Pharmacological Isolation and Characterization of NMDA Receptor-Mediated Synaptic Potential in the Dentate Gyrus of Rat Hippocampal Slices

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ABSTRACT—We attempted to observe the dentate gyrus field potential evoked by low-frequency stimulation of the perforant path in Mg²⁺-free medium and identify the N-methyl-D-aspartate (NMDA) receptor-mediated synaptic potential using rat hippocampal slices. When perfusing solution was changed from normal medium (1.3 mM Mg²⁺) to Mg²⁺-free medium, the evoked potential was greatly increased and secondary population spikes appeared following a primary population spike. The evoked potential recorded in Mg²⁺-free medium was only partly blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-NMDA receptor antagonist. The CNQX-resistant component of the evoked potential in Mg²⁺-free medium was completely abolished by 30 μM dl-2-amino-5-phosphonovalerate, a NMDA receptor antagonist, indicating that the NMDA receptor-mediated synaptic response can be isolated by masking the non-NMDA receptor-mediated component of the evoked potential under the Mg²⁺-free condition. The isolated NMDA receptor-mediated synaptic potential was also blocked by 7-chlorokynurenate, an antagonist at the glycine site of the NMDA receptor, and restored by the concomitant presence of glycine. Observation of pharmacologically isolated NMDA receptor-mediated synaptic potential is a simple and good method for studying the direct effects of drugs on NMDA receptor-mediated responses.

Keywords: N-methyl-D-aspartate (NMDA) receptor, Synaptic potential, Dentate gyrus, Mg²⁺

In the central nervous system, the N-methyl-D-aspartate (NMDA) type of glutamate receptor plays important roles in processes such as the generation of long-term potentiation (1–5), spatial learning (6–8), epilepsy (9, 10) and glutamate neurotoxicity (11–13). Recent autoradiographic studies have revealed that NMDA receptors are present at higher density in the CA1 region and the dentate gyrus of the hippocampus (14–16). Properties of NMDA receptor-mediated synaptic responses in the hippocampus have been studied extensively in the Schaffer collateral-CA1 pyramidal cell synapses, but are not well studied in the perforant path-dentate gyrus granule cell synapses.

NMDA receptors do not contribute appreciably to excitatory synaptic responses evoked by low-frequency stimulation under normal conditions, because Mg²⁺ blocks the cation influx through the NMDA receptor-associated channel in a voltage-dependent manner (17, 18). Coan and Collingridge (19) have reported that low-frequency, NMDA receptor-mediated synaptic responses can be reliably observed in the Schaffer collateral-CA1 pyramidal cell synapses when Mg²⁺ is omitted from the extracellular medium. This Mg²⁺-free model is a simple and good method for studying direct effects of drugs on the NMDA receptor-mediated synaptic responses. In the present study, we attempted to observe the dentate gyrus field potentials evoked by low-frequency stimulation of the perforant path in Mg²⁺-free medium and identify the NMDA receptor-mediated synaptic responses using a NMDA receptor antagonist, dl-2-amino-5-phosphonovalerate (APV), and a non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). As a result, we succeeded to isolate the NMDA receptor-mediated synaptic potentials evoked by low-frequency stimulation in the dentate gyrus. Furthermore, it is known that the NMDA receptor channel complex contains a glycine modulatory site and that modulation by glycine is essential for the opening of the NMDA receptor-associated cation channel (20–22). Therefore, we further characterized the pharmacological properties
of the isolated NMDA receptor-mediated synaptic potentials in the dentate gyrus including the regulation by Mg$^{2+}$ and glycine.

MATERIALS AND METHODS

The hippocampi were quickly isolated from male Wistar rats, 8- to 10-week-old, and cut manually into transverse slices of 400- to 500-µm thickness. The slices were allowed to recover for more than 1 hr in an incubation chamber containing artificial cerebrospinal fluid (ACSF) which was maintained at 34°C and continuously oxygenated with 95% O$_2$–5% CO$_2$. The composition of ACSF was as follows: 124.0 mM NaCl, 5.0 mM KCl, 2.4 mM CaCl$_2$, 1.3 mM MgSO$_4$, 1.24 mM KH$_2$PO$_4$, 26.0 mM NaHCO$_3$ and 10.0 mM glucose.

