The Journal of Neuroscience, January 1991, 11(1): 123–133

Acetylcholine Release from the Rabbit Retina Mediated by NMDA Receptors

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The cholinergic amacrine cells of the rabbit retina may be labeled with ^{3}H-choline, and the activity of the cholinergic population may be monitored by following the release of ^{3}H-ACh. In magnesium-free medium, the glutamate analog NMDA caused massive ACh release, up to 50× the basal efflux. Magnesium blocked the NMDA-evoked release of ACh with an IC_{50} of 151 μM. The NMDA-evoked release of ACh was unchanged in glycine-free medium or in the presence of 500 μM glycine. However, the block of NMDA-evoked release by 7-chlorokynurenic acid (7-Cl-Kyn) was reversed by exogenous glycine. This suggests that the NMDA receptors mediating ACh release possess an allosteric glycine binding site, but under normal conditions, it is saturated by endogenous glycine.

Submaximal doses of NMDA were used to determine the potency of NMDA antagonists and their specificity was established with submaximal doses of other glutamate agonists. DL-2-amino-7-phosphonoheptanoate (DL-AP-7) was a competitive NMDA antagonist, with an IC_{50} of 33 μM, and (+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) was a noncompetitive NMDA antagonist, with an IC_{50} of 10.6 nM. Neither antagonist blocked the light-evoked release of ACh from the retina. Furthermore, light stimulation did not activate the use-dependent block characteristic of MK-801, indicating that the endogenous transmitter did not open the NMDA channel. These results suggest that NMDA receptors do not mediate the physiological input to cholinergic amacrine cells in the rabbit retina.

Glutamate is gaining acceptance as the major excitatory neurotransmitter in the CNS, including the retina (Mayer and Westbrook, 1987; Massey, 1990). Both physiological and biochemical evidence support the concept of multiple postsynaptic glutamate receptors (Foster and Fagg, 1984; Mayer and Westbrook, 1987). One of these receptors is selectively activated by the glutamate analog NMDA. NMDA receptor activation leads to the gating of an ion channel permeable to monovalent cations and calcium (MacDermott et al., 1986). There are several regulatory sites on this receptor complex in addition to the primary NMDA binding site (Reynolds and Miller, 1988; MacDonald et al., 1989; Monaghan et al., 1989; Thomson, 1989). The analysis of these sites has provided specific pharmacological tools to investigate NMDA receptor function.

The first clue to the complexity of the NMDA receptor came from the study of magnesium, which blocks NMDA responses in a voltage-dependent manner (Nowak et al., 1984; Mayer and Westbrook, 1985). This is considered the hallmark of the NMDA receptor, and it is not observed with other glutamate receptors. More recently, a strychnine-insensitive glycine binding site has been discovered that potentiates the action of NMDA (Johnson and Ascher, 1987). This is a large effect: at a concentration of 1 μM or less, glycine potentiates the response to NMDA, or glutamate acting at NMDA receptors, by a factor of 10 or more. In fact, glycine may be an absolute requirement for NMDA receptor activation (Kleckner and Dingledine, 1988). Kynurenic acid (Kyn) and the quinoxalinediones 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) are competitive antagonists at the glycine binding site of the NMDA receptor complex in several systems (Birch et al., 1988; Kessler et al., 1989). Another analog, 7-chlorokynurenic acid (7-Cl-Kyn), is much more potent and has greater selectivity for the glycine site (Kemp et al., 1988).

The physiological role of NMDA receptors has been examined in various parts of the CNS, and while they may play a significant role in such processes as long-term potentiation or kindling (Collingridge et al., 1983), there is general agreement that NMDA receptors do not mediate conventional, fast synaptic transmission (Crunelli et al., 1983). However, this exclusion does not eliminate NMDA receptors from an important physiological role in the nervous system. NMDA receptors are the most sensitive to glutamate, and it has been suggested that their tonic activation by ambient glutamate enhances the excitability of neurons (Sah et al., 1989). In some systems, there are dual postsynaptic glutamate receptors, and NMDA receptors mediate a slow component of the postsynaptic response (Dale and Roberts, 1985; Forsythe and Westbrook, 1988; Mittman and Taylor, 1988).

In the vertebrate retina, NMDA receptors appear to be absent or rare on second-order neurons in the outer retina (Lasater and Dowling, 1982; Slaughter and Miller, 1983, 1985; Coleman et al., 1986; Massey and Miller, 1987). In contrast, NMDA receptors are found on nearly all third-order neurons in the inner retina (Slaughter and Miller, 1983; Bloomfield and Dowling, 1985; Massey and Miller, 1988, 1990), but they contribute, at best, only a minor portion of the light-driven input (Coleman and Miller, 1989; Massey and Miller, 1990). The cholinergic amacrine cells of the rabbit retina are a well-described subset of amacrine cells whose activity can be monitored by following

Received Mar. 29, 1990; revised Aug. 30, 1990; accepted Sept. 5, 1990.

This work was supported by National Eye Institute Grant EY-06515 and Texas Higher Education Coordinating Board Grant 1953. We wish to thank Dr. Robert Marc for discussion.

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ACh release (Masland and Livingstone, 1976; Massey and Redburn, 1982; Masland et al., 1984; Tauchi and Masland, 1984). Perfusion with NMDA, in magnesium-free medium, causes massive ACh release from the rabbit retina. However, the light-evoked release of ACh seems to be mediated predominantly by kainate (KA) receptors (Cunningham and Neal, 1985; Linn et al., 1991).

One goal of these experiments was to establish the dose and specificity of some recently developed NMDA antagonists in the mammalian retina. Second, we used these pharmacological tools to determine the contribution of NMDA receptors to the light-evoked release of ACh. Our results indicate that NMDA receptors mediating ACh release are regulated by magnesium and glycine, though the glycine site seems to be saturated under normal conditions. DL-2-Amino-7-phosphonoheptanoate (DL-AP-7) and (+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) are specific NMDA antagonists with different sites of action. Neither antagonist reduced the light-evoked release of ACh from the retina, so we were unable to demonstrate that NMDA receptors carry any part of the physiological input to cholinergic amacrine cells.

Materials and Methods

Preparation. The procedures followed in this paper are identical to those in the previous, companion paper (Linn et al., 1991), with minor modifications. Briefly, rabbits were deeply anesthetized with urethane (loading dose, 1.5 gm/kg, i.p.), and the orbit was infused with Xylocaine (2%). An *in vivo* retinal eyecup was prepared under dim red light (Massey and Redburn, 1982), and cholinergic amacrine cells were labeled with ^{3}H-choline (^{3}H-Ch). After loading, the eyecup was perfused with Krebs' solution, at 37°C, until a steady baseline was obtained. Subsequently, 1-min fractions were collected directly into vials and prepared for liquid scintillation counting. The retina was stimulated by 3-Hz flashing light for 4-min periods or perfused with test solutions as described in Results. Increments in the release of radioactivity were shown in the previous paper (Linn et al., 1991) to indicate ACh release.

Solutions. All drugs were dissolved directly in choline-free Krebs' bicarbonate medium (Ames and Nesbett, 1981). Most experiments were conducted in magnesium-free medium to promote NMDA responses. MK-801 was generously provided by Merck, Sharp, and Dohme. All other drugs and reagents were obtained from commercial sources.

