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Effects of Divalent Cations on Heteromeric NMDA Receptor Channels Expressed in *Xenopus* Oocytes

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Summary. **The effects of Ca^{2+}, Ba^{2+}, Mg^{2+} and Zn^{2+} on the activity of heteromeric N-methyl-D-aspartate (NMDA) receptor channels ($\varepsilon1/\zeta1$, $\varepsilon2/\zeta1$, $\varepsilon3/\zeta1$ and $\varepsilon4/\zeta1$) expressed in *Xenopus laevis* oocytes were examined using the whole-cell clamp technique. The whole-cell current in response to 10 μM glutamate plus 10 μM glycine consisted of two phases: an initial spike-like phase and a subsequent sustained phase. Profiles of the sustained phase were almost a plateau in oocytes expressing the $\varepsilon2/\zeta1$, $\varepsilon3/\zeta1$ and $\varepsilon4/\zeta1$ channels and in one third of those expressing the $\varepsilon1/\zeta1$ channel. In two thirds of the oocytes expressing the $\varepsilon1/\zeta1$ channel, the sustained phase gradually increased. The initial spike-like phase was enhanced as the extracellular Ca^{2+} concentration increased, but the enhancement was suppressed by an intracellular injection of ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA). Therefore, this spike-like current may be induced by intracellular Ca^{2+}-dependent processes: Ca^{2+}-dependent Cl^--conductance. Extracellular Ca^{2+} suppressed the flat sustained phase in oocytes expressing the $\varepsilon1/\zeta1$, $\varepsilon3/\zeta1$ and $\varepsilon4/\zeta1$ channels in a concentration-dependent manner. However, no comparable effect was observed in oocytes expressing the $\varepsilon2/\zeta1$ channel in which the whole-cell current was unchanged at 0.2, 0.6 and 2.0 mM extracellular Ca^{2+}. This sustained phase was unchanged by EGTA injection into oocytes expressing the $\varepsilon1/\zeta1$, $\varepsilon2/\zeta1$ and $\varepsilon3/\zeta1$ channels and only partially inhibited in oocytes expressing the $\varepsilon4/\zeta1$ channel. Ba^{2+} also inhibited the flat sustained phase in oocytes expressing the $\varepsilon1/\zeta1$, $\varepsilon3/\zeta1$ and $\varepsilon4/\zeta1$ channels, but not those expressing the $\varepsilon2/\zeta1$ channel. Mg^{2+} and Zn^{2+} suppressed the sustained current in a concentration-dependent manner in oocytes expressing every heteromeric channel. However, the $\varepsilon3/\zeta1$ and $\varepsilon4/\zeta1$ channels were more resistant to Mg^{2+} than the $\varepsilon1/\zeta1$ and $\varepsilon2/\zeta1$ channels. The inhibitory effects of Zn^{2+} on heteromeric NMDA channels were similar to those of Mg^{2+}, except for the $\varepsilon1/\zeta1$ channel. The gradually increasing current observed in oocytes expressing the $\varepsilon1/\zeta1$ channel was very sensitive to Zn^{2+}, and the sustained phase showed a plateau in the presence of 1 μM Zn^{2+}. These diverse sensitivities of heteromeric channels to divalent cations may depend on properties of the ε subunits. The characterization of these heteromeric NMDA channels based on their sensitivities to divalent cations, which is demonstrated in this paper, may be useful in understanding the physiological significance of the molecular diversity of the NMDA receptor channels.**

INTRODUCTION

The N-methyl-D-aspartate (NMDA) receptor channel plays an essential role in functional changes in neurons, including synaptic plasticity, neuronal diseases, and ischemic neuronal damage.[1-3] These functional changes are known to be induced by Ca^{2+}-influx mediated by the NMDA receptor, which is highly permeable to Ca^{2+}.[4-7] However, Ca^{2+} and Ba^{2+} themselves have also been reported to suppress NMDA receptor-mediated current.[5,7]

The activity of the NMDA receptor channel is known to be modified by Mg^{2+} and Zn^{2+}. Mg^{2+}, an open-channel blocker, acts on the NMDA recepror in a voltage-dependent manner. Depolarization of the plasma membrane weakens the Mg^{2+}-blockade, and triggers the Ca^{2+}-influx.[8,9] On the other hand, Zn^{2+} blocks the NMDA receptor channel mainly by an allosteric effect.[10-13] It has been reported that Zn^{2+} is present in synaptic vesicles and is co-released with neurotransmitters during neuronal excitation.[14-17] Some studies have suggested that Zn^{2+} is a putative modulator for the NMDA receptor in the central nervous system (CNS).[11,13,18]

Recently, subunit-specific cDMAs of the NMDA receptor channel have been cloned and analyzed, and

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the physiological significance of their molecular diversity has been investigated.[19–25] However, there are as yet no comprehensive studies of the effect of divalent cations on heteromeric NMDA receptor channels. To investigate the physiological significance of molecular diversity, the effects of divalent cations Ca^{2+}, Ba^{2+}, Mg^{2+} and Zn^{2+} on the heteromeric NMDA receptor channels expressed in *Xenopus* oocytes were characterized by analyses of evoked whole cell currents in response to glutamate plus glycine stimulation.

