Competitive Inhibition of NMDA Receptor–Mediated Currents by Extracellular Calcium Chelators

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Calcium chelators have been widely used in electrophysiological recordings of N-methyl-D-aspartate (NMDA) receptor–mediated currents, as well as in studies of excitotoxicity. Intracellularly applied calcium chelators are known to inhibit, at least in part, such calcium-dependent processes as calmodulin-dependent inactivation, calscein-dependent desensitization, and rundown of NMDA receptors. On the other hand, the functional consequences and potential nonspecific effects of extracellularly applied chelators have not been extensively investigated. In whole-cell patch-clamp recordings from human embryonic kidney (HEK) 293 cells transiently transfected with recombinant NMDA receptors, we found that addition of calcium chelators such as EGTA shifted the glutamate dose-response curve to the right, from an EC50 for NR1A/NR2A of 8 μM in 1.8 mM Ca2+ to ~24 μM in a solution containing nominal 0 Ca2+/5 mM EGTA and further to ~80 μM in 20 mM EGTA. A similar shift in glutamate dose-response was observed for NR1A/NR2B currents. This dose-response shift was not due to a decrease in extracellular Ca2+ concentration because there was no change in the glutamate EC50 at Ca2+ concentrations ranging from 10 mM to nominal 0/200 μM EGTA. Moreover, addition of 5 mM EGTA fully chelated with 6.8 mM Ca2+ did not produce any shift in the glutamate dose-response curve. We propose that calcium chelators, containing four free carboxy moieties, competitively inhibit glutamate binding to NMDA receptors.

INTRODUCTION

N-methyl-D-aspartate (NMDA)-type glutamate receptors play a key role in synaptogenesis, synaptic plasticity, and some neuropathological conditions, largely due to the receptor’s high permeability to Ca2+ (Dingledine et al. 1999; Lipton and Rosenberg 1994; McBain and Mayer 1994). In the CNS, free Ca2+ levels both inside and outside of cells are highly regulated. The extracellular Ca2+ concentration under physiological conditions is approximately 2 mM (Ghosh and Greenberg 1995) but can drop as low as 0.5 mM during periods of intense neuronal activity (e.g., Heinemann et al. 1977).

Calcium chelators have been widely used to investigate the role of free Ca2+ on NMDA receptor function. For example, buffering intracellular Ca2+ concentration by perfusing 10 mM EGTA or 10 mM bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA) via the recording pipette helped to reduce inactivation (Legendre et al. 1993), indicating inactivation is Ca2+-dependent, a result further supported by biochemical analysis and patch-clamp recordings (Ehlers et al. 1996; Hiatsune et al. 1997; Krupp et al. 1996, 1999; Wyszynski et al. 1997; Zhang et al. 1998). This effect of Ca2+ on NMDA receptor–mediated currents occurs rapidly since 10 mM BAPTA, a much more rapid and selective Ca2+-chelator, was shown to be more effective in preventing inactivation (Legendre et al. 1993). Reducing extracellular Ca2+ could also inhibit inactivation, suggesting that the effect of intracellular Ca2+-chelators was most likely due to chelating Ca2+ rather than directly acting on NMDA receptors (e.g., Legendre et al. 1993). Similarly, experiments in which Ca2+-chelators were applied intracellularly helped demonstrate that NMDA receptor–mediated rises in intracellular Ca2+ cause the depolymerization of the actin cytoskeleton and rundown of NMDA receptor–mediated peak current (Rosenmund and Westbrook 1993a,b; for review, McBain and Mayer 1994). Therefore, Ca2+-chelators are routinely included in intracellular recording solutions to ensure stable NMDA receptor–mediated current by inhibiting inactivation and rundown. Ca2+-chelators are also included in extracellular recording solutions for whole-cell patch-clamp recording (e.g., Zhang et al. 1998) and especially for inside-out and cell-attached single channel recordings (e.g., Gibb and Colquhoun 1992) to control Ca2+ level and achieve long-lasting recordings. As well, extracellular Ca2+-chelators have been needed to help define the role of Ca2+ in glutamate-induced excitotoxicity (e.g., Murphy et al. 1988).

NMDA receptors are most likely a tetrameric complex of two NR1 subunits in combination with NR2A, NR2B, NR2C, and/or NR2D (reviewed by Dingledine et al. 1999). Expression of these receptors in heterologous systems has been a fruitful approach to characterizing their structure, function, and modulation (Dingledine et al. 1999). In this report, we have investigated the effects of extracellular Ca2+ on NMDA receptor function. As previously reported by others, we show large potentiation of NMDA receptor–mediated currents with decreasing concentrations of added extracellular Ca2+. Surprisingly, we have found that Ca2+-chelators such as EGTA act as competitive antagonists and shift the glutamate dose response curve to the right. These results have important implications in

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interpreting data from experiments in which external Ca\(^{2+}\) concentration is manipulated by chelators such as EGTA.