Results

Magnesium dependence

When the retina was perfused with increasing doses of NMDA in the presence of normal physiological levels of magnesium (1.2 mM), there was no change in the release of ACh. Figure 1 demonstrates the complete absence of NMDA responses in the presence of magnesium, as previously reported by Cunningham and Neal (1985). At concentrations as high as 10 mM, no NMDA responses could be detected, even though the release of ACh in response to light and other glutamate agonists [e.g., α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)] was normal. NMDA was the only glutamate analog to exhibit this magnesium dependent block; KA, quisqualate (QQ), and AMPA responses were unchanged in the presence or absence of magnesium (Linn et al., 1991).

To determine the concentration of magnesium required to block the response to NMDA, we applied a submaximal dose of NMDA (300 μM) in 1-min pulses against a decreasing staircase of magnesium concentrations and measured the release of labeled ACh. We found that the IC_{50} for magnesium versus NMDA was 151 μM, the threshold dose was around 50 μM, and 0.7 mM magnesium caused a 95% reduction in the NMDA-evoked release of ACh (see Fig. 1, inset).

In magnesium-free medium, perfusion with NMDA caused massive ACh release much like other glutamate agonists (Linn et al., 1991). Figure 2 shows the release of radioactivity in response to increasing doses of NMDA, reaching saturation at 25–30 times the basal efflux. The threshold dose for NMDA was 50 μM with an ED_{50} of 112 μM. Thus, NMDA was a moderately potent glutamate analog, approximately an order of magnitude less potent than KA or QQ (ED_{50}s, 12.4 and 10.3 μM, respectively). From the dose–response curve, we chose to use a submaximal concentration of 300 μM NMDA in subsequent experiments, which were all performed in magnesium-free medium. Perfusate analysis identified the NMDA released radioactivity as ^{3}H-ACh, and this indicates that the release originated from cholinergic amacrine cells (Linn et al., 1991).

Requirement for glycine

Glycine potentiates NMDA responses in several systems (Johnson and Ascher, 1987; Thomson, 1989), and it may be required for NMDA receptor activation (Kleckner and Dingledine, 1988). The Krebs' solution used in these experiments contained 6 μM glycine (Ames and Nesbett, 1981), so we made a glycine-free version. However, the response to NMDA in glycine-free medium was unchanged, and the addition of up to 1 mM exogenous glycine failed to potentiate the release of ACh by NMDA. These results suggest that the high-affinity glycine binding site of the NMDA receptor complex may be saturated by endogenous glycine.

Alternatively, it could be argued that glycine had no effect because the NMDA receptors in this system are not associated with glycine binding sites. One way to investigate this possibility is to use antagonists recently demonstrated to act at this site, such as 7-Cl-Kyn (Kemp et al., 1988; Monaghan et al., 1989). Figure 3 shows that 100 μM 7-Cl-Kyn completely blocked the NMDA- (300 μM) evoked release of ACh in magnesium-free medium. The partial attenuation of the light response is due to the Kyn-like activity of 7-Cl-Kyn. Co-perfusion with excess glycine reversed the NMDA antagonism due to 7-Cl-Kyn so that the same dose of NMDA produced a 17-fold increase over the basal efflux. This indicates that 7-Cl-Kyn is a competitive antagonist at the glycine binding site of the NMDA receptor. The addition of glycine also blocked the light-evoked release of ACh, from which, recovery was slow. After removing the glycine, 7-Cl-Kyn once again blocked the NMDA-evoked release of ACh, and when all drugs were washed out, a light response and a control response to NMDA were obtained. Using this pharmacological approach, it appears that the NMDA receptors mediating ACh release possess a glycine modulatory site as in other systems (Thomson, 1989).

Antagonists

DL-AP-7

Biochemical and physiological studies have established that DL-AP-7 is a specific NMDA antagonist, and these results have been confirmed in the rabbit retina (Perkins et al., 1982; Olverman et al., 1984; Coleman and Miller, 1988; Massey and Miller, 1990). To establish the optimal dose of DL-AP-7 in this system, a submaximal dose of NMDA (300 μM) was tested against an increasing staircase of DL-AP-7 concentrations (Fig. 4). After the first 2 light-evoked peaks to confirm retinal viability, a control response to NMDA was obtained. By 200 μM, perfusion with DL-AP-7 completely abolished the NMDA-evoked release of ACh, and after washout, a control response was obtained.

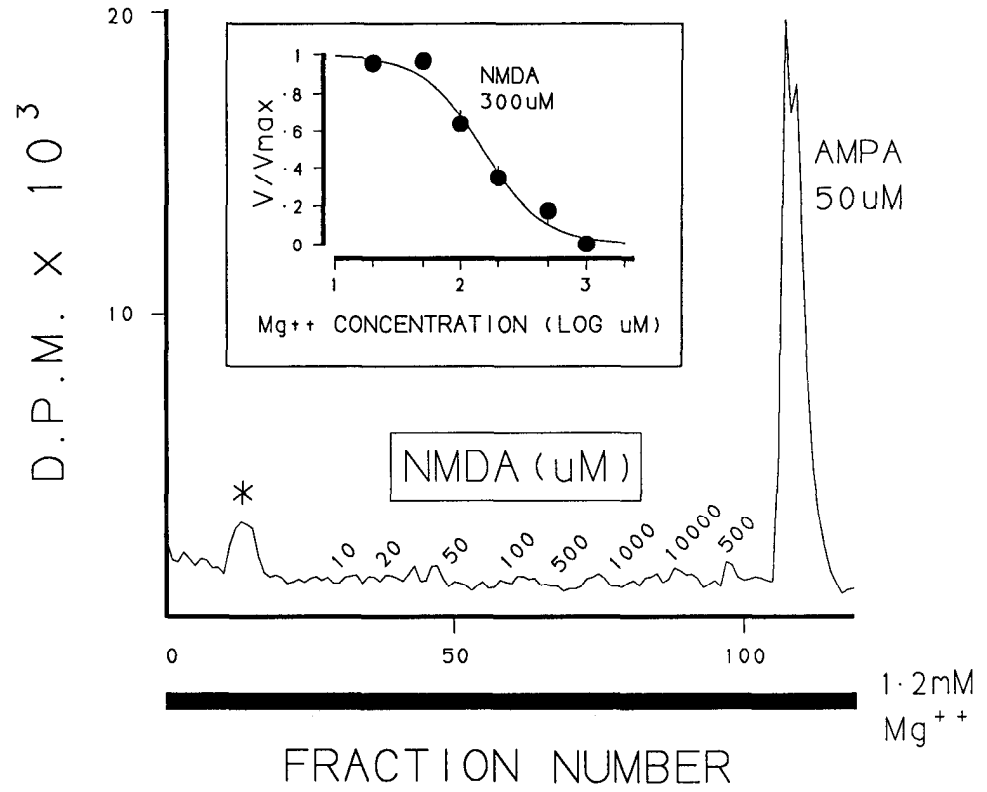

Figure 1. NMDA-evoked release of ACh from rabbit retina is completely blocked by physiological concentrations of magnesium. In this and all other figures, release data in DPMs were plotted against the fraction number. One-minute fractions were collected, and glutamate analogs were applied for 1 min. *Oblique numbers* indicate the concentration of NMDA (μM), and *asterisks* indicate light stimulation at 3 Hz for 4 min. During perfusion with 1.2 mM magnesium, light stimulation produced a normal response, but NMDA responses were blocked up to a concentration of 10 mM. Other glutamate analogs were unaffected by magnesium, and AMPA (50 μM) caused an 18-fold increase in release. *Inset,* Dose–response curve for magnesium dependence of NMDA responses. Peak : base ratios from responses to 300 μM NMDA were normalized to controls in magnesium-free medium and fitted to the equation V/V_{max} = [antagonist]^n/($IC_{50}{}^n$ + [antagonist]^n), where V is the response, V_{max} is the control response in magnesium-free medium, IC_{50} is the concentration of magnesium reducing the response by half, and n is the Hill coefficient. *Vertical bars* indicate SEMs; where not shown, they are smaller than the symbol. The IC_{50} for magnesium versus NMDA was 151 μM. These results suggest that, under normal physiological conditions, NMDA responses are blocked.

