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The calcium-dependent transient inactivation of recombinant NMDA receptor-channel does not involve the high affinity calmodulin binding site of the NR1 subunit

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Abstract

N-methyl-d-aspartate (NMDA) receptor function can be regulated by direct binding of calmodulin to a low and high affinity (C1 exon cassette) site in the C-terminal region of the NR1 subunit. To evaluate the involvement of the high affinity binding site in the transient inactivation of the NMDA receptor-channels by intracellular calcium, several splice variants of the NR1 subunit have been individually co-transfected with the NR2A subunit in HEK 293 cells. The transient Ca^{2+} induced inactivation (40–50%) of the heteromeric receptors was similar whether the NR1 variants contained (NR1–1a, 1b) or lacked (NR1–2a, 2b, 4a, 4b) the C1 exon cassette bearing the high affinity binding site for calmodulin. This demonstrates that this site is not involved in the Ca^{2+} dependent transient inactivation of NMDA receptors. © 1997 Elsevier Science Ireland Ltd.

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The transient inactivation of *N*-methyl-d-aspartate (NMDA) receptor channels by increased intracellular calcium concentration ($[Ca^{2+}]_i$) is a general property found in both native [4,6,8,12,13] and recombinant [9] receptors. The mechanisms of this inactivation as well as the potential site(s) of action of calcium ions on NMDA receptor subunits are yet unknown. Calmodulin is involved in Ca^{2+} inactivation of NMDA receptors and is thus potentially involved in the transient inactivation of these receptors. Indeed, a low and high (C1 exon cassette) affinity binding site for calmodulin have been identified on the C-terminal domain of the NR1 subunit of NMDA receptors [2]. In contrast to the low affinity site which is present on all NR1 splice variants, the C1 exon cassette is present only in some NR1 splice variants [3]. We thus examined the possible involvement of the high affinity calmodulin site in the Ca^{2+} dependent transient inactivation of NMDA receptors by comparing the NR1 splice variants containing (NR1–1a, NR1–1b) or lacking (NR1–2a, NR1–2b, NR1–4a and NR1–4b) the C1 exon cassette. These were individually cotransfected with the NR2A subunit in HEK 293 cells. Our results show that each heteromeric receptor, including those composed of NR1 variants lacking the C1 exon cassette, were transiently inactivated by Ca^{2+}, indicating that this cassette is not involved in Ca^{2+} dependent transient inactivation of NMDA receptor channels.

cDNAs encoding the various NR1 splice variants used in this study (NR1–1a, NR1–1b, NR1–2a, NR1–2b, NR1–4a and NR1–4b, according to the nomenclature of Hollmann and Heinemann [3]) were spliced out from their native pBluescript plasmids (Stratagene) and subcloned into the eukaryotic expression vector pcDNA I/Amp (Invitrogen). Constructs were verified by specific polymerase chain reaction (PCR) reactions amplifying DNA fragments that span exon 5 (encoding the N-terminal insertion of NR1) and exons 21 and 22 (encoding the C-terminal C1 and C2 cassettes), restriction mapping and finally, partial sequencing. Immortalized human embryonic kidney cells (HEK 293) were cultured in 10 mm glass culture wells (1×10^5–2×10^5 cells/well) in complete medium (Dulbecco's modified Eagle's medium, DMEM; Gibco BRL)

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containing 10% calf serum. Transfections were realized using standard phosphate calcium transfection as previously described [1] with mixtures of cDNAs encoding the NR1 and NR2A subunits and green fluorescent protein (GFP). Following 12 h incubation, the cells were rinsed in phosphate-buffered saline (PBS) and fresh complete medium containing 10% calf serum was added. Cells were recorded 24–48 h later.

