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Kinetics of NMDA Channel Opening

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The period required for NMDA channels to open for the first time after agonist binding (the first latency) was estimated in outside-out patch recordings from rat hippocampal neurons using fast-application techniques and the open channel blocker MK-801. In the presence of MK-801, brief applications of L-glutamate or the low-affinity agonist L-cysteate resulted in a similar amount of block despite the much shorter period of channel activation by L-cysteate. A brief coapplication of L-glutamate and MK-801 resulted in a block similar to that found with an application of L-glutamate in a background of MK-801. These results, along with our findings that MK-801 does not block desensitized receptors, indicate that NMDA channels have a mean first latency of ~10 msec, consistent with a peak open probability near 0.3. If NMDA channels at synapses behave similarly, relatively few channels would be required to produce the postsynaptic calcium transient associated with synaptic plasticity and developmental regulation.

Key words: ion channels; NMDA; kinetics; open probability; first latency; EPSC time course

In the vertebrate CNS, synaptic release of the excitatory neurotransmitter L-glutamate results in activation of NMDA receptor channels in the postsynaptic membrane that can last for several hundred milliseconds (Hestrin et al., 1990; Lester et al., 1990). The high affinity of the NMDA receptor for L-glutamate results in this prolonged channel activity because of the slow unbinding of agonist (Lester et al., 1990; Patneau and Mayer, 1990; Clements and Westbrook, 1991; Gibb and Colquhoun, 1991, 1992; Lester and Jahr, 1992). Two schemes describing the single-channel events that underlie a macroscopic response to a brief pulse of L-glutamate have developed in parallel.

In the first scheme, NMDA channels open, on average, ~10 msec after agonist binding. The scheme is based on experiments using MK-801 (Jahr, 1992), which blocks NMDA channels very rapidly after they open but unblocks very slowly at negative holding potentials, and then only when the agonist is bound (Huettner and Bean, 1988). In the presence of 20 μM MK-801, a brief application of a saturating concentration of L-glutamate results in channel activity reflecting the first openings of individual channels. This is because any channel that opens for more than ~2 msec will be blocked essentially irreversibly by 20 μM MK-801. Therefore, in a patch containing many NMDA channels, the macroscopic current recorded in MK-801 approximates a first-latency distribution (Jahr, 1992).

This distribution can be fitted with a single exponential with a time constant of ~13 msec, indicating that most channels open for the first time soon after agonist binding. If this first-latency distribution is deconvolved from a response in the absence of MK-801, the resulting distribution represents the conditional probability that a channel is open at time t given that it was open at $t = 0$. This distribution decays slowly, lasting several hundred milliseconds. Taken together, these distributions describe channel behavior in which openings occur with moderately high probability soon after agonist binding and repeatedly open and close until dissociation of the agonist hundreds of milliseconds later (Jahr, 1994). It also was estimated that at the peak of the patch response to L-glutamate, ~30% of the channels in the patch were open simultaneously. This agrees well with other patch and whole-cell measurements (Lin and Stevens, 1994; Benveniste and Mayer, 1995; Rosenmund et al., 1995) and some synaptic measurements (Hessler et al., 1993).

In the second scheme, most NMDA channels open after a considerable delay. The distribution describing the conditional probability that a channel is open at time t given it was open at $t = 0$ was constructed for this scheme by aligning groups of channel openings, called "super-clusters" (Gibb and Colquhoun, 1991, 1992), recorded at low agonist concentrations (Edmonds and Colquhoun, 1992). In contrast to the first scheme, this conditional open-probability distribution decays quickly, from 1 to 0.26 in 10 msec, indicating that after the initial burst or cluster of openings, the likelihood of subsequent openings is very low (Edmonds and Colquhoun, 1992). Deconvolving the conditional open-probability distribution from the response of a patch to a brief pulse of L-glutamate gives rise to a first-latency distribution that predicts that the majority of the channels open for the first time > 100 milliseconds after the binding agonist and that the channels have a very low P_o. The low open probability is consistent with estimates using steady-state agonist applications (Huettner and Bean, 1988; Traynelis and Cull-Candy, 1990) and some synaptic measurements (Rosenmund et al., 1993, 1995).

