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Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels

(excitatory amino acid receptors/visual cortex/cell culture/single channels)

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ABSTRACT **Whole-cell and single-channel recording techniques were used to study the action of the anticonvulsant drug MK-801 {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate} on responses to excitatory amino acids in rat neocortical neurons in cell culture. MK-801 caused a progressive, long-lasting blockade of current induced by *N*-methyl-D-aspartate (*N*-Me-D-Asp). However, during the time that *N*-Me-D-Asp responses were inhibited, there was no effect on responses to quisqualate or kainate, suggesting that *N*-Me-D-Asp receptors and kainate/quisqualate receptors open separate populations of ion channels. Binding and unbinding of MK-801 seems to be possible only if the *N*-Me-D-Asp-operated channel is in the transmitter-activated state: MK-801 was effective only when applied simultaneously with *N*-Me-D-Asp, and recovery from MK-801 blockade was speeded by continuous exposure to *N*-Me-D-Asp [time constant (τ) ≈ 90 min at −70 to −80 mV]. Recovery from block during continuous application of *N*-Me-D-Asp was strongly voltage dependent, being faster at positive potentials (τ ≈ 2 min at +30 mV). Mg^{2+}, which is thought to block the *N*-Me-D-Asp-activated ion channel, inhibited blockade by MK-801 at negative membrane potentials. In single-channel recordings from outside-out patches, MK-801 greatly reduced the channel activity elicited by application of *N*-Me-D-Asp but did not significantly alter the predominant unitary conductance. Consistent with an open-channel blocking mechanism, the mean channel open time was reduced by MK-801 in a dose-dependent manner.**

L-Glutamate is thought to serve as the major excitatory neurotransmitter in the central nervous system (1, 2). Most central neurons express two distinct receptors for excitatory amino acids. The *N*-methyl-D-aspartate (*N*-Me-D-Asp) receptor (1–3) is selectively activated by *N*-Me-D-Asp, whereas kainate and quisqualate serve as selective agonists of non-*N*-Me-D-Asp receptors (1–3). Both receptors activate ion channels that increase the membrane permeability to monovalent cations (4–6). In addition, the channel controlled by *N*-Me-D-Asp receptors conducts Ca^{2+} (6–8).

Electrophysiological responses to *N*-Me-D-Asp are blocked by competitive antagonists of the *N*-Me-D-Asp receptor (9) and by a number of divalent cations, including Mg^{2+} (6, 10–12). A third group of compounds that antagonize responses to *N*-Me-D-Asp, but that do not compete for the *N*-Me-D-Asp binding site, include the dissociative anesthetics ketamine and phencyclidine (13–17) and the σ opioid SKF 10,047 (17, 18). Recently, Wong and colleagues (19, 20) have shown that the anticonvulsant compound MK-801 {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate} belongs to this third class of antagonists. MK-801 competes with the binding of SKF 10,047, ketamine, and phencyclidine to rat cortical membranes and it antagonizes the depolarization of cortical tissue by bath-applied *N*-Me-D-Asp (19).

We have investigated the action of MK-801 on currents elicited by excitatory amino acids in rat cortical neurons maintained in dissociated tissue culture. When applied simultaneously with *N*-Me-D-Asp, MK-801 produced a long-lasting blockade of the current evoked by *N*-Me-D-Asp but did not affect responses to kainate and quisqualate. The properties of blockade by MK-801 suggest that the drug cannot bind or unbind unless the channel has been opened by transmitter and that the drug probably binds within the channel pathway itself.

METHODS

Neurons were dissociated from the visual cortex of 2- to 6-day-old Long Evans rat pups and grown in culture for 5–43 days as described (21). Currents activated by excitatory amino acids were measured in the whole-cell and outside-out patch-clamp configurations (22). Pipettes contained an internal solution (in mM) of 120 cesium methanesulfonate, 5 CsCl, 10 Cs_2EGTA, 5 $Mg(OH)_2$, 5 MgATP, 1 Na_2GTP, and 10 Hepes (pH adjusted to 7.4 with CsOH). The external solution (in mM) was 160 NaCl, 2 $CaCl_2$, and 10 Hepes (pH 7.40). In whole-cell experiments, 300 nM tetrodotoxin and 10 μM bicuculline methiodide were added to the external solution to suppress spontaneous activity. MK-801, the kind gift of Paul Anderson (Merck Sharp & Dohme), was added from stock solutions of 2–50 mM in ethanol, stored at −20°C. Final concentrations of ethanol were <0.1%. Cells or patches were bathed in control or agonist-containing external solution flowing from one of a linear array of 7–10 microcapillary tubes fed by gravity (23). Rapid solution changes were made by moving the array of tubes relative to the cell (whole-cell) or by moving the pipette relative to the tubes (patch). All experiments were done at 20–25°C.

RESULTS

Whole-Cell Recordings. Fig. 1 illustrates the blocking action of MK-801 on inward current activated by *N*-Me-D-Asp. In Fig. 1*A*, the cell was held at −70 mV in the whole-cell recording mode, bathed in the Mg^{2+}-free external solution. Application of 200 μM *N*-Me-D-Asp elicited an inward current that rose rapidly to a peak and then decayed to a steady current. When *N*-Me-D-Asp and 10 μM MK-801 were applied simultaneously, the current reached nearly the same peak but was then progressively blocked with a time constant (τ) of about 11 sec. The blockade by MK-801 persisted when the cell was washed with control solution for 20 sec and then tested with a subsequent application of *N*-Me-D-Asp. Fig. 1*B* shows a similar experiment, but with *N*-Me-D-Asp applied together with 1 μM glycine. Glycine potentiated the response to *N*-Me-D-Asp (23); however, it

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Abbreviation: *N*-Me-D-Asp, *N*-methyl-D-aspartate.

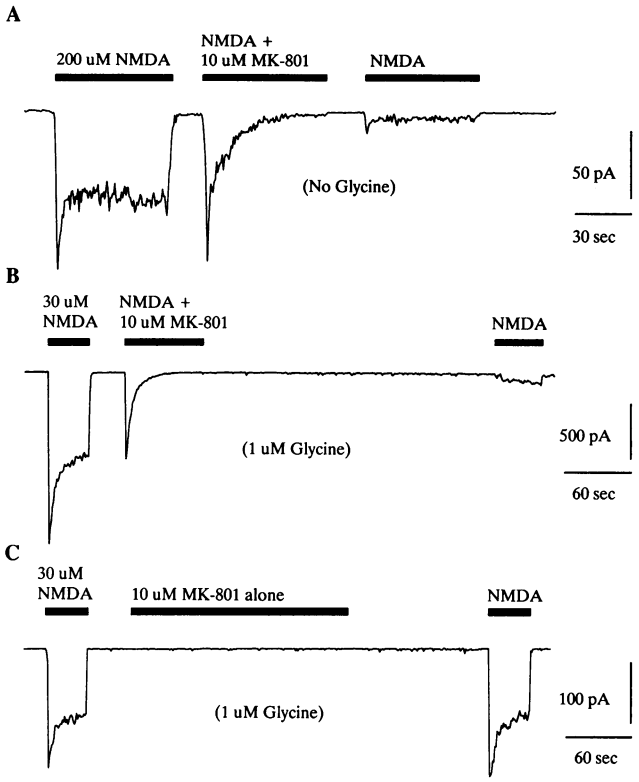


FIG. 1. Whole-cell recordings from three different cultured cortical neurons. (*A*) Block of *N*-Me-D-Asp (NMDA)-activated current by 10 μM MK-801; no glycine. Holding potential, −70 mV. (*B*) Block in the presence of glycine. Holding potential, −70 mV. (*C*) Application of 10 μM MK-801 without *N*-Me-D-Asp. Holding potential, −70 mV. Currents were filtered at 1 kHz and noise was further reduced by averaging current over 5-msec intervals. Membrane potentials are corrected for a junction potential of −10 mV between the external and internal solutions.

did not affect the sensitivity of the current to block by MK-801. Glycine was included in most further experiments so that relatively large currents could be obtained with low concentrations of *N*-Me-D-Asp. Fig. 1*B* shows that the blocking action of MK-801 was long lasting; there was little response to a subsequent application of *N*-Me-D-Asp even after 4 min of washing with control solution (see also Fig. 3 and Table 1). MK-801 was effective, however, only when applied simultaneously with *N*-Me-D-Asp (Fig. 1*C*). Application of MK-801 alone had no effect on the holding current and had little effect on the response of the cell to a subsequent application of *N*-Me-D-Asp. This result suggests that the MK-801 can exert its blocking action only when the receptor has been activated by agonist.

As shown in Fig. 2, the action of MK-801 was specific for responses to *N*-Me-D-Asp. Application of two other excitatory amino acids, quisqualate and kainate, elicited inward currents that were unchanged even during a period when MK-801 had blocked the response to *N*-Me-D-Asp by >90%. In addition, when MK-801 was applied simultaneously with either quisqualate or kainate it did not reduce the response to these compounds and it did not cause a reduction in the response to a subsequent application of *N*-Me-D-Asp alone.

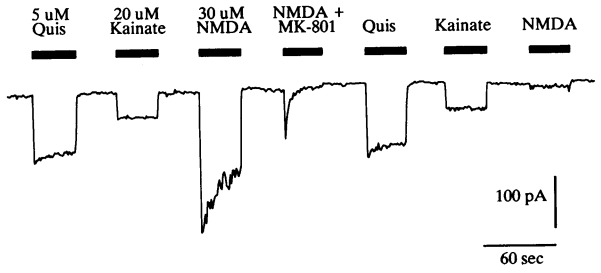


FIG. 2. MK-801 blocks the current induced by *N*-Me-D-Asp (NMDA) but not that induced by quisqualate (Quis) or kainate. Holding potential, −80 mV. This specificity of action was confirmed in six other neurons.

Recovery from MK-801 blockade was very slow in the absence of *N*-Me-D-Asp. In six cells, brief pulses of *N*-Me-D-Asp were applied every 5–10 min to test for recovery from MK-801 blockade (e.g., Fig. 3*A*). After 20–30 min of perfusion with control solution, the current induced by *N*-Me-D-Asp recovered to only about 10% of the initial response. Recovery was much faster when *N*-Me-D-Asp was applied continuously to the cell; in this case, substantial recovery could be seen over 4–5 min in some cells (Fig. 3*B*), with a mean τ (in seven cells) of about 90 min at −70 mV. A dramatic acceleration of recovery from blockade was observed when cells were held at +30 mV during the application of *N*-Me-D-Asp; in this case (where current flow was outward), nearly complete recovery was seen in 4–5 min (Fig. 3*C*). Holding at +30 mV in the absence of *N*-Me-D-Asp did not enhance the rate of recovery from MK-801. Table 1 shows the τ values of onset of blockade by MK-801 and of recovery from blockade by MK-801 at +30 mV and −70 mV (the fits with single exponentials were usually good but not perfect). Despite the pronounced voltage dependence of recovery, the rate of development of block was no different at +30 mV than at −70 mV. Table 1 also shows that the rate of development of block was faster in 10 μM than 2 μM MK-801. The concentration dependence of the rate of block extended to lower concentrations of MK-801 as well, with 100 nM blocking with $\tau \approx 1.5$ min; clear effects could be seen

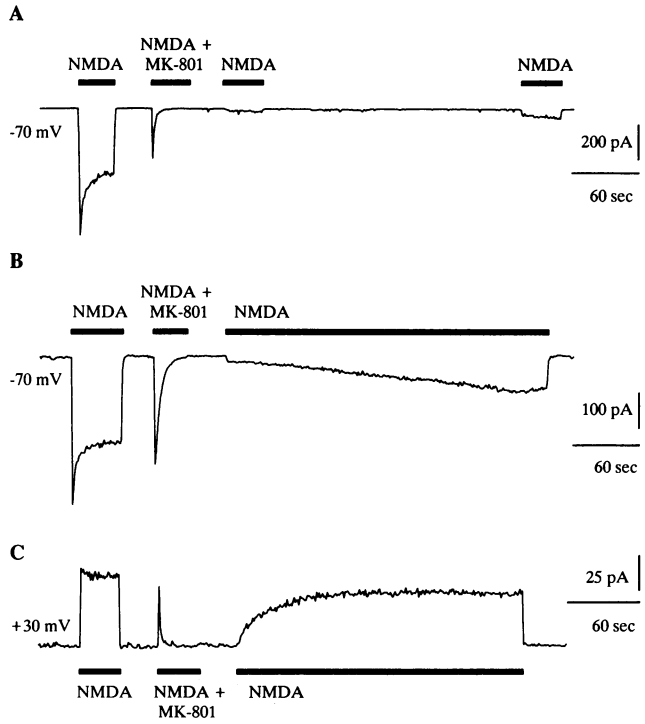


FIG. 3. Recovery from blockade by MK-801. (*A*) After blocking the response to 30 μM *N*-Me-D-Asp (NMDA) with 10 μM MK-801, the recovery of the *N*-Me-D-Asp-induced current was monitored at −70 mV by applying short pulses of 30 μM *N*-Me-D-Asp at 5-min intervals. The recovery of this cell was evaluated for 30 min but only the first two test applications of *N*-Me-D-Asp are shown. (*B*) Following blockade by 20 μM MK-801, 30 μM *N*-Me-D-Asp was applied continuously for 5 min at −70 mV. (*C*) MK-801 (10 μM) block of current elicited at +30 mV by 30 μM *N*-Me-D-Asp.

Table 1. Time constants (τ) of onset of blockade by MK-801 and recovery from blockade by MK-801

Holding potential, mV	MK-801, μM	τ of onset, sec	n	Holding potential, mV	τ of recovery, min	n
+30	2	25 ± 3	5	+30	1.8 ± 0.3	14
−70	2	24 ± 5	4	−70	92 ± 40	7
−70	10	8.1 ± 0.9	12			

Onset: A single exponential was fit to the relaxation phase of current induced by application of 30 μM *N*-Me-D-Asp, 1 μM glycine, and MK-801. Recovery: Cells were exposed to 2–20 μM MK-801, together with 30 μM *N*-Me-D-Asp and 1 μM glycine, until a complete blockade of *N*-Me-D-Asp-induced current was achieved. A single exponential was fit to the rising phase of current induced by a subsequent, continuous application of 30 μM *N*-Me-D-Asp and 1 μM glycine alone. The τ values are presented as mean ± SEM of determinations.

with concentrations as low as 10 nM, but the development of block was too slow to quantitate.

Effect of Mg^{2+}. In agreement with previous work (6, 8, 11), physiological levels of Mg^{2+} caused a substantial reduction in the current activated by *N*-Me-D-Asp at holding potentials of −70 to −80 mV (Fig. 4*A*), but MK-801 still produced a long-lasting blockade of the *N*-Me-D-Asp-induced current in 0.5–1 mM Mg^{2+}. In solutions containing 10 mM Mg^{2+} (Fig. 4*B*) the *N*-Me-D-Asp-induced current was completely blocked at holding potentials of −70 to −80 mV. As shown in Fig. 4*B*, 10 mM Mg^{2+} prevented MK-801 from blocking the *N*-Me-D-Asp-induced current, even when MK-801 was applied for a long time in the presence of *N*-Me-D-Asp. This result suggests that when Mg^{2+} has blocked the *N*-Me-D-Asp-induced current, it prevents the interaction of MK-801 with the channel. The action of Mg^{2+} seems directly related to its blockade of the current activated by *N*-Me-D-Asp (Fig. 4*C*): when cells were depolarized to +30 mV, the blockade of *N*-Me-D-Asp-induced current by Mg^{2+} was relieved, and MK-801 was able to produce a long-lasting blockade of the *N*-Me-D-Asp-induced current in the presence of Mg^{2+}.