MATERIALS AND METHODS

Expression of heteromeric NMDA receptor channels in *Xenopus* oocytes

Messenger RNAs specific for the $\varepsilon 1$, $\varepsilon 2$, $\varepsilon 3$, $\varepsilon 4$ and $\zeta 1$ subunits were synthesized *in vitro* with SP6 RNA polymerase (Ambion MEGAscript) in the presence of cap dinucleotide ^{7}mGpppG using *Eco*RI-cleaved pSPGR$\varepsilon 1$, pSPGR$\varepsilon 2$, pSPGR$\varepsilon 3$ and pSPGR$\varepsilon 4$, and *Not*I-cleaved pSPGR$\zeta 1$ as templates, respectively.[19,25,26] *Xenopus laevis* oocytes were injected with the $\zeta 1$ subunit-specific mRNA and the ε subunit-specific mRNA in a molar ratio of 1 to 1; total amounts of mRNAs injected per oocyte were ~0.6 ng, ~0.3 ng, ~30 ng and ~30 ng for the $\varepsilon 1$ and $\zeta 1$, the $\varepsilon 2$ and $\zeta 1$, the $\varepsilon 3$ and $\zeta 1$ and the $\varepsilon 4$ and $\zeta 1$ subunits, respectively.

The oocytes were incubated at 19°C for 3–4 days in modified Barth's medium[27] containing gentamicin (0.1 mg/ml). On the second day, the follicular cell layer was mechanically removed after treatment with 1 mg/ml collagenase for 1 h.[28] In this paper, the terms "$\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels" represent the heteromeric NMDA receptor channels of the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ subunits, respectively.

Analysis of glutamate-induced current in NMDA receptors

Whole-cell currents evoked by bath-application of 10 μM L-glutamate plus 10 μM glycine for 30 or 60 s were recorded at a membrane potential of −70 mV using a microelectrode amplifier (Nihon Kohden, Co., Tokyo, MEZ-8201), a voltage clamp amplifier (Nihon Kohden, Co., Tokyo, CEZ-1100) and a thermal array recorder (Nihon Kohden, Co., Tokyo, WS-641G) with two conventional glass micropipettes for voltage-clamping. The micropipettes were filled with 3 M KCl. The modified frog Ringer's solutions bathing the oocytes consisted of 115 mM NaCl, 2.5 mM KCl, 10 mM HEPES-NaOH (pH 7.2), and the indicated concentration of divalent cations. The temperature of the bathing solution was 19°C. In this experiment, 0.2 mM-Ca^{2+} frog Ringer's solution was used as a standard solution, since it was difficult to clamp the membrane potential at −70 mV in divalent cation-free solutions. The effects of Mg^{2+} or Zn^{2+} on the current were examined with modified frog Ringer's solutions containing 0.2 mM Ca^{2+} to minimize the effect of endogenous Ca^{2+}-activated Cl^- current in *Xenopus* oocytes.[29,30]

Intracellular EGTA injection

To evaluate how much of the evoked current consisted of endogenous Ca^{2+}-activated Cl^- current, the effect of intracellular injection of ethyleneglycol-bis-(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), a Ca^{2+}-chelator, was examined. EGTA was injected ionophoretically into the oocytes using a micropipette filled with 100 mM EGTA adjusted to pH 8.0 with KOH. Based on the results obtained in 2.0 mM-Ca^{2+} frog Ringer's solution, a direct electric current (0.6 μA) was applied through a negatively charged electrode containing EGTA for 4 min. The effectiveness of EGTA injection was verified by the disappearance of the initial spike current in 2.0 mM-Ca^{2+}. Data for analysis of the evoked current were obtained from oocytes in which a spike-like component disappeared after EGTA injection.

Data analysis

The evoked current was measured in duplicate experiments. Data are represented as the mean± SEM, and the statistical significance was verified by a one-way analysis of variance. A p-value of less than 0.05 was considered significant.

RESULTS

The effects of extracellular Ca^{2+} concentrations on the evoked current in heteromeric NMDA receptor channels

Profiles of whole-cell current induced by 10 μM L-glutamate plus 10 μM glycine for 30 s in *Xenopus* oocytes expressing the heteromeric NMDA channels $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ in the presence of various extracellular Ca^{2+} concentrations are shown in Fig. 1A. The evoked current consisted of two phases: an initial spike-like phase that desensitized quickly, and a subsequent sustained phase. The initial spike-like phase was very small at 0.2 mM-Ca^{2+}, but increased in each NMDA receptor channel as the extracellular Ca^{2+} concentration increased. On the