Each slice was transferred into a recording chamber (1.5 ml) in which warmed (34°C) and oxygenated (95% O$_2$–5% CO$_2$) ACSF was continuously perfused at a rate of 1.4 ml/min. Stimulation to the perforant path was delivered through bipolar tungsten electrodes positioned across the fibers, and the evoked potential was extracellularly recorded from the granule cell layer of the dentate gyrus (Fig. 1A). A glass capillary microelectrode filled with 0.9% NaCl (tip resistance 5–7 Mohms) was used for the recording. A single test stimulation (0.05 msec duration) was applied at intervals of 60 sec. The stimulus intensity was set so that the amplitude of the population spike was 50% of the maximum in normal ACSF and was not changed throughout the experiment. To observe NMDA receptor-mediated synaptic responses, we used the ACSF with no added MgSO$_4$ (Mg$^{2+}$-free ACSF). All drugs were delivered by perfusion.

As shown in Fig. 1B, the evoked potential recorded from the dentate gyrus granule cell layer is composed of slow positivity and sharp negativity. The slow positivity reflects mainly excitatory postsynaptic potential (EPSP), which is generated in dendritic spines and spreads along the dendrites, but is termed the positive component in this report, because possible contamination of the inhibitory somatic response cannot be completely neglected. The sharp negativity is a result of the synchronous firing of the granule cells, and this is generally termed the population spike. To quantify changes of evoked potentials, we employed the following parameters: 1) the amplitude of positive component, 2) the amplitude of the population spike, 3) latency of the population spike peak, 4) the number of population spikes, 5) half-decay time of the positive component. The way of measuring these parameters is described in Fig. 1B in detail. APV was purchased from Sigma Chemical Co. (St. Louis, MO, USA). CNQX and 7-chlorokynurenate (7-Cl-Kyn) were purchased from Tocris Neuramin, Ltd. (Bristol, UK). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka).

RESULTS

First we investigated the influence of omitting extracellular Mg$^{2+}$ on synaptically evoked potentials in the dentate gyrus. The typical result is shown in Fig. 2. When perfusing solution was changed from normal ACSF (1.3 mM

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**Fig. 1.** Recording of evoked potential in the dentate gyrus of the rat hippocampal slice. A: schematic illustration of a transverse slice of rat hippocampus showing locations of stimulating and recording electrodes. B: a typical field potential recorded from the dentate gyrus granule cell layer. The time of application of test stimulation is indicated by a black arrow head. The voltage difference between the first positive peak and the succeeding negative peak was measured (a). Next, the voltage difference between the negative peak and the second positive peak was measured (b). The amplitude of the population spike was defined as (a+b)/2. The amplitude of the positive component was defined as the voltage difference between the basal potential level and the positive peak (c). The latency of the population spike was defined as the time difference from the start of stimulation to the negative peak (t). The half-decay time of the positive component was defined as h.
Fig. 2. The effect of omitting Mg\(^{2+}\) on the dentate gyrus field potential evoked by single stimulation of the perforant path at intervals of 60 sec. The stimulus intensity was adjusted so that the amplitude of the primary population spike was 50% of the maximum in normal ACSF and was not changed throughout the experiment. A: typical single records, taken 1 min before and 8, 20, 34, 60 and 120 min after the start of perfusion with Mg\(^{2+}\)-free ACSF. Stimulus artifacts have been blanked for clarity, and the time of stimulation is indicated by black arrow heads. Calibration bars: vertical, 2 mV; horizontal, 10 msec. B: time-course of changes of evoked potential in a typical experiment. The upper graph shows the amplitude of the positive component, and the lower shows the amplitude of the first (1st), second (2nd) and third (3rd) population spikes. Perfusing medium was changed from normal ACSF to Mg\(^{2+}\)-free ACSF at time 0.