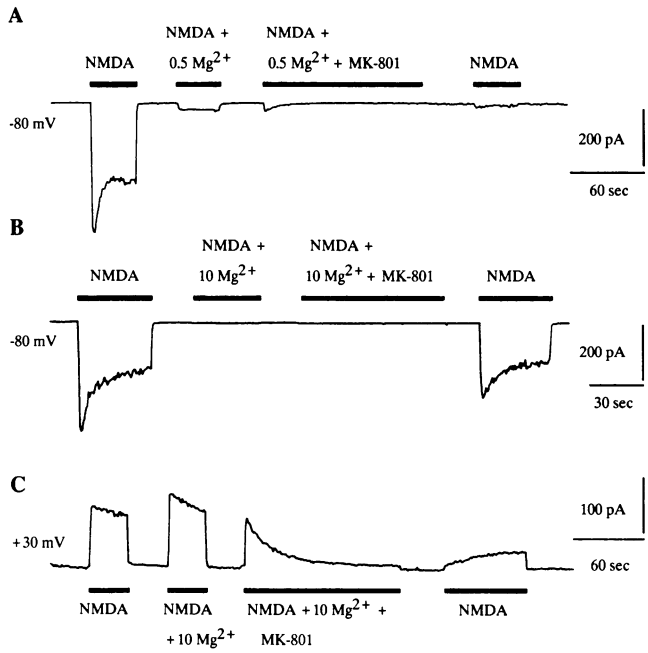


FIG. 4. Effect of Mg^{2+} on the blockade of *N*-Me-D-Asp (NMDA)-induced current by MK-801. (*A*) Ten micromolar MK-801 applied together with 30 μM *N*-Me-D-Asp plus 0.5 mM Mg^{2+} gradually blocked the current elicited by *N*-Me-D-Asp and prevented a response to a subsequent application of 30 μM *N*-Me-D-Asp in Mg^{2+}-free saline. (*B*) In a solution containing 10 mM Mg^{2+}, 30 μM *N*-Me-D-Asp did not elicit inward current at −80 mV. Application of 2 μM MK-801 with 30 μM *N*-Me-D-Asp and 10 mM Mg^{2+} did not block current elicited by a subsequent application of 30 μM *N*-Me-D-Asp in Mg^{2+}-free saline. (*C*) At +30 mV, 30 μM *N*-Me-D-Asp and 30 μM *N*-Me-D-Asp with 10 mM Mg^{2+} gave roughly equivalent responses. Two micromolar MK-801 produced a long-lasting block of the current evoked by *N*-Me-D-Asp. Traces in *A–C* were obtained from three different cells.

Single-Channel Recordings. MK-801 was also effective at blocking *N*-Me-D-Asp-activated single-channel activity in outside-out patches. In Fig. 5*A*, 50 μM *N*-Me-D-Asp elicited a burst of channel activity that partially desensitized in 5 or 10 sec to a relatively steady rate of opening; subsequent application of *N*-Me-D-Asp with 2 μM MK-801 evoked a smaller initial burst of openings followed by progressively diminishing channel activity. In this and other applications of MK-801, channel activity declined to virtually zero after exposure for several minutes. In all cases, channel block seemed to be all-or-none; the openings in the presence of the drug had the same amplitude as those in the control (Fig. 5 *A* and *B*) but were much less frequent. In some patches, multiple sizes of unitary events activated by *N*-Me-D-Asp were evident (cf. refs. 8 and 26), but, in all cases, application of 10 μM MK-801 with *N*-Me-D-Asp and glycine completely abolished all sizes of events. In patches, as in the whole-cell recordings, recovery from MK-801 block was faster with *N*-Me-D-Asp application to the patch and faster at positive potentials.

Channel openings were, on the average, shorter in the presence of MK-801. For the patch shown in Fig. 5*C*, the mean open time of 7.1 msec in the control was reduced by MK-801 to 2.5 msec. The reduction in mean open time was reversible; after recovering channel activity (by exposing the patch to *N*-Me-D-Asp at a holding potential of +50 mV), the mean open time was 8.2 msec. In applications of 10 μM MK-801 to three patches, the mean open time (± SEM) was reduced to 38% ± 2% of the control (6.2 ± 1.1 msec in the control; 2.3 ± 0.4 msec in MK-801). In applications of 2 μM MK-801, reductions in open times were also seen consistently; the mean open time was reduced in each of seven patches to an average of 54% ± 5% of control values (7.3 ± 1.0 msec in the control; 3.9 ± 0.6 msec in MK-801).

