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Report

Evidence That Xenon Does Not Produce Open Channel Blockade of the NMDA Receptor

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Weigt HU, Adolph O, Georgieff M, Georgieff EM, Föhr KJ. Evidence that Xenon does not produce open channel blockade of the NMDA receptor. *J Neurophysiol* 99: 1983–1987, 2008. First published January 30, 2008; doi:10.1152/jn.00631.2007. Previous studies had not excluded the possibility that the mechanism by which Xenon (Xe) blocks *N*-methyl-D-aspartate (NMDA) receptors might be that of an open-channel blocker. We tested this possibility on mutant NMDA receptors carrying an alanine (A) to cysteine (C) mutation located within the SYTANLAAF-motif of the third transmembrane region (TM3). This mutation was shown to yield constitutively open ion channels after modification with a thiol-modifying reagent. We expressed such mutant channels in Neuro2A cells and recorded glutamate (50 μM)-induced currents in the whole cell recording mode. Although Xe (3.5 mM) blocked the currents through the wild-type receptor NR1-1a/NR2A and NR1-1a/NR2B by ~40% and those through the mutant receptors NR1-1a/NR2A(A650C) or NR1-1a/NR2B(A651C) by ~30%, it was unable to block the currents through the methane thiosulfonate etyhlammonium-modified mutant receptors. On the other hand, established open-channel blockers of the NMDA receptor such as MK-801 (1 μM) or Mg ions (Mg^{2+}; 1 mM) were able to block these permanently open channels. These results suggest that Xe does not act as a classical open-channel blocker at the NMDA receptor.

INTRODUCTION

Although the *N*-methyl-D-aspartate (NMDA) receptor has long been known to be a molecular target for Xenon (Xe) (Franks et al. 1998), the knowledge as to how the noble gas interacts with this ion channel is still scarce. Several groups have shown that Xe interacts with glutamate receptors in a noncompetitive manner, indicating that Xe might bind to the receptor apart from the ligand binding site (Dinse et al. 2005; Franks et al. 1998). In the case of ion channels, the term "noncompetitive" also includes sites located within the channel pore. Indeed we and others found hints that led to the speculation that Xe might interact with glutamate receptors by a channel blocking mechanism (Colloc'h et al. 2007; Dinse et al. 2005). Others, however, have disclaimed this speculation (de-Sousa et al. 2000).

In search of an appropriate approach to substantiate or discard these speculations, we went back to previous studies in which the substituted cysteine accessibility method was used to identify residues within NMDA receptors lining the channel-pore or structural elements coupling ligand binding with channel opening (Beck et al. 1999; Jones et al. 2002; Sobolevsky et al. 2002b). In these studies, multiple cysteine substitutions within the highly conserved 9-amino-acid motif SYTANLAAF at the C-terminal end of TM3, partly forming the extracellular vestibule of the ion channel pore, have been investigated. A single cysteine (C) substitution of alanine (A) at position 7 (A-7-C) showed unique properties as it revealed constitutive open channels after modification with the thiol-modifying reagent MTSEA. This covalent modification occurred only in the presence of the agonists glutamate and glycine, indicating that this process required the activation of the ion channel. After removal of both the modifying reagent and the agonists, the channels stayed open. Yuan et al. tested this mutant with different blockers and found that the modified channels were insensitive to competitive antagonists and less sensitive to allosteric modulators but retained their sensitivity to open channel blockers, although the effects varied depending on the presence or absence of agonists (Yuan et al. 2005). Taking advantage of this particular receptor mutant, we were able to address the question whether Xe is capable of blocking open NMDA receptor ion channels directly.

METHODS

The neuroblastoma cell line Neuro2A (ATCC#CCL 131) was used for transient transfection. The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM, supplemented with 2 mM L-glutamine (Boehringer, Mannheim, Germany), 10% (vol/vol) fetal calf serum (Gibco, Eggenstein, Germany), 50 U penicillin/ml, and 50 μg/ml streptomycin (Gibco) on polyornithine-coated culture dishes. After the cells had reached 40% confluency, they were transfected with the cDNAs encoding NMDA receptors. For wild-type receptors, the rat NR1-1a subunit (Moriyoshi et al. 1991) GeneBank Accession No. (NM_017010) was combined either with the mouse NR2A (NM_008170), the mouse NR2B (NM_008171), the rat NR2C (M91563), or the rat NR2D (L31611) subunit. To achieve constitutively open channels, NR1-1a wild-type subunits were combined with the NR2A(A650C) or the analogous NR2B(A651C) mutant (A-7-C), which had alanine (A) at position 7 within the SYTANLAAF-motif substituted for cysteine (C). The mutant channels were then modified by co-application of MTSEA (0.4 mM), glutamate (50 μM) and glycine (10 μM) for 2 min. The substitutions were engineered using the Stratagene quickchange site-directed mutagenesis kit and confirmed by sequence analysis (Stratagene, Amsterdam). Cysteine substitutions were also performed at the adjacent sites to position 7 (L-6-C and A-8-C). These were NR2A(L649C), NR2A(A651C), NR2B(L650C), and NR2B(A652C).

For experimentation, the cells were rinsed with extracellular solution composed of (in mM): 140 NaCl, 2.7 KCl, 1.5 $CaCl_2$, 10 glucose, and 12 HEPES; pH 7.3. Glutamate-evoked membrane currents were determined in the whole cell recording mode (Hamill et al. 1981) using an EPC-9 amplifier and TIDA software (HEKA, Lambrecht,

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Germany). Patch pipettes were drawn from borosilicate glass. Their tip resistances were between 3 and 6 MΩ when filled with (in mM) 140 $CsCl_2$, 2 $MgCl_2$, 2 ATPx2Na, 2 EGTA, and 10 HