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Use-, Concentration- and Voltage-Dependent Limitation by MK-801 of Action Potential Firing Frequency in Mouse Central Neurons in Cell Culture

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ABSTRACT

The anticonvulsant, (±)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine (MK-801), blocked single postsynaptic responses of mouse spinal cord neurons in cell culture to N-methyl-D-aspartate (NMDA). The block was concentration dependent with $IC_{50} = 10^{-7}$ M against 10^{-5} M NMDA and 2×10^{-7} M against 10^{-3} M NMDA. Serial responses were blocked in use-dependent manner by 10 times lower doses of MK-801, depending on rate of NMDA application. MK-801 (approximate IC_{50}, 8×10^{-8} M) also limited sustained high-frequency repetitive firing of sodium-dependent action potentials (AP) elicited by long (400 msec) depolarizing pulses. Without changing resting membrane potential, single AP elicited by short (0.5–1 msec) depolarizing current pulses were blocked in voltage-, use- and frequency-dependent manner. Maximal rate of rise of individual AP elicited by short or long pulses decreased progressively until failure to fire. The time constant of recovery of AP from inactivation in a paired pulse protocol was prolonged from about 1 msec in control solution to 12 msec in solution containing 3×10^{-7} M MK-801. These characteristics suggest that MK-801 blocks voltage-sensitive sodium current, which generates the upstroke of the AP. Overlap of concentrations blocking NMDA responses and sustained repetitive firing suggests that both actions may contribute to anticonvulsant efficacy of MK-801.

The anticonvulsant, MK-801, has been shown to protect against electroshock-induced tonic seizures and tonic hind limb extension induced by bicuculline (Clineschmidt *et al.*, 1982; McNamara *et al.*, 1988) and against amygdaloid-kindled seizures in rats (McNamara *et al.*, 1988; Sato *et al.*, 1988; Gilbert, 1988; Young *et al.*, 1989). These animal models simulate generalized tonic-clonic and partial seizures in man. As add-on therapy in a clinical trial, MK-801 reduced the frequency of partial seizures with or without secondary generalization in about half of a small group of patients whose seizures were incompletely controlled by other anticonvulsants (Troupin *et al.*, 1986). Binding of [^{3}H]MK-801 was displaced by antagonists of NMDA in a noncompetitive manner (Wong *et al.*, 1986; Foster and Wong, 1987). Also, in patch clamp experiments, inward currents activated by NMDA were blocked in use-dependent manner by MK-801 (Huettner and Bean, 1988; Halliwell *et al.*, 1989). Blockade of excitation mediated by NMDA-preferring glutamate receptors is one mechanism that could contribute to the anticonvulsant efficacy of MK-801. But, are there other important actions?

Therapeutically relevant concentrations of several clinically used anticonvulsant compounds that protect against electroshock-induced seizures in animals limited SRF of sodium-dependent AP by mammalian central neurons in cell culture. Examples include phenytoin, carbamazepine and valproic acid (McLean and Macdonald, 1983, 1986a,b, respectively), all of which are useful in the treatment of generalized tonic-clonic and some partial seizures. On the basis of structural similarity to carbamazepine, we expected that MK-801 also would limit SRF. In the present experiments, MK-801 limited SRF at concentrations between 10^{-8} and 10^{-6} M, overlapping the range of concentrations that inhibited postsynaptic responses to NMDA in the cultured neurons and suggesting that both actions may contribute to therapeutic efficacy. These findings have been presented in preliminary form (McLean and Wamil, 1989).

Methods

Cell Culture

Cell cultures were prepared by methods adapted from Ransom *et al.* (1977; see McLean and Macdonald, 1983). In brief, spinal cords were

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ABBREVIATIONS: AP, action potential(s); E_m, transmembrane potential(s); IRI, inter-response interval(s); NMDA, N-methyl-D-aspartate; P, pressure application(s); SRF, sustained repetitive firing; $\dot{V}_{max}$, maximal rate of rise of action potentials; MK-801, (±)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine; mDPBS, modified Dulbecco's phosphate-buffered saline; CPP, 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid; I_{Na}, voltage-activated sodium current.

removed from mouse embryos on g.13 to g.14. To prepare cortical cultures, lateral cerebral cortex, free of hippocampus and basal ganglia, was dissected from brains of g.16 to g.19 embryos. Pooled tissues were minced and then mechanically dissociated by trituration. The dissociated cells and small clumps were suspended in plating medium consisting of equal parts (v/v) Eagle's minimal essential medium (supplemented with 1.5 g/l sodium bicarbonate and 5.5 g/l glucose) and Hank's balanced salt solution to which the following were added: 5% (v/v) fetal calf serum, 5% horse serum, 10 ng/ml 7S nerve growth factor and 1 ml/l Mito Serum Extender (all supplements from Collaborative Research, Bedford, MA). Aliquots of the suspension were placed in sterile collagen-coated 35-mm plastic dishes and incubated at 35°C in an atmosphere of 90% room air and 10% CO_2 to maintain the pH at 7.4. Growth of non-neuronal cells was suppressed by addition of 5-fluoro-2'deoxyuridine and uridine (50 μg/ml each) to the culture medium on the 7th day *in vitro*. After 24 h of exposure to 5-fluoro-2'deoxyuridine, medium without fetal calf serum was used to "feed" cultures twice weekly for 3 to 20 weeks before experiments.

Experimental Protocols

General. A culture dish mounted on the stage of an inverted phase contrast microscope was continuously perfused at about 1 ml/min with mDPBS containing elevated Mg^{++} concentrations to suppress spontaneous activity (composition in mM: 143.4 NaCl; 2.2 KCl; 0.8 $CaCl_2$; 0–7.0 $MgCl_2$ and 5.6 glucose in 9.5 mM sodium-phosphate buffer; pO_2 100–120 mm Hg, pH 7.4). This solution was used to allow comparison with previously published data. Temperature was 37°C. Glass micropipettes filled with 3 M KAc (50–80 megohms tip resistance) were used to penetrate somata of selected neurons. A bridge circuit in the Axoclamp amplifier (Axon Instruments; Burlingame, CA) allowed simultaneous measurement of E_m and injection of current through the microelectrode. The amplified E_m was displayed on a Tektronix (Beaverton, OR) R5113 storage oscilloscope for on-line monitoring and recorded on one channel of a video tape after digitization by a modified audio processor (Unitrade Inc., Philadelphia, PA). Intracellularly applied current was recorded on the second channel.

Membrane potential was differentiated electronically with a DDT amplifier (World Precision Instruments; Cambridge, MA) and the $\dot{V}_{max}$ was proportional to the peak of the differentiated signal. In the figures, the differentiated trace is shown inverted, as $-dV/dt$.

Assessment of repetitive firing of action potentials. Methods have been detailed previously (McLean and Macdonald, 1983). In drug-free solution, all neurons with stable resting membrane potentials fired AP throughout 400-msec depolarizing pulses (*i.e.*, they demonstrated SRF). During superfusion with solutions containing MK-801 at some concentrations, AP fired for less than 200 msec of 400-msec depolarizing steps and $\dot{V}_{max}$ declined progressively until firing stopped. This response is referred to as limitation of SRF or limited repetitive firing. In the continued presence of MK-801 for up to 1 hr, SRF could be restored in most neurons by steady-state hyperpolarization to potentials negative to −65 mV, thereby confirming viability. In population studies, neurons were exposed to MK-801 for less than 1 hr to minimize cumulative effects. Very large depolarizing steps elicited limited repetitive firing, even in drug-free solution. However, if SRF was observed at moderate depolarizations, the neuron was judged to be capable of SRF overall, whether superfused with drug-free or drug-containing solutions. Effects on SRF have been presented here as the percentage of neurons exhibiting SRF at E_m ranging between −45 and −70 mV during exposure to a given concentration of a drug. In some experiments, trains of short (1 msec) pulses were used to elicit individual AP at various frequencies.

Assessment of NMDA-induced depolarization. The excitatory amino acid, NMDA, was applied to quiescent neurons (E_m negative to −45 mV; all manifested SRF) by pressure ejection from blunt-tipped (heat polished to a diameter of about 3 μm) micropipettes. After filling with mDPBS (same composition as superfusate) containing NMDA alone or in combination with MK-801, a pipette was mounted on a separate manipulator and positioned 50 to 250 μm from the soma of the neuron selected for study. A Picospritzer (General Valve; Fairfield, NJ) was used to apply discrete pressure pulses of variable duration to the blunt end of the micropipette. To adjust for variability of the micropipettes, pressure was increased until there was 1) streaming of solution from the tip of the pipette, 2) movement of debris on the bottom of the dish, or 3) movement of the fluid meniscus in the micropipette barrel. Excessive ejection pressure dislodged the intracellular electrode or caused an apparent hyperpolarization and movement of the impaled neuron, which terminated abruptly upon cessation of the pulse. Pressure application of 5 nM tetrodotoxin, approximately the IC_{50} of tetrodotoxin against sodium current of axons of several different species (see Ritchie and Rogart, 1977 for review), reversibly abolished AP of central neurons in cell culture. This suggests that the drug concentration in the vicinity of the soma was close to that in the drug-containing pipette. Maximal NMDA-induced depolarizations occurred within a few seconds after pressure pulses ended and were followed by gradual repolarization to the original E_m. Peak depolarization was defined as the difference between the stable E_m and the largest depolarization if no AP fired, or the peak of a curve connecting the take-off potentials of AP. Action potential firing continued as long as depolarization exceeded threshold. Drug effects were reversed by termination of pressure pulses, removal of the drug-containing pipette from the bathing solution, and continuous superfusion with mDPBS. Data were excluded if drug effects were not reversible. Results qualitatively similar to those reported here have been obtained in bicarbonate buffers with and without added glycine and with or without tetrodotoxin to block action potentials. Data obtained in phosphate buffer are reported here for comparison with previously published data.

The interval between serial applications, referred to here as the IRI, was timed from complete repolarization of one response to the point of triggering the next application. This approach was used to avoid superimposition of responses and variable time of recovery between applications, because response duration shortened with use-dependent blockade. Also, waiting longer than full recovery limited cumulative effects of NMDA. Similar responses were obtained repeatedly (up to 40 times without desensitization) with application of 10^{-5} M NMDA at IRI of 2 to 20 sec. Desensitization was observed in response to repeated applications of to 10^{-4} to 10^{-3} M NMDA at 2 to 5 sec, but not 20 sec or longer, IRI. Trains of 10 pressure applications were ample in most experiments to demonstrate stable, nondesensitizing responses and reversible use-dependent block.

Photographs of individual responses were assembled into montages to prepare data figures. In the figures, the onset of each P was indicated with an arrowhead ± a number (*e.g.*, P_1 and P_{10}, first and tenth pressure application).

Experimental compounds. Drugs were diluted in mDPBS from concentrated stock solutions prepared as follows: 10 mM NMDA (Sigma Chemical Co., St. Louis, MO) in distilled water; 1 mM MK-801 (Merck, Sharp and Dohme Research Laboratories, West Point, PA or Research Biochemicals Inc., Natick, MA) or AP-7 (Sigma) in mDPBS; CPP (Research Biochemicals) in mDPBS. The pH of the superfusate was not altered by adding compounds in the range of concentrations studied. At 2% (v/v), methyl sulfoxide did not alter electrophysiological properties or NMDA responses measurably.

Statistics. Paired and unpaired *t* tests were used to evaluate population data (expressed as mean ± 1 S.E.M.).