The present experiments were undertaken to determine which of these schemes best describes NMDA channel behavior in outside-out patches.

MATERIALS AND METHODS

Cell culture. Experiments were conducted on outside-out patches of rat hippocampal neurons grown in primary culture. Cells were taken from P1–3 rats and maintained in culture for 1–3 weeks (Lester et al., 1989).

Solutions. Recording pipettes were filled with a solution containing (in mM): cesium gluconate 140, NaCl 10, HEPES 10, EGTA 10, and Mg-ATP 5, adjusted to pH 7.2 with CsOH, and kept on ice until use. External

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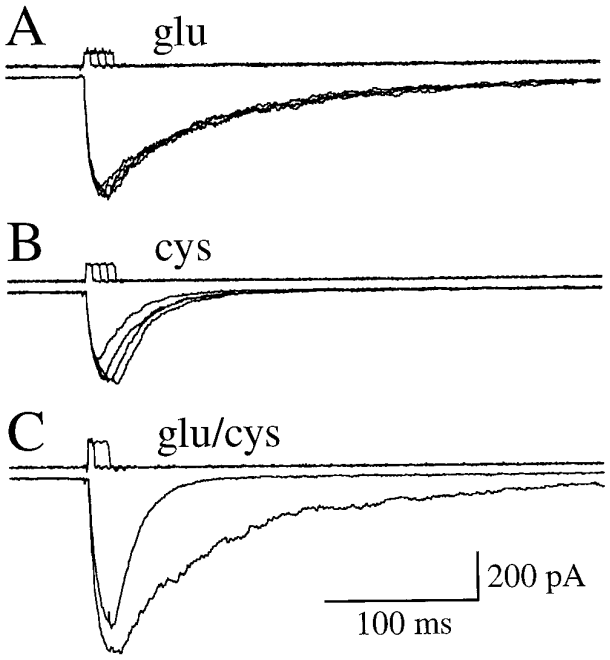

Figure 1. Decay time of NMDA channel responses depends on the agonist. *A*, Superimposed responses of a patch to 5, 10, 15, and 20 msec applications of 10 mM L-glutamate (*glu*). Each trace is an average of five sweeps; the different length applications were made successively and then repeated in a cyclic manner. *B*, Superimposed responses to 5, 10, 15, and 20 msec applications of 10 mM L-cysteate (*cys*) using the same protocol as in *A*. *C*, The average NMDA receptor response to a 15 msec application of 10 mM L-cysteate superimposed on the average response to a 5 msec application of 10 mM L-glutamate in the same patch. *A–C* are from different patches. Open-tip traces of the various length applications are superimposed above the patch responses. $V_h = -60$ mV.

solutions contained (in mM): NaCl 160, KCl 3, HEPES 10, $CaCl_2$ 0.2, and 5 μM NBQX and 20 μM glycine, adjusted to pH 7.4 with NaOH. High-purity salts were used in the external solution to minimize contaminating divalents. Internal and external solution osmolalities were 305–315 mOsms. Chemicals were obtained from the following sources: Sigma (St. Louis, MO; L-glutamate, L-cysteate, HEPES, EGTA, Mg-ATP, and gluconic acid); Mallinckrodt [Paris, France; KT (NaCl)]; Aldrich Chemical [Milwaukee, WI; high-purity (Gold Label) NaCl, KCl, and cesium hydroxide]; Johnson Matthey (Ward Hill, MA; $CaCl_2$); Bio-Rad Laboratories (Hercules, CA; glycine); RBI (Natick, MA; MK-801). NBQX was a gift from Novo Nordisk (Denmark).

Recording and perfusion techniques. Outside-out patch recordings were made with borosilicate glass pipettes (WPI, Sarasota, FL) pulled to a "bubble number" of 7.4–7.8 and occasionally lightly polished to final tip resistance of 1–4 MΩ. Currents were sampled at 2 kHz and low-pass-filtered at 1 kHz using an Axopatch 200A, AxoBasic software, and a TL-1 DMA interface (Axon Instruments, Foster City, CA). Solution exchanges were made with flow tubes attached to piezoelectric bimorphs (Vernitron, Bedford, OH), as described previously (Lester and Jahr, 1992; Tong and Jahr, 1994). Open-tip measurements were made at the end of each experiment to test the speed and consistency of the solution exchange. Data from patches with questionable exchange were not analyzed. The open-tip solution exchanges had a 10–90% rise time of < 500 μsec, the sampling interval. Patches were held at 0 mV between agonist applications and at -60 mV during responses to agonists. Trials were separated by 15–20 sec to allow recovery from desensitization. Statistical analysis was performed using InStat (Graph Pad Software, San Diego, CA). Reported values are given as mean ± SD. All experiments were performed at room temperature.

RESULTS

Block by MK-801 is independent of agonist affinity

NMDA channels activated by short pulses of high-affinity agonists remain active for longer periods than when bound by low-affinity agonists (Lester and Jahr, 1992), because ligand-gated ion channels generally can open only while agonists are bound (Hille, 1992). Responses activated by brief applications of the low-affinity agonist L-cysteate decay much faster ($\tau_1 = 31$ msec, 95%; $\tau_2 = 164$ msec) than responses to L-glutamate ($\tau_1 = 68$ msec, 80%; $\tau_2 = 553$ msec) (Lester and Jahr, 1992). If NMDA channels open only after prolonged bound times (the long first-latency scheme), then far fewer channels would be blocked by MK-801 during an L-cysteate response than during an L-glutamate response, because L-cysteate would unbind before most channels could open for the first time. However, if NMDA channels open soon after agonist binding (the short first-latency scheme), receptor activation by L-cysteate and L-glutamate should result in a similar degree of block. This prediction requires the behavior of the channels to be comparable while either agonist is bound, as evinced by the similarity of responses to long applications of the two agonists (Lester and Jahr, 1992).

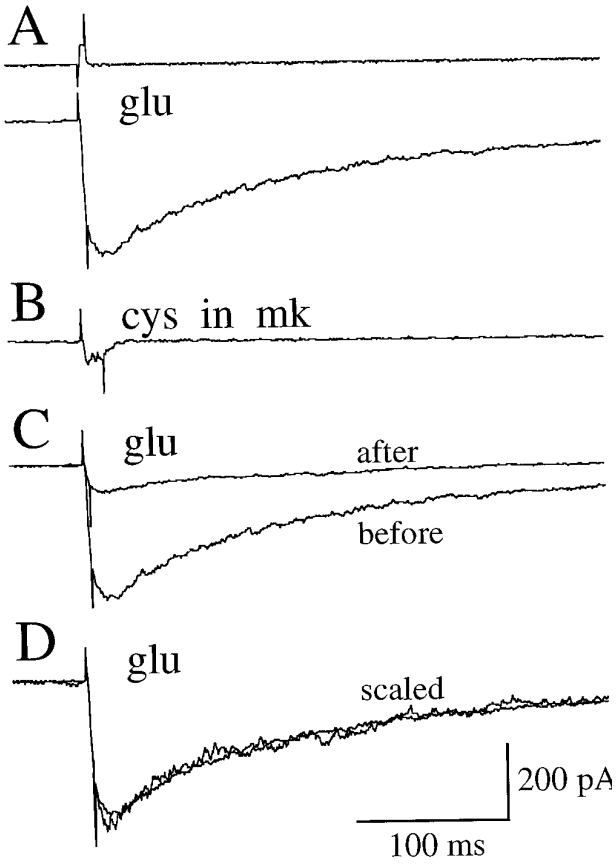

Figure 2. The magnitude of block by MK-801 is comparable for L-cysteate and L-glutamate responses. *A*, The average NMDA receptor response of an outside-out patch to a 5 msec pulse of 10 mM L-glutamate (*glu*) before MK-801 exposure. Above the response is the open-tip trace measured at the end of the experiment. *B*, A 15 msec application of 10 mM L-cysteate in a background of 20 μM MK-801, the blocking trial. *C*, Averaged responses to 5 msec pulses of 10 mM L-glutamate before and after the MK-801 exposure, superimposed. *D*, Responses in *C* scaled. All traces are from the same patch.