DISCUSSION

Our results show that MK-801 is a potent and selective blocker of *N*-Me-D-Asp-activated channels with no effect (at least up to 10 μM) on kainate- or quisqualate-activated currents. Recent single-channel experiments (8, 26) have shown that *N*-Me-D-Asp, kainate, and quisqualate can all activate channels with the same multiple conductance levels with different levels favored by different agonists. One possibility raised by these experiments is that *N*-Me-D-Asp and non-*N*-Me-D-Asp receptors are coupled to the very same channel-forming proteins. Our results argue against this possibility: MK-801 induces a long-lived block of *N*-Me-

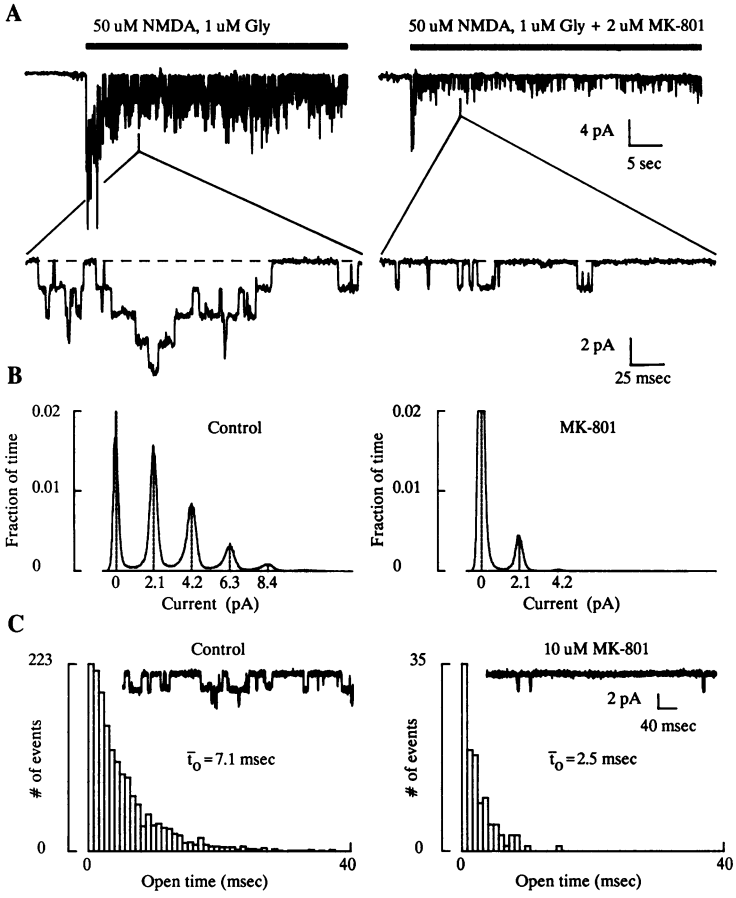
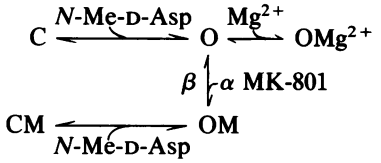