**Methods**

Cell culture and transfection

Human embryonic kidney 293 (HEK 293) cells (CRL 1573; ATCC, Rockville, MD) were plated on 10-cm culture dishes (Falcon, Becton Dickson, Franklin Lakes, NJ) and maintained at 37°C in humidified 95% O\(_2\)-5% CO\(_2\) as described previously (Chen et al. 1997). Culture medium was prepared from minimal essential medium (MEM; Life Technologies, Burlington, Ontario, Canada) and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 mM sodium pyruvate (GIBCO, Canadian Life Technologies, Burlington, Ontario, Canada), 100 U/ml penicillin-streptomycin (GIBCO), and 1 mM glutamine. For transient transfection using the calcium-phosphate method (Chen and Okayama 1987), the cDNA plasmid ratio for NR1A and NR2 subunits was 1:1 with a total amount of 10 \(\mu\)g. Cells were transfected in humidified 97% O\(_2\)-3% CO\(_2\) at 37°C for 6–8 h. After transfection, cells were transferred onto glass coverslips in 35-mm culture dishes (Falcon). The NMDA receptor antagonist (+)-2-amino-5-phosphono pentanoic acid (APV, 1 mM; Research Biochemicals, Natick, MA) and/or memantine (100 \(\mu\)M; Research Biochemicals) were added to the medium to enhance cell survival (Chen et al. 1997; Raymond et al. 1996).

Electrophysiology

Approximately 20–36 h after transfection, cells on coverslips were transferred to the recording chamber on the stage of an inverted microscope (Axiovert 100, Carl Zeiss, Thornburg, NY). Extracellular solution, which contained 145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 11 mM glucose, and 10 mM HEPES and titrated with NaOH to pH 7.4, flowed constantly through the chamber. Intracellular solution contained 145 mM KCl, 5.5 mM BAPTA, 4 mM adenosine 5’-triphosphate magnesium salt (MgATP), and 10 mM HEPES, titrated to pH 7.25 with KOH. Recording pipettes were pulled from borosilicate glass (Warner Instruments, Hamden, CT) with the Narishige (Tokyo) PP-83 electrode puller. Pipettes with open tip resistance of ~1–5 M\(\Omega\) were used.

Agonist-evoked currents were recorded using the patch-clamp technique in the whole-cell voltage clamp configuration with a holding potential of −60 mV. After forming the whole-cell recording configuration, the cell was lifted from the chamber floor. Agonists were applied rapidly by piezo-controlled switching of a tube, which was positioned ~100 \(\mu\)m from the cell. Solutions were gravity fed through the two sides of the \(\theta\) tube. The 10–90% rise time of solution exchange at the open pipette tip was ~0.5 ms (Chen et al. 1999). Currents were filtered at 5 kHz and sampled at 2 kHz using pCLAMP6 software and the Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Analyses were conducted with Clampfit (Axon Instruments) and Origin (Microcal Software, Northampton, MA) software.

Chemicals and plasmid CDNs

All chemicals were from Sigma (St. Louis, MO) unless otherwise stated. NR1A (also known as NR1–1α; Hollmann et al. 1993) and NR2B cDNAs were gifts from Dr. S. Nakanishi, Kyoto University, Kyoto, Japan. NR2A (from mouse brain, also known as e1) was a gift from Dr. M. Mishina, University of Tokyo, Tokyo. These cDNAs were subcloned into eukaryotic expression vectors with the cytomegalovirus promoter, as described (Chen et al. 1997; Raymond et al. 1996).

**Results**

Reducing extracellular Ca\(^{2+}\) concentration potentiates NMDA receptor–mediated current

At physiological extracellular Ca\(^{2+}\) concentrations, the Ca\(^{2+}\) influx resulting from NMDA receptor activation induces partial pore block, Ca\(^{2+}\)-dependent inactivation, and peak current rundown (Ascher and Nowak 1988; Ehlers et al. 1996; Hisatsune et al. 1997; Krupp et al. 1996, 1999; Legendre et al. 1993; Rosenmund and Westbrook 1993a,b; Wyllie et al. 1996; Zhang et al. 1998). We applied 1 mM glutamate and 50 \(\mu\)M glycine for 2 s at intervals of 30 s and a holding potential of −60 mV.