It was necessary to determine the minimum application duration of saturating L-cysteate required to give a maximal response, because the unbinding rate of L-cysteate is much faster than that of L-glutamate. A response with maximal amplitude was achieved with a 15 msec pulse of 10 mM L-cysteate (Fig. 1). The charge transfer during the first 20 msec of the response was 89 ± 10% of

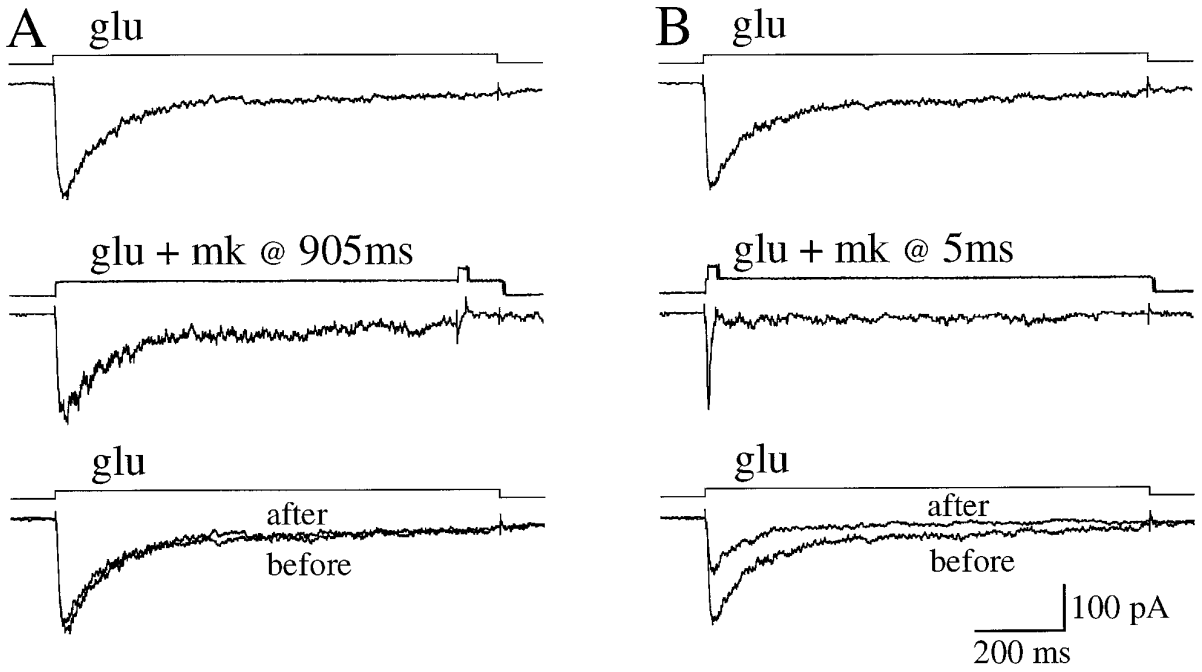

Figure 3. MK-801 does not block desensitized NMDA channels. *A*, *Top*, The average NMDA receptor response of an outside-out patch to a 1 sec application of 10 mM L-glutamate (*glu*). *Middle*, A 1 sec application with a concomitant 20 msec jump into 10 mM L-glutamate plus 20 μM MK-801 (*mk*), 905 msec after the beginning of the long application. The open-tip current is displayed above the response. *Bottom*, The superimposed averages of five responses to 1 sec applications of 10 mM L-glutamate before and after the single MK-801 sweep. *B*, The same experiment as shown in *A*, except the 20 msec jump into L-glutamate plus MK-801 is 5 msec after the start of the long L-glutamate application. The responses in this figure are all from a single patch.

that produced by a 5 msec pulse of 10 mM L-glutamate, whereas the total charge transfer with L-cysteate was only 23 ± 9% of that with L-glutamate ($n = 6$). L-cysteate appears to be slightly less efficacious than L-glutamate at NMDA receptors, because 10 mM L-cysteate is a saturating concentration (Patneau and Mayer, 1990).