Nonlinear least-squares analysis of data from these experiments yielded the dose–response curve in Figure 5 with an IC_{50} of 33 μM for DL-AP-7 versus 300 μM NMDA.

Once the optimal dose was established, we wished to confirm that DL-AP-7 was a competitive antagonist in this system. Therefore, we tested increasing concentrations of NMDA against a fixed concentration of DL-AP-7 (200 μM). As shown in Figure 6, DL-AP-7 caused a parallel shift in the NMDA dose–response curve of one log unit. When the concentration of NMDA was raised sufficiently, from 500 μM to 5 mM, the same maximum release of ACh was obtained in the presence of DL-AP-7. Although the data points in this figure were normalized to 1, the underlying peak : base ratios were also the same. These results indicate that DL-AP-7 is a competitive NMDA antagonist in the retina, in close agreement with previous results in the retina and other systems. The NMDA dose–response curve in DL-AP-7 is dramatically different from the analogous curve in the presence of MK-801 (see below). This suggests that the 2 antagonists operate by different mechanisms.

The goal of this study was to identify the input to the cholinergic amacrine cells, so to determine the contribution of NMDA receptors, we tested DL-AP-7 against the light-evoked release of ACh. Figure 7 illustrates a self-contained experiment that may be functionally divided into 2 parts. The first half shows that DL-AP-7 had no antagonistic effect on the light response. Conversely, the light-evoked release of ACh was slightly enhanced by 14.2 ± 9.3% (mean ± SEM; n = 8). The significance of this small increase is unknown. The second half of this experiment is a demonstration of the pharmacological specificity of DL-AP-7. After the light-evoked controls were completed, DL-AP-7 was tested against equivalent submaximal doses of glutamate agonists. In the presence of 200 μM DL-AP-7, KA (15 μM) caused a 17-fold increase in the release of ACh, and QQ (15 μM), a 20-fold increase. In contrast, the response to 300 μM NMDA was abolished. After the washout of DL-AP-7, the NMDA response was comparable to those evoked by KA or QQ controls. These results demonstrate, in the same experiment, that DL-AP-7 is a specific NMDA antagonist that does not block the light-evoked release of ACh.

MK-801

The tricyclic compound MK-801 is not structurally related to glutamate, and it belongs to a different class of NMDA antagonist than DL-AP-7. MK-801 is thought to bind to a site within the open channel of the NMDA-receptor complex instead of interacting directly with the glutamate receptor like DL-AP-7 (Wong et al., 1986). To identify the optimal dose for MK-801, a submaximal dose of NMDA (300 μM) was tested against an

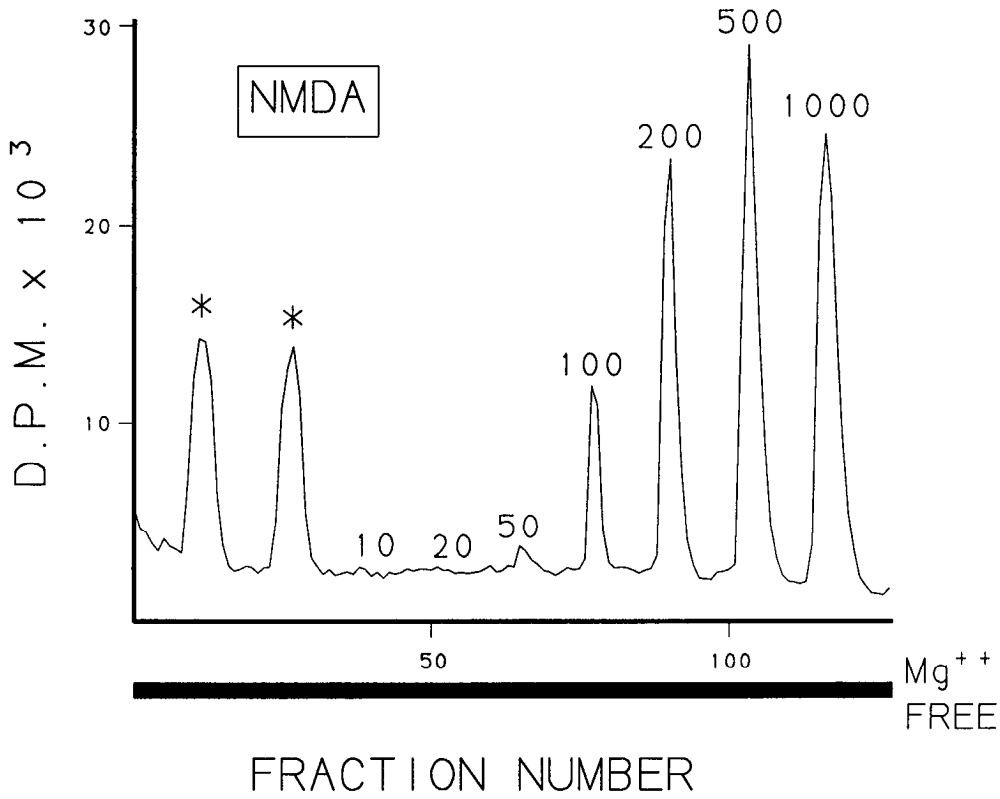

Figure 2. Release of ACh from rabbit retina in response to increasing series of NMDA concentrations in magnesium-free medium. *Asterisks* indicate light stimulation at 3 Hz for 4 min. *Numbers* show the dose of NMDA in μM. NMDA had a threshold dose of 50 μM, reaching saturation at 500 μM.

increasing staircase of MK-801 concentrations. In Figure 8, 2 light peaks were obtained and then a control response to NMDA. Perfusion with MK-801 revealed that the threshold dose was between 2 and 5 nM, and by 20 nM, the NMDA-evoked release of ACh was abolished. It should be noted that a light response and a KA response were obtained in 500 nM MK-801, 25 times the dose sufficient to eliminate the NMDA response. Nonlinear least-squares analysis of data from these experiments produced