![Figure 1](https://example.com/figure1.png)
−60 mV, while recording from NR1A/NR2A- or NR1A/NR2B-transfected cells in the whole-cell mode. Within seconds of reducing extracellular Ca\(^{2+}\) concentration by switching from solutions containing 1.8 to 0.2 mM, then nominal zero, then 5 mM EGTA, we observed a graded increase of the peak current amplitudes mediated by both NR1A/NR2A (Fig. 1A) and NR1A/NR2B (data not shown). In 5 mM EGTA, peak current amplitudes were approximately sixfold larger than those recorded in control conditions (1.8 mM Ca\(^{2+}\)) for both subtypes [5.8 ± 2.1-fold, n = 7 for NR1A/NR2A (Fig. 1A) and 6.3 ± 0.8-fold, n = 5 for NR1A/NR2B subtypes].

Zn\(^{2+}\) has been shown to be a potent NMDA receptor antagonist and Zn\(^{2+}\) contamination in the nanomolar range in the recording solution can inhibit NR1A/NR2A-type NMDA receptor–mediated current significantly (Chen et al. 1997; Paolelli et al. 1997; Williams 1996). Therefore, we investigated whether the potentiation of NR1A/NR2A-mediated currents observed on switching solution to 5 mM EGTA with no added Ca\(^{2+}\) was partly contributed by the relief of Zn\(^{2+}\) block. Addition of the Zn\(^{2+}\) chelator N,N,N’,N’-tetakis(2-pyridylmethyl)-ethylendiamine (TPEN) (1 μM) in the extracellular recording solution with 1.8 mM Ca\(^{2+}\) caused an increase in current amplitude to 1.6 ± 0.2-fold of control (P < 0.001 by paired t-test; n = 5), consistent with previously reported results (Paolelli et al. 1997). Thus, the effect of decreasing extracellular zinc accounted for only ~25% of the increase in peak amplitude for NR1A/NR2A-mediated currents observed in recording solution containing Ca\(^{2+}\) chelators and played no role in the potentiation observed for NR1A/NR2B-mediated currents (since the IC\(_{50}\) for zinc inhibition is in the micromolar range; Chen et al. 1997; Paolelli et al. 1997; Williams 1996).

We then recorded currents from NR1A/NR2A-transfected cells evoked by a subsaturating concentration of glutamate (10 μM, with 50 μM glycine). Under these conditions, we observed the same trend of increasing current amplitude with decreasing concentration of added extracellular calcium (Fig. 1B). However, with the addition of 5 mM EGTA (with no added Ca\(^{2+}\)) to the extracellular solution, we made two surprising observations. The first was that the current amplitude decreased significantly compared with the no added calcium condition (Fig. 1B). The second was that the current activation time course was significantly slowed (Fig. 1B). The 10–90% rise time was 34.5 ± 1.6 ms in 1.8 mM Ca\(^{2+}\) and 81.4 ± 9.5 ms in 5 mM EGTA with no added Ca\(^{2+}\) (P < 0.01, paired t-test; n = 6). Recordings from NR1A/NR2B-transfected cells also revealed significant slowing of the activation time course in 5 mM EGTA/no added Ca\(^{2+}\) to 126 ± 13 ms from 65.4 ± 7.2 ms in 1.8 mM Ca\(^{2+}\) (P < 0.01, paired t-test; n = 5). As well, apparent desensitization was abolished (Fig. 1B). The slowed rise time, decreased peak amplitude, and lack of apparent desensitization of glutamate-evoked current observed on addition of EGTA suggested to us that NMDA receptors might exhibit decreased sensitivity to agonist under these conditions.

Glutamate dose-response curve is shifted to the right in extracellular solution containing 5 mM EGTA/no added Ca\(^{2+}\)

To test the possibility that extracellular Ca\(^{2+}\) modulates the glutamate EC\(_{50}\), we generated glutamate dose-response curves for both NR1A/NR2A and NR1A/NR2B in the presence of 1.8 mM extracellular Ca\(^{2+}\) or no added Ca\(^{2+}\) plus 5 mM EGTA (Fig. 2). Consistent with our prediction, glutamate dose-response curves of both subtypes were shifted significantly to the right for 5 mM EGTA/no added Ca\(^{2+}\) compared with 1.8 mM extracellular Ca\(^{2+}\) (Fig. 2). The glutamate EC\(_{50}\) for NR1A/NR2A was 8.1 ± 0.6 and 24.0 ± 0.7 μM for extracellular Ca\(^{2+}\) of 1.8 mM versus 5 mM EGTA with no added Ca\(^{2+}\), respectively. For NR1A/NR2B, the glutamate EC\(_{50}\) was 1.6 ± 0.1 and 3.6 ± 0.6 μM for the same two conditions. Since the glutamate dose-response curve shift was observed under conditions of very low extracellular Ca\(^{2+}\) concentration and in the presence of the Ca\(^{2+}\) chelator EGTA, we next addressed whether the shift was an effect of extracellular Ca\(^{2+}\) or EGTA.