Control responses were elicited with a 5 msec pulse of 10 mM L-glutamate before and then after a single trial consisting of a 15 msec pulse of 10 mM L-cysteate applied after equilibrating the patch in 20 μM MK-801 (Fig. 2). L-glutamate was used for the control responses to allow comparison with the block using L-glutamate (Jahr, 1992). Charge transfer (Q) was measured by integrating the averages of five of the control responses before and after the MK-801 trial, and the percent block was calculated as $(Q_{before} - Q_{after})/Q_{before}$. Consistent with the short first-latency scheme, the block observed using L-cysteate (79 ± 11%, $n = 7$) was not significantly different ($p > 0.1$, Student's unpaired two-tailed t test) to that using L-glutamate (70 ± 10%, $n = 8$) (Jahr, 1992).

MK-801 does not block desensitized channels

A concern with the previous experiments, in which MK-801 was continuously present, is the possibility that MK-801 may block receptors that are bound by ligand but in a nonconducting (e.g., desensitized) state, thereby resulting in an overestimate of the number of channels that open before agonist unbinding. To address this concern, a 1 sec application of L-glutamate (10 mM) was used to desensitize a population of receptors. An application of this length results in an NMDA response that decays markedly while agonist is present (Fig. 3*A*, *top trace*). During this long application of L-glutamate, 20 μM MK-801 was coapplied for 20 msec either 905 msec into the application (Fig. 3*A*) or 5 msec into the application (Fig. 3*B*). Five control responses to L-glutamate before and after the MK-801 trial were averaged and integrated to calculate the reduction in charge transfer caused by the exposure to MK-801 at the two times. The amount of block $[(Q_{before} - Q_{after})/Q_{before}]$ was significantly less when MK-801 was applied at 905 msec (19 ± 16% block) than at 5 msec (51 ± 17% block; $p = 0.009$, Student's two-tailed paired t test, $n = 6$). The amount of block was correlated with the charge transfer at the time of the block, as would be expected if MK-801 could only block open receptors. The ratio of the block at 905 msec to the block at 5 msec (38 ± 32%) was not significantly different from the ratio of the charge transfer from 905–925 msec to the charge transfer from 5–25 msec (26 ± 14%; $p = 0.27$, Student's two-tailed paired t test, $n = 6$). These results indicate that desensitized receptors are unavailable for block by MK-801.

MK-801 blocks most channels in the first 10 msec

To estimate more directly the average first latency of NMDA channels, patches were exposed to MK-801 only during a brief agonist application. If the short first-latency scheme is correct, a transient simultaneous exposure to L-glutamate and MK-801 should produce a significant block. However, if the long first-latency scheme is correct, the majority of openings occur later in the response and little block should occur. The amount of block caused by a single 10 msec pulse of 10 mM L-glutamate and 20 μM MK-801 was measured as above by integrating the charge transfer under control conditions (5 msec pulse of 10 mM L-glutamate, five sweeps in each average) before and then after the exposure to MK-801 (Fig. 4). This protocol resulted in an average block of 62 ± 10% measured in 10 patches. This amount of block is comparable ($p > 0.10$, Student's two-tailed unpaired t test) to the 70 ± 10% ($n = 8$) (Jahr, 1992) measured when the MK-801 was present

