3 H]MK-801, 5 μM L-glutamate, 5 μM glycine and 10 μM MgCl_2 (final concentrations). Orphenadrine, and for comparison memantine, were added at increasing concentrations ranging up to 300 μM . Protein concentration was around 0.40 mg protein/ml. After 16 h incubation bound ligand was separated by rapid filtration through Whatman GF/B filters using a Titertek cell harvester followed by a 10 sec wash with cold assay buffer. Filters were transferred into plastic vials and upon addition of 5 ml of a toluene-based scintillation cocktail, they were monitored after 2 h for tritium in a Beckman LS 1801 counter at 54% efficiency. The binding displaced by 100 μM of unlabelled MK-801 was taken as specific binding. Pseudo-Hill coefficients and preliminary estimates of binding parameters from displacement experiments were provided by the EBDA program (McPherson, 1983). The K_i -values were determined with the iterative non-linear curve-fitting program developed by Munson and Rodbard (1980). Mean values are given \pm S.D.

Patch clamp recordings from cultured superior colliculus neurones were performed as described previously (Parsons et al., 1993). In brief, superior colliculi were isolated from embryonic rats (E20–21) and maintained in culture for 7–14 days in NaHCO_3 /HEPES-buffered minimum essential medium supplemented with 5% foetal calf serum and 5%

horse serum (Gibco) and incubated at 37°C with 5% CO₂ (95% humidity). The superior colliculus culture was chosen for these experiments as it provides very stable recording conditions which are an absolute prerequisite for voltage-dependency and kinetic experiments. Moreover, the relatively small neurones (soma 15–20 μmØ) are ideally suited to minimise problems of buffered diffusion for concentration clamp experiments.

Voltage-clamp recordings were made from these neurones in the whole cell mode at room temperature (20–22°C) with the aid of an EPC-7 patch clamp amplifier (List). Polished glass recording electrodes were pulled with an horizontal puller (DMZ) and had an internal tip diameter between 1.0 and 1.2 μm and a tip resistance of 4–8 mΩ. Most recordings were made at a membrane potential of –70 mV. Cells were continuously superfused via one of eight channels of a custom made fast superfusion system with a common outflow. Test substances were applied by rapidly switching channels – complete exchange of the superfusion solution around the neurone was achieved within 10–20 ms as confirmed by the speed of block of voltage-activated calcium channels when switching to solutions containing cadmium. The application of solutions and the synchronised on-line electronic acquisition of data were controlled by the program PCLAMP for IBM PCs. Subsequently, AUTESP for IBM PCs (Garching Instruments, Munich) was utilised to analyse the data semi-automatically off-line. Only results from stable cells were accepted for inclusion in the final analysis i.e. following recovery of responses to NMDA by at least 50% of their depression by orphenadrine.

The contents of the intracellular solution were as follows (mM): CsCl (120), TEACl (20), EGTA (10), MgCl₂ (1), CaCl₂ (0.2), glucose (10), ATP (2), cAMP (0.25); pH was adjusted to 7.3 with CsOH or HCl. All extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), CaCl₂ (0.2), glucose (10), HEPES (10), sucrose (4.5), glycine (1*10⁻²), (pH 7.3). Neurones were pharmacologically isolated from one another by the inclusion of 0.3 μM TTX to block voltage-activated sodium currents. NMDA and/or orphenadrine were added to this basic solution in concentrations detailed in Results. In addition, the bath solution and one superfusion solution contained 1.5 mM CaCl₂ to aid formation of a “giga Ohm seal”.

[³H]MK-801 was purchased from New England Nuclear, cold MK-801 from RBI. The protein-assay solutions and protein standards were purchased from Bio-Rad (München, FRG). All other compounds, including orphenadrine, were obtained from Sigma.

Results

Orphenadrine nearly completely inhibited [³H]MK-801 binding at 100 μM (Fig. 1). The displacement curves produced by orphenadrine had Hill coefficients near unity (1.1 ± 0.1) and were best fitted using a one-site model. The K_i-value of orphenadrine was 6.0 ± 0.7 μM, which is between that of amantadine and budipine on the one hand and memantine on the other hand (Table 1) (Kornhuber et al., 1991, 1995).

