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The mechanism of action and pharmacological specificity of the anticonvulsant NMDA antagonist MK-801: a voltage clamp study on neuronal cells in culture

Robert F. Halliwell, John A. Peters & [1]Jeremy J. Lambert

Neuroscience Research Group, Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY

1 Some possible molecular mechanisms of action of the anxiolytic, anticonvulsant and neuroprotective agent MK-801 have been examined in 'whole-cell' voltage clamp recordings performed on rat hippocampal and cortical neurones, bovine adrenomedullary chromaffin cells and N1E-115 neuroblastoma cells maintained in cell culture.

2 Transmembrane currents recorded from rat hippocampal and cortical neurones in response to locally applied N-methyl-D-aspartate (NMDA) were antagonized by MK-801 (0.1–3.0 μM). Blockade was use-dependent, and little influenced by transmembrane potential. MK-801 (3 μM) had no effect on currents evoked by kainate (100 μM).

3 The antagonism of NMDA-induced currents by MK-801 was only slowly and incompletely reversed when the cell membrane potential was clamped at -60 mV during washout. Prolonged applications of NMDA at $+40$, but not -60 mV during washout, markedly accelerated recovery from block.

4 In contrast to MK-801, ketamine (10 μM) blocked NMDA-induced currents in a voltage-dependent manner. Blockade increased with membrane hyperpolarization and was completely reversible upon washout.

5 MK-801 (1–10 μM) produced a voltage- and concentration-dependent block of membrane currents elicited by ionophoretically applied acetylcholine (ACh) recorded from bovine chromaffin cells. The block was readily reversible upon washout.

6 γ-Aminobutyric acid$_A$ ($GABA_A$) receptor-mediated chloride currents of chromaffin cells were unaffected by MK-801 (1–100 μM). In contrast, such currents were potentiated by diazepam (1 μM). MK-801 (100 μM) had no effect on currents evoked by GABA on hippocampal neurones.

7 MK-801 (10 μM) had little effect on membrane currents recorded from N1E-115 neuroblastoma cells in response to ionophoretically applied 5-hydroxytryptamine (5-HT). Such currents were antagonized by the 5-HT_3 receptor antagonist GR 38032F (1 nM) and also by MK-801 at high concentration (100 μM).

8 Voltage-activated, tetrodotoxin-sensitive, sodium currents of chromaffin cells were unaffected by 10 μM MK-801. However, at a relatively high concentration (100 μM), MK-801 reduced the amplitude of such currents to approximately 77% of control.

9 The relevance of the present results to the central actions of MK-801 is discussed.

Introduction

The excitatory actions of the amino acids L-glutamate and L-aspartate on the central nervous system are mediated through up to four distinct receptor subtypes (Cotman & Iversen, 1987; Watkins & Olverman, 1987). Of these, the receptor selectively activated by N-methyl-D-aspartate (NMDA) has been particularly well characterized. Electrophysiological studies performed on central neurones in cell culture indicate that NMDA activates a cation selective ion channel which conducts

[1] Author for correspondence.

calcium in addition to sodium and potassium ions (Ascher & Nowak, 1987). Membrane currents evoked by NMDA are blocked in a voltage-dependent manner by magnesium ions (Nowak *et al.*, 1984, Mayer & Westbrook, 1985) and are potentiated by glycine, which appears to act through a strychnine-insensitive allosteric regulatory site on the NMDA receptor (Johnson & Ascher, 1987).

Antagonists of the NMDA-ion channel complex are potentially important therapeutic agents; the treatment of certain neurodegenerative disorders being one possible application (Meldrum, 1985). Whilst competitively-acting NMDA receptor antagonists such as D-2-amino-5-phosphonovalerate (APV) have proved useful as *in vitro* research tools, their therapeutic potential is limited since they do not readily cross the blood brain barrier. Recently, the lipophilic compound MK-801 ((+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) has been shown to be a potent non-competitive antagonist of the NMDA receptor-channel complex (Wong *et al.*, 1986). This compound displays anticonvulsant, sympathomimetic and anxiolytic properties (Clineschmidt *et al.*, 1982) and in some animal models can protect against central neuronal loss induced by NMDA and ischaemic episodes (Gill *et al.*, 1987). Biochemical studies have demonstrated high-affinity stereoselective binding sites for radiolabelled MK-801 in rat brain (Wong *et al.*, 1986) with a topography similar to that described for NMDA receptors (Monaghan & Cotman, 1985; Bowery *et al.*, 1988). The binding of [^{3}H]-MK-801 to rodent brain membranes is enhanced by excitatory amino acids (Foster & Wong, 1987) and by glycine (Wong *et al.*, 1987). Selective non-competitive antagonists of the NMDA receptor channel complex, including the dissociative anaesthetics phencyclidine (PCP) and ketamine (Anis *et al.*, 1983; Harrison & Simmonds, 1985; Martin & Lodge, 1985; Honey *et al.*, 1985), displace [^{3}H]-MK-801 from its binding site with potencies which correlate well with their blocking actions in functional tests (Wong *et al.*, 1986). Such data are consistent with MK-801 binding to a site within the NMDA-gated ion channel rather than the agonist recognition site, a notion supported by the demonstration of agonist-dependent antagonism by MK-801 of NMDA-induced depolarizations in slices of rat cerebral cortex (Kemp *et al.*, 1987). Studies performed on voltage-clamped rodent central neurones have demonstrated that the antagonism of NMDA-induced currents by ketamine is use- and voltage-dependent, suggesting that ketamine also binds to a site within the NMDA-gated ion channel (Honey *et al.*, 1985; MacDonald *et al.*, 1987).

In the present study, the actions of MK-801 and ketamine on NMDA-induced currents recorded from voltage-clamped hippocampal and cortical neurones in cell culture were compared. As ketamine is known to block the nicotinic receptor ion channel (Maleque *et al.*, 1981; Volle *et al.*, 1982), the possibility that MK-801 shares this action was investigated in experiments on voltage-clamped bovine adrenomedullary chromaffin cells. Due to the postulated involvement of γ-aminobutyric acid$_A$ ($GABA_A$) receptors, 5-hydroxytryptamine$_3$ ($5\text{-}HT_3$) receptors and voltage-activated sodium channels in the mode of action of some anxiolytic and anticonvulsant drugs (Willow, 1986; File, 1987; Jones *et al.*, 1988) we additionally evaluated whether or not MK-801 interacts with these sites. A preliminary account of a part of this work has appeared in abstract form (Callachan *et al.*, 1988). Whilst this report was in preparation, a complementary study performed on rodent visual cortical neurones (Heuttner & Bean, 1988) came to our attention.