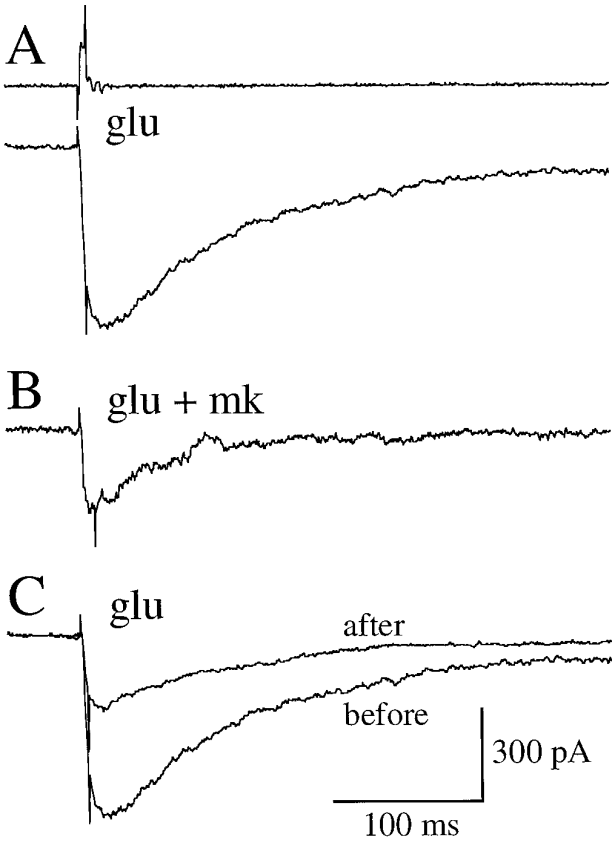

Figure 4. Brief exposure to L-glutamate plus MK-801 produces an amount of block similar to that found by applying L-glutamate in a background of MK-801. *A*, An average of five NMDA receptor responses of an outside-out patch to 5 msec pulses of 10 mM L-glutamate (*glu*) before exposure to MK-801. The open-tip current is displayed above the response. *B*, The response of the same patch to a 10 msec pulse of 10 mM L-glutamate and 20 μM MK-801 (*mk*). *C*, The average in *A* and an average of five responses after exposure to MK-801, superimposed.