Fig. 3. The effect of 30 \(\mu\)M APV on the synaptic response recorded in Mg\(^{2+}\)-free ACSF. The field potential evoked by half maximum stimulation was first recorded in normal ACSF, and the slice was perfused with Mg\(^{2+}\)-free ACSF. After the response reached the steady state (approximately 60 min after introduction of Mg\(^{2+}\)-free ACSF), 30 \(\mu\)M APV was added in Mg\(^{2+}\)-free ACSF. A: typical single records of evoked potentials in Mg\(^{2+}\)-free ACSF before and 15 min after addition of 30 \(\mu\)M APV. Half-decay time of the positive component was indicated by the white arrow head. Vertical and horizontal calibration bars indicate 2 mV and 10 msec, respectively. B: the time scale of the records in A was expanded, and the records in the absence and presence of APV were superimposed. Vertical and horizontal calibration bars indicate 2 mV and 2 msec, respectively. C: the influences of 30 \(\mu\)M APV on the amplitude of the positive component, the amplitude of the first population spike, the latency of the first population spike, the number of the population spikes and the decay-half time of the positive component. Solid black and stippled columns are the data before and 15 min after addition of 30 \(\mu\)M APV, respectively. The data are represented as the mean \(\pm\) S.E.M. of 5 observations. Asterisks indicate significant differences from the respective controls (solid black columns): *\(P<0.01\), paired \(t\)-test.
Mg$^{2-}$) to Mg$^{2+}$-free ACSF, the amplitude of the positive component and population spike greatly increased. Furthermore, the decay of the positive component became much slower, and secondary population spikes, which were never observed in normal ACSF, appeared following a primary population spike. The increased responses usually took 40–60 min to reach the steady state. The amplitude of the positive component and primary population spike became 226.2±18.5% (n=12) and 382.7±40.4% (n=12) of those in normal ACSF, respectively. The maximum number of population spikes elicited varied among the slices from 2 to 16, and the mean number of population spikes observed in Mg$^{2+}$-free ACSF was 5.6±1.4 (n=12). The responses in Mg$^{2+}$-free ACSF could be observed stably for more than 2.5 hr in all of the 12 tested slices.

Next we investigated the effects of APV, a selective NMDA receptor antagonist, and CNQX, a selective non-

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**Fig. 4.** The effect of 10 μM CNQX on the synaptic response recorded in Mg$^{2+}$-free ACSF. After the slices were perfused with Mg$^{2+}$-free ACSF for 60 min, 10 μM CNQX was added. A: typical single records of evoked potentials in Mg$^{2+}$-free ACSF before and 15 min after addition of 10 μM CNQX. Vertical and horizontal calibration bars indicate 2 mV and 10 msec, respectively. B: the time scale of the records in A was expanded and superimposed to clarify the change of the latency of population spikes. Vertical and horizontal calibration bars indicate 2 mV and 2 msec, respectively. C: time-course of change of evoked potential was shown in terms of the latency of the first (1st) and second (2nd) population spike. CNQX was added to Mg$^{2+}$-free ACSF at time 0. D: the influences of 10 μM CNQX on the amplitude of the positive component, the amplitude of the first population spike, the latency of the first population spike, the number of population spikes and the half-decay time of the positive component. Solid black and stippled columns are the data before and 15 min after addition of 10 μM CNQX, respectively. The data are represented as the mean±S.E.M. of 9 observations. Asterisks indicate significant differences from the respective controls (solid black columns): *P<0.01; paired t-test.
NMDA receptor antagonist, on the evoked potential recorded in Mg\(^{2+}\)-free ACSF. Figure 3 shows the effect of APV on the evoked potential recorded in Mg\(^{2+}\)-free ACSF. When APV was added in Mg\(^{2+}\)-free ACSF, the decay of the positive component became much faster and secondary population spikes completely disappeared, indicating that development of slow EPSP and appearance of secondary population spikes in the Mg\(^{2+}\)-free condition are mediated by NMDA receptors. The onset of the positive component and the amplitude and latency of the first population spike were not influenced by APV at all. Interestingly, the positive component and the primary population spike in Mg\(^{2+}\)-free ACSF supplemented with APV were larger than those in normal ACSF, indicating that the responses not mediated by NMDA receptors were also enhanced by omitting extracellular Mg\(^{2+}\). On the other hand, CNQX at 10 \(\mu\)M completely blocked the evoked potentials in normal ACSF (data not shown), but the evoked potential recorded in Mg\(^{2+}\)-free ACSF was only partly blocked by 10 \(\mu\)M CNQX (Fig. 4). After addition of CNQX, the onset of the positive component became slower and the latency to the population spike became longer, confirming that development of fast EPSP is mediated by non-NMDA receptors. The amplitude of the positive component partly decreased and the half-decay time of the positive component was apparently elongated. Secondary population spikes were not blocked by CNQX. These changes were immediately produced after addition of CNQX and reached the steady state within 15 min (Fig. 4C). The component resistant to 10 \(\mu\)M CNQX was also not blocked by a higher concentration (25 \(\mu\)M) of CNQX. As shown in Fig. 5, APV blocked the CNQX-resistant component of the evoked potential in Mg\(^{2+}\)-free ACSF in a concentration-dependent manner. The IC\(_{50}\) values for APV in terms of the amplitude of the positive component and population spikes were 7.41 ± 1.22 \(\mu\)M (n = 5) and 1.99 ± 0.49 \(\mu\)M (n = 5), respectively, and the CNQX-resistant component was completely abolished by APV at 30 \(\mu\)M. These results clearly suggest that NMDA receptor-mediated synaptic responses can be isolated as a CNQX-resistant component of evoked potential in the absence of extracellular Mg\(^{2+}\).