Methods

Dissociation and culture of hippocampal and cortical neurones

Embryonic rat hippocampal neurones were isolated and cultured essentially as described by Heuttner & Baughman (1986) with minor modifications. E18 embryos, dissected from Sprague-Dawley rats which had been killed by cervical dislocation, were decapitated and the whole brain quickly removed. The cerebral cortices or hippocampi were isolated in Hank's balanced salt solution (HBSS) at ambient temperature (17–21°C), chopped into fragments and incubated for 60 min at 37°C in an enzyme solution containing (in mM): NaCl 116, KCl 5.4, $NaHCO_3$ 26, NaH_2PO_4 1, $CaCl_2$ 1.5, $MgSO_4$ 1, EDTA 0.5, glucose 25, cysteine 1 and papain (20 units ml^{-1}) (pH 7.4). Subsequently, tissue fragments were rinsed in 5 ml of HBSS supplemented with 1 mg ml^{-1} bovine serum albumen (BSA) and 1 mg ml^{-1} ovomucoid. The tissue was transferred into a further 3–4 ml of this solution and dissociated into a cell suspension by gentle trituration with a fire-polished pasteur pipette. Dissociated cells were layered onto 5 ml of HBSS containing BSA (10 mg ml^{-1}) and ovomucoid (10 mg ml^{-1}) and centrifuged at 100 *g* for 10 min. The supernatant was discarded and the cells resuspended in a growth medium composed of Minimal Essential Medium supplemented with 5% (vol/vol) foetal calf serum (FCS), 5% horse serum, streptomycin (50 mg l^{-1}) and penicillin (5×10^4 iu l^{-1}). The final concentrations of glutamine and glucose in the medium were adjusted to 2 and 20 mM respectively. Approximately $3–5 \times 10^5$ cells were plated into

35 mm diameter (Falcon) 'Primaria' culture dishes and incubated in 1.5 ml of growth medium at 37°C in an atmosphere of 95% air, 5% CO_2 at 100% relative humidity. Cultures were fed at 3–4 day intervals by replacing approximately two thirds of the volume of growth medium. The proliferation of non-neuronal cells was suppressed as they approached confluency by the inclusion of cytosine arabinoside (10 μM) in the growth medium for a period of 48 h. In some instances, dissociated hippocampal cells were plated onto confluent monolayers of non-neuronal cells isolated from neonatal rat cerebral cortices by methods similar to those described above. Hippocampal and cortical neurones were used in experiments 10 to 30 days after plating.

Dissociation and culture of chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated and cultured by the method of Fenwick *et al.* (1982) with minor modifications (Cottrell *et al.*, 1987) and used in electrophysiological experiments 1–7 days after plating.

Culture of N1E-115 neuroblastoma cells

N1E-115 cells were cultured according to Peters *et al.* (1988a) and used 2 to 7 days after plating.

Electrical recordings

Agonist- and voltage-activated currents were recorded by the 'whole-cell' clamp mode of the patch-clamp technique (Hamill *et al.*, 1981) with a List Electronics L/M EPC-7 converter headstage and amplifier. Currents were low pass filtered (Bessel characteristic) at the cut-off frequencies indicated in the Figure legends, and recorded onto either magnetic tape with an FM tape recorder (Racal Store 4DS), or onto video tape with a video recorder (Ferguson Videostar) in conjunction with a Sony PCM 701 digital pulse code modulator. In all experiments except those utlizing the agonist NMDA, cells were continuously superfused with a saline containing (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2, $CaCl_2$ 1 and HEPES-NaOH 10, (pH 7.2). NMDA-induced currents were recorded in a saline nominally free of Mg^{2+} and supplemented with glycine (1 μM) and tetrodotoxin (300 nM–1 μM) to maximize the NMDA-induced response (Nowak *et al.*, 1984; Johnson & Ascher, 1987) and suppress ongoing synaptic activity respectively. Voltage-activated sodium currents were recorded from cells bathed with a nominally Ca^{2+}-free saline containing Co^{2+} (1 mM) to eliminate contamination by voltage-activated Ca-currents. In all experiments K-currents were suppressed by dialysing the cell interior with a Cs-rich pipette solution comprising (in mM): CsCl 140, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 1.1 and HEPES-NaOH 10 (pH 7.2). The pipette solution was supplemented with 2 mM Mg-ATP in experiments when GABA-evoked currents were recorded from hippocampal neurones. All recordings were made at room temperature (17°–21°C).

Drug application

Unless specified otherwise, antagonist compounds were introduced to the bath via the superfusion system. In the majority of experiments, agonists were applied locally to cells by pressure ejection (1.4×10^5 Pa) from modified patch pipettes. Alternatively, acetylcholine (ACh), 5-HT and NMDA were applied by ionophoresis from high-resistance (40–180 MΩ) micropipettes by use of a constant-current device based on the design of Dreyer & Peper (1974). Pipettes were back-filled with aqueous solutions containing either ACh bromide (1.0 M), 5-HT creatinine sulphate (20 mM; pH 3.5) or NMDA (20 mM; pH 8.0). ACh and 5-HT were ejected by positive rectangular pulses of current and their diffusional release opposed by retaining currents of −2 to −10 nA. NMDA was applied by negative ejection currents superimposed on a retaining current of 0.6 to 1.6 nA.

Data analysis

Agonist activated currents were analysed either manually from pen recorder traces, or by semi-automated programs (Dempster, 1988) run on a PDP 11-73 minicomputer essentially as described by Peters *et al.* (1988b). Briefly, up to 5 agonist-evoked currents were digitised into 512 or 1024 data points at an appropriate sampling rate, and subsequent to validation, averaged to yield a mean current. For illustration, such averaged currents were then plotted on a Hewlett Packard 7470A plotter. All quantitative data are expressed as the mean ± s.e.mean.

Reagents

All biological and synthetic media employed in cell culture were obtained from Gibco (Paisley, Scotland). Sera were heat-inactivated at 56°C for 30 min before use. Papain, ovomucoid and cysteine were supplied by Sigma. Drugs employed in electrophysiological experiments were obtained from the following sources: acetylcholine bromide, adenosine 5′-triphosphate (Mg salt), γ-aminobutyric acid, DL-2-amino-5-phosphonovaleric acid (APV), glycine,

kainic acid, 5-hydroxytryptamine creatinine sulphate, N-methyl-D-aspartic acid (Sigma); MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate) (Merck, Sharp and Dohme); GR 38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazol-4-one hydrochloride dihydrate) (Glaxo); diazepam (Roche); ketamine (Parke Davis). With the exception of diazepam, which was prepared as a concentrate in ethanol, all drugs were freshly dissolved into either recording saline or twice-distilled deionized water. Ethanol, at the final concentration (0.01% vol/vol) used in experiments, had no effect upon the relevant agonist-induced currents.

Results

Inward currents evoked by local applications of NMDA and kainate were recorded under voltage-clamp in neurones from the hippocampus or cerebral cortex. All neurones sampled were sensitive to the agonists and displayed spontaneous synaptic currents if TTX was omitted from the perfusate. There were no obvious differences in characteristics of the agonist-evoked currents recorded from cells of cortical or hippocampal origin. Unless specifically stated otherwise, experiments were conducted at a holding potential of −60 mV.