Transfected cells were visualised using an inverted microscope equipped with a fluorescence unit to detect GFP expression. For the electrophysiological studies, cells were perfused with an external solution containing (in mM): NaCl 140, KCl 1, glucose 10, $CaCl_2$ 2, Na-HEPES 10, glycine 0.02 (pH 7.4). Whole-cell patch-clamp recordings were made from isolated HEK 293 cells using borosilicate glass pipettes with resistances of 2–5 MΩ when filled with the internal solution containing (in mM): CsCl 130, $CaCl_2$ 0.25, EGTA 1.1, Cs-HEPES 10, Mg-ATP 4, creatine phosphate 6, (pH 7.2) $[Ca^{2+}]_i$ $\sim 5 \times 10^{-8}$ M. The calcium-induced inactivation of NMDA receptors was assessed as previously reported [7,9], by measuring changes in the amplitude of the glutamate test currents following $[Ca^{2+}]_i$ increases induced by conditioning applications of glutamate. The test currents were induced by brief (50–150 ms) pulses of glutamate (100 μM) (dissolved in the external solution but containing 0.2 mM calcium) through a micropipette (tip diameter 2 μm) using a picospritzer. Conditioning applications (1–20 s) of glutamate (100 μM) dissolved in the external solution containing 0.2 or 2 mM Ca^{2+} were realised using a fast perfusion system. The degree of calcium-induced inactivation was calculated as $(I_o - I)/I_o$, where I_o is the average amplitude of 3–5 test currents before conditioning applications of glutamate and I, the amplitude of the test current measured 4 s after the end of the conditioning application of glutamate.

Fig. 1 illustrates whole-cell currents recorded from HEK 293 cells expressing NR1–4a/NR2A subunits of the NMDA receptor lacking the high affinity binding site for calmodulin. Brief (50 ms) applications of glutamate through the micropipette every 10 s induced currents with stable amplitude. A long (5 s) application of glutamate in the presence of 0.2 mM $[Ca^{2+}]_o$ induced a slight desensitisation (ratio steady state/peak = 0.84), but had no effect on the test current, elicited 4 s latter by a brief application of glutamate. In contrast, a conditioning appli-