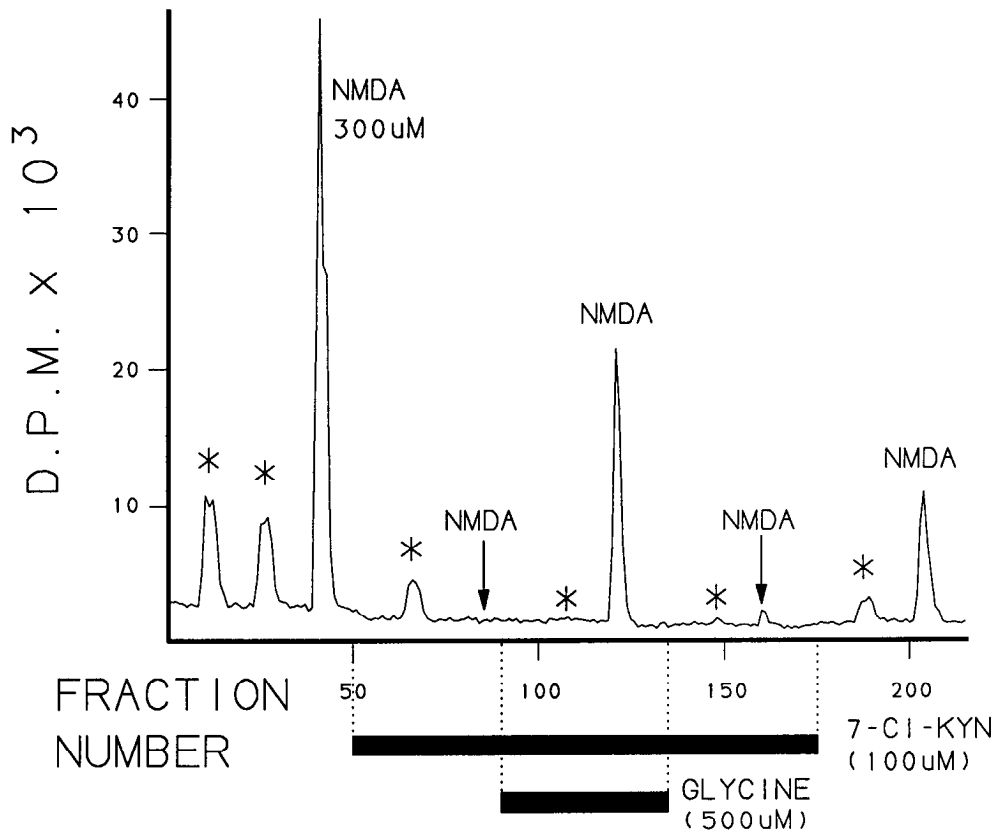

Figure 3. Glycine reverses antagonism of NMDA responses by 7-Cl-Kyn. After 2 light responses (*asterisks*), a control response to NMDA was obtained (peak : base ratio, 18.5). Perfusion with 7-Cl-Kyn partly reduced the light-evoked release of ACh but eliminated the NMDA response. Co-perfusion with glycine blocked the light-evoked release of ACh, as previously reported, but now, in the presence of 7-Cl-Kyn, NMDA caused a 17-fold increase in release. After washing out the glycine, NMDA was blocked once more by 7-Cl-Kyn. On return to control medium, light stimulation produced a normal response, and NMDA caused a 10-fold increase over the base line. The apparent reduction of the NMDA response through the experiment is normal, due to the exponential rundown of release and the depletion of a finite pool of labeled ACh. These results suggest that 7-Cl-Kyn is an antagonist at the allosteric glycine site of the NMDA receptor complex.

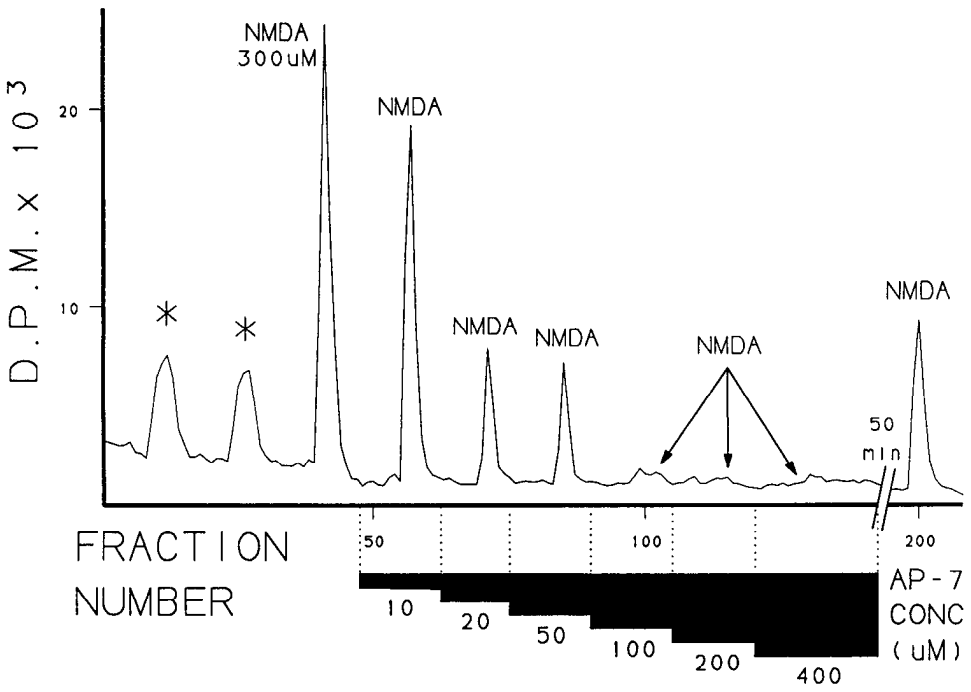

Figure 4. Blockade of NMDA-evoked ACh release by DL-AP-7. After 2 light responses (*asterisks*), a control response to a submaximal dose of NMDA (300 μM) was obtained. Then, pulses of NMDA were applied against an increasing staircase of DL-AP-7 concentrations, indicated by the *solid bars* and *dotted lines*. The threshold dose for DL-AP-7 was 20 μM, and *arrows* indicate a residual response at 100 μM and complete blockade at 200 and 400 μM DL-AP-7. A break in the x-axis indicates a 50-min break before obtaining a control NMDA response at fraction number 200. This experiment indicates that DL-AP-7 is a reversible NMDA antagonist.

the curve in Figure 5 with an IC_{50} of 10.6 nM for MK-801 against NMDA. From this data, we picked a working dose of 50 nM for MK-801. A comparison of the dose–response curves in Figure 5 shows that MK-801 is over 3 log units more potent than DL-AP-7. Thus, MK-801 is easily the most potent antagonist we have tested in the rabbit retina.

After establishing an optimal dose of MK-801, we perfused with increasing concentrations of NMDA to see if the blockade could be overcome. However, as seen in Figure 6, in the presence of 50 nM MK-801, even concentrations of NMDA as high as 10 mM failed to elicit ACh release. Thus, the NMDA dose–response curve is flat in the presence of MK-801. This is clearly different from DL-AP-7, and it is typical of noncompetitive antagonism.