Figure 1a–c illustrates the influence of the antagonists MK-801 (300 nM), ketamine (10 μM) and APV (10 μM) on inward currents evoked by ionophoretically applied NMDA. Although a quantitative comparison of these three blockers was not attempted, differences in their potency and reversibility were obvious. When applied against responses evoked by pulsatile applications of NMDA (40–80 nA, 1 s, 0.05 Hz), the antagonism produced by low concentrations of MK-801 (100–300 nM) often developed slowly, such that a 'steady-state' block was difficult to establish with certainty. This may, at least in part, have been due to the method of agonist application employed, since subsequent experiments indicated that the antagonist action of MK-801 is largely use-dependent (see below). In 4 cells superfused with solution containing 300 nM MK-801 for at least 15 min, the amplitude of the NMDA-evoked current was estimated to be reduced to 16.8 ± 3.5% of its control value. A more rapid, and essentially complete, blockade of ionophoretically induced responses (NMDA current being reduced to 1.3 ± 1.3% of control, $n = 3$) was observed when MK-801 was bath-applied at a concentration of 3 μM. The blockade produced by MK-801 was very poorly reversed upon washout. In the exemplar cell (Figure 1a) a modest degree of recovery was observed during a 20 min wash with MK-801-free medium, but in other cells no recovery was discernible within this time. Incomplete recovery from blockade by MK-801 has previously been reported (Wong *et al.*, 1986) and will be considered further below. In contrast to MK-801, ketamine (10 μM, Figure 1b) and APV (10 μM, Figure 1c) antagonized responses evoked by ionophoretically applied NMDA in a completely reversible manner. Blockade of NMDA-induced currents by MK-801 and ketamine was selective, as neither compound had any effect upon inward currents evoked by pressure-applied kainate (100 μM) when bath-applied at concentrations of 3 and 10 μM respectively (Figure 1d).

Use-dependent block of NMDA-induced currents by MK-801

It has previously been demonstrated that antagonism of NMDA-evoked currents in hippocampal neurones by ketamine (20 μM) is a use-dependent phenomenon (MacDonald *et al.*, 1987). Figure 2 illustrates the results of experiments designed to test whether or not MK-801 shares this property. On four separate cells, control inward currents to pressure-applied NMDA (100 μM; 0.05 Hz) were recorded at a holding potential of −60 mV. Once a stable response had been observed over several minutes, agonist application was discontinued and the cells superfused with recording medium containing MK-801 (3 μM). If activation of the NMDA-ion channel complex is a prerequisite for blockade by MK-801, it would be anticipated that no antagonism of the response would develop in the absence of agonist. This prediction was approximately borne out by the results of the representative experiment depicted in Figure 2a, where the first of a train of responses of NMDA, recorded following a 10 min pre-exposure to MK-801, was only slightly depressed relative to its control value. Subsequent responses recorded in the presence of MK-801 progressively declined, as would be expected if use-dependent block were occurring. Figure 2b shows the pooled data obtained from the four cells in which the protocol shown in Figure 2a was followed, together with the results of control experiments which rule out the possibility that the 10 min interval during which agonist application was suspended was insufficient to allow the build-up of an effective concentration of MK-801 in the bath. From inspection of the mean data, it is clear that the antagonist action of MK-801 is largely use-dependent. However, the first response in a pulse train initiated after 10 min of pre-exposure to MK-801 was consistently depressed relative to control (by 15.0 ± 5.5%, $n = 4$), and this

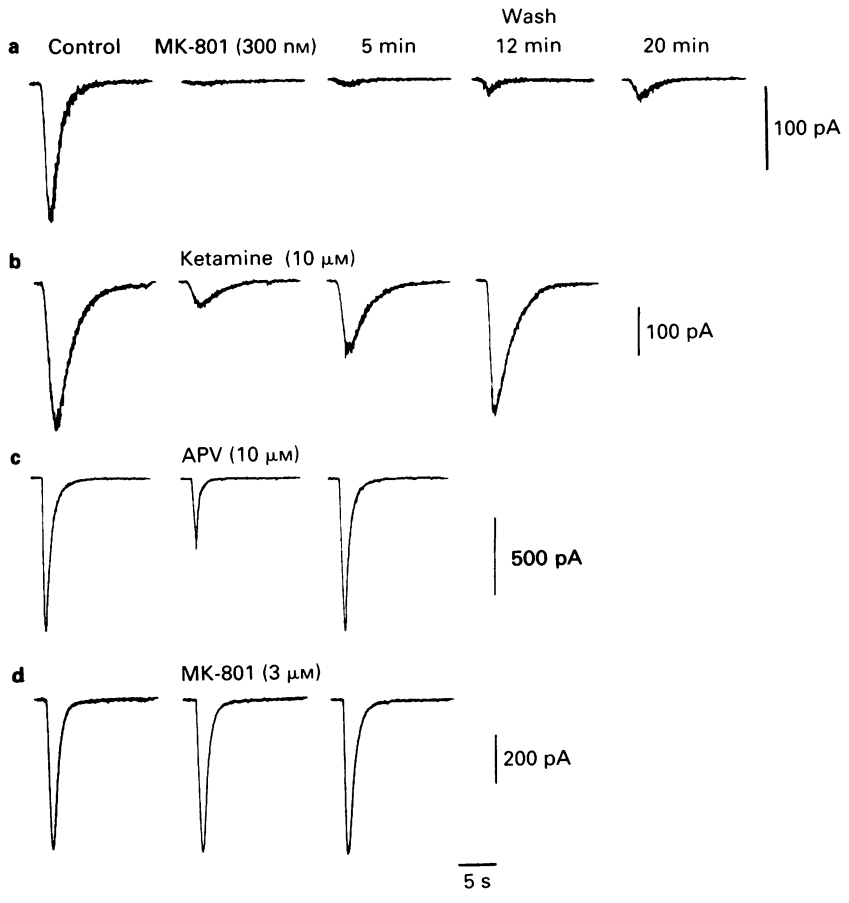

Figure 1 The influence of bath-applied MK-801, ketamine or DL-2-amino-5-phosphonovaleric acid (APV) on membrane currents evoked by N-methyl-D-aspartic acid (NMDA) or kainate. (a) MK-801 (300 nM) antagonizes inward currents elicited by ionophoretically applied NMDA (50 nA, 1 s, 0.05 Hz). Note that blockade was poorly reversed upon washout. (b) Traces illustrating the reversible blockade by ketamine (10 μM) of currents evoked by ionophoretically applied NMDA (40 nA, 1 s, 0.05 Hz). (c) Reversible suppression of NMDA-induced responses by APV (10 μM). NMDA was applied ionophoretically (60 nA, 500 ms, 0.05 Hz). (d) MK-801 (3 μM) has no effect upon inward currents evoked by pressure applied kainate (100 μM, 30 ms, 0.05 Hz). All currents were recorded from hippocampal neurones voltage-clamped at −60 mV. Each trace is the computer generated average of 4 agonist-evoked currents which were in all cases low-pass filtered at 500 Hz.

might suggest that a small component of the block occurs via a use-independent mechanism (see Discussion).

Use-dependent blockade of NMDA-evoked currents by MK-801 was also observed when the agonist was applied by ionophoresis. In contrast, APV (10 μM) antagonized currents induced by ionophoretically applied NMDA in a use-independent manner (data not illustrated), consistent with its proposed role as a competitive antagonist of the NMDA receptor (Harrison & Simmonds, 1985).