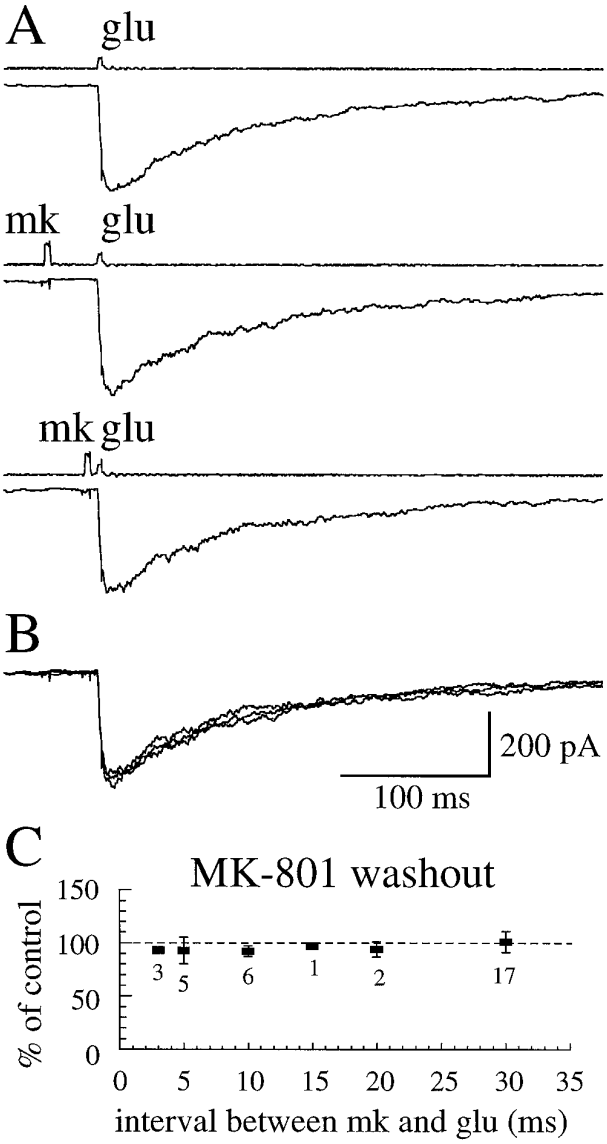

Figure 5. Rapid washout of MK-801. *A*, Three types of trials made in succession and then repeated cyclically. *Top*, Control trial, a single 4 msec pulse of 10 mM L-glutamate (*glu*). *Middle*, A 4 msec pulse of 20 μM MK-801 (*mk*) followed by a 4 msec pulse of 10 mM L-glutamate with a 30 msec interval between the two pulses. *Bottom*, Same as the *middle trace*, but with a 3 msec interval between the MK-801 pulse and the L-glutamate pulse. Each trace is the average of three sweeps. The open-tip currents are displayed above each trace. *B*, The three traces shown in *A*, superimposed. All traces are from the same patch. *C*, A plot of the mean response for each time interval, expressed as percent of the control response in the patch in which the measurement was made. The numbers below the data points indicate the number of patches contributing to each mean, and the error bars are SDs.

throughout the blocking trial, and indicates that a large percentage of channels open within 10 msec of binding L-glutamate.

It is possible that MK-801 can bind to a low-affinity site on the closed channel (e.g., in the external vestibule) from which it could block the channel once the pore opens tens of milliseconds after the end of the application. This would result in an underestimate of the average first latency. To address this possibility, a 4 msec application of 10 mM L-glutamate was preceded at decreasing intervals by a 4 msec pulse of 20 μM MK-801 (Fig. 5). With each patch, three types of applications were made in succession and then repeated in a cyclical fashion three to six times: first a control trial with no pulse of MK-801, followed by a trial with a pulse of MK-801 completed 30 msec before the start of the L-glutamate pulse, and finally a test trial with a pulse of MK-801 ending either 20, 15, 10, 5, or 3 msec before the pulse of L-glutamate. Except for two long-lived patches, only one of the test times was attempted per patch.

In 17 patches, the trials with an application of MK-801 ending 30 msec before the L-glutamate test pulse were 101 ± 9.6% of the control response. A one-way ANOVA was performed with post hoc comparisons between the decreasing test intervals and the responses of the 30 msec interval, all expressed as percentages of the control response in the same patch. The ANOVA gave a p value of 0.182, indicating the difference among the means was not significant. In addition, the Bonferroni p values for individual comparisons with the 30 msec interval were not significant.

DISCUSSION

Our results using concentration jumps with outside-out patches support the short first-latency scheme presented above. We have found that block by MK-801 is independent of agonist affinity; transient NMDA channel activity evoked by L-cysteate results in a block by MK-801 comparable to that of longer-lasting L-glutamate-induced activity. We also have shown that a brief

coapplication of MK-801 and L-glutamate is sufficient to produce a block similar to that found when MK-801 is present throughout the trial. In addition, we have demonstrated that the block of desensitized receptors by MK-801 is unlikely to account for the large block produced by MK-801 during a patch response. These results indicate that most of the channels that open before agonist dissociation do so for the first time within 10 milliseconds, and that the long decay of NMDA channel responses to brief pulses of L-glutamate is attributable to repeated openings of channels and not to long first latencies. This is consistent with the channels having a peak open probability near 0.3 in response to brief saturating concentrations of agonist (Jahr, 1992, 1994).

The conclusions of this study contrast with those of low-concentration steady-state studies that require a low probability of opening and long first latencies to explain the slow NMDA response (Edmonds and Colquhoun, 1992). The disparity may be attributable to differences in the behavior of the channels under contrasting recording conditions. As has been suggested previously (Edmonds and Colquhoun, 1992), quick jumps into high concentrations may give rise to activations with a higher P_o than those found in steady-state low-concentration experiments. Preliminary evidence to this effect was reported by Edmonds and Colquhoun (1992) and was explained by possible differences in the initial occupancies of the various kinetic states. An example of a kinetic model that could account for the differences between the experimental results is one that includes openings of receptors with one ligand bound.