Results

Responses to NMDA. During superfusion with mDPBS containing 7 mM Mg^{++} to suppress spontaneous activity, resting E_m was −65 ± 1.4 mV (mean ± S.E.; N = 49). Responses to 10^{-5} M NMDA were reversibly blocked by 10^{-8} M CPP (N = 4) or 10^{-8} M AP-7 (N = 5) in use-dependent manner, or by a single application of 10^{-5} M CPP (N = 12) or AP-7 (N = 12). Figure 1 shows responses elicited by single pressure applications of 10^{-5} M NMDA and the reduction of amplitude of depolarization and AP firing by coapplication of MK-801 at some concentrations. Figure 2 shows dependence of amplitude of

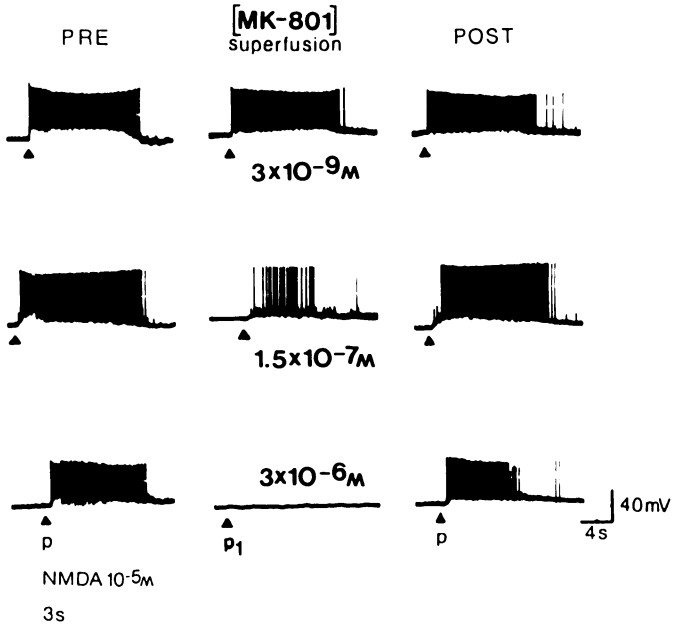

Fig. 1. Concentration-dependent block by MK-801 of responses to single applications of NMDA. Each panel shows the response to a 3-sec P of 10^{-5} M NMDA during superfusion with drug-free solution or solution containing MK-801 at the indicated concentration. Each row shows traces from the same spinal cord neuron. *Top row:* In drug-free solution (PRE), NMDA produced depolarization and fast firing of AP. The response persisted during superfusion with 3×10^{-9} M MK-801 (middle) and after washout (POST). *Middle row:* During superfusion with 1.5×10^{-7} M MK-801, depolarization and AP firing were reduced (middle). *Bottom row:* During superfusion with 3×10^{-6} M MK-801, the response to NMDA was abolished (middle) reversibly (POST). Calibrations at bottom right applied throughout.

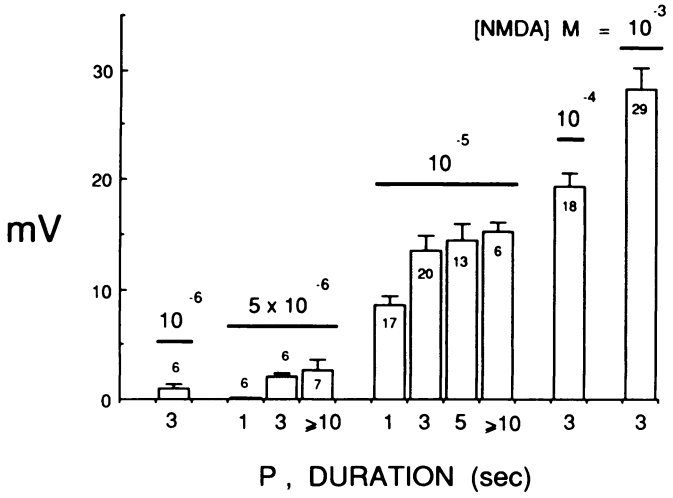

Fig. 2. Amplitude of depolarization in response to P of NMDA. Amplitude (mV) varied with concentration of NMDA (underlined) and duration of P (sec).

depolarization on NMDA concentration and duration of application. The lowest concentration producing a response was 10^{-6} M.

Consistency of repeated responses was tested by 10 serial pressure applications of NMDA at 1 to 2-sec IRI (fig. 3). Despite a tendency for responses to 10^{-3} M NMDA to desensitize, the response to P_{10} did not differ significantly from that to P_1 ($P > .1$ by unpaired t test). The most consistent responses were obtained with 10^{-5} M NMDA (92 of 97 neurons, or 95% of neurons, responded). Three-sec duration and a concentration of 10^{-5} M NMDA were used for most experiments involving serial applications because response amplitude did not differ significantly from that of responses to longer applications (fig. 2, $P > .05$ for 3-sec P *vs.* 5-sec or ≥10-sec P) and drug effects on amplitude were easily detectable.

Effect of MK-801 on resting membrane properties. Pressure application of 3×10^{-5} M MK-801 for 30 sec did not change resting E_m (-59.7 ± 4.5 mV before and after application, $N = 6$). Input resistance was 64.2 ± 3.7 megaohms ($N = 28$) in control solution and 64.6 ± 2.8 megaohms ($N = 14$) after 10 applications of 3×10^{-5} M MK-801.

Effects of MK-801 on responses to NMDA. In control solution and at resting E_m of -65.0 ± 1.4 mV ($N = 43$), single applications of 10^{-5} M NMDA at long IRI (>1 min) produced depolarizations of 13.5 ± 1.5 mV ($N = 20$) (fig. 1, PRE; fig. 4). At concentrations greater than 3×10^{-9} M, MK-801 applied by superfusion (fig. 1, center) or by pressure ejection together with 10^{-5} M NMDA (figs. 4 and 5) reduced the amplitude of depolarization. The IC_{50} for reduction of depolarization was approximately 10^{-7} M, and all cells failed to depolarize when MK-801 was coapplied at concentrations between 3×10^{-7} ($N = 6$) and 3×10^{-6} M ($N = 6$).