Figure 9 illustrates some other characteristics of MK-801 besides its high potency. After 2 light-evoked responses, perfusion with 300 μM NMDA elicited a large control response, as expected. After 12 min of perfusion with MK-801, the response to NMDA was reduced but still substantial, 8.6× the resting release rate. However, the effect of a second and subsequent applications of NMDA were abolished. In other words, the onset of MK-801 is slow, in contrast to the competitive antagonist

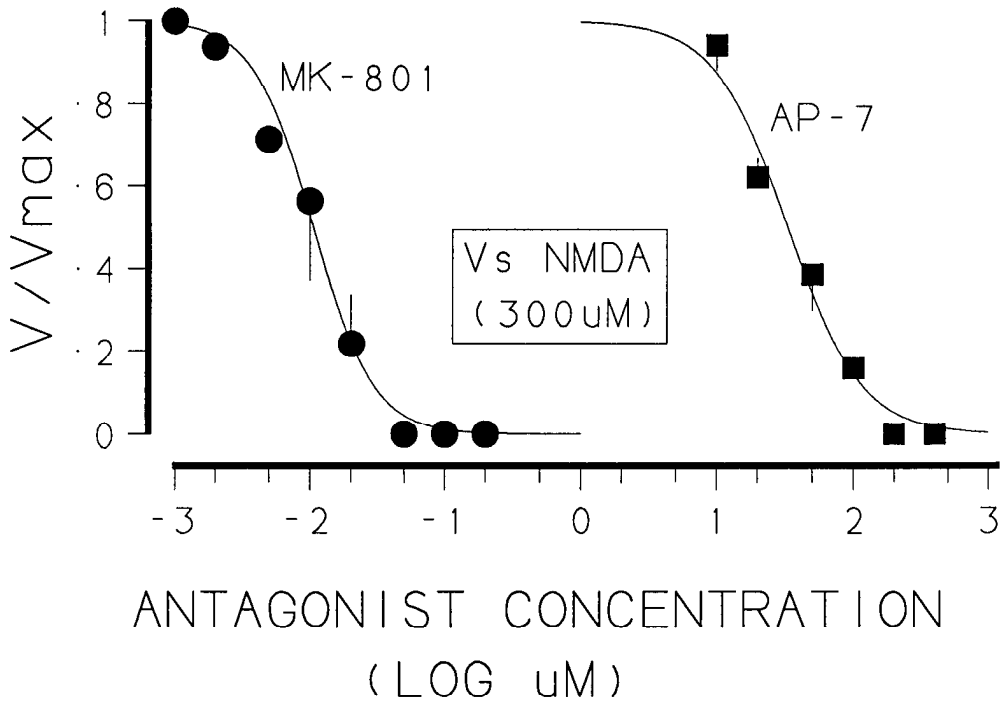

Figure 5. Semilog dose–response curves for DL-AP-7 and MK-801 versus NMDA. The data were derived from experiments such as those shown in Figures 4 and 8, where increasing doses of antagonist were tested against a fixed submaximal dose of NMDA (300 μM). *Vertical bars* indicate SEMs; where not shown, they are smaller than the symbol. The IC_{50}s are 10.6 nM for MK-801 and 33 μM for DL-AP-7. Thus, MK-801 is over 3 log units more potent than DL-AP-7.

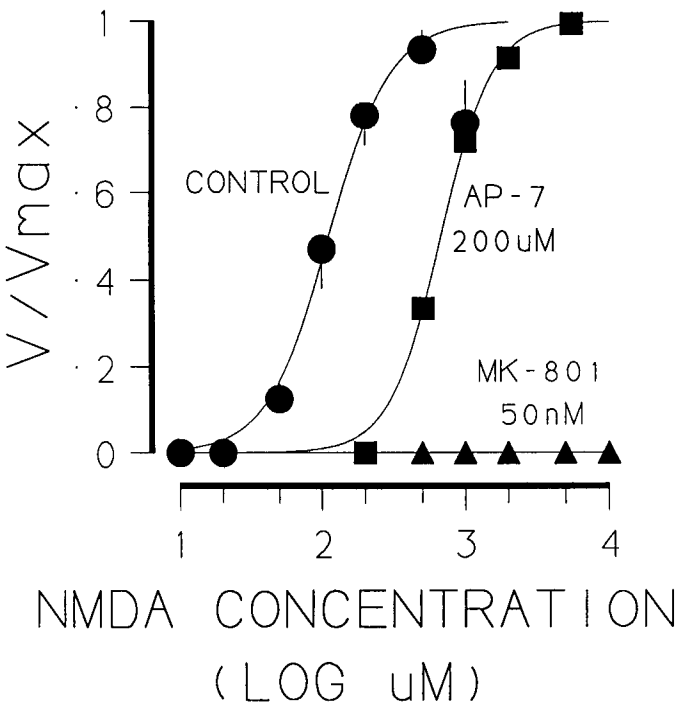

Figure 6. Semilog dose–response curves for NMDA against fixed doses of DL-AP-7 (200 μM) and MK-801 (50 nM). *Vertical bars* indicate SEMs; where not shown, they are smaller than the symbol. *Control* indicates the dose–response curve for the NMDA-evoked release of ACh in magnesium-free medium. DL-AP-7 caused a parallel shift of approximately 1 log unit in the dose–response curve, and the maximum response was not reduced. In contrast, the dose–response curve in MK-801 was flat, indicating that 50 nM MK-801 blocked the response of up to 10 mM NMDA. These curves suggest that DL-AP-7 is a competitive NMDA antagonist, and that MK-801 is a noncompetitive NMDA antagonist.

DL-AP-7, which was almost immediately effective. This observation is consistent with the idea that MK-801 is a use-dependent, open-channel blocker (Wong et al., 1986).

Once an effective block of NMDA responses was established, we tested MK-801 against the light-evoked release of ACh. The effects of MK-801 on the light-evoked response were not significant (−2.35 ± 7.72%, mean ± SEM; $n = 8$). After the light responses, we wished to demonstrate the pharmacological specificity of MK-801, so equivalent doses of KA and QQ were applied. In the presence of MK-801, KA and QQ still caused massive ACh release, 27× and 29× the basal efflux, respectively. These results show, in the same experiment, that MK-801 is a specific NMDA antagonist in the rabbit retina that has no effect on the light-evoked release of ACh.

Finally, because MK-801 is reported to bind within the open NMDA channel in an irreversible manner, we examined the recovery of NMDA responses after the washout of MK-801. In the last part of Figure 9, the NMDA block was almost total 30 min after returning to control medium. After 60 min, the NMDA-evoked release of ACh was only 5× the basal efflux, approximately 25% of the predrug control and subsequent applications of KA and QQ that produced peaks of 20× the resting release. The long-lasting effects of MK-801 prevented us from obtaining postdrug controls, but this slowly irreversible action is clearly different from DL-AP-7 and typical of noncompetitive antagonists.

The use-dependent block of NMDA receptors by MK-801 offered a unique way to see if NMDA receptors were activated during light stimulation. We wondered if light stimulation in the presence of MK-801 would prevent subsequent responses to NMDA. In other words, can light stimulation open the NMDA channel to permit the access of MK-801 and the resulting development of a use-dependent block? We were unable to design