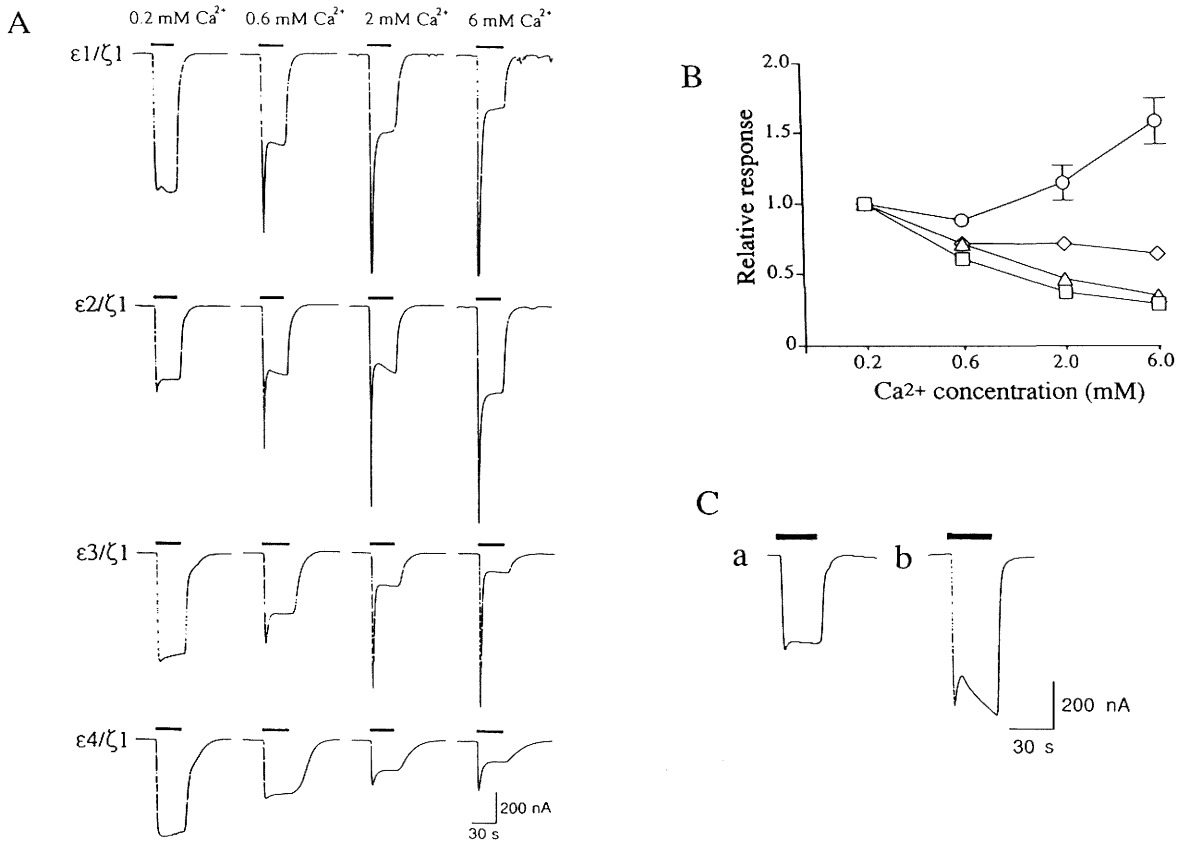

Fig. 1. Effects of extracellular Ca^{2+} concentration on the evoked current induced by 10 μM L-glutamate plus 10 μM glycine in *Xenopus* oocytes expressing four types of heteromeric NMDA receptor channels. **A.** Evoked currents in the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels were measured sequentially in Ringer's solutions that contained either 0.2 mM, 0.6 mM, 2.0 mM or 6.0 mM Ca^{2+}, in this order. Membrane potential was clamped at -70 mV. Inward current is downwards. The duration of the bath-application of glutamate and glycine is indicated by the bar. These conditions also apply to the following figures. **B.** Effects of Ca^{2+} on the amplitude of the sustained current in the $\varepsilon 1/\zeta 1$ (◇), $\varepsilon 2/\zeta 1$ (○), $\varepsilon 3/\zeta 1$ (△) and $\varepsilon 4/\zeta 1$ (□) channels. Each point represents the mean of relative responses obtained from 11-18 oocytes; SEM are indicated by bars when larger than the symbols. **C.** Traces of the evoked current in the $\varepsilon 1/\zeta 1$ channels in 0.2 mM-Ca^{2+} Ringer's solution. (**a**) One third of the oocytes (n=71) showed a flat sustained phase, and the amplitude of this current was considered in the analysis. (**b**) The sustained current gradually increased and did not level off during stimulation in the resting two thirds (n=137) of the oocytes expressing the $\varepsilon 1/\zeta 1$ channel. This increasing current was excluded from the analysis in Fig. 1B.

other hand, the sustained phase decreased as the extracellular Ca^{2+} concentration increased in oocytes exressing the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. In oocytes expressing the $\varepsilon 2/\zeta 1$ channel, the amplitude of the sustained phase was almost unchanged at 0.2, 0.6 and 2.0 mM-Ca^{2+}, and was increased at 6.0 mM-Ca^{2+}(Fig. 1B). The amplitudes of the sustained phase in oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels in 0.2 mM-Ca^{2+} Ringer's solution were 173-658 nA, 133-590 nA, 192-615 nA and 131-573 nA, respectively.

The sustained phase was flat in almost all of the oocytes expressing the $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels, and in about one third (71/208) of those expressing the $\varepsilon 1/\zeta 1$ channel. However, the current in the sustained phase underwent a gradual increase in the remaining two thirds (137/208) (Fig. 1C). The rate and amount of increase varied in each oocyte. Moreover, the gradual increase in the current was profoundly suppressed by a trace amount of Zn^{2+}, as described below. Therefore, we analyzed the current in only the flat sustained phase to characterize the activities of