Extracellular Ca\(^{2+}\) concentration does not modulate the glutamate dose-response using cells transfected with NR1/NR2A

We constructed a series of glutamate dose-response curves under conditions in which we varied either extracellular Ca\(^{2+}\) or EGTA concentration (Fig. 3). In the absence of added Ca\(^{2+}\), contamination of standard extracellular recording solutions by this ion has been estimated in the range of 20 μM (Hoth 1995; Xiong et al. 1997). Therefore, if the rightward shift of the glutamate dose response curve was due to elimination of extracellular calcium, we would predict a similar shift with a lower but effective concentration of EGTA (200 μM with no added Ca\(^{2+}\), as we observed for 5 mM EGTA/no added Ca\(^{2+}\). Instead, the glutamate dose-response curve in the presence of
Our major finding is that Ca\(^{2+}\) chelators are competitive antagonists at the glutamate-binding site of NMDA receptors. This conclusion is based on three observations. First, the decrease in current amplitude evoked by submaximal glutamate concentrations observed in the presence of 5 mM EGTA was associated with a slowing in rise-time to peak and lack of glutamate-induced desensitization, typical of responses to lower concentrations of glutamate (e.g., see Fig. 1). Second, the glutamate dose-response curve was shifted to the right by EGTA in a dose-dependent manner, without a decrease in the maximal response to saturating concentrations (i.e., 1 mM) of glutamate. Third, the effect of calcium chelators on the glutamate dose-response was not a result of altering extracellular Ca\(^{2+}\) concentration, since Ca\(^{2+}\) concentrations in the range of ~20 \(\mu\)M up to 1.8 mM had no effect on glutamate potency. Furthermore, the amount of chelator necessary to produce a change in the glutamate affinity is several hundred-fold in excess of what is required to effectively chelate any residual extracellular Ca\(^{2+}\) (~20 \(\mu\)M Ca\(^{2+}\) and 5 mM EGTA). Consistent with our findings, Gu and Huang (1994) also reported no effect of extracellular Ca\(^{2+}\) on tritium NMDA receptor dose-response to NMDA.

Xiong et al. (1997) demonstrated that a transient decrease in extracellular Ca\(^{2+}\) concentration alone could evoke currents in patch-clamp recordings from cells ranging from neurons to HEK 293 cells. These currents were mediated by a cation selective, Ca\(^{2+}\) sensing channel. Our results could not be complicated by that effect because extracellular Ca\(^{2+}\) was maintained at a constant level when switching between control and agonist-containing solutions to evoke NMDA receptor-mediated currents. In addition, although a decrease in extracellular Ca\(^{2+}\) concentration would alter charge-shielding effects on the membrane surface, this effect would be predicted to decrease, rather than increase, current amplitude through the cation-selective NMDA receptor channel.

Recent studies suggest that there may be differences in mechanisms of modulation of recombinant versus native neuronal NMDA receptors, since tyrosine phosphorylation by src affects zinc inhibition of recombinant (NR1A/NR2A- and NR1A/NR2B-type) but not native neuronal NMDA receptors (Xiong et al. 1999). However, this interaction depends on alterations made to receptor structure by intracellular proteins or second messengers, which may differ between neurons and the nonneuronal cells used to analyze recombinant receptors. On the other hand, results of studies restricted to correlations between structure (e.g., subunit composition) and binding/gating properties of recombinant NMDA receptors have been remarkably informative with regard to native neuronal NMDA receptors (for review, see Dingledine et al. 1999). Therefore, we predict that our finding of competitive inhibition of glutamate binding by EGTA for recombinant NMDA receptors can be extended to native neuronal NMDA receptors composed of NR1A, NR2A, and/or NR2B subunits.

The fact that EGTA acts as a competitive antagonist for glutamate binding is not a complete surprise, since EGTA has two pairs of carboxyl residues, whereas glutamate has one pair. Recordings made in the presence of other extracellular Ca\(^{2+}\) chelators that contain free carboxyl groups, including EDTA and BAPTA, showed similar shifts in the NMDA receptor glutamate dose-response curve (Chen, unpublished data). Moreover, effects consistent with competitive inhibition by calcium chelators were also observed for glutamate-evoked currents mediated by the non-NMDA receptors GluR2 and GluR6 (Chen, unpublished data). These results suggest that
inclusion of Ca\(^{2+}\) chelators in the extracellular solution during patch-clamp recording or excitotoxicity experiments will have an effect other than lowering Ca\(^{2+}\) concentration, complicating the interpretation of data.

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