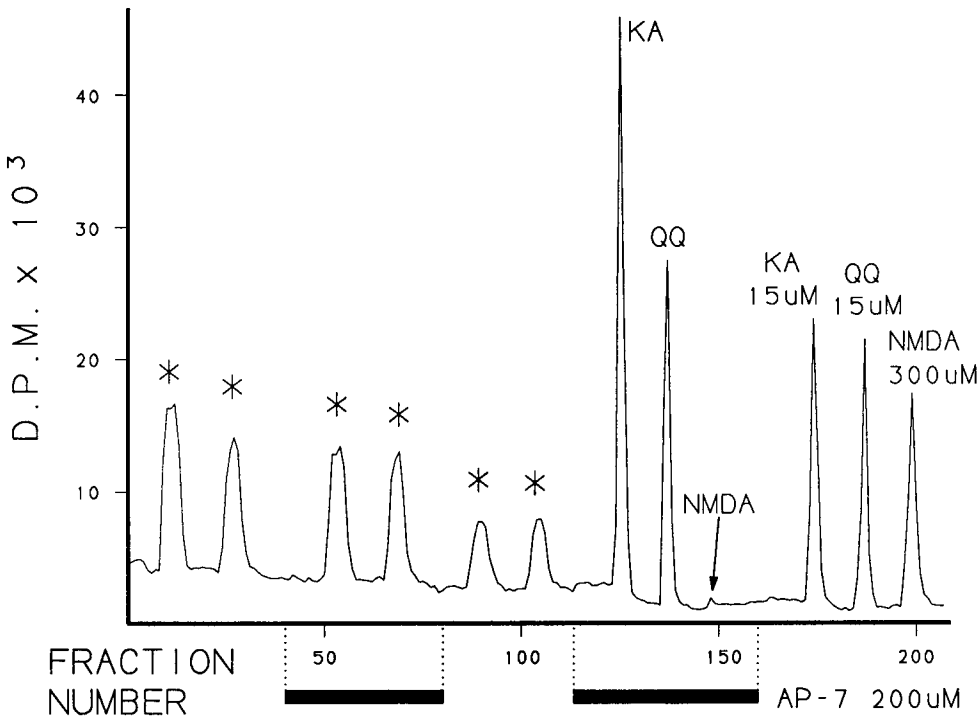

Figure 7. DL-AP-7: effect on light-evoked release of ACh and pharmacological specificity. In the first half of this experiment, DL-AP-7 (200 μM; indicated by the *solid bar* and *dotted lines*) did not block the light-evoked release of ACh. *Asterisks* indicate light stimulation for 4 min at 3 Hz. The second half of this experiment is a demonstration of the pharmacological specificity of DL-AP-7. Perfusion with DL-AP-7 had no effect on the response to a submaximal dose of KA (15 μM) or QQ (15 μM; peak : base ratios, 17 and 20, respectively). In contrast, the NMDA-evoked release of ACh was abolished by DL-AP-7. On return to control medium, similar responses were obtained for NMDA, QQ, and KA (peak : base ratios: 16, 19, and 16, respectively). As usual, control peaks at the end of the experiment are small due to the exponential decline in release, but the peak : base ratios are approximately constant. These results show, in a self-contained experiment, that DL-AP-7 is a specific NMDA antagonist that does not block the light-evoked release of ACh.

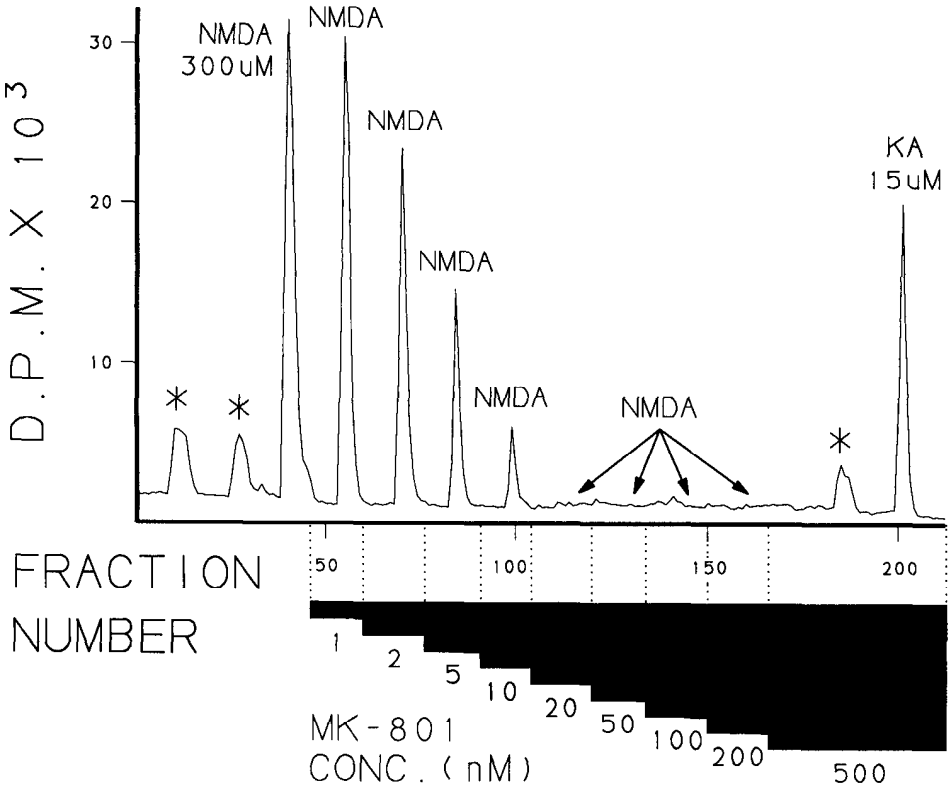

Figure 8. Blockade of NMDA-evoked ACh release by MK-801. After 2 light responses (*asterisks*), a control response to a submaximal dose of NMDA (300 μM) was obtained. Then, pulses of NMDA were applied against an increasing staircase of MK-801 concentrations indicated by the *solid bars* and *dotted lines*. The threshold dose for MK-801 was 2–5 nM, and by 20 nM, the response to NMDA was abolished. *Arrows* indicate applications of NMDA that produced no response. Finally, a light response and a KA response were obtained in 500 nM MK-801, 25× the dose needed to block the NMDA-evoked release of ACh. This experiment indicates that MK-801 is a potent NMDA antagonist.