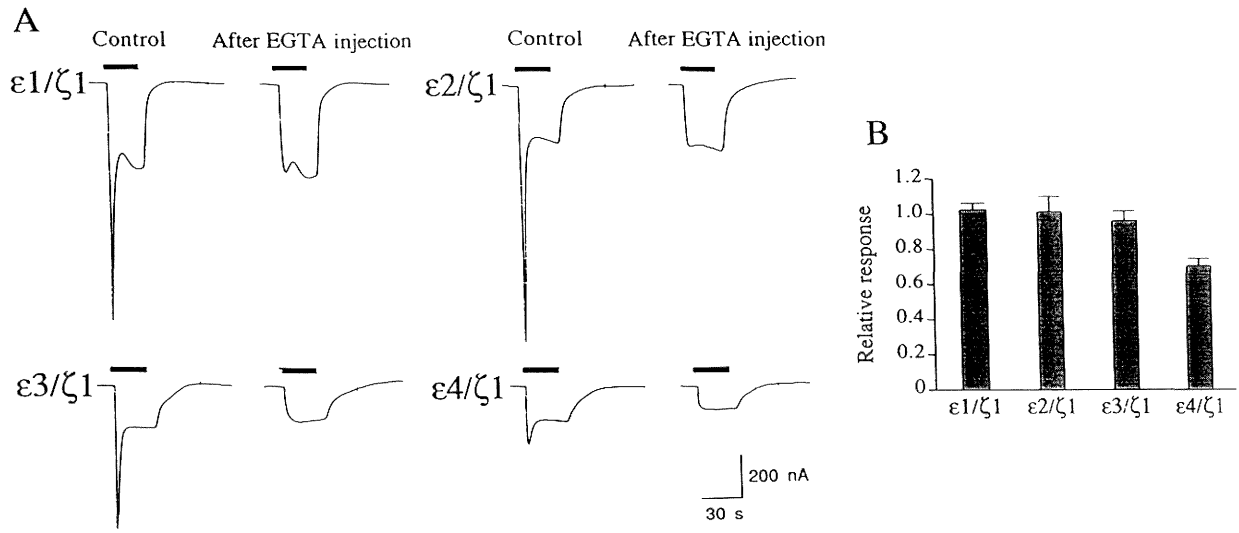

Fig. 2. Effects of intracellular EGTA injection on the current responses induced by 10 μM L-glutamate plus 10 μM glycine in 2.0 mM-Ca^{2+} Ringer's solutions in *Xenopus* oocytes expressing four types of heteromeric NMDA receptor channels. **A.** Traces of the evoked current in the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels before and after EGTA injection. EGTA was injected ionophoretically into the oocytes using a micropipette filled with 100 mM EGTA (0.6 μA, 4 min). **B.** Effects of EGTA injection on the amplitude of the sustained current in the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. Data represent the mean±SEM of fractional responses obtained from 12-32 oocytes.

heteromeric NMDA channels.

The $\varepsilon 4/\zeta 1$ channel was the most sensitive to extracellular Ca^{2+} in the four types of heteromeric channels, followed by the $\varepsilon 3/\zeta 1$ channel and the $\varepsilon 1/\zeta 1$ channel. The $\varepsilon 2/\zeta 1$ channel was resistant to Ca^{2+} at 0.2, 0.6 and 2.0 mM, while the evoked current showed a significant increase at 6.0 mM-Ca^{2+}.

Effect of EGTA injection on the evoked current in oocytes expressing heteromeric NMDA channels

Fig. 2A shows the evoked current in oocytes expressing heteromeric NMDA channels in 2.0 mM-Ca^{2+} before and after EGTA injection. The initial spike-like phase disappeared after EGTA injection in most of the oocytes. Amplitudes of the sustained currents were not affected by intracellular EGTA injection in the oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$ and $\varepsilon 3/\zeta 1$ channels, and they were reduced to 70.6 ± 4.2% in the oocytes expressing the $\varepsilon 4/\zeta 1$ channel (Fig. 2B). The amplitudes of the sustained current in the oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels at 2.0 mM-Ca^{2+} before EGTA injection were 142-664 nA, 163-771 nA, 69-217 nA and 67-230 nA, respectively.

Effects of Ba^{2+} on the evoked current

The profiles of the evoked current in oocytes expressing heteromeric NMDA channels in the presence of various concentrations of extracellular Ba^{2+} (0.2-6.0 mM) are shown in Fig. 3A. The initial spike-like phase almost disappeared. The sustained phase in oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels were reduced by extracellular Ba^{2+} in a concentration-dependent manner. However, the responses of oocytes expressing the $\varepsilon 2/\zeta 1$ channel were not significantly changed by extracellular Ba^{2+} concentrations. Although oocytes expressing the $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels were more sensitive than those expressing the $\varepsilon 1/\zeta 1$ channel at 6.0 mM-Ba^{2+}, there were no significant differences in sensitivity among oocytes expressing these three channels at 0.6 and 2.0 mM (Fig. 3B). The amplitudes of the sustained current in oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels in 0.2 mM-Ba^{2+} Ringer's solution were 217-442 nA, 82-594 nA, 84-148 nA and 129-339 nA, respectively.

Effects of Mg^{2+} on the evoked current

The effects of Mg^{2+} on the evoked current in oocytes expressing the heteromeric channels in a bathing