Monoliganded receptor openings are not likely to occur at physiological concentrations of transmitter (Patneau and Mayer, 1990; Clements and Westbrook, 1991). However, the low agonist concentrations used in the steady- state experiments may increase the likelihood of such events, analogous to what is found with acetylcholine receptors (Dionne et al., 1978; Colquhoun and Sakmann, 1985; Colquhoun and Ogden, 1988) and $GABA_A$ receptors (Twyman et al., 1990). The saturating concentrations of agonists used in the present study result in doubly liganded receptors that may be more likely than monoliganded receptors to enter into the "high-P_o periods" seen by many researchers (Jahr and Stevens, 1987; Howe et al., 1988; Gibb and Colquhoun, 1991, 1992). These periods of intense activity, with a P_o of ~0.8 (Gibb and Colquhoun, 1992), can last for hundreds of milliseconds and may contribute significantly to the macroscopic response evoked by brief applications of high concentrations of L-glutamate. In steady-state experiments using low concentrations of L-glutamate but high concentrations of glycine, the periods of high P_o occurred about once a minute during continuous recording and yet contributed 22% of the openings (Gibb and Colquhoun, 1991). It is not known whether high-P_o periods are dependent on agonist concentration, but this could explain the brevity of the aligned single-channel activations observed by Edmonds and Colquhoun (1992). In that study, high-P_o periods were not reported, perhaps because of the very low concentrations of both L-glutamate and glycine used. These periods of intense activity could result in significant charge transfer late in the response, slowing the decay of the conditional open probability distribution.

Correlations in channel activity apparent in single-channel recordings (Gibb and Colquhoun, 1992; Edmonds et al., 1995) may be indicative of channel properties that could result in the discrepancy between the concentration jump experiments and those under steady-state conditions. Strong correlation between adjacent openings, between adjacent shut periods, and an inverse correlation between adjacent open and shut periods indicate that long openings more often are found near other long openings and brief closings. The extreme of this trend would be the high-P_o periods mentioned above, and this behavior may result from a kinetic state favored by jumps into high agonist concentrations.

A recent study by Benveniste and Mayer supports the short first-latency–high-P_o scheme. Using coapplications of the open channel blocker 9-aminoacridine (9-AA) and L-glutamate, they measured an absolute limit of 75 msec on the first latency of NMDA receptors (Benveniste and Mayer, 1995). Coapplications of longer durations collected no additional channels in the open-blocked state, as measured by the amplitude of tail currents evoked by a depolarizing pulse at the end of the application. In addition, after a 20 msec coapplication, they noted a rise in inward current on returning to the control solutions that they interpreted as first openings of channels, similar to a response to L-glutamate in the absence of 9-AA. This response was only 27% of control, which suggests that 73% of the channels that would open in response to L-glutamate opened within 20 msec of agonist presentation.

The single-channel behavior in agreement with our findings consists of an average first latency of ~10 msec, a peak P_o near 0.3, and a significant number of channels exhibiting long-lasting bursting. If synaptic NMDA channels behave similarly to those in outside-out patches (Hessler et al., 1993) (see also Rosenmund et al., 1993, 1995), this short first-latency–high-P_o scheme suggests that relatively few channels (~5–30) are required at individual synaptic sites to account for the small NMDA component of miniature excitatory synaptic currents (Bekkers and Stevens, 1989; Robinson et al., 1991; McBain and Dingledine, 1992; Silver et al., 1992). Very recently, it has been reported that calmodulin can regulate the P_o of NMDA channels (Ehlers et al., 1996). This may account for the differences in estimates of P_o in different preparations (Jahr, 1992; Hessler et al., 1993; Rosenmund et al., 1993, 1995).

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