We further characterized the pharmacologically isolated NMDA receptor-mediated synaptic potential recorded...
Fig. 6. The effect of increasing concentrations of Mg²⁺ on the isolated NMDA receptor-mediated synaptic potential. To observe the NMDA receptor-mediated component, the slices had been perfused with Mg²⁺-free ACSF for 40 min and subsequently with Mg²⁺-free ACSF plus 10 μM CNQX for 20 min. After the stability of the responses was confirmed for a further 10 min, the influence of Mg²⁺ was examined by cumulatively introducing MgSO₄ in the perfusing medium. The style of the graph is the same as in Fig. 5. The data in C are the mean ± S.E.M. of 6 observations.

Fig. 7. The effect of 7-Cl-Kyn on the isolated NMDA receptor-mediated synaptic potential. The experimental procedure and the graph style were the same as in Fig. 6. In the experiment shown in A and B, 200 μM glycine was further added after 30 μM 7-Cl-Kyn. The data in C are the mean ± S.E.M. of 5 observations.
in Mg\(^{2+}\)-free ACSF containing 10 \(\mu\)M CNQX. Figure 6 shows the effect of Mg\(^{2+}\) on the isolated NMDA receptor-mediated synaptic response. Increasing concentration of Mg\(^{2+}\) blocked the isolated NMDA receptor-mediated synaptic response in a concentration-dependent manner. The inhibitory effect of Mg\(^{2+}\) was seen from 0.02 mM, and the population spikes almost disappeared in the presence of 0.3 mM Mg\(^{2+}\). Complete inhibition of the positive component required a higher concentration (0.6 mM) of Mg\(^{2+}\). The IC\(_{50}\) values for Mg\(^{2+}\) in terms of the amplitude of the positive component and population spikes were 0.159 ± 0.036 mM (n = 6) and 0.075 ± 0.019 mM (n = 6), respectively. As shown in Fig. 7, the isolated NMDA receptor-mediated synaptic potential was blocked by 7-Cl-Kyn, a selective antagonist at the glycine site of the NMDA receptor, in a concentration-dependent manner. The positive component and population spikes completely disappeared in the presence of 100 \(\mu\)M 7-Cl-Kyn. The blocking effect of 7-Cl-Kyn was reversed by addition of glycine, indicating that 7-Cl-Kyn acted selectively and competitively on the glycine site. The IC\(_{50}\) values for 7-Cl-Kyn in terms of the amplitude of the positive component and population spikes were 14.20 ± 0.79 \(\mu\)M (n = 5) and 5.22 ± 1.16 \(\mu\)M (n = 5), respectively.