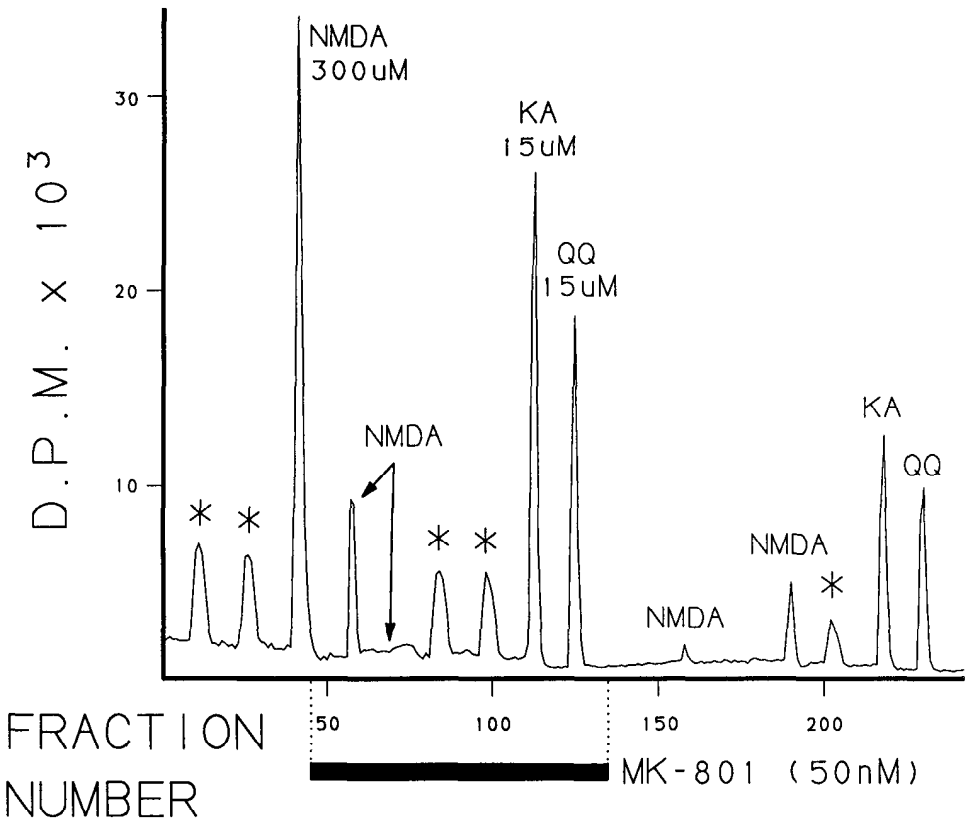

Figure 9. MK-801: effect on light-evoked release of ACh and pharmacological characteristics. Control responses were obtained to light stimulation (*asterisks*), and then, a submaximal dose of NMDA (300 μM) caused a 22-fold increase over the base release. Perfusion with 50 nM MK-801, indicated by the *solid bar* and *dotted lines*, reduced the next NMDA response to 8× the base release, and the following pulse of NMDA caused no release. This is consistent with a use-dependent block by MK-801. MK-801 had no effect on the light-evoked release of ACh (*asterisks*) even after the NMDA block was established. To test the pharmacological specificity of MK-801, we also applied submaximal doses of other glutamate agonists, but the KA- (15 μM) and QQ- (15 μM) evoked release of ACh was unchanged (peak : base ratios, 27 and 29, respectively). Sixty minutes after the washout of MK-801, the NMDA response was still blocked, but the responses to light were normal, and KA and QQ produced a 20-fold increase in release. The rundown in the gross size of responses through the experiment is normal, but the peak : base ratios are approximately the same. This experiment shows that MK-801 is a long-lasting, pharmacologically specific NMDA antagonist that does not block the light-evoked release of ACh.

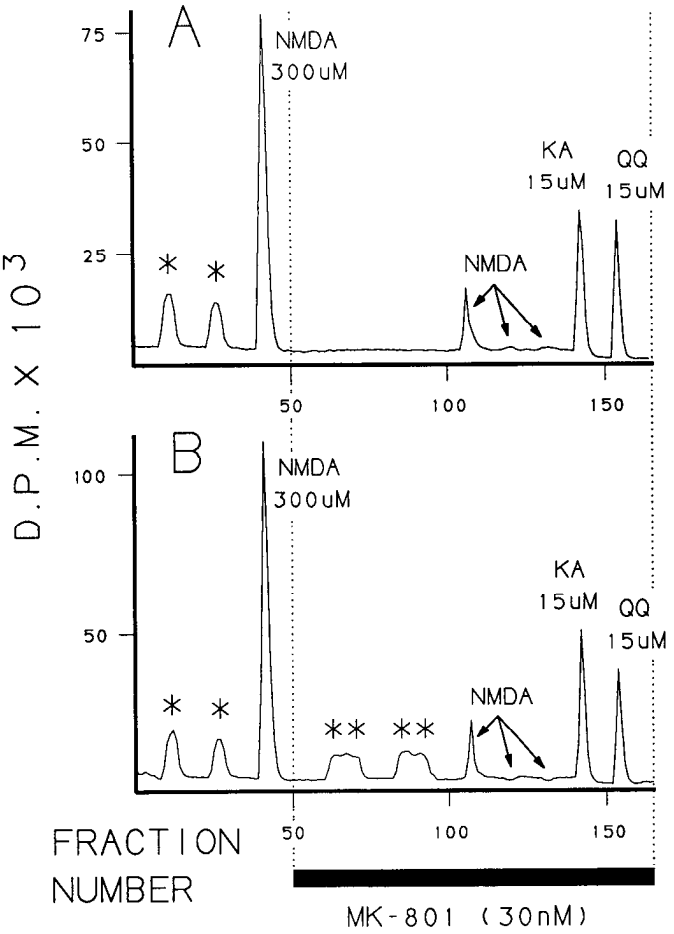

Figure 10. Does light stimulation open NMDA channels, allowing access to MK-801? *A,* Control. Two light peaks (*single asterisks*) and a control response to NMDA were obtained before perfusion with 30 nM MK-801, shown by the *solid bar* and *dotted lines.* After 60 min, a pulse of NMDA was required before subsequent responses to NMDA were blocked. This is consistent with a use-dependent block by MK-801. The release of ACh in response to KA and QQ was not blocked by MK-801. *B,* Test experiment, identical to the control except for 2 periods of light stimulation (10 min, 3 Hz; *double asterisks*), which produced broad peaks during perfusion with MK-801. The next application of NMDA still produced a partial response, almost identical to the control, indicating that light stimulation, which effectively released ACh, did not potentiate the use-dependent block of NMDA by MK-801. Subsequent pulses of NMDA caused no release once a use-dependent block was established by the test pulse of NMDA. This result implies that the endogenous transmitter fails to open NMDA channels, so that access to MK-801 is prevented.

a self-contained experiment to test this question because the effects of MK-801 were practically irreversible. Therefore, it was necessary to conduct a separate control experiment, shown in Figure 10*A*. After 2 light peaks, a control response to NMDA was obtained (peak : base ratio, 25), and then, the perfusate was switched to MK-801 (30 nM) for the rest of the experiment. After 60 min, another pulse of NMDA produced a 6-fold increase over the base release, and subsequently, the NMDA evoked release of ACh was abolished. This is typical of the slow-onset, use-dependent block previously described for MK-801. The final responses to KA and QQ were not affected by MK-801, showing that the preparation was still functional.

The test experiment in Figure 10*B* was identical to the control, except for light stimulation at 3 Hz for 2 periods of 10 min during perfusion with MK-801. However, these 3600 light flashes did not potentiate the action of MK-801. The following pulse of NMDA produced a 7-fold increase over the basal efflux, as in the control experiment, and then, subsequent responses to NMDA were abolished. This result implies that the endogenous transmitter did not open NMDA channels to allow the access of MK-801, and therefore, light stimulation did not produce a use-dependent block of NMDA responses. This is consistent with our previous observations, which suggest that the physiological input to cholinergic amacrine cells is not mediated by NMDA receptors.

Discussion

We have examined the effect of NMDA and NMDA antagonists on the release of ACh from the rabbit retina. Our results lead to 4 major conclusions: (1) NMDA causes massive ACh release in magnesium-free medium. (2) The NMDA receptors mediating ACh release possess a glycine binding site that appears to be saturated under normal conditions. (3) DL-AP-7 is a competitive NMDA antagonist, and MK-801 is a noncompetitive NMDA antagonist in the retina. (4) Four types of NMDA antagonist (Mg^{2+}, 7-Cl-Kyn, DL-AP-7, and MK-801) failed to block the light-evoked release of ACh. Therefore, we have been unable to demonstrate that the receptors mediating the NMDA-evoked release of ACh are activated by physiological stimulation.

NMDA

In magnesium-free medium, the glutamate agonist NMDA caused massive ACh release from the rabbit retina. The cholinergic amacrine cells are undoubtedly the source of this release because they are the only cells in the retina to synthesize ACh (Masland and Mills, 1979). In addition, NMDA had no effect on horizontal cells in the rabbit retina (Massey and Miller, 1987), and pure NMDA antagonists such as DL-AP-7 did not reduce the b-wave of the electroretinogram, an indicator of bipolar cell function (Coleman and Miller, 1988; Massey and Miller, 1990). Together, these results suggest that the receptors mediating the NMDA-evoked release of ACh are in the inner retina.