Comparison of the influence of membrane potential on blockade of NMDA-induced currents by MK-801 and ketamine

Figure 3 illustrates the results of various experiments in which the influence of membrane potential upon the antagonism of NMDA-evoked currents by MK-801 and ketamine was examined. In initial experiments, MK-801 (300 nM), or ketamine (10 μM), were bath-applied to hippocampal neurones voltage-clamped at a constant holding potential of either

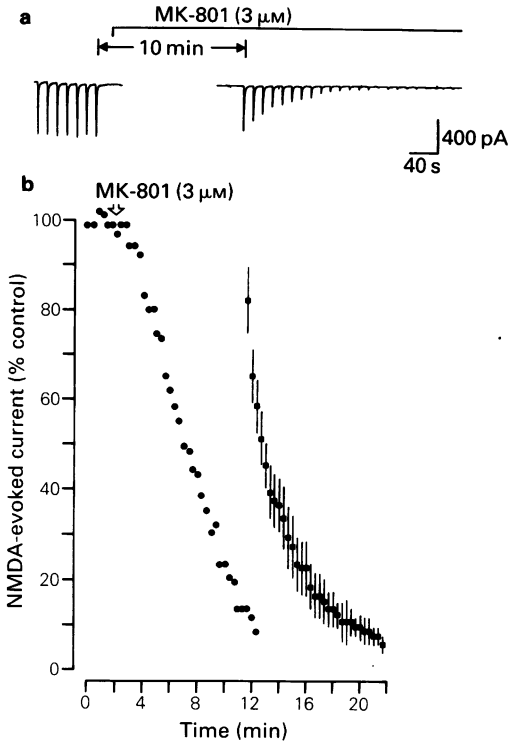

Figure 2 Blockade of N-methyl-D-aspartic acid (NMDA)-evoked currents by MK-801 is use-dependent. (a) Chart recorder trace showing inward currents evoked by pressure applied NMDA (100 μM, 20 ms, 0.05 Hz). The gap in the trace indicates that agonist application was suspended during the introduction of MK-801 (3 μM) to the bath. Following a 10 min equilibration period, agonist application was recommenced. The progressive decline of NMDA-evoked currents in the presence of MK-801 reflects use-dependent block. (b) Plot of NMDA-induced current amplitude, as a percentage of control, against time. The arrow indicates the time at which MK-801 (3 μM) was applied to the bath: (■) represents the mean data obtained from 4 cells in which the protocol described in (a) was followed; (●) show the mean data obtained from 2 cells in which agonist application was continued throughout the application of MK-801. The latter experiment was performed as a control to ensure that a 10 min equilibration period was sufficient to allow an effective concentration of MK-801 to develop in the bath. All experiments were performed at a holding potential of −60 mV and NMDA-evoked currents low-pass filtered at 500 Hz.

−60 or +40 mV. The amplitude of the current evoked by NMDA (100 μM), pressure applied (1.4×10^5 Pa) for 20–30 ms at 20 s intervals, was expressed relative to its control value following a 10 min application of the blocking drug (Table 1). It

Table 1 Influence of holding potential on block by MK-801 and ketamine

	Response amplitude (% control)	
Antagonist (μM)	−60 mV	+40 mV
Unpaired observations		
MK-801 (0.3)	52 ± 4.5 (6)	62 ± 5.0 (5)
Ketamine (10.0)	20 ± 2.5 (4)	89 ± 6.5 (4)
Paired observations		
MK-801 (0.3)	28 ± 4.3 (3)	28.5 ± 7.6 (3)
Ketamine (10.0)	17 ± 2.2 (3)	75 ± 5.5 (3)

should be stressed that whilst blockade by ketamine reached a steady-state within this time, the antagonism occurring to MK-801 did not (see discussion). The results presented in Table 1 suggest that antagonism by MK-801 is little influenced by membrane potential, whereas blockade by ketamine is highly voltage-dependent, the degree of suppression increasing with membrane hyperpolarization.

To examine further the effect of holding potential, experiments were performed in which the influence of MK-801 or ketamine upon the NMDA current-voltage (I–V) relationship was examined over the potential range −60 to +40 mV. In the absence of antagonists, the I–V relationship obtained with either ionophoretic or pressure applications of NMDA to hippocampal or cortical cells was usually linear, or demonstrated a small degree of outward rectification (Figure 3a). In a minority of cells, a complex I–V relationship was observed in which a marked inflection, due to a decrease in slope conductance, was apparent over the potential range −40 to 0 mV. Such 'voltage-dependent inactivation' of NMDA-evoked currents has been described previously by Mayer & Westbrook (1985) and may possibly be linked to an influx of Ca^{2+}. Cells displaying this feature were not studied further.

The results of a representative experiment examining the effect of MK-801 (100 nM) upon the NMDA I–V relationship are illustrated in Figure 3a2. The magnitude of the block produced by MK-801 appeared similar at all holding potentials examined. In contrast, antagonism by ketamine (10 μM) was pronounced only at negative holding potentials as previously observed (MacDonald *et al.*, 1987). Paired observations on the influence of MK-801 and ketamine on the NMDA-evoked response after prolonged application, at holding potentials of −60 and +40 mV, are given in Table 1. The reversal potential of the NMDA-induced current in the presence of either ketamine (10 μM − 8.0 ± 3.0 mV, $n = 3$) or MK-801 (100 nM, −6.0 mV; 300 nM, −4.0 mV) was similar to its control value (−6.0 ± 1.4 mV, $n = 7$).

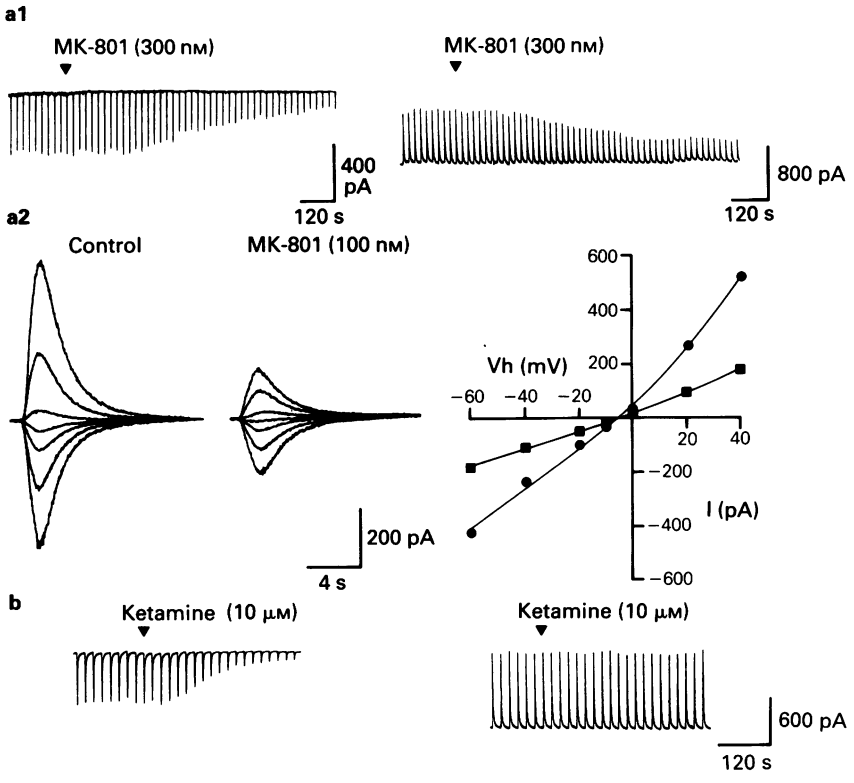

Figure 3 The influence of holding potential on blockade of N-methyl-D-aspartic acid (NMDA)-induced currents by MK-801 and ketamine. (a1) Chart recorder traces illustrating the antagonism of responses to pressure applied NMDA (100 μM, 20 ms, 0.05 Hz) by MK-801 (300 nM) at holding potentials of −60 mV (left) and +40 mV (right). Note that inward and outward currents are similarly depressed. The two recordings shown were performed on different hippocampal neurones. (a2) NMDA-induced currents recorded at holding potentials ranging between −60 and +40 mV from a hippocampal neurone in the absence and presence of MK-801 (100 nM). In order to achieve an approximately steady-state block, MK-801 was applied for 25 min against currents induced by ionophoretically applied NMDA (60 nA, 1 s, 0.05 Hz). Each trace is the average of 4 responses to NMDA and leakage currents have been subtracted by computer programme. The data are shown graphically to the right, where the relationship between NMDA-induced current amplitude and holding potential in the absence (●) and presence (■) of MK-801 is plotted. Note that antagonism appears voltage-independent and is not associated with any change in the reversal potential of the response. (b) Chart recorder traces illustrating the influence of ketamine (10 μM) upon responses to locally applied NMDA (100 μM, 20 ms, 0.05 Hz) recorded at holding potentials of −60 (left) and +40 mV (right). Note that blockade is voltage-dependent. Recordings were obtained from different hippocampal neurones. All currents were low-pass filtered at 0.5 kHz.