**DISCUSSION**

The most remarkable changes of evoked potential in the dentate gyrus after omitting extracellular Mg\(^{2+}\) were 1) increase of positive component, 2) increase of population spike amplitude, 3) elongation of decay of positive component and 4) appearance of secondary population spikes. Pharmacological characterization with APV and CNQX of the synaptic potential in Mg\(^{2+}\)-free medium revealed that the NMDA receptors participate in the slow EPSP component and the appearance of secondary spikes. Blockade of fast EPSP by CNQX resulted in a slowed onset of the positive component and delayed generation of action potential (population spike). The CNQX-resistant component was identified as the synaptic responses purely mediated by NMDA receptors, since it was completely blocked by APV. It appears that activation of NMDA receptors alone can produce a depolarization large enough to generate action potentials in the dentate gyrus granular neurons. NMDA receptors largely contribute to neuronal excitability if only it is released from the Mg\(^{2+}\) block.

Coan and Collingridge (19) have reported that, when the evoked potential was recorded from the CA1 region in Mg\(^{2+}\)-free medium, spontaneous population spike discharges were sometimes observed, and the evoked potential suddenly disappeared in rare cases. In preliminary experiments, we also observed some cases where the increased responses in the CA1 region in Mg\(^{2+}\)-free ACSF gradually decreased or suddenly disappeared. However, such an event was never observed in the case of the dentate gyrus synapses. These differences may be due to vulnerability of the CA1 pyramidal neurons or synaptic properties including neural networks. Since properties of NMDA receptor-mediated synaptic potential in the dentate gyrus seemed to be virtually the same as those in the CA1 region, vulnerability of CA1 pyramidal neurons may be determined by differences of intracellular mechanisms. Comparison of the evoked potentials in the Mg\(^{2+}\)-free condition between the CA1 region and the dentate gyrus may provide useful information on the cause of epileptiform activity or neuronal cell death.

Furthermore, we investigated the effects of Mg\(^{2+}\) and 7-Cl-Kyn on the pharmacologically isolated NMDA receptor-mediated synaptic potential in the dentate gyrus. Mg\(^{2+}\) blocked the isolated NMDA receptor-mediated response in the low micromolar range. Mg\(^{2+}\) is known to affect neurotransmitter release, but the effect observed in the present study is probably due to blocking of NMDA receptor-associated cation channels, because it requires millimolar concentrations to reduce neurotransmitter release (23). The isolated NMDA receptor-mediated synaptic potential was completely blocked by 7-Cl-Kyn and restored by concomitant application of exogenous glycine, indicating that the NMDA receptor-mediated synaptic response in the dentate gyrus was absolutely supported by endogenous glycine. The concentrations of Mg\(^{2+}\) and 7-Cl-Kyn effective for blocking the isolated NMDA receptor-mediated response in the dentate gyrus were very similar to those effective for blocking the evoked potential in the CA1 region in Mg\(^{2+}\)-free medium (19, 24). Modulation of NMDA receptors by Mg\(^{2+}\) and glycine in the perforant path-dentate granule cell synapses seems to be virtually the same as that in the Schaffer collateral-CA1 pyramidal cell synapses.

To study the drug effects on the NMDA receptor-mediated events, investigators have often examined influences on the responses induced by application of glutamate or NMDA. However, it remains possible that the responses induced by exogenously applied amino acids are different from those by endogenous glutamate released during synaptic transmission. Observing the synaptic field potential evoked by low-frequency stimulation in Mg\(^{2+}\)-free medium is a simple method for directly studying synaptically-evoked, NMDA receptor-mediated responses. Furthermore, we successfully isolated the NMDA receptor-mediated component of synaptic transmission in the dentate gyrus by masking the non-NMDA receptor-mediated component with CNQX. Observation of the isolated NMDA receptor-mediated synaptic field potential should be useful for studying the effects of...
drugs on NMDA receptor-mediated synaptic responses or the functional changes of the NMDA receptor channel complex during some physiological and pathological events.

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