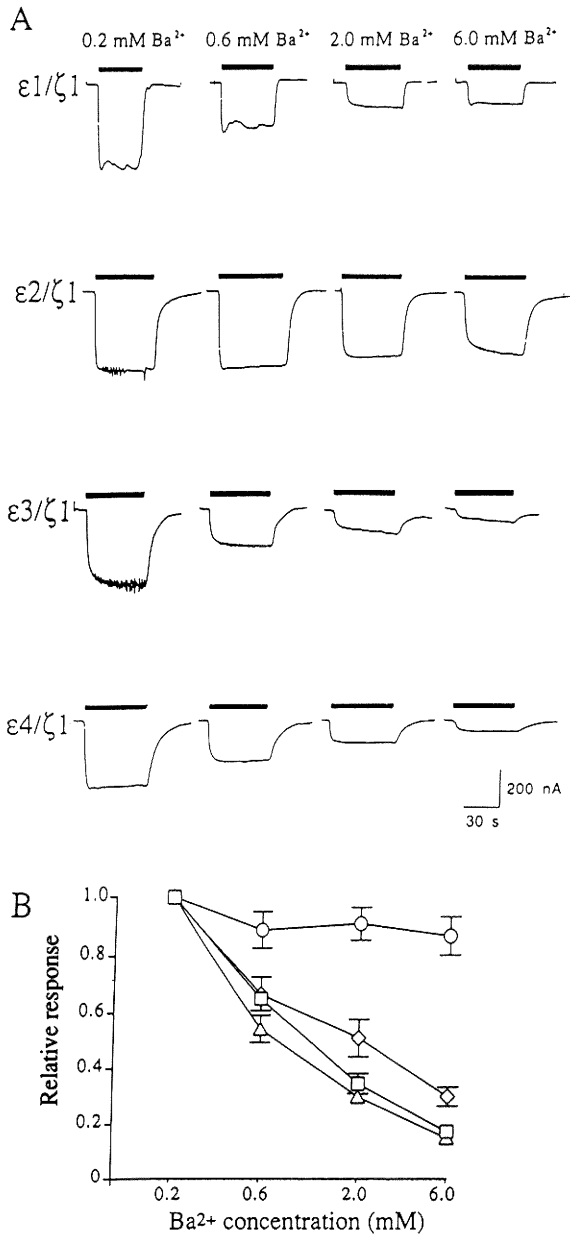

Fig. 3. Effects of extracellular Ba^{2+} on the evoked current induced by 10 μM L-glutamate plus 10 μM glycine in 0.2 mM-Ca^{2+} Ringer's solution in *Xenopus* oocytes expressing four types of the heteromeric NMDA receptor channels. **A.** Evoked current in the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels expressed on oocytes was measured sequentially in Ca^{2+}-free Ringer's solutions that contained either 0.2 mM, 0.6 mM, 2.0 mM or 6.0 mM of Ba^{2+}, in this order. **B.** Effects of Ba^{2+} on the amplitude of the sustained current in the $\varepsilon 1/\zeta 1$ (◇), $\varepsilon 2/\zeta 1$ (○), $\varepsilon 3/\zeta 1$ (△) and $\varepsilon 4/\zeta 1$ (□) channels. Data have been obtained from 3-5 oocytes.

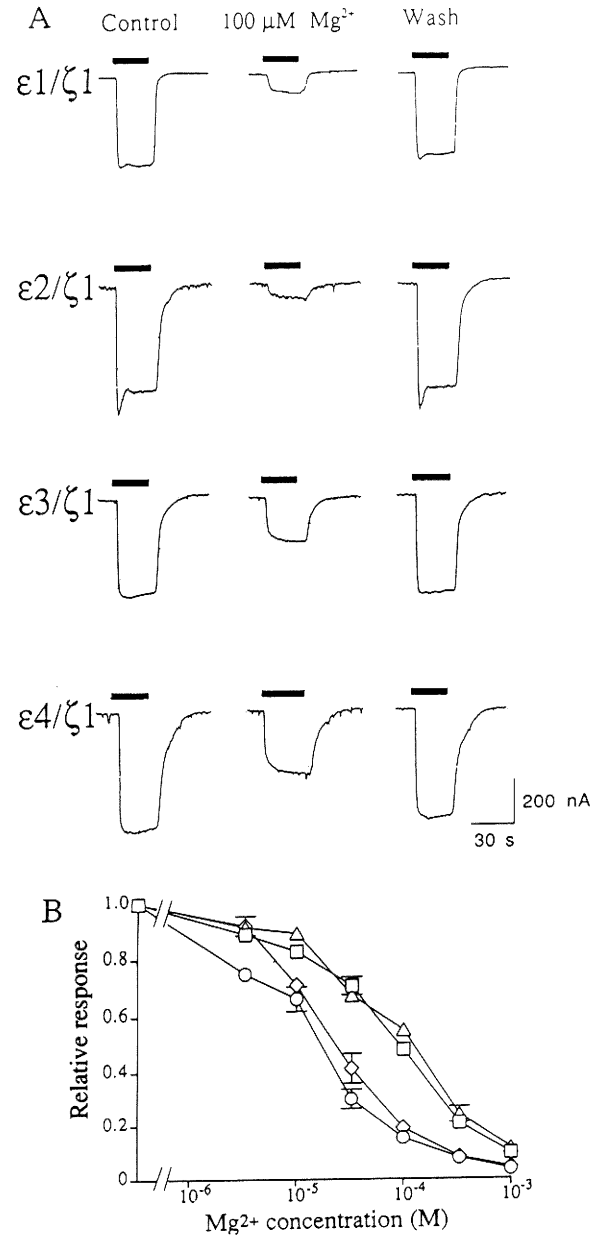

Fig. 4. Effects of extracellular Mg^{2+} on the evoked current induced by 10 μM L-glutamate plus 10 μM glycine in 0.2 mM-Ca^{2+} Ringer's solution in *Xenopus* oocytes expressing four types of the heteromeric NMDA receptor channels. **A.** Effects of 100 μM Mg^{2+} on the traces of the current in the oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. **B.** Effects of Mg^{2+} on the amplitude of the sustained current in the $\varepsilon 1/\zeta 1$ (◇), $\varepsilon 2/\zeta 1$ (○), $\varepsilon 3/\zeta 1$ (△) and $\varepsilon 4/\zeta 1$ (□) channels. The evoked current in each oocyte was measured at only one of the indicated concentrations of Mg^{2+}. Data have been obtained from 3-17 oocytes.