Voltage-dependent recovery of NMDA-induced currents from blockade by MK-801

As noted previously, the antagonism of NMDA-induced currents produced by MK-801 (300 nM–3 μM) was very poorly reversed when the membrane potential was held constant at −60 mV and NMDA applied ionophoretically as repetitive 'brief' pulses (1 s duration, 0.05 Hz) during washout (e.g. Figure 1a). Figure 4 illustrates the results of an experiment which demonstrates that recovery from such blockade could be accelerated by prolonged applications of NMDA at a positive holding potential (+40 mV). Briefly, MK-801 (300 nM) was bath-applied against responses induced by brief ionophoretic applications of NMDA. Once substantial antagonism of the response had developed (e.g. Figure 4a), MK-801 was washed out of the bath for at least 10 min. Essentially no recovery occurred during this time. The influence of prolonged (30 or 60 s) ionophoretic application of NMDA upon recovery was then assessed at holding potentials of either −60 or +40 mV. Whilst a prolonged application of agonist at −60 mV had little or no effect upon subsequent

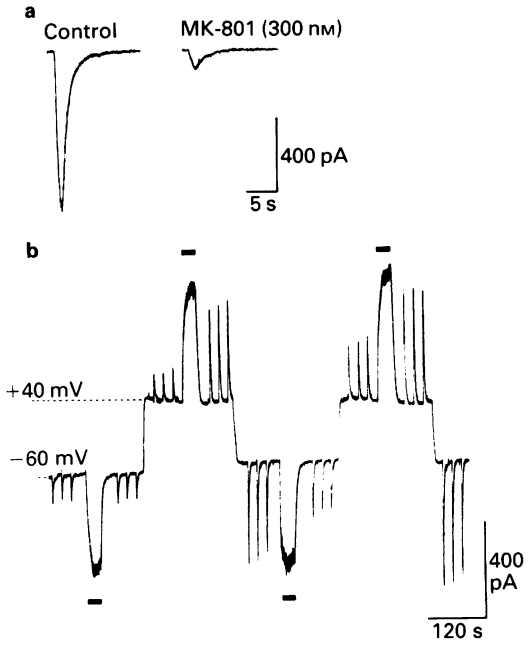

Figure 4 Reversal of MK-801-induced blockade shows voltage-sensitivity. (a) Control responses to ionophoretically applied N-methyl-D-aspartic acid (NMDA) (60 nA, 1 s, 0.05 Hz) and the response recorded following the application of MK-801 (300 nM) for 20 min. Traces are the average of 4 NMDA-induced currents recorded at a holding potential of −60 mV. (b) Chart recorder trace illustrating responses to NMDA during washout of MK-801. The trace starts after 10 min of wash with MK-801-free solution, during which time no recovery of NMDA-evoked response was observed. Prolonged ionophoretic applications of NMDA (60 nA, 30 s) are indicated by the bars adjacent to the trace. Note that the initial prolonged application of agonist at a holding potential of −60 mV has little effect on the amplitude of subsequent responses to brief pulses of NMDA (60 nA, 1 s, 0.05 Hz). In contrast, at a holding potential of +40 mV, a similar prolonged application of NMDA markedly alleviates the block produced by MK-801. Some redevelopment of the block occurs upon returning to a holding potential of −60 mV (see text for further details). Currents were recorded from a hippocampal neurone and low-pass filtered at 0.5 kHz.

responses to brief test pulses of NMDA, a similar treatment at a holding potential of +40 mV enhanced the amplitude of repetitive test responses. Upon returning to a holding potential of −60 mV, a relief of the MK-801-induced blockade was apparent, but this tended to fade with either brief repetitive or prolonged applications of NMDA (Figure 4b). The cycle of relief of blockade by agonist application at +40 mV, and partial redevelopment of block at −60 mV, could be demonstrated many times in an individual cell and was a consistent finding between cells ($n = 5$). In control experiments it was established that: (i) in the absence of MK-801 a prolonged application of NMDA at +40 mV has little effect upon subsequent responses to brief applications recorded at −60 mV, and (ii) depolarization of the cell *per se* is ineffective in relieving blockade evoked by MK-801. Collectively, these observations suggest that recovery from blockade by MK-801 is a use- and voltage-dependent process.

Voltage-dependent blockade of ACh-evoked currents by MK-801

Bovine chromaffin cells possess nicotinic receptors that are pharmacologically similar to those of mammalian autonomic ganglia (Chiapinelli *et al.*, 1988) the activation of which induces the opening of a relatively non-selective cation channel (J.J. Lambert, J.M. Nooney & J.A. Peters, unpublished observations). MK-801 suppressed ACh-evoked currents in a concentration-dependent manner, with an approximate IC_{50} of 1.7 μM at −60 mV (Figure 5a). In contrast to the block of NMDA-evoked currents, the antagonism of ACh-evoked currents by MK-801 was readily reversible at all concentrations tested (1–10 μM) usually within 10 min of starting washout. The block of NMDA-induced currents by MK-801 appeared voltage-independent (Figure 3a). In contrast, the blockade of ACh-induced currents by MK-801 was clearly voltage-dependent, the block increasing with hyperpolarization (Figure 5b).