FIG. 5. MK-801 block of unitary currents in outside-out patches. (*A*) Currents activated in an outside-out patch by 50 μM *N*-Me-D-Asp (NMDA) and 1 μM glycine (left) and by 50 μM *N*-Me-D-Asp, 1 μM glycine, and 2 μM MK-801 (right). The first 35–40 sec of each record is shown; the control application lasted for a total of 54 sec, after which the patch was returned to *N*-Me-D-Asp-free solution for 18 sec before the MK-801-containing solution was applied. Currents were filtered at 1 kHz (−3 decibels) with an 80-decibel/decade low-pass Bessel filter and digitized every 60 μsec. (*B*) Amplitude histograms for patch in *A*. Histograms were constructed (with a bin size of 0.02 pA) from 21 sec of the current record beginning about 5 sec after each application of *N*-Me-D-Asp-containing solution. Patch J09A. (*C*) Open-time histograms from a different patch. Histograms were made from idealized records constructed by using a half-amplitude threshold criterion (24) and the channel amplitude obtained from the amplitude histogram; in cases of overlapping events, a minimum open time was measured from the first opening from baseline to the first closing. However, values for mean open time were calculated from $\bar{t}_0 = \Sigma j t_j / N$, where j is the current level (expressed as number of channels open), t_j is the dwell time (over the whole record) at current level j, and N is the total number of channel openings in the record (cf. ref. 25). The control application (left) of 30 μM *N*-Me-D-Asp and 1 μM glycine lasted 145 sec. There were 3307 openings during this time, of which 1654 were from baseline and were included in the histogram. The value of 7.1 msec for mean open time was calculated for all of the openings, as described above; the mean of the individual open times in the histogram was 5.3 msec. The application of solution containing 30 μM *N*-Me-D-Asp, 1 μM glycine, and 10 μM MK-801 (right) lasted 350 sec, during which there were 113 openings (101 of which occurred during the first 30 sec); none of the openings overlapped, so all were included in the histogram, and the value of 2.5 msec for the mean open time was the same whether calculated by the equation above or by averaging the individual openings. (*Insets*) Current records from 6 sec after application of control *N*-Me-D-Asp solution (left) and 6 sec after application of MK801-containing solution (right). Patch J12C.

D-Asp-activated current, and during the period that *N*-Me-D-Asp-linked channels are blocked, the responses to kainate and quisqualate are unaffected (Fig. 2). This result suggests that *N*-Me-D-Asp receptors and kainate/quisqualate receptors are coupled to different populations of channels. One possibility consistent with all of the results is that the two types of receptors are coupled to completely separate subsets of the same type of ion channel.

Selective Binding to Open Channels. Our results permit several conclusions about the molecular nature of the MK-801–channel interaction. (*i*) Channels must be opened by agonist to be blocked by MK-801; exposure to MK-801 in the absence of *N*-Me-D-Asp produces no block (Fig. 1). (*ii*) Open channels that have been blocked by MK-801 can lose their transmitter and close with the drug still bound; this is shown by the fact that blocked channels must be activated again by exposure to *N*-Me-D-Asp for recovery to occur (Fig. 3). (*iii*) MK-801 most likely binds within the channel pathway itself. This conclusion comes from the ability of Mg^{2+}, which produces ionic blockade of the channel (6, 8, 11), to prevent MK-801 block from occurring (Fig. 4). These observations suggest the following model in which binding and unbinding of drug molecules cannot occur unless the channel is in the transmitter-activated, open state:



N-Me-D-Asp transforms closed channels (C) into the open state (O), which is blocked by MK-801 to yield an open, but nonconducting, state (OM). Dissociation of *N*-Me-D-Asp allows the channel to close, which traps the MK-801 (CM). Mg^{2+} can also block the open state, and MK-801 is unable to bind to a Mg-blocked channel (OMg^{2+}).

The K_d for MK-801 binding to the channel can be estimated by using this model. We found it impossible to measure the K_d directly, since equilibration of block with low concentrations of drug was so slow. If equilibration between closed and open states is fast compared to drug binding, then block by a relatively large concentration of drug will proceed with a τ given by:

$$1/(\alpha \cdot p_0 \cdot [\text{MK-801}]), \qquad [1]$$

where p_0 is the probability of a channel being open in the absence of MK-801. Recovery from block will proceed with a τ of $1/(\beta \cdot p_0)$. The K_d for MK-801 binding to open channels is then given by:

$$K_d = \beta/\alpha = (\tau_{block} \cdot [\text{MK-801}])/\tau_{recovery}. \qquad [2]$$