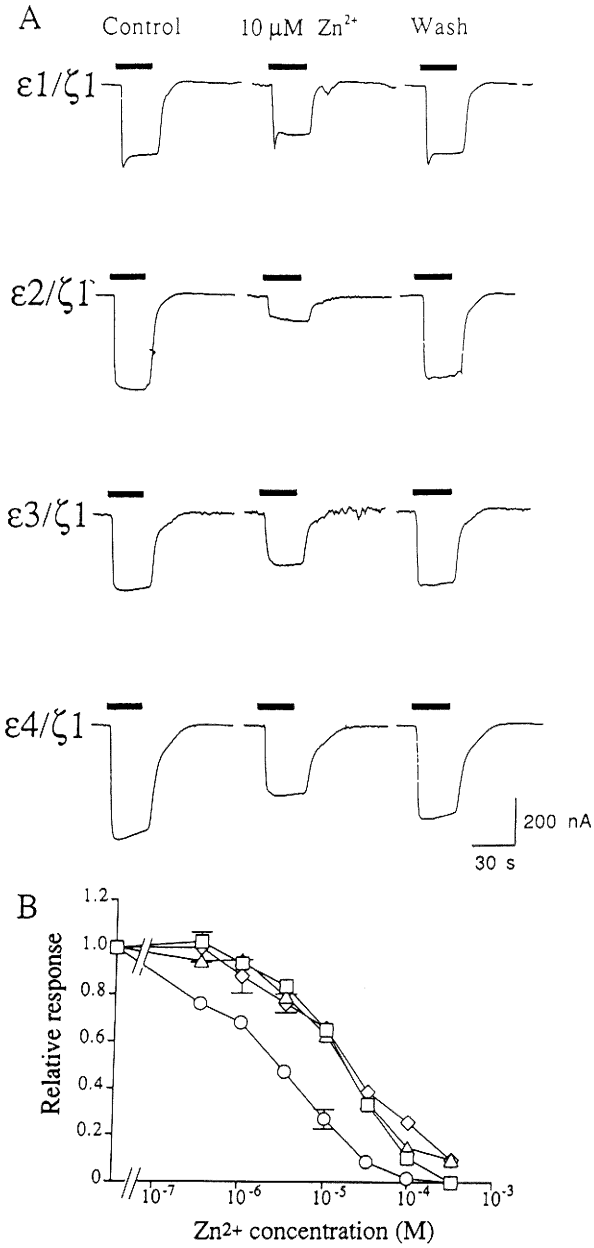

Fig. 5. Effects of extracellular Zn^{2+} on the evoked current induced by 10 μM L-glutamate plus 10 μM glycine in 0.2 mM-Ca^{2+} Ringer's solution in *Xenopus* oocytes expressing four types of the hetermeric NMDA receptor channels. **A.** Effects of 10 μM Zn^{2+} on the traces of the evoked current in the oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. **B.** Effects of Zn^{2+} on the amplitude of the sustained current in the $\varepsilon 1/\zeta 1$ (◇), $\varepsilon 2/\zeta 1$ (○), $\varepsilon 3/\zeta 1$ (△) and $\varepsilon 4/\zeta 1$ (□) channels. The evoked current in each oocyte was measured at only one of the indicated concentrations of Zn^{2+}. Data have been obtained from 3-17 oocytes.

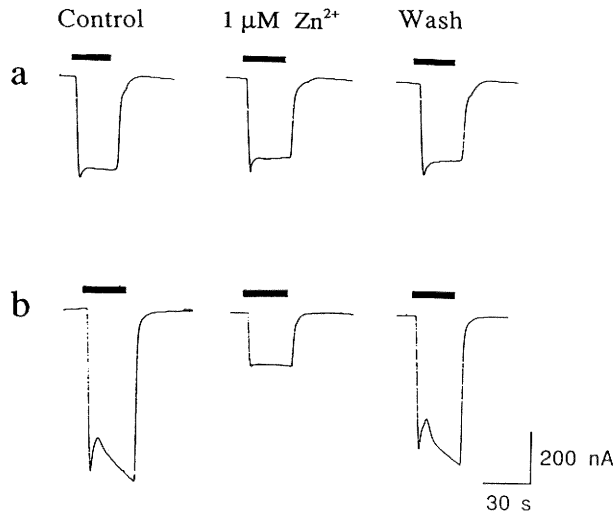

Fig. 6. Effects of 1 μM Zn^{2+} on two types of the sustained current induced by 10 μM L-glutamate plus 10 μM glycine in *Xenopus* oocytes expressing the $\varepsilon 1/\zeta 1$ channels. **(a)** 1 μM Zn^{2+} show little effect on the flat type of the sustained current. **(b)** The gradually increasing type of the sustained current is profoundly inhibited and its profile shows a plateau in the presence of 1 μM Zn^{2+}.

solution containing 0.2 mM Ca^{2+} are shown in Fig. 4A. The initial spike-like phase was suppressed by Mg^{2+}. The sustained phase was blocked by Mg^{2+} in a concentration-dependent manner in oocytes expressing all of the heteromeric channels. The $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels were more resistant to Mg^{2+} than the $\varepsilon 1/\zeta 1$ and $\varepsilon 2/\zeta 1$ channels. There were no significant differences in the sensitivity to Mg^{2+} between the $\varepsilon 1/\zeta 1$ and $\varepsilon 2/\zeta 1$ channels, nor between the $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels (Fig. 4B). The amplitude of the sustained current in oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels in 0.2 mM-Ca^{2+} Ringer's solution were 71-635 nA, 83-683 nA, 101-657 nA and 81-641 nA, respectively.

Effects of Zn^{2+} on the evoked current

Profiles of the evoked current in oocytes expressing the heteromeric NMDA channels in the presence of various concentrations of extracellular Zn^{2+} are shown in Fig. 5A. The sustained phase was suppressed by Zn^{2+} in a concentration-dependent manner in oocytes expressing all of the heteromeric NMDA channels. The $\varepsilon 2/\zeta 1$ channel was more sensitive to inhibition by Zn^{2+} than the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. There were no significant differences in sensitivities among the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels at a Zn^{2+} concentration of 10^{-6} to 10^{-4} M (Fig. 5B). The ampli-

tude of the sustained phase in oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels in 0.2 mM-Ca^{2+} Ringer's solution were 73-657 nA, 124-671 nA, 130-642 nA and 131-601 nA, respectively.