MK-801 and $GABA_A$ receptors

Bovine chromaffin cells possess $GABA_A$ receptors pharmacologically similar to those of mammalian central neurones (Bormann & Clapham, 1985; Cottrell *et al.*, 1985; 1987). MK-801, at concentrations of 1, 10 and 100 μM had no effect on GABA-evoked whole cell currents in chromaffin cells ($n = 4$ for each concentration examined), whereas the anxiolytic diazepam produced the expected potentiation of GABA-evoked currents (Figure 6a). In acutely isolated CA1 hippocampal neurones, NMDA and the competitive NMDA receptor antagonist APV, have been observed to potentiate membrane currents elicted by GABA (Stelzer & Wong, 1987). However, in experiments on cultured hippocampal neurones, MK-801 (100 μM) had no effect upon GABA-evoked currents ($n = 4$). In common with Stelzer & Wong (1987), we found it necessary to include 4 mM Mg^{2+} and 2 mM ATP in the recording pipette solution in order to avoid a progressive run-down of GABA responses in hippocampal neurones. Interestingly,

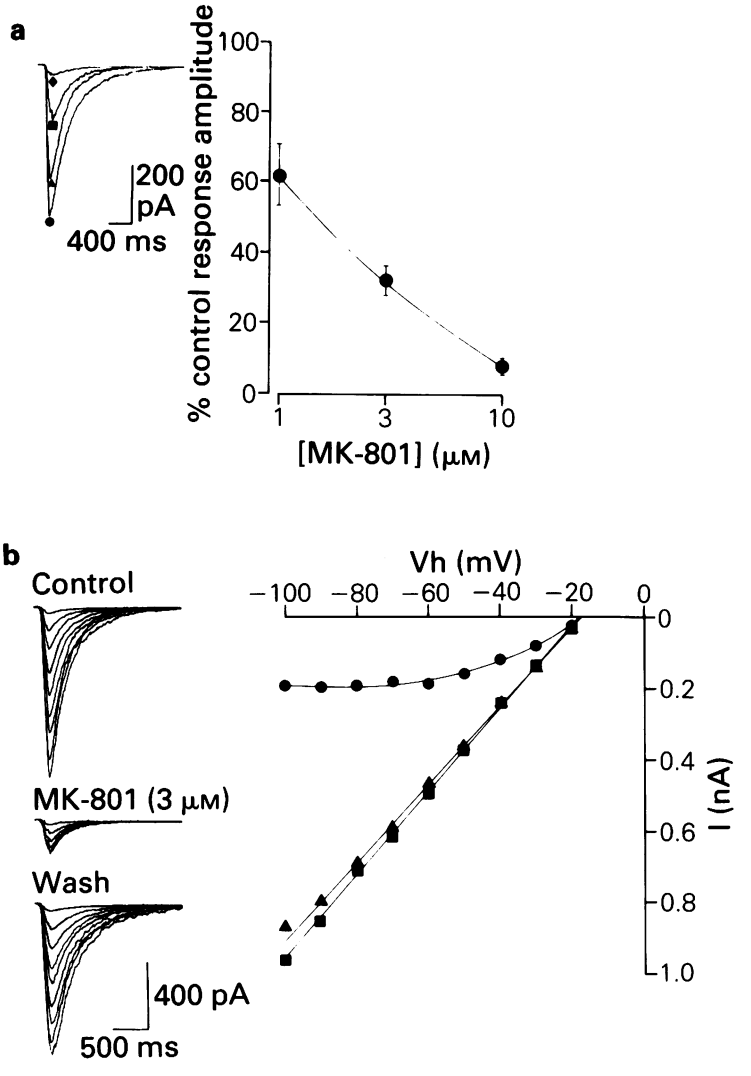

Figure 5 MK-801 antagonizes acetylcholine (ACh)-evoked currents recorded from bovine chromaffin cells. (a) Concentration-dependent blockade of ACh-induced currents by MK-801. Currents-evoked by ionophoretically applied ACh (30 nA, 100 ms, 0.1 Hz) were recorded at a holding potential of −60 mV. Traces, which are the average of 4 ACh-induced currents, illustrate a control response to ACh (●) and responses in the presence of 1 (▲), 3 (■) and 10 (◆) μM MK-801. The blockade produced by all concentrations of MK-801 tested was readily reversible upon washout. (b) Plot of the amplitude of whole cell currents evoked by ionophoretically applied ACh, as a percentage of control, against the log of the concentration of bath-applied MK-801. Data points are the mean of experiments from 4 to 6 cells. The vertical lines indicate s.e.mean. The curve, fitted to the data points by eye, yields an estimated IC_{50} for MK-801 of 1.7 μM. (c) Voltage-dependent blockade of ACh-induced whole-cell currents by MK-801. Membrane currents were evoked by ionophoretically applied ACh (60 nA, 100 ms, 0.1 Hz). The membrane potential was held at −60 mV and 1 s before applying ACh, was transiently stepped for 3 s to the test potential. In each panel the traces illustrate, from uppermost to lowermost, the average of 4 whole-cell currents elicited by ACh at holding potentials of −20 to −100 mV in 10 mV increments. Note that in the presence of MK-801 (3 μM), the ACh-induced current amplitude is essentially constant between holding potentials of −60 and −100 mV, indicating a voltage-dependent block. (d) Graphical representation of the data depicted in (c). The amplitude of the ACh-evoked current is plotted against holding potential before (■) and during (●) the application of MK-801 (3 μM). Virtually complete recovery was apparent upon washout (▲). All currents were low-pass filtered at 500 Hz.

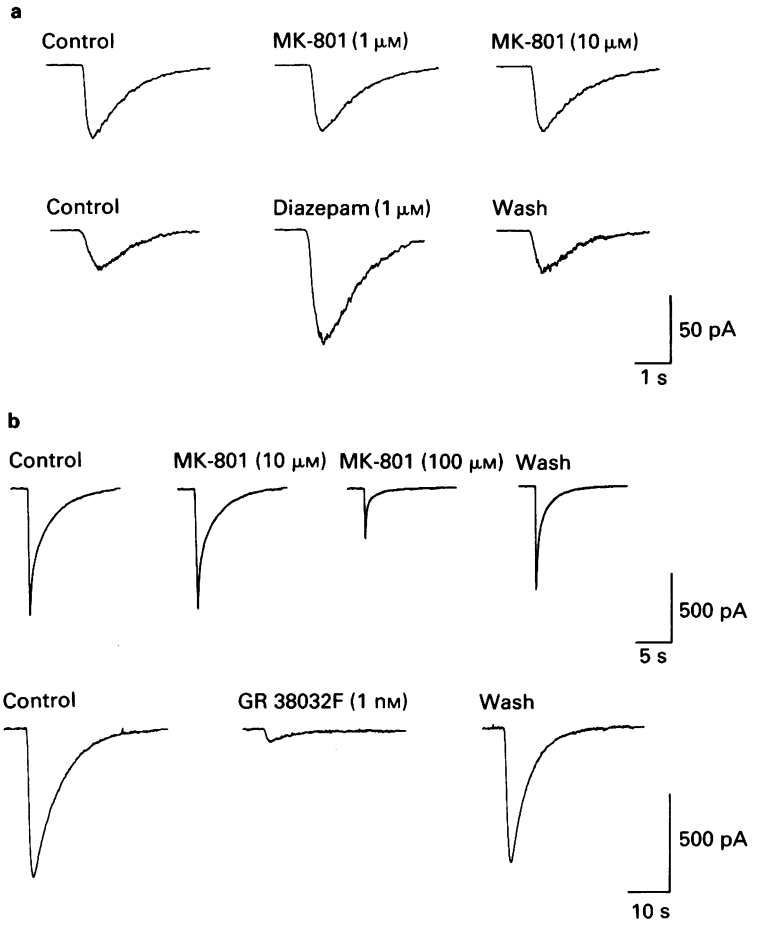

Figure 6 MK-801 does not affect GABA-evoked currents, but at high concentration depresses 5-HT_3 receptor-mediated responses. (a) GABA-induced whole cell currents recorded from bovine chromaffin cells. Membrane currents were evoked by pressure applied GABA (100 μM, 20 ms, 0.05 Hz) and recorded at a holding potential of −60 mV. MK-801 (1 and 10 μM) had no effect on GABA-evoked currents but diazepam (1 μM) reversibly potentiates the response to GABA. (b) 5-HT-induced whole cell currents recorded from N1E-115 cells. Membrane currents were elicited by ionophoretically applied 5-HT (40 nA, 50 ms, 0.02 Hz) and recorded at a holding potential of −60 mV. MK-801 (10 μM) produces only a slight depression of the inward current evoked by 5-HT, but at a concentration of 100 μM, exerts a substantial and reversible antagonism of the response. (c) GR 38032F (1 nM) reversibly antagonizes the 5-HT-induced current in an N1E-115 cell. Membrane currents were evoked by pressure-applied 5-HT (10 μM, 20 ms, 0.011 Hz) and recorded at a holding potential of −60 mV.