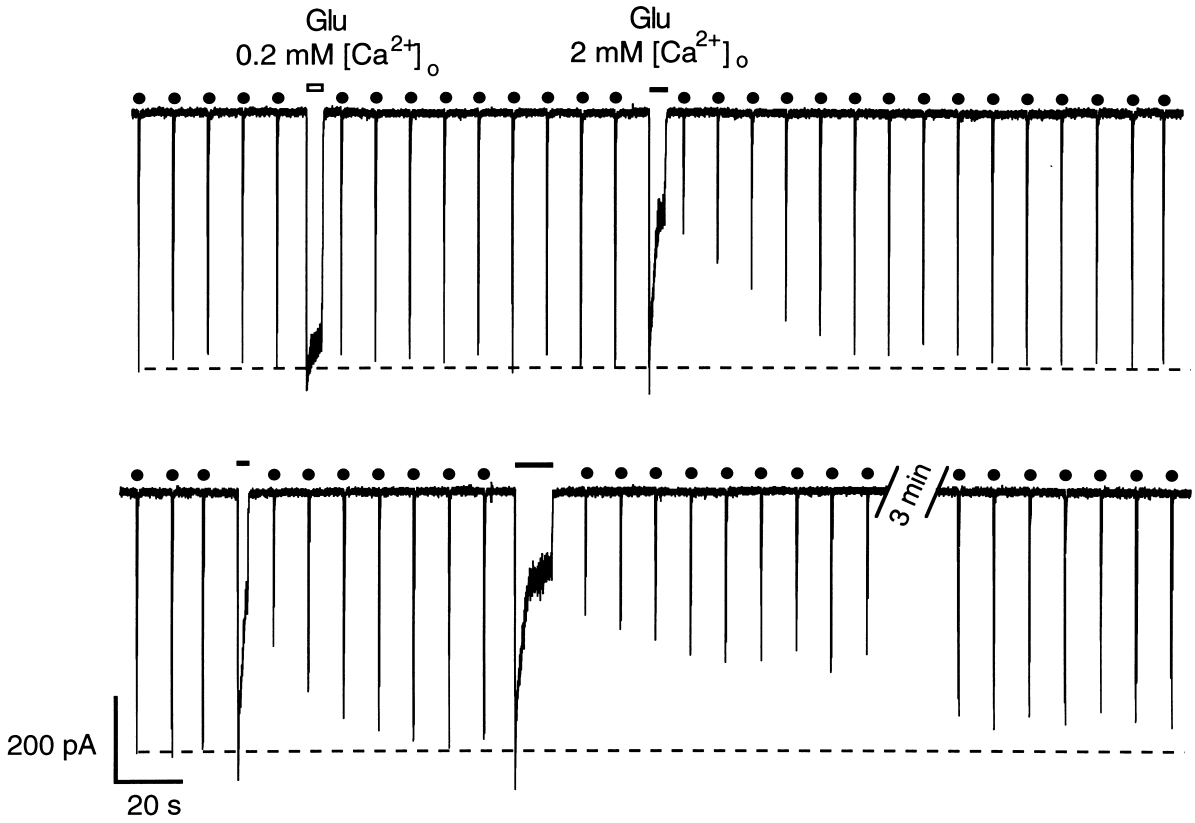

Fig. 1. Transient inactivation of the heteromeric NR1–4a/NR2A NMDA receptor-channels expressed in HEK 293 cells by $[Ca^{2+}]_i$. Filled points mark the applications of the test glutamate pulses (150 ms) using a micropipette approached to a single cell. The application of conditioning pulses of glutamate using the fast perfusion system is shown by open (0.2 mM Ca_o^{2+}) and filled (2 mM Ca_o^{2+}) bars. The duration of glutamate applications with 2 mM Ca_o^{2+} was 5 s (upper trace) and 3 and 12 s (bottom trace). Bottom trace is the prolongation of the current record shown in the upper trace.

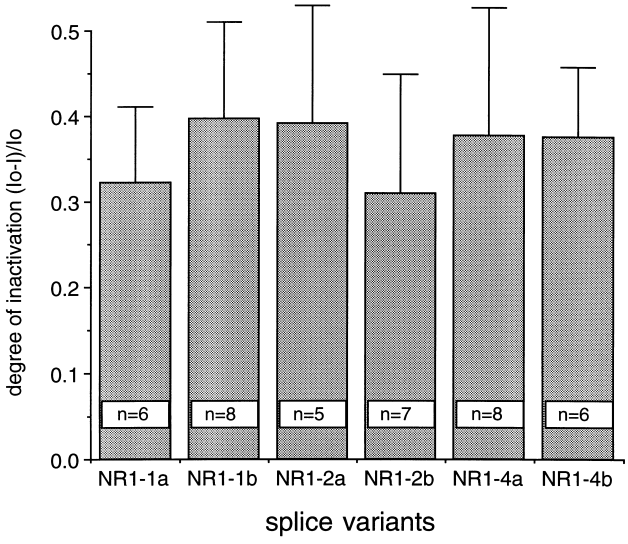

Fig. 2. The mean degree of transient inactivation induced by conditioning applications of glutamate (charge more than 1 nC) studied for different splice variants. The degree of inactivation was measured as explained in the text and as reported previously [7,9]. All results are presented as mean values ± SD. The values obtained for each of the splice variants were not significantly different. Significance was tested using Student's *t*-test with level 5%.

cation (5 s) of glutamate in the presence of 2 mM Ca^{2+}, induced a higher desensitization (ratio steady state/peak = 0.35) and decreased by 50% the amplitude of the test response measured 4 s latter. The time of recovery of NMDA receptor channels from inactivation depended on the duration of the conditioning applications of glutamate (i.e. the amount of calcium influx through the NMDA receptor channel). Thus, the times of the recovery of NMDA test pulses from inactivation increased from 50 to 60 s and to more than 270 s with increases of charge ranging from 0.9 to 1.5 and 3 nC, respectively (Fig. 1). Taken together, these results demonstrate that $[Ca^{2+}]_i$ transiently inactivates NR1–4a/NR2A receptor currents. Similar effects of conditioning applications of glutamate in the presence of 2 mM Ca^{2+} in the external solution were observed with the other NR1 splice variants NR1–4b, NR1–2a and NR1–2b, which also lack the high affinity site for calmodulin. Furthermore, there was no significant difference in the sensitivity to $[Ca^{2+}]_i$ between receptors containing these subunits and the NR1–1a/NR2A and NR1–1b/NR2A receptors containing the C1 exon cassette (Fig. 2).

The mechanism of the transient inactivation still remains to be clarified but it should be emphasized that this mechanism could be distinct from the other actions of $[Ca^{2+}]_i$ on NMDA receptors. The NMDA channel can be modulated by several mechanisms including a calcium-calcineurine-dependent dephosphorylation [5,11] an interaction with the cytoskeleton [10] or via the interaction of calmodulin with the NR1 subunit at two distinct binding sites in the C-terminal region [2]. The transient inactivation of NMDA channels observed in neurones is different from the modulation induced by phosphorylation and elements of the cytoskeleton [7]. In the present study we have also excluded the possibility that the high affinity binding site for calmodulin on NR1 [2] could participate in the transient inactivation of NMDA receptors by $[Ca^{2+}]_i$. The implication in this process of the low affinity calmodulin binding site on NR1 remains to be analyzed as well as the contribution of the NR2 subunits.

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