The calculations suggest a profound voltage dependence of the binding affinity of MK-801. The τ values in Table 1 yield an estimate of about 10 nM for the K_d at −70 mV, which is in excellent agreement with the values of 2–30 nM derived from direct radiolabeled drug binding to cortical membranes (19, 20). At +30 mV, the calculated K_d is 50 times higher, about 500 nM. (Since, as will be discussed, drug binding at the single-channel level is quite rapid, the

assumption that drug binding is slow compared with channel opening and closing is probably not met. In this case, time courses for onset and recovery are expected to be somewhat slower than given by the expressions above, but simulations show that the K_d calculated by Eq. 2 should still be accurate within a factor of 2 or 3.) The voltage dependence of recovery from MK-801 block is consistent with the MK-801 binding site being inside the channel. Since MK-801 has a pK of 8.2, most drug molecules are in the cationic form at physiological pH; it is tempting to speculate that the voltage dependence arises from a direct effect of electric field on binding of charged drug molecules to a site in the channel pathway, perhaps the same site to which Mg^{2+} binds. Ketamine and phencyclidine, which compete for the MK-801 binding site (19), also show a marked voltage dependence (14, 16).

Single-Channel Behavior. The single-channel results are consistent with a mechanism of open-channel block. Such a mechanism predicts a dose-dependent decrease in the mean open time of single-channel events, since with drug present openings can be terminated by drug block as well as by normal closing (cf. ref. 27). Quantitatively, the data are reasonably consistent with a value for α, the forward rate constant for MK-801 binding, of 3×10^7 $M^{-1} \cdot sec^{-1}$. With a control mean open time of 7 msec, this open-channel blocking rate predicts that 2 μM MK-801 should reduce $\bar{t}_0$ to 70% of the control and that 10 μM drug should reduce $\bar{t}_0$ to 32%; the measured reductions were to 53% and 37%, respectively. The forward rate constant that we estimate, 3×10^7 $M^{-1} \cdot sec^{-1}$, is nearly identical with the value (2×10^7 $M^{-1} \cdot sec^{-1}$) obtained by Neher and Steinbach (27) for local anesthetic block of open acetylcholine receptor channels; as they pointed out, such values are characteristic of a wide variety of open-channel blockers and are close to the theoretical maximum from diffusion limitation.

Interpreting the decrease in mean open time as reflecting drug block of open channels implies that open-channel block occurs on a millisecond time scale, in contrast to the rather slow development of block seen in the whole-cell recording. Since the rate of block of macroscopic currents (according to the simple open-channel blocking model) is given by the product of α and the fraction of open channels, the comparison implies that the probability of a channel being open must be quite low. Simulations show that an α of 3×10^7 $M^{-1} \cdot sec^{-1}$ would yield the τ values given in Table 1 for macroscopic block (and recovery) if p_0 were about 0.002. This value at first seems surprisingly low, but it is consistent with direct measurements in control patches with no overlapping channel activity, where p_0 was estimated as 0.002–0.007 (upper limits, since there may well have been more than one channel in the patches). A mechanism of open-channel block thus suggests that MK-801 blocks open channels quite rapidly, so that a channel spending more than a few milliseconds open has a good probability of being blocked but that channels spend so little time open that macroscopic block is slower by a factor of 1000.

These findings have interesting implications for how MK-801 is likely to affect synaptic transmission in the brain. Fast transmission of single excitatory postsynaptic potentials, largely mediated by kainate/quisqualate receptors (28, 29), will be relatively unaffected by MK-801, whereas more complicated synaptic events involving *N*-Me-D-Asp receptors—including long-term potentiation (29) and epileptic activity—would be susceptible to block. Because of the slow onset and a long duration of blockade by MK-801, the drug's effect will accumulate in a use-dependent way with repeated channel activation (cf. refs. 14 and 19); the depth of block will depend in a complicated way on the rate and pattern of synaptic activation. *N*-Me-D-Asp receptors have been implicated in neuronal damage due to anoxia and hyperexcitation (30–33); the properties of blockade by MK-801 may underlie its potential clinical value for treatment of stroke and epilepsy while minimizing disruption of normal brain function.

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