ATP-free recording solution appears to support relatively stable GABA responses in bovine chromaffin cells.

MK-801 and 5-HT receptors

Preliminary reports have demonstrated that antagonists of 5-HT_3 receptors such as GR 38032F (Butler *et al.*, 1988) appear to be anxiolytic in some animal models of anxiety (Jones *et al.*, 1988). 5-HT induces currents in N1E-115 neuroblastoma cells which are mediated by 5-HT_3 receptors (Neijt *et al.*, 1988; Peters *et al.*, 1988a). In 3 of 4 cells tested, MK-801 (10 μM) produced a slight suppression of such currents, but was without effect in the remaining cell of this sample. On average, 5-HT-induced currents were reduced to 88.8 ± 2.8% of their control value in the presence of 10 μM MK-801. At a concentration of 100 μM, MK-801 reversibly reduced the amplitude of

the response to 5-HT to 33.8 ± 2.7% ($n = 4$) of control. The 5-HT-induced current was potently antagonized by GR 38032F (1 nM), confirming that such responses are mediated via the 5-HT_3 receptor subtype (Figure 6b).

MK-801 and voltage-activated sodium currents

The anticonvulsants carbamazepine and phenytoin are known to block voltage-activated sodium channels in a use-dependent manner, and such an action may contribute to their anticonvulsant effects (Willow, 1986). In initial experiments on hippocampal neurones, MK-801 (100 μM) reduced the amplitude of inward currents elicited by transiently stepping the membrane potential to 0 mV from a holding potential of −80 mV. Such currents were abolished by tetrodotoxin (0.3 μM), and were therefore due to the opening of voltage-activated sodium channels. No attempt was made to analyse the effect of MK-801 in detail in hippocampal neurones; we simply note that sodium currents were reduced to between 80 and 60% of their control value when the compound was applied at a concentration of 100 μM. Further quantification of this effect in hippocampal neurones was avoided, since we considered the time constant of the voltage-clamp in these cells, relative to the kinetics of the sodium current, insufficient for accurate voltage control. As an alternative, the effect of MK-801 on voltage-activated sodium currents of bovine chromaffin cells was examined, since in such cells adequate voltage control of even rapidly changing currents may be easily attained by use of the whole-cell recording technique (Hamill *et al.*, 1981). Voltage-activated sodium currents of chromaffin cells were completely blocked by tetrodotoxin (0.3 μM, $n = 4$). In contrast, MK-801 (10 μM) had no discernible effect on the sodium current, but at a relatively high concentration (100 μM), rapidly and reversibly reduced it to 77 ± 3.4% ($n = 5$) of control (Figure 7).

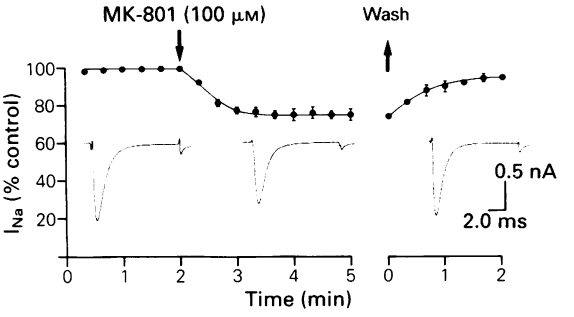

Figure 7 Only relatively high concentrations of MK-801 inhibit voltage-activated sodium currents. Voltage-activated sodium currents were elicited from bovine chromaffin cells by transiently stepping the holding potential from −80 mV to 0 mV (0.5 Hz, 10 ms). Experiments commenced 30 min after forming the whole cell clamp to allow for any change in the sodium current induced by pipette dialysis. Control currents were recorded for 2 min at which time tetrodotoxin (TTX) (0.3 μM) or MK-801 (10–100 μM) was applied to the cell surface by microperfusion for 5 min before removal of the microperfusion pipette and washout of the bath. Such currents were completely blocked by TTX (0.3 μM), unaffected by MK-801 (10 μM) and reduced to 77 ± 3.4% of control by 100 μM MK-801 ($n = 5$). The graph shows the effect of 100 μM MK-801 on voltage-activated sodium current amplitude as a function of time. Each point is the mean of 5 experiments with s.e.mean shown by vertical lines. Computer averaged currents ($n = 10$) are shown before, during, and after washout of 100 μM MK-801. The relatively small capacitative and leakage currents were not subtracted. Currents were low-pass filtered at 2.0 kHz.

Discussion

MK-801 is a potent antagonist of NMDA-induced depolarizations in rat cortical slices (Wong *et al.*, 1986; Kemp *et al.*, 1987; Davies *et al.*, 1988) and spinal cord (Childs *et al.*, 1988). In experiments employing voltage-clamp techniques, MK-801 blocks NMDA-induced currents in rat visual cortical neurones (Huettner & Bean, 1988), and in *Xenopus* oocytes injected with rat brain messenger RNA (Kushner *et al.*, 1988). In agreement with these studies, we now show that MK-801 antagonizes currents evoked by NMDA, but not kainate, in voltage-clamped hippocampal and cortical neurones in cell culture.

It has been suggested that MK-801 might act at the level of the NMDA-activated ion channel, blocking ion conduction (Kemp *et al.*, 1987) in a manner analogous to that proposed for some drugs at the nicotinic receptor of the neuromuscular junction (e.g. Lambert *et al.*, 1983), or autonomic ganglia (e.g. Gurney & Rang, 1984). In support of this suggestion, the block of NMDA-induced currents by MK-801 was largely agonist-dependent. A similar use-dependent block has been obtained in voltage-clamped rat visual cortical neurones (Huettner & Bean, 1988), and in studies employing extracellular recording techniques in rat cortical slices (Wong *et al.*, 1986; Kemp *et al.*, 1987). Consistent with these observations, the binding of radiolabelled MK-801 to rat brain membranes is potentiated by NMDA receptor agonists and by glycine (Foster & Wong, 1987; Wong *et al.*, 1987). However the antagonism by MK-801 of depolarizing responses to NMDA recorded extracellularly from rat spinal cord *in vitro*

does not show agonist-dependency (Childs *et al.*, 1988) and the degree of use-dependency in the cortical slice preparation appears to be temperature-dependent (Davies *et al.*, 1988). In the present work, a small depression of the NMDA-induced current was apparent following a 10 min pre-exposure to MK-801 (3 μM), despite the fact that the application of NMDA was discontinued as MK-801 was introduced to the bath. Such a result might suggest that a small component of the block by MK-801 occurs via a use-independent mechanism. Although the present data do not allow the exclusion of the latter possibility, a more parsimonious explanation is that use-dependent blockade by MK-801 occurs during the rising phase of the initial response, leading to a depression of its amplitude (cf. Lingle, 1983; MacDonald *et al.*, 1987). In this respect it is interesting that the use-dependent nature of blockade by MK-801 was originally demonstrated with comparatively slowly rising agonist responses recorded extracellularly from a cortical slice preparation (Kemp *et al.*, 1987). Incompletely suppressed synaptic activity, and possibly diffusional release of NMDA from the drug application pipette, might in the present experiments, have supplied sufficient agonist for some degree of use-dependent blockade to develop during the 10 min incubation period with MK-801.