As described above, the sustained phase in oocytes expressing the $\varepsilon 1/\zeta 1$ channel gradually increased in two thirds of the cases (Fig. 1C). This gradual increase in the sustained current was profoundly suppressed by Zn^{2+} even at 1 μM (Fig. 6). However, the flat sustained current was little suppressed at this concentration.

DISCUSSION

Profiles of whole-cell currents in response to the glutamate plus glycine stimulation of oocytes expressing heteromeric NMDA channels showed two distinct phases. For each channel type, the initial spike-like phase of the evoked current was increased by extracellular Ca^{2+} in a concentration-dependent manner and depressed by an intracellular injection of EGTA. The profiles of the evoked current in EGTA-injected oocytes were quite similar to those observed in Ba^{2+}-Ringer's solution. Therefore, the first spike-like component may be induced by a rise in intracellular Ca^{2+}, which in turn may be due to a Ca^{2+}-influx caused by the activation of NMDA channels.

Xenopus oocytes are known to have at least two types of endogenous Ca^{2+}-dependent Cl^- conductance: one type shows a fast onset and is desensitized quickly, thus producing a spike-like phase in evoked current; the other is activated more slowly, producing a sustained phase.[29,30] Ca^{2+}-influx through heteromeric NMDA receptor channels may induce an intrinsic Ca^{2+}-dependent Cl^- current and result in the addition of these Cl^- currents to the inward whole-cell current.[29] Boton et al.[30] reported that when the fast Cl^- conductance was completely suppressed by the injection of EGTA, more than 80% of the slowly activated Cl^- conductance was also inhibited. In the present experiment, the sustained phase was insensitive to EGTA injection of oocytes that expressed the $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$ and $\varepsilon 3/\zeta 1$ channels. More than 70% of the amplitude of the sustained current induced by activation of the $\varepsilon 4/\zeta 1$ channel was maintained after EGTA injection. These findings indicate that the contribution of the slowly activated Cl^--conductance may be negligible in the sustained current induced by the activation of $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$ and $\varepsilon 3/\zeta 1$ channels. Although the cause of the slight inhibition in the current induced by stimulation of the $\varepsilon 4/\zeta 1$ channel is not clear, most of the current may not be produced by the slowly activated Cl^--conductance. Therefore, it is conceivable that the sustained phase of the whole-cell current induced by glutamate plus glycine stimulation primarily reflects the inward current through heteromeric NMDA channels.

It has been reported that both Ca^{2+} and Na^+ are permeable through the NMDA receptor channel.[5-7] In this experiment, however, the inward current through the NMDA receptor channel was thought to be mainly due to Na^+-conductance, since the membrane potential of oocytes was held at -70 mV and the extracellular Ca^{2+} concentration was 0.2 mM. We observed that extracellular Ca^{2+} inhibited the current through the heteromeric NMDA channels in a concentration-dependent manner, except for the $\varepsilon 2/\zeta 1$ channel, and that more than 60% of the current decreased in oocytes expressing the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels at 6.0 mM-Ca^{2+}. It would be unreasonable for the Ca^{2+} current through the heteromeric channels to decrease at higher extracellular Ca^{2+} concentrations. Therefore, extracellular Ca^{2+} may suppress Na^+ current through the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. Only the current through the $\varepsilon 2/\zeta 1$ channel was not suppressed by extracellular Ca^{2+}, being instead rather increased at 6.0 mM-Ca^{2+}.

The effect of extracellular Ba^{2+} on the current in heteromeric NMDA receptor channels was similar to that of extracellular Ca^{2+}. Ba^{2+} also suppressed currents in the $\varepsilon 1/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels in a concentration-dependent manner, but not in the $\varepsilon 2/\zeta 1$ channel. The similar effects of Ca^{2+} and Ba^{2+} on heteromeric NMDA channels suggest that these divalent cations possess a similar inhibitory mechanism. Moreover, the fact that the $\varepsilon 2/\zeta 1$ channel was resistant to both extracellular Ca^{2+} and Ba^{2+} indicates that there may be no functinal site affected by Ca^{2+} and Ba^{2+} in the $\varepsilon 2/\zeta 1$ channel.

It has been reported that the heteromeric NMDA channels expressed in the *Xenopus* oocyte have different sensitivities to Mg^{2+} depending on their ε subunits.[20,23] These observations were made in 2.0 mM-Ba^{2+} Ringer's solution. However, Ba^{2+} is not a physiological divalent cation. In the present study, the responses of four types of heteromeric NMDA channels to various concentrations of Mg^{2+} were observed in a Ca^{2+} containing solution, although the concentration of Ca^{2+} was prepared at 0.2 mM to minimize the influence of Ca^{2+}-induced Cl^- current and to easily maintain the membrane potential at -70 mV. The glutamate plus glycine-induced currents in oocytes expressing the $\varepsilon 1/\zeta 1$ and $\varepsilon 2/\zeta 1$ channels were strongly suppressed by extracellular Mg^{2+}, whereas the $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels were less sensitive to Mg^{2+}. Accordingly, heteromeric NMDA receptor channels may be categorized into two groups based on their

sensitivities to Mg^{2+}. This categorization in 0.2 mM-Ca^{2+} Ringer's solution is basically similar to that in 2.0 mM-Ba^{2+} Ringer's solution.[20,23]