The release of ACh by NMDA has been described in other systems, most notably from striatal slices (Lehmann and Scatton, 1982; Scatton and Lehmann, 1982), and this system has frequently been used to evaluate new NMDA antagonists (e.g., Lehmann et al., 1988). However, the NMDA-evoked release of ACh from the retina is different in several ways. Striatal ACh release seems to be mediated exclusively by NMDA receptors, but in the retina, other glutamate agonists are more potent than NMDA (Linn et al., 1991). Furthermore, the NMDA-evoked release of ACh from striatal slices was only 3-fold, but in the retina, peaks up to 50 times the resting release were obtained in response to NMDA. We do not know why the release from the retina is so large, but it may be related to the high coverage of cholinergic amacrine cells and the resulting density of cholinergic terminals (Tauchi and Masland, 1984, 1985). Alternatively, ACh release from the retina may be stronger because the tissue is intact, whereas the cells in a striatal slice have had at least some of their processes cut. It may be of general interest that ACh release from the retina is such a sensitive measure of glutamate receptor activation.

Magnesium dependence

The NMDA-evoked release of ACh from the rabbit retina was completely blocked by physiological levels of magnesium (1.2

mM), as previously reported by Cunningham and Neal (1985). The IC_{50} for magnesium was 151 μM, which is in close agreement with earlier results (Ault et al., 1980; Lehmann and Scatton, 1982). Therefore, even if the free magnesium concentration is partly reduced by protein binding (Foldes, 1981), there is still enough magnesium to block the response to NMDA. In contrast, the light-evoked release of ACh and the response to other glutamate agonists were not changed by magnesium, and this suggests that NMDA receptors do not carry the physiological input to cholinergic amacrine cells.

Ganglion cells in the rabbit retina are directly excited by NMDA (Bloomfield and Dowling, 1985; Massey and Miller, 1990), but in contrast to the release of ACh, this effect of NMDA is not magnesium dependent. This is puzzling because the ganglion cell dendrites and the cholinergic processes are intermingled in the inner plexiform layer, so they are probably exposed to the same concentration of exogenous NMDA or magnesium. We cannot explain this difference, but we note that there is preliminary evidence for multiple NMDA receptor subtypes (Young and Fagg, 1990).

Requirement for glycine

The NMDA-evoked release of ACh from the rabbit retina was not potentiated by additional glycine or reduced in glycine-free medium. However, 7-Cl-Kyn, an antagonist at the glycine site of the NMDA receptor (Kemp et al., 1988), blocked the response to NMDA. Furthermore, the action of 7-Cl-Kyn was reversed by exogenous glycine (Fig. 3). This indicates that 7-Cl-Kyn and glycine compete for the same site in the retina and demonstrates the presence of an allosteric glycine binding site. Glycine was also shown to potentiate the response to NMDA in ganglion cells under culture conditions where the level of glycine is low (Fain et al., 1989). From these results, we deduce that the glycine binding site is normally saturated by endogenous glycine.

Antagonists

The major goal of this study was to determine the contribution of NMDA receptors to the light-evoked release of ACh. We chose to use DL-AP-5 as an NMDA antagonist because the homologue DL-AP-7 has some activity at the 2-amino-4-phosphonobutyrate (APB) receptor (Slaughter and Miller, 1985; Coleman and Miller, 1988; Massey and Miller, 1990). Our results show that DL-AP-7 is a specific and competitive NMDA antagonist (IC_{50}, 33 μM), in agreement with previous work in the retina and other systems (Olverman et al., 1984; Massey and Miller, 1990). In contrast to Cunningham and Neal (1985), we found no evidence that NMDA antagonists could block the KA-evoked release of ACh.

MK-801 (IC_{50}, 10.6 nM) was a pharmacologically selective NMDA antagonist over 3 log units more potent than DL-AP-7. In addition, the blockade of NMDA responses by MK-801 was slow in onset, irreversible, and could not be overcome by excess NMDA. These observations are consistent with previous work indicating that MK-801 is a noncompetitive, use-dependent NMDA antagonist (Wong et al., 1986; Karschin et al., 1988).

Once the dose and specificity were established, we tested these antagonists against the light-evoked release of ACh. However, even in magnesium-free medium, neither antagonist reduced the light response, though in the same experiments, the NMDA-evoked release of ACh was abolished. It is convincing that we obtained the same result with 2 antagonists that act at different sites, DL-AP-7 at the NMDA receptor and MK-801 at the open channel. Furthermore, 7-Cl-Kyn, which is an antagonist at the glycine site, and magnesium did not block the light-evoked release of ACh. These observations indicate that NMDA receptors do not mediate the light-evoked release of ACh from the rabbit retina. This result is complementary to our previous work with the KA antagonist DNQX, which blocked the light-evoked release of ACh but not the NMDA response (Linn et al., 1991). This indicates that KA receptors, and not NMDA receptors, mediate the physiological input to the cholinergic amacrine cells.

One of the counterintuitive properties of MK-801 is that the blockade is dependent on the presence of NMDA. This use-dependent block, which we have observed in the retina (Fig. 9), suggests that MK-801 interacts with the open state of the NMDA-associated channel (Wong et al., 1986). However, prolonged light stimulation was not sufficient to activate the use-dependent block of NMDA responses by MK-801 (Fig. 10). From this, we deduce that light stimulation, though it causes ACh release, does not activate NMDA receptors in this pathway. In other words, the endogenous transmitter does not open the NMDA channel to permit the access of MK-801. Again, this indicates that the input to the cholinergic amacrine cells is not mediated by NMDA receptors.

Function of NMDA receptors

From the results described above, we are confident that NMDA receptors do not mediate the light-evoked release of ACh under our experimental conditions. However, we were constrained to use a diffuse light as a stimulus. Is it possible that a spatially optimal stimulus could generate a response mediated by NMDA receptors? The obvious case is the response to a moving stimulus because directionally selective ganglion cells in the rabbit retina are well known to receive input from cholinergic amacrine cells (Masland and Ames, 1976; Ariel and Daw, 1982), and NMDA receptors could be well suited for this kind of nonlinear response.

We think this is unlikely because a drifting grating produced the same amount of ACh release as diffuse stimulation (O'Malley and Masland, 1989). In addition, directional selectivity in ganglion cells is not blocked by NMDA antagonists (Massey and Miller, 1990). Instead, the mechanism of directional selectivity appears to be dependent on asymmetrical GABA inhibition (Wyatt and Daw, 1976; Daw et al., 1989).

Ganglion cells in the rabbit retina receive part of their input via NMDA receptors, and a slow NMDA component has been reported in the salamander retina (Mittman and Taylor, 1988; Massey and Miller, 1990). Thus, ganglion cells appear to have dual postsynaptic glutamate receptors, as reported in other systems. In addition, there are many functions for NMDA receptors besides fast synaptic transmission. NMDA receptors may be important for slow modulation, calcium regulation, and the control of plasticity, among other possibilities. At this time, our understanding of the physiological significance of NMDA receptors appears to lag behind our knowledge of the receptor and its pharmacology (Dale, 1989).

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