Application of 10^{-3} M NMDA resulted in a mean depolarization of 21.9 ± 2.5 mV ($N = 19$; fig. 4). The IC_{50} for reduction of this depolarization by MK-801 was about 2×10^{-7} M ($N = 12$).

During serial applications of NMDA and MK-801 at 1- to 2-sec IRI, responses were blocked reversibly in use-dependent manner (fig. 5). Action potential $\dot{V}_{max}$ decreased until the peak of the depolarization and recovered to the initial rate by the time AP firing ceased before complete repolarization (fig. 5, top row, PRE). Amplitude of depolarization and duration of AP firing decreased progressively with successive applications until no response was obtained (fig. 5, top center; reduced response to P_5 and absence of response to P_{10} shown). By P_{10}, 3×10^{-8} M MK-801 was about as potent as 3×10^{-7} M at P_1. After washout, application of NMDA alone elicited a response similar to PRE (fig. 5, top row, POST). Similar findings were obtained in 27 other neurons exposed to 3×10^{-8} M MK-801. Three times 10^{-10} ($N = 6$) and 3×10^{-9} ($N = 6$) M MK-801 had no effect.

Effect of MK-801 on SRF. The amplitude of current pulses (400 msec) was adjusted to a level which elicited SRF throughout pulses applied at 0.5 to 1 Hz. After control recordings, MK-801 was applied by pressure (fig. 5, bottom row), or the superfusate was changed to mDPBS containing MK-801 at selected concentrations and back to drug-free solution (Fig. 6). Figure 6 shows recordings from the same neuron exposed to multiple concentrations of MK-801. At 1.5×10^{-7} M or higher, limitation of SRF occurred with progressive diminution of $\dot{V}_{max}$ until firing failed for the remainder of the pulse. Limitation of SRF was obtained in 18 neurons superfused with MK-801 at concentrations $\geq 1.5 \times 10^{-7}$ M. Limitation of SRF was reversed by washing with drug-free solution for 3 to 15 min. Exposures longer than 1 hr required up to 3 hr for the return of SRF.

Additional groups of neurons with initial E_m of -55 to -65 mV were superfused with control mDPBS ($N = 80$) or solution containing MK-801 (total $N = 332$; fig. 5). Concentration-dependent reduction in percentage of neurons capable of SRF was observed in solutions containing MK-801 (fig. 7).

Characteristics of AP blockade by MK-801. In the

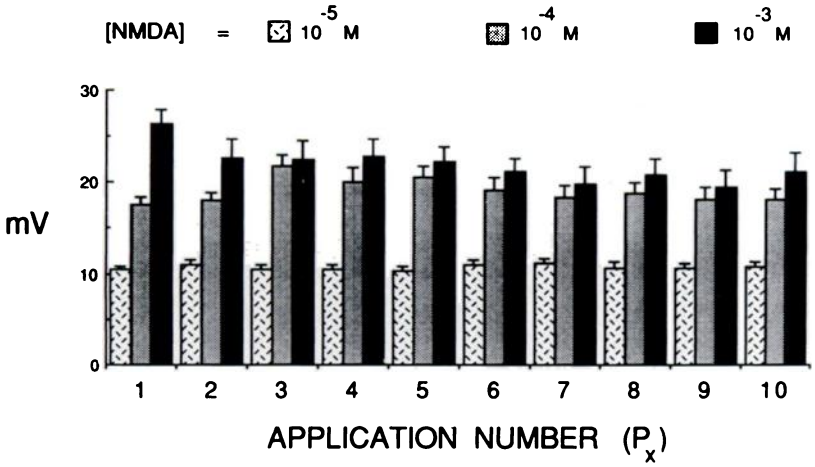

Fig. 3. Consistency of responses to P of NMDA. Ordinate: amplitude of depolarization (mV). Abscissa: ordinal number of pressure applications (P_x). Results of application of three concentrations of NMDA shown (code above graph; N = 20 at 10^{-5} M, 18 at 10^{-4} M and 29 at 10^{-3} M).

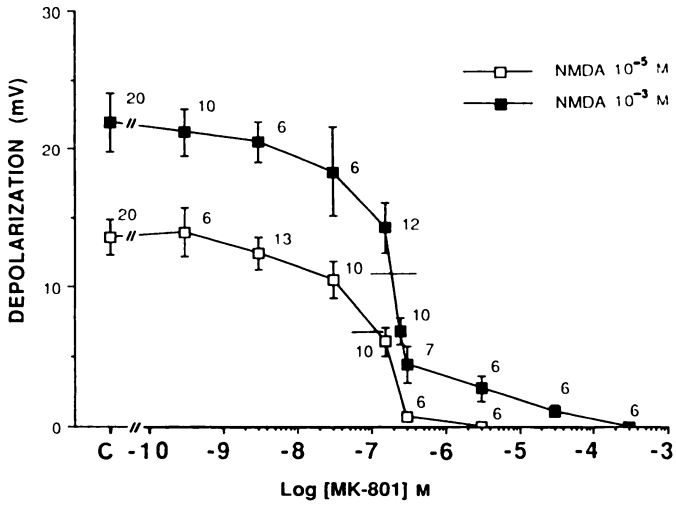

Fig. 4. Concentration-dependent reduction of depolarizing responses to NMDA by MK-801. Amplitude of depolarizing responses (mean mV ± S.E.M., N) to single applications of 10^{-5} and 10^{-3} M NMDA plotted against MK-801 concentration in the superfusate. IC_{50} indicated by horizontal bar.

continuing presence of MK-801 at a concentration that limited SRF at E_m from −55 to −65 mV, hyperpolarization to between −70 and −80 mV resulted in the restoration of repetitive firing in most neurons (N = 98 of 106 neurons exposed to MK-801 by superfusion). To explore the protective effect of hyperpolarization against the effect of MK-801 further, 100-Hz trains of 1-msec depolarizing pulses were used (fig. 8). All stimuli elicited AP in control solution (fig. 8; CONTROL, −55 mV). During the last few seconds of a 30-sec pressure application of 3×10^{-7} M MK-801, many stimuli failed to elicit AP (15 of 20 failed in fig. 8, MK-801, −55 mV), and AP that fired occurred at longer latencies with slower $\dot{V}_{max}$ (fig. 8, MK-801, −55 mV). After hyperpolarization of the neuron, stimulus intensity was adjusted so that no failures occurred at 100 Hz (fig. 8, CONTROL, −80 mV). During pressure application of 10^{-7} M MK-801, many stimuli failed to elicit AP (6 of a train of 12, fig. 8, MK-801, −80 mV) even with increased stimulus intensity, but the $\dot{V}_{max}$ of those which fired was about the same as that of control AP (fig. 8, MK-801, −80 mV; compare $-dV/dt$ trace with CONTROL). Use-dependent reduction of $\dot{V}_{max}$ and block of AP were evident after return to the original E_m. Resting membrane potential was not altered by MK-801. Qualitatively similar findings were observed in four other neurons.

Use dependence. The effect of MK-801 on sodium-dependent AP of the spinal cord neurons in cell culture was also use dependent (*i.e.*, maximal effect on $\dot{V}_{max}$ or cessation of firing occurred after multiple AP fired). In control solution, AP with roughly constant $\dot{V}_{max}$ and some failures occurred during 150-Hz stimulation with 1-msec current pulses (fig. 9, CONTROL). During pressure application of 3×10^{-7} M MK-801 for 90 sec, the number of failures in response to trains of stimuli increased, and $\dot{V}_{max}$ of AP that did fire were progressively reduced (fig. 9, MK-801). After cessation of the pressure application and washout of MK-801, the number of AP fired by each train (triggered every 30 sec) increased to approximately the original number,

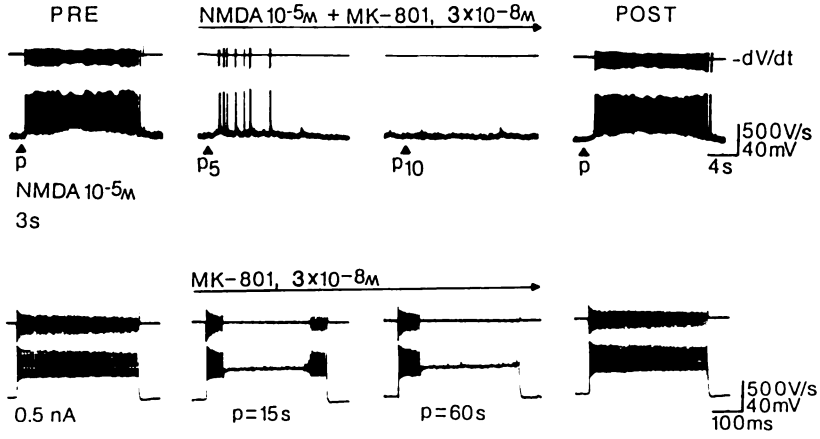

Fig. 5. MK-801 blocked NMDA responses and limited sustained repetitive firing of action potentials (S.R.F.) in a use-dependent manner. *Top row:* 3-sec pressure application of 10^{-5} M NMDA produced depolarization and firing of AP (PRE). Response to P_5 of NMDA plus MK-801 3×10^{-8} M had reduced depolarization and duration of AP firing. No response to P_{10} was observed. Recovery occurred after washout (POST). *Bottom row:* Depolarizing current pulses (400-msec duration, 0.5 nA, 1 Hz) elicited SRF (PRE). Limitation of SRF began within 15 sec and severe limitation with steep reduction of $\dot{V}_{max}$ (peak excursion of $-dV/dt$; top trace) before cessation of firing occurred within 60 sec (*i.e.*, during the fourth 15-sec application) of P of 3×10^{-8} M MK-801 (center panels). Reversal of blockade occurred with time-dependent washout of MK-801 (POST). Top traces shows $-dV/dt$, bottom traces show transmembrane potential. Calibrations at right apply to respective rows.

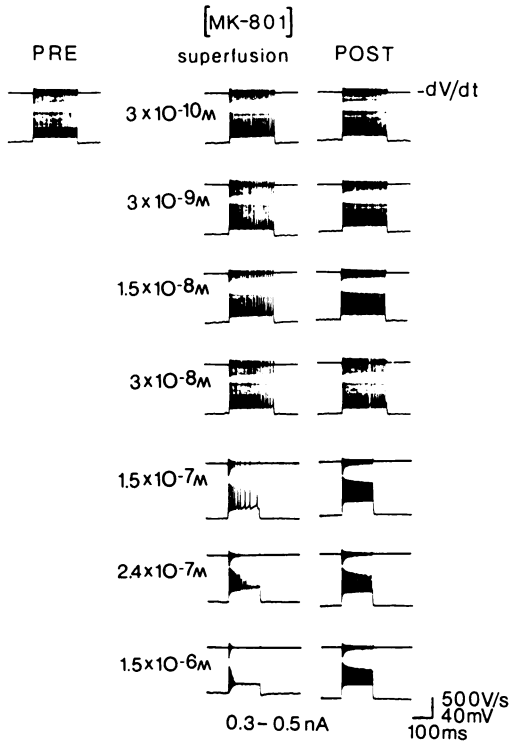

Fig. 6. Concentration-dependent limitation of SRF of a spinal cord neuron superfused with MK-801. Same neuron throughout; 7 mM Mg^{++} mDPBS superfusion to suppress spontaneous activity. In control solution, 400-msec depolarizing current pulses elicited SRF (top row, PRE). Limitation of SRF occurred during superfusion with mDPBS containing MK-801 (center column). After washout with drug-free solution until full recovery of SRF (POST), superfusate was changed to one containing the next concentration of MK-801. MK-801 concentrations of 1.5×10^{-7} to 1.5×10^{-6} M limited SRF reversibly. Top trace shows $-dV/dt$; bottom trace shows transmembrane potentials. Calibrations at bottom right apply throughout.

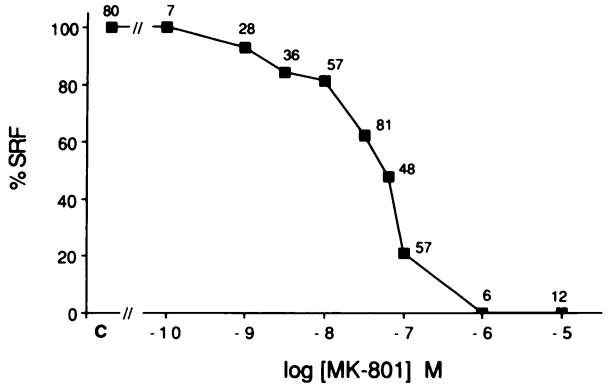

Fig. 7. Concentration-dependent limitation of SRF by MK-801. Percentage of neurons capable of SRF (number of neurons given each concentration) plotted against the concentration of MK-801 in the superfusate.

and $\dot{V}_{max}$ recovered gradually (fig. 9, RECOVERY, 30–90 sec). Similar results were observed in 10 other spinal cord neurons.

Frequency dependence. The effect of 3×10^{-7} M MK-801 on trains of AP depended on the frequency of stimulation. Recordings from one spinal cord neuron are shown in figure 10. In control solution (fig. 10, left column) at 1- and 50-Hz stimulation rates, there were no failures in response to 12 brief (1 msec) pulses, and $\dot{V}_{max}$ was roughly constant. During repeated trains of pulses at 100 Hz, AP fired with interspersed failures and $\dot{V}_{max}$ decreased noticeably, but less than 25%. At

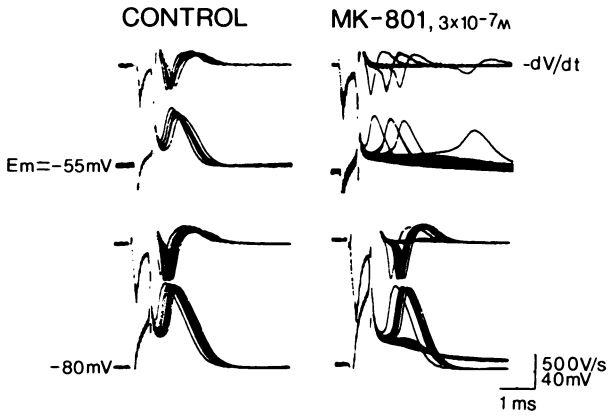

Fig. 8. Voltage-dependent effect of MK-801 on action potential firing. *Control (left column):* At $E_m = -55$ mV (top) and $E_m = -80$ mV (bottom), AP fired with minor fluctuation of $\dot{V}_{max}$ (peak excursion of $-dV/dt$ trace) and no failures. During the last few seconds of pressure application of 3×10^{-7} M *MK-801 (right column):* at −55 mV (top), $\dot{V}_{max}$ declined, latency increased and failures occurred; at −80 mV (bottom) $\dot{V}_{max}$ was stable and few failures occurred. Stimulation frequency was 100 Hz in all panels. Top trace shows $-dV/dt$, bottom trace shows intracellularly recorded transmembrane potentials. Calibrations to bottom right apply throughout.

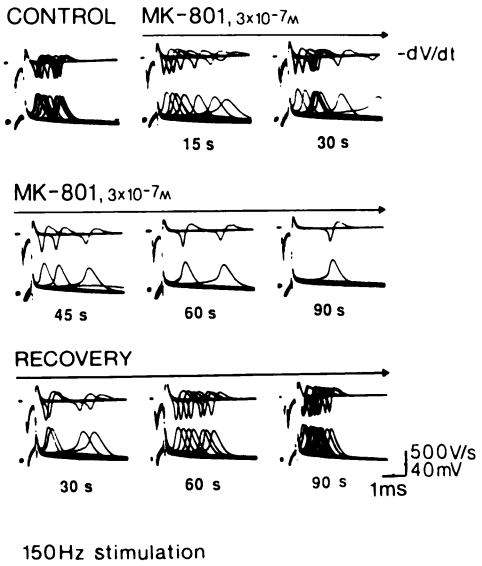

Fig. 9. Use-dependent reduction of $\dot{V}_{max}$ and failure of AP firing during pressure application of MK-801. In each panel, the same neuron was stimulated at 150 Hz during superfusion with 7 mM Mg^{++} mDPBS (CONTROL). Recordings at 15, 30, 45, 60 and 90 sec (top row, center and right; middle row) of pressure application of 3×10^{-7} M MK-801 show reduction of $\dot{V}_{max}$ (peak amplitude of dV/dt) and failure of most AP. After cessation of MK-801 application, AP and $\dot{V}_{max}$ recovered progressively with time (RECOVERY; bottom row). Top trace shows $-dV/dt$, bottom trace shows transmembrane potentials. Calibrations to bottom right apply throughout.

150 Hz, approximately the same number of failures occurred, and reduction of $\dot{V}_{max}$ remained about the same as at 100 Hz.

During the last few seconds of 30-sec pressure applications of 3×10^{-7} M MK-801 (fig. 10, right column), no failures occurred during 1-Hz trains and $\dot{V}_{max}$ was unchanged from control. At 50 Hz, $\dot{V}_{max}$ decreased slightly compared to control and AP duration was increased along with some loss of spike height. Resting potential was unchanged and no failures occurred. At 100 Hz, up to 50% of stimuli failed to elicit AP and $\dot{V}_{max}$ decreased steeply with firing of successive AP. At the slowest rates of rise, latency was prolonged, AP amplitude

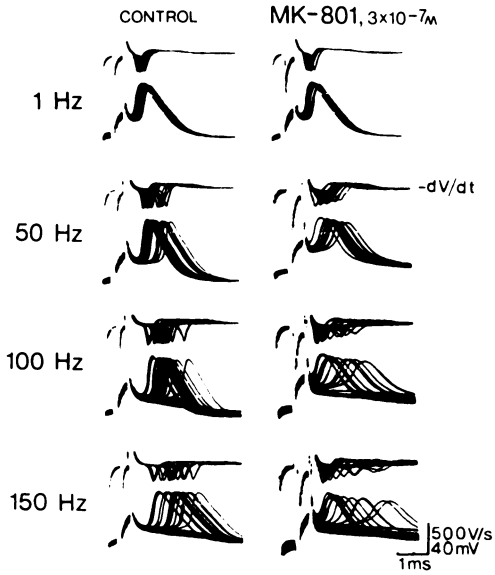

Fig. 10. Frequency-dependent effect of MK-801 on AP firing. *1 Hz (top row)*: Compared to CONTROL, MK-801 had no effect on $\dot{V}_{max}$ or AP firing during stimulation with trains of 1-msec pulses. *50 Hz (second row)*: MK-801 produced fluctuation of $\dot{V}_{max}$ and latency, but no AP failures. *100 Hz (third row)* and 150 Hz (*bottom row*): $\dot{V}_{max}$ of AP in CONTROL was nearly constant at different latencies. MK-801 application caused marked reduction of $\dot{V}_{max}$ and more AP failures than in CONTROL. Right traces were obtained at the end of 30-sec P of 3×10^{-7} M MK-801. Top trace shows $-dV/dt$, bottom trace shows transmembrane potentials. Calibrations to bottom right apply throughout.

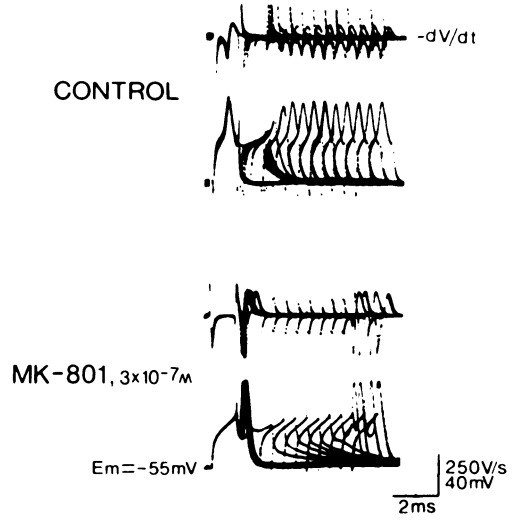

Fig. 11. MK-801 prolonged refractoriness. *Top:* AP elicited by pairs of stimuli delivered at different intervals were superimposed. With resting membrane potential = −55 mV, AP failed at intervals less than 1.5 msec. At 1.5- to 4-msec intervals, $\dot{V}_{max}$ recovered progressively to a maximum of 200 to 250 V/sec. *Bottom:* With resting membrane potential = −55 mV, a second AP could not be elicited at intervals less than 9 msec. Top trace shows $-dV/dt$, bottom trace shows transmembrane potentials. Calibrations to bottom right applied throughout.

decreased and duration increased more noticeably than at 50 Hz. At 150 Hz, similar changes in AP parameters and more failures were observed. These effects were reversed after washout of MK-801. Qualitatively similar findings were obtained in 12 other spinal cord neurons.

Effect of MK-801 on refractoriness. Intracellular application of pairs of 1-msec depolarizing current pulses at varying intervals were used to test the effect of MK-801 on refractoriness. In control solution, a second AP could not be obtained by a pulse delivered less than 1.9 ± 0.4 (N = 13; timed from end of previous pulse) msec after the first pulse in a pair (fig. 11, CONTROL). At interpulse intervals of 1.5 to 4 msec, $\dot{V}_{max}$ progressively recovered to the rate of rise of AP elicited by the first pulse of the pairs with a time constant of recovery of 2.3 ± 0.3 msec (N = 8). After a 30-sec pressure application of 3×10^{-7} M MK-801, a second AP could not be elicited at intervals less than 4.9 ± 0.4 msec (N = 12; fig. 11, MK-801). Mean time constant for recovery of $\dot{V}_{max}$ after pressure application of MK-801 was 12.5 ± 1.6 msec (N = 8). Both the duration of the absolute refractory period and the time constant of recovery of $\dot{V}_{max}$ were significantly different from control ($P < .001$). After washout of MK-801, absolute refractoriness was 2.2 ± 0.1 msec (N = 8), and $\dot{V}_{max}$ recovered with a time constant of 3.0 ± 0.3 msec (N = 8). The recovery values differed significantly from those during exposure to MK-801 ($P < .001$), but not from control values ($P > .1$).