Orphenadrine antagonized inward current responses to NMDA (200 μM) in a concentration-dependent manner (IC₅₀ on steady state currents of 16.2 ± 1.6 μM, n = 6, SEM) with e.g. 7 times lower potency than memantine but 4 times greater potency than amantadine (Figs. 2 and 3a, see Parsons et al., 1995b). The Hill coefficient of 1.12 ± 0.05 was close to unity and thus gives no indication for interactions of orphenadrine with multiple sites on the NMDA receptor complex.

The effects of orphenadrine (100 μM) were strongly voltage-dependent (Fig. 3b). Moreover, relatively fast use-dependence (Fig. 2a) was confirmed in experiments where orphenadrine was applied either (1) only with NMDA (2) only between NMDA application or (3) both between and with NMDA

applications (data not shown). Thus, the effects of orphenadrine seem to be mediated via open channel blockade.

Kinetic experiments were performed by applying various concentrations of orphenadrine (30–300 μM) for 5 seconds in the continuous presence of NMDA (200 μM). Single exponential fits were made using the program AUTESP. Orphenadrine exhibited relatively fast, concentration-dependent open channel blocking kinetics (K_{on} $0.013 \pm 0.002 \text{ } 10^6 \text{ M}^{-1} \text{ S}^{-1}$) whereas the offset rate was concentration-independent (K_{off} $0.230 \pm 0.004 \text{ S}^{-1}$). Calculation of the ratio $K_{\text{off}}/K_{\text{on}}$ revealed an apparent K_{d} -value of 17.2 μM which is nearly identical to the IC_{50} calculated at equilibrium (Fig. 4).

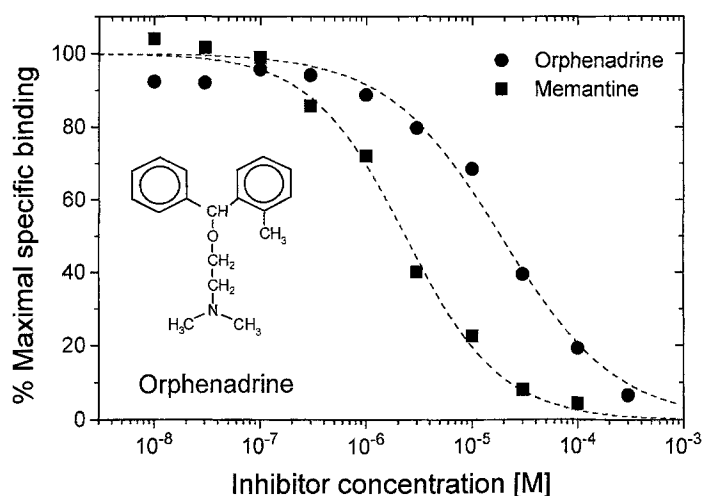


Fig. 1. Inhibition of specific binding of $[^3\text{H}]\text{MK-801}$ (3 nM) to homogenates of postmortem human frontal cortex by orphenadrine. A competition curve for memantine is given for comparison. The values in this figure are the means of triplicate determinations of a single representative experiment. The experiment was repeated 3 times with very similar results using homogenates from different brains. The K_{I} -values and Hill numbers for the binding sites are given in Table 1

Table 1. Binding characteristics of orphenadrine to the PCP binding site. The data for memantine, amantadine and budipine are given for comparison

	K_{I} -value (μM)	Pseudo-Hill coefficient
Orphenadrine	6.0 ± 0.7	1.1 ± 0.1
Memantine*	0.5 ± 0.2	1.0 ± 0.2
Amantadine*	10.5 ± 6.1	0.9 ± 0.3
Budipine*	11.7 ± 0.2	1.3 ± 0.3

Data are given as mean \pm SD values ($n = 3-7$). For further details see text. *Data are taken from Kornhuber et al., 1991, 1995

Discussion

The results of the present short study provide the first data indicating that orphenadrine is an uncompetitive NMDA receptor antagonist. In line with its relatively low affinity, the open NMDA receptor channel blockade by orphenadrine showed fast kinetics and strong voltage-dependency. These properties may partially account for the clinical efficacy and good tolerability of orphenadrine in the treatment of Parkinson's disease (Carlsson and Carlsson, 1990; Schmidt et al., 1992; Riederer et al., 1992; Chen et al., 1992; Parsons et al., 1993; Klockgether and Turski, 1993; Danysz et al., 1994, 1995).

The serum concentrations of orphenadrine are in the low micromolar range under therapeutic conditions (Altamura et al., 1986a,b; Contin et al.,

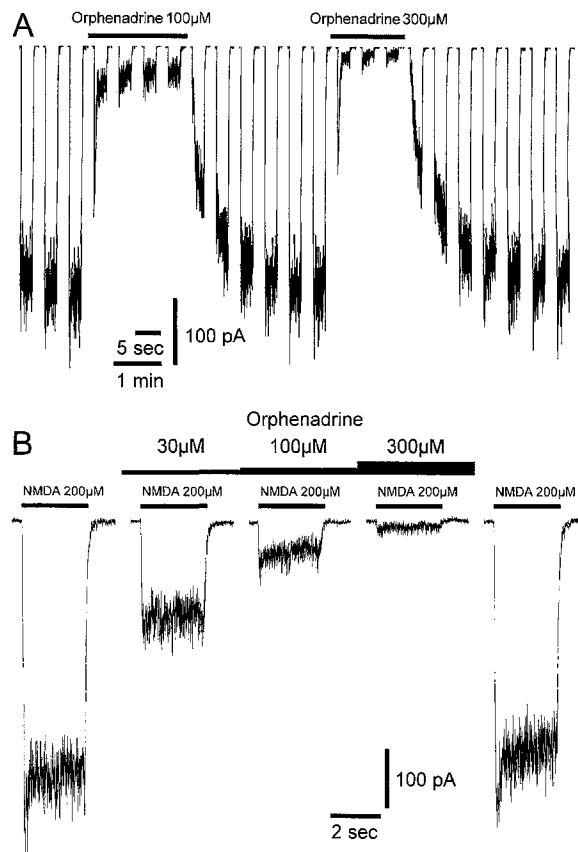


Fig. 2. **A** Use-dependence of orphenadrine on a single superior colliculus neurone. NMDA (200 μM) was applied for 2.5 seconds every 30 seconds in the continuous presence of glycine (1 μM) and at a constant membrane potential of -70 mV. This inter-response interval has been omitted for clarity (two time scales). Orphenadrine (100 μM and 300 μM) was continuously present for 2 and 1.5 minutes respectively as indicated by the solid bars. Note that the first peak response following a 10–20 second preincubation with orphenadrine (100 μM) was less affected. Recovery from the effects of orphenadrine was relatively rapid, but also use-dependent. **B** Concentration-dependence of the blockade of NMDA receptors by orphenadrine on a single superior colliculus neurone. The left and right panels show control and recovery responses to NMDA (200 μM). The middle three panels show equilibrium agonist responses in the continuous presence of orphenadrine (30, 100 and 300 μM respectively)

1987; Labout et al., 1982). Thus, therapeutic concentrations are much higher than the K_i -value of orphenadrine at central muscarinic receptors ($0.1 \mu\text{M}$, Syvälahti et al., 1988) but are close to the K_i -value at the PCP binding site ($6.0 \mu\text{M}$). This suggests that orphenadrine has, in addition to anticholinergic effects, NMDA receptor antagonistic properties. While NMDA receptor antagonism *alone* might explain antiparkinsonian, antispastic and antinociceptive properties of orphenadrine, the additional interference with cholinergic transmission might have synergistic effects e.g., in the treatment of Parkinson's disease. The present results not only provide an alternative explanation for the antiparkinson, antispastic and antinociceptive effects but also suggest neuroprotective properties of orphenadrine similar to those of amantadine and memantine (Kornhuber et al., 1994).

It should be noted that the potency of orphenadrine in the present study on cultured superior colliculus neurones was about 5 fold lower than thera-

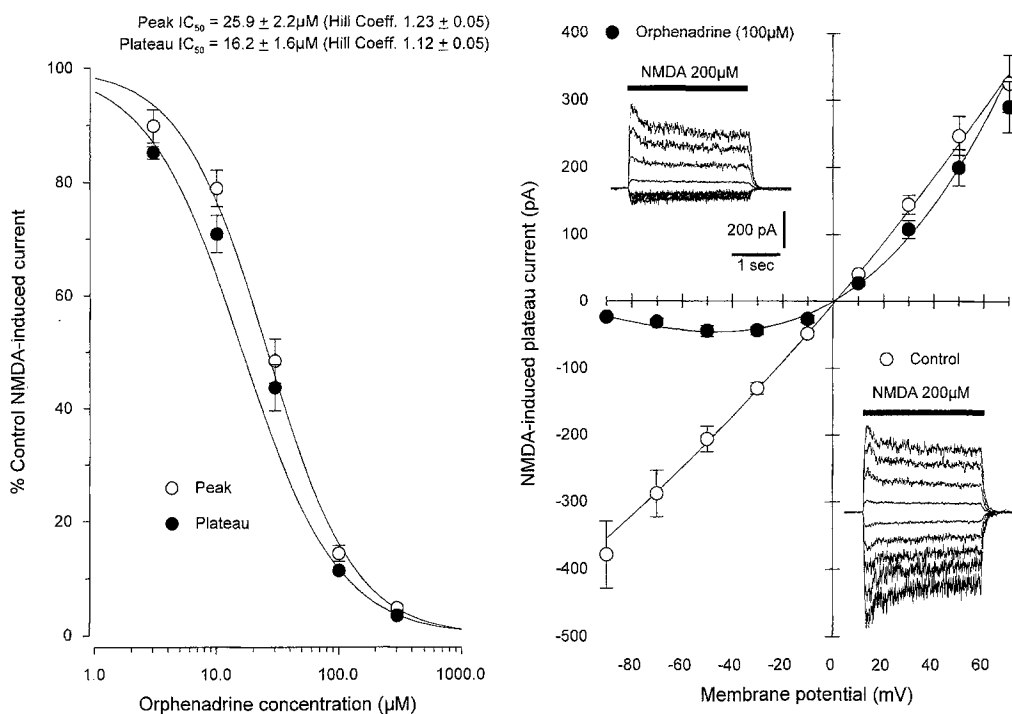


Fig. 3. **Left:** Concentration-dependence of the blockade of NMDA receptors by orphenadrine. Peak and plateau (steady state) NMDA current response were normalised to control levels and plotted as means (\pm SEM) against orphenadrine concentration ($n = 6$ per concentration). Estimation of IC_{50} s and curve fitting were made according to the 4 parameter logistic equation (Grafit, Erithacus Software). **Right:** Voltage-dependence of the blockade of NMDA receptors by orphenadrine. NMDA ($200 \mu\text{M}$) was applied for 2.5 seconds every 30 seconds in the continuous presence of glycine ($1 \mu\text{M}$) at various membrane potentials. Plateau NMDA current responses in the absence and presence of orphenadrine ($100 \mu\text{M}$) have been plotted as means \pm S.E.M. against membrane potential ($n = 3$). The upper left insert shows original data for the i.v. curve in the presence of orphenadrine ($100 \mu\text{M}$). The lower right insert shows original data for control NMDA responses in the absence of antagonist

apeutically-relevant concentrations and 3 fold lower than for the displacement of [^3H]MK-801 binding in cortical membranes. It is possible that this difference is due to the differential expression of NMDA receptor subtypes in e.g. cultured superior colliculus neurones and the basal ganglia *in vivo*. Thus, the antiparkinsonian drug amantadine was also found to be more potent against NMDA-induced currents in freshly dissociated striatal neurones than in hippocampal or superior colliculus neurones whereas the opposite was true for other uncompetitive NMDA receptor antagonists with less favorable profiles in animal models of Parkinson's disease (Danysz et al., 1994, 1995; Parsons et al., 1995a,b). Moreover, biochemical studies also indicate that the relative potency of amantadine, compared e.g. to MK-801 or memantine, is higher in striatal slices (Lupp et al., 1992; Stoof et al., 1992). This raises the possibility that the good clinical tolerability of orphenadrine and amantadine in the treatment of Parkinson's disease is related to relatively selective actions at NMDA receptor subtypes (Monyer et al., 1992) expressed selectively in basal ganglia and less to their fast, strongly voltage-dependent open channel blocking kinetics. Indeed, if hyperactivity of glutamatergic pathways in the basal ganglia is responsible for Parkinsonian symptoms (Carlsson and Carlsson, 1990; Schmidt et al., 1992; Riederer et al., 1992; Klockgether and Turski, 1993; Danysz et al., 1994, 1995), then such properties might rather be

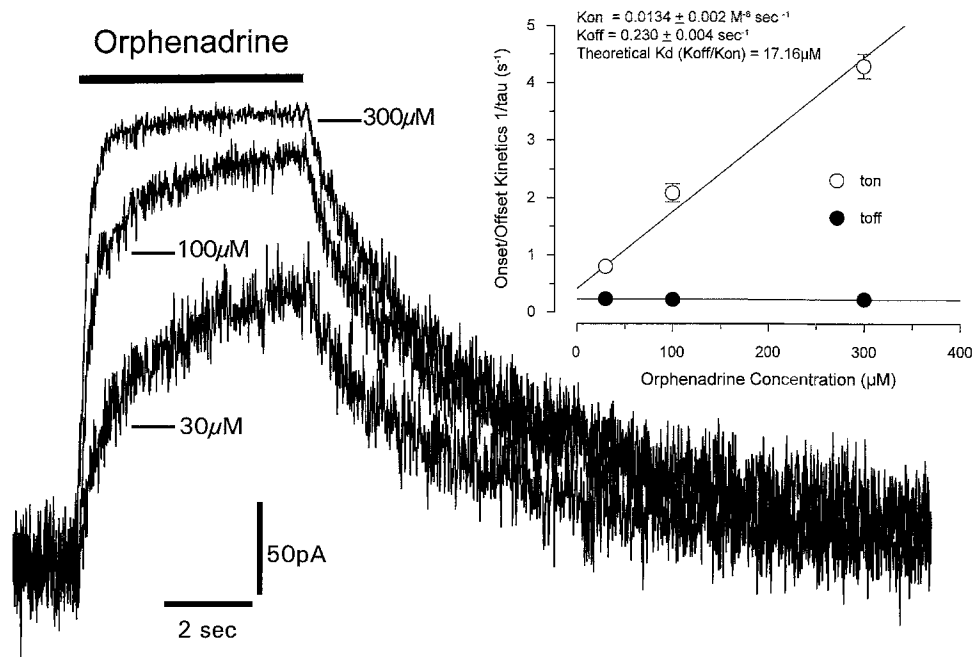


Fig. 4. Kinetics of NMDA open-channel block by orphenadrine. NMDA (200 μM) was applied continuously. Orphenadrine (30, 100 and 300 μM) was then coadministered for 5 seconds with NMDA as indicated by the bar. The insert shows the concentration-dependence of the rate of onset orphenadrine blockade and the concentration-independence of the offset rate. K_{on} was $0.013 \pm 0.002 \cdot 10^6 \text{ M}^{-1} \text{ S}^{-1}$ and K_{off} was $0.230 \pm 0.004 \text{ sec}^{-1}$. Calculation of affinity as the ratio $K_{\text{off}} / K_{\text{on}}$ gave a K_{d} -value of $17.2 \mu\text{M}$ which is close to the IC_{50} calculated at equilibrium ($16.2 \mu\text{M}$)

predicted to decrease the efficacy of amantadine or orphenadrine i.e. they should effect such transient synaptic transmission less than prolonged low level pathological activation (Parsons et al., 1993, 1995b). In this regard, it is important to note that the voltage-dependency of amantadine may be less pronounced in striatal than in hippocampal neurones (Parsons et al., 1995a) and that relevant concentrations do indeed block synaptic transmission in this same structure (Rohrbacher et al., 1994).

In conclusion, the present data show for the first time that orphenadrine is active at the PCP binding site of the NMDA receptor at therapeutic concentrations. Orphenadrine is a strongly voltage-dependent uncompetitive NMDA receptor antagonist with fast, open channel blocking kinetics – at least in cultured superior colliculus neurones.

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