While inhibition of the NMDA receptor by Mg^{2+} is voltage-dependent, that by Zn^{2+} has been shown to be mainly voltage-independent.[8,9,12,13] The whole-cell current in oocytes that expressed the $\varepsilon 2/\zeta 1$ channel was profoundly inhibited by Zn^{2+} in 0.2 mM-Ca^{2+} Ringer's solution, while the $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels were less sensitive to Zn^{2+}. Similar differences in sensitivity to Zn^{2+} have also been found between the $\varepsilon 2/\zeta 1$ and $\varepsilon 3/\zeta 1$ channels in 2.0 mM-Ba^{2+} Ringer's solution.[23]

The flat sustained current in response to glutamate plus glycine stimulation in the $\varepsilon 1/\zeta 1$ channel was somewhat resistant to inhibition by Zn^{2+}, as well as the $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. On the other hand, the gradually increasing current in the sustained phase, which was observed in two thirds of the oocytes expressing the $\varepsilon 1/\zeta 1$ channel, was profoundly suppressed even at low doses of Zn^{2+}(1 μM), and the profiles in the sustained phase became a plateau (Fig. 6). Therefore, it is conceivable that the sustained phase in the $\varepsilon 1/\zeta 1$ channel consists of two components: a flat current with low sensitivity to Zn^{2+}, and a gradually increasing current with high sensitivity to Zn^{2+}. The proportion of the latter component may vary among oocytes. This gradually increasing current is not likely to be Ca^{2+}-dependent, since it was observed even after intracellular EGTA injection and in 2.0 mM-Ba^{2+} Ringer's solution (data not shown). Although the mechanism of the changes in inward current is not yet clear, these changes may occur through Ca^{2+}-independent processes.

The CNS, especially the hippocampus, cerebral cortex and cerebellum, is known to contain a large amount of Zn^{2+}.[31] It has also been reported that Zn^{2+} is present in synaptic vesicles and is co-released with neurotransmitters during excitation of the neurons, and acts as a modulator of synaptic transmission.[14-17] On the other hand, NMDA receptor subunits have been shown to follow distinct distribution patterns in the CNS by *in situ* hybridization studies.[20-22,32-34] The $\varepsilon 1$ subunit is widespread throughout the cerebrum and cerebellum, especially in the cerebral cortex and hippocampus. Interestingly, the $\varepsilon 1$ subunit shows a distribution that is quite similar to that of Zn^{2+} in the CNS. Moreover, Zn^{2+} is known to accumulate in the brain in the postnatal stage, when the $\varepsilon 1$ subunit-specific mRNA predominantly increases.[35] This similarity in the distribution and developmental course of the $\varepsilon 1$ subunit and Zn^{2+} suggests that Zn^{2+} is closely related to the function of the $\varepsilon 1$ subunit, and modulates synaptic transmission in these areas. The current in the $\varepsilon 2/\zeta 1$ channel is also strongly inhibited by Zn^{2+}. The $\varepsilon 2$ subunit is selectively expressed in the forebrain and is abundant in the cerebral cortex and hippocampus, where long-term potentiation (LTP) has been observed. Accordingly, it is considered that Zn^{2+} may act as a potent modulator of synaptic transmission.

On the other hand, the $\varepsilon 3$ subunit is predominant in the cerebellum, especially in the granular cell layer, and the $\varepsilon 4$ subunit is expressed in the diencephalon and brain stem. The heteromeric $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels shannels show similar responses to divalent cations, and currents through these channels are generally inhibited by divalent cations, although their sensitivities to Mg^{2+} and Zn^{2+} are relatively low. However, the roles of the $\varepsilon 3$ and $\varepsilon 4$ subunits are still unclear.

Recent studies have shown that an aspargine residue in the putative transmembrane segment M2 in the ε and ζ subunits is the functional site that determines the Ca^{2+}-permeability, the sensitivity to Mg^{2+}, and, to some extent, the sensitivity to Zn^{2+}, of the NMDA receptor channel and distinguishes it from the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor.[23,36,37] However, differences in channel activity to Ca^{2+} or Ba^{2+} between the $\varepsilon 1/\zeta 1$ and $\varepsilon 2/\zeta 1$ channels cannot be explained in terms of the sequences in the M2 region, since the $\varepsilon 1$ and $\varepsilon 2$ subunits have the same sequence of amino acids in this region.[20] Therefore, the M2 region may not be the functional site for blocking by Ca^{2+} or Ba^{2+}. Other sites, which are sensitive to divalent cations, may exist in another location and determine the effects of Ca^{2+} or Ba^{2+} on ε subunits.

In summary, the present study has comprehensively characterized the effects of divalent cations on heteromeric NMDA channels expressed on *Xenopus* oocytes. The initial spike-like phase in the whole-cell current can be ascribed to Ca^{2+}-dependent Cl^- conductance induced by Ca^{2+}-influx through heteromeric NMDA channels. The flat sustained phase, which is thought to reflect the activity of the heteromeric channels, was generally suppressed by divalent cations. Only the $\varepsilon 2/\zeta 1$ channel was not inhibited by Ca^{2+} or Ba^{2+}. On the other hand, the $\varepsilon 2/\zeta 1$ channel was more sensitive to Mg^{2+} and Zn^{2+} than the $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ channels. The gradually increasing current in the sustained phase observed in two thirds of the oocytes expressing the $\varepsilon 1/\zeta 1$ channel was very sensitive to Zn^{2+}. The different sensitivities to divalent cations may be closely related to the physiological significance of the molecular diversity of the NMDA receptor.

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