Discussion

Concentration dependence of blockade of NMDA responses and limitation of SRF of AP by mouse central neurons in cell culture were compared here to test the relevance of SRF to the therapeutic efficacy of MK-801. The primary effect of MK-801 on NMDA responses was to reduce depolarization. Characteristics of the blockade of responses to NMDA were consistent with published data. Similarity of IC_{50} values (1 to 2×10^{-7} M) of MK-801 against different concentrations of NMDA suggested that the blockade was not competitive, as shown previously in binding studies (Wong *et al.*, 1986). Also, the blockade was use dependent, as shown in patch clamp experiments (Huettner and Bean, 1988). An important aspect of use dependence observed here was that MK-801 was about 10 times more potent at 2-sec than at 20-sec, or longer, intervals. Responses to NMDA included AP firing as long as depolarization exceeded threshold. Firing varied with the degree of depolarization, as indicated by fluctuations in $\dot{V}_{max}$, and decreased during exposure to MK-801 before complete block. Effects on AP were observed directly in the SRF test. The range of MK-801 concentrations which blocked single responses to NMDA coincided with that which limited SRF (10^{-8} to 10^{-6} M). Increased sensitivity of NMDA responses to MK-801 was apparent with serial applications at short IRI. But, even at 3×10^{-8} M MK-801, a concentration which produced use-dependent block of NMDA responses in all neurons, limitation of SRF occurred in about 30% of neurons. These findings suggest that limitation of SRF also might contribute to the anticonvulsant efficacy of MK-801. To put these findings in context, the IC_{50} of phenytoin against SRF was about 4×10^{-6} M (McLean and Macdonald, 1983), and in the present investigations, that of MK-801 was about 10^{-7} M, about 40 times more potent. Coincidentally, this is roughly the same ratio of potency of the two drugs against maximal electroshock-induced seizures in mice, an assay in which phenytoin has been considered a principal reference compound (12.7 mg/kg for phenytoin and 0.35 mg/kg for MK-801; Clineschmidt *et al.*, 1982).

Limitation of SRF is an *in vitro* effect shared by MK-801 with several anticonvulsant compounds at clinically relevant concentrations. In addition to phenytoin (McLean and Macdonald, 1983), these include carbamazepine (McLean and Mac-

donald, 1986a), valproic acid (McLean and Macdonald, 1986b) and concentrations of benzodiazepines encountered in treating *status epilepticus* (McLean and Macdonald, 1988). Limitation of SRF by anticonvulsant compounds has not been demonstrated directly in the intact brain. However, high-frequency firing, simulated in neurons in cell culture by SRF, has been recorded along projection pathways from a chemically induced seizure focus (Sypert and Reynolds, 1974; Ward 1983). Limitation of high-frequency firing could disrupt the spread of abnormal activity from the focus and contribute to anticonvulsant efficacy (see McLean and Macdonald, 1983, and Macdonald and McLean, 1986, for discussion).

Activation of a potassium conductance could limit SRF. However, hyperpolarization was not observed during the 400-msec depolarizing steps, and AP duration increased, rather than decreasing, before failure. Furthermore, in experiments with trains of brief stimuli, $\dot{V}_{max}$ of AP was reduced, whereas resting membrane potential did not change. Thus, increase of one or more potassium conductances is unlikely to account for the observed reduction of $\dot{V}_{max}$ and limitation of SRF by MK-801.

Limitation of SRF by MK-801 was characterized by progressively decreasing $\dot{V}_{max}$ until firing stopped, enhancement of the effect at low membrane potentials and prolongation of refractoriness. In addition, the $\dot{V}_{max}$ of AP elicited by 100 to 150-Hz trains of brief (1 msec) stimuli declined abruptly until most stimuli failed to elicit AP. The upstroke velocity of AP of spinal cord neurons in cell culture was reduced by lowering $[Na^+]_o$, but not $[Ca^{++}]_o$, and by exposure to tetrodotoxin (Ransom and Holz, 1977; Heyer and Macdonald, 1982; McLean and Macdonald, 1983). Although the relationship between I_{Na} and $\dot{V}_{max}$ of nerve AP is not linear at low membrane potentials (Hondeghem, 1978), when sodium is the predominant charge carrier and when comparison is made at the same resting potential, $\dot{V}_{max}$ reflects the relative amplitude of I_{Na} (Hondeghem, 1985; Courtney, 1984; Cohen *et al.*, 1984; Roberge and Drouhard, 1987; Sheets *et al.*, 1988). Thus, the present findings suggest that limitation of SRF by MK-801 probably involved use-dependent block of voltage-sensitive sodium current. In support of this interpretation, voltage clamp studies with clinically used anticonvulsants (Schwartz and Vogel, 1977; Courtney and Etter, 1983; Matsuki *et al.*, 1984; VanDongen *et al.*, 1986), which also limited SRF, demonstrated use-dependent reduction of I_{Na} and slow recovery of I_{Na} from inactivation.

In rat hippocampal neurons in cell culture, 3×10^{-3} M MK-801 reduced amplitude and $\dot{V}_{max}$ without preventing AP firing (Rothman 1988). Also, under whole cell patch clamp recording conditions at a slow (0.5 Hz) rate of stimulation, 10^{-4} M MK-801 reduced I_{Na} of bovine chromaffin cell by only 23% (Halliwell *et al.*, 1989). Because of the high concentrations of MK-801 required for the partial block observed, the authors of both studies concluded that the effects of MK-801 on $\dot{V}_{max}$ and I_{Na} were unlikely to contribute to therapeutic efficacy. Two technical considerations limit this interpretation. First, both studies were performed at room temperature. Higher concentrations of MK-801 were required to limit SRF at a reduced temperature (unpublished observations). Both receptors and ligands could be altered at low temperature. For example, the affinity of tetrodotoxin for one type of squid axon sodium channel increased with cooling, whereas affinity for a second type increased with warming (Sevcik, 1982). Lidocaine became less lipid-soluble due to alkaline shift of pK_a with cooling. The concentration of the charged form increased at low temperature, thereby favoring hydrophilic (open-channel) block and eliminating hydrophobic interaction (inactivated-channel block) of sodium channels (Sanchez *et al.*, 1987; Courtney, 1987). Second, frequency dependence of MK-801 effects was not tested in either study. The importance of frequency-dependent effects in the mechanism of action of anticonvulsants has been stressed (Courtney, 1975; Courtney and Etter, 1983). Performance of the present investigations at physiological temperature and testing specifically for the effect of frequency of stimulation could account for the greater sensitivity of sodium-dependent AP to MK-801 observed here.

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