In agreement with previous reports (Honey *et al.*, 1985; MacDonald *et al.*, 1987) ketamine blocked NMDA-induced currents in a voltage-dependent manner, the block increasing with hyperpolarization. In contrast, under identical recording conditions, MK-801 appeared equieffective in blocking inward and outward NMDA-induced currents. In this context, it should be noted that the time constant of onset of blockade of NMDA-induced currents by MK-801 is independent of membrane potential (Huettner & Bean, 1988). Like MK-801, the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV) blocks NMDA-induced currents in a voltage-independent manner (MacDonald *et al.*, 1987). However, the agonist-dependency of both the blocking action, and binding of MK-801, make it unlikely that the drug binds to the agonist recognition site of the NMDA-receptor-channel complex, as has been suggested for APV. If alternatively, MK-801 blocks the NMDA-activated ion channel, the above results and others (Huettner & Bean, 1988), clearly demonstrate that it does so in a voltage-independent manner.

The recovery from blockade by MK-801 was both use- and voltage-dependent (see also Huettner & Bean, 1988). Similar observations have been made for the block of NMDA ion channels by ketamine (MacDonald *et al.*, 1987), nicotinic ion channels by some methonium compounds (Gurney & Rang, 1984) and ACh-activated channels in lobster muscle fibres by chlorisondamine (Lingle, 1983). To explain these observations, it has been suggested that the drug binds in the lumen of the ion channel and becomes trapped therein when the channel closes (Lingle, 1983; Gurney & Rang, 1984; MacDonald *et al.*, 1987).

In the instances cited above, both blocking and unblocking of the ion channel have been found to be voltage-dependent. An important difference in the present work with MK-801 is the dissimilar influence of membrane potential upon blockade and recovery. MK-801 has a pKa of 8.2 (P. Anderson, unpublished observation) and is therefore mainly in the cationic form at pH 7.2. One interpretation of the voltage-independence of the development of blockade by MK-801 is that it is the minority uncharged form of the drug which blocks the ion channel. The same reasoning suggests that the voltage-dependence of recovery reflects the expulsion of the protonated molecule from the ion channel. One possible explanation of this paradox might be that MK-801 converts to the protonated state once bound to its site within the channel. Alternatively, it may be speculated that the NMDA ion channel undergoes some voltage-dependent conformational change which specifically facilitates unblocking, or that it is the net direction of ion flow through the channel, rather than membrane potential *per se*, which influences recovery.

Both ketamine and PCP are known to block the nicotinic receptor-channel complex in a non-competitive manner. The mechanism of blockade is not well understood, but is thought to involve a blockade of both open and closed nicotinic ion channels by the drug (e.g. Maleque *et al.*, 1981; Volle *et al.*, 1981; Aguayo & Albuquerque, 1986; but see Changeux *et al.*, 1986). The present study demonstrates that MK-801 is a potent antagonist of currents evoked by nicotinic receptor activation in bovine chromaffin cells. Unlike the antagonism of NMDA-induced responses, blockade of ACh-evoked currents by MK-801 reached a steady-state relatively quickly (within approximately 2–3 min), was fully reversible upon washout, and was at least partly voltage-dependent with an IC_{50} of 1.7 μM (at -60 mV). The latter of these characteristics implicates the protonated form of MK-801 in the blockade of ACh-induced currents. Whether or not the phenomena of use-dependent blocking and recovery observed with MK-801 at the NMDA-ion channel complex occur at the nicotinic channel also, remains to be determined. Although a high concentration (100 μM) of MK-801 was observed to antagonize 5-HT_3 receptor-mediated currents in N1E-115 cells, this system and kainate-induced currents were minimally affected by doses of MK-801 (1–10 μM) which markedly suppress ACh-evoked currents in chromaf-

fin cells and NMDA-evoked currents in central neurones. Thus the blockade of ACh-induced responses by MK-801 is at least partially selective, and could be due to an association of the drug with a specific site on the nicotinic-channel complex. The primary amino acid sequence of the nicotinic receptor-ion channel complex of the *Torpedo* electroplaque organ is known and progress in identifying the amino acids to which PCP binds has been reported (Changeux & Revah, 1987). When the amino acid sequence of the NMDA ion channel is determined, it will be of interest to compare the PCP/MK-801 binding site with that of its counterpart on the nicotinic receptor.

MK-801 is a potent anticonvulsant, anxiolytic and neuroprotective agent (Clineschmidt, 1982; Clineschmidt *et al.*, 1982; Gill *et al.*, 1987). This behavioural profile may be due entirely to the known antagonism of the NMDA-receptor-channel complex by MK-801. However, the possibility exists that other receptors or ion channels may contribute to its effects. Anxiolytics of the benzodiazepine type are thought to act primarily by binding to a site associated with the $GABA_A$ receptor (e.g. Martin, 1987). A recent report has demonstrated that the NMDA receptor antagonist carboxypiperazine phosphonic acid (CPP) is a potent displacer of flunitrazepam binding (White *et al.*, 1988), although MK-801 binding is unaffected by clonazepam (Wong *et al.*, 1986). In the present study, MK-801 had no effect on GABA-induced currents which were, however, potentiated by the anxiolytic diazepam. This result is consistent with radioligand binding studies (Wong *et al.*, 1986) and the insensitivity of the anticonvulsant action of MK-801 to the benzodiazepine antagonist Ro15-1788 (Clineschmidt, 1982), an agent which reverses the flunitrazepam-induced potentiation of GABA-induced currents in bovine chromaffin cells (Cottrell *et al.*, 1987). These results suggest that the central effects of MK-801 are unlikely to be mediated directly by $GABA_A$ receptors.

In preliminary studies, antagonists of the $5\text{-}HT_3$ subclass of receptor have been reported to be active in animal tests predictive of anxiolytic effects in man (Jones *et al.*, 1988). In the present study, $5\text{-}HT_3$ receptor-mediated currents recorded from N1E-115 cells were minimally affected by MK-801 at a concentration of 10 μM, but substantial antagonism was observed with a ten fold higher concentration of the drug. To place this action in perspective, it should be noted that the $5\text{-}HT_3$ receptor antagonist and putative anxiolytic GR 38032F virtually abolished the 5-HT-induced current at a concentration of 1 nM. In contrast to the present results, MK-801 has recently been shown to potentiate 5-HT-induced depolarizations in a rat spinal cord preparation, possibly due to an effect of MK-801 on 5-HT uptake (Childs *et al.*, 1988).

Carbamezepine and phenytoin are known to block voltage-activated sodium channels in a use-dependent manner, a molecular mechanism thought to contribute to their anticonvulsant actions (Willow, 1986). In the present study, although high doses of MK-801 did block voltage-activated sodium channels, it seems unlikely that such an action is important in its anticonvulsant effect when compared with the potent blockade of NMDA-induced currents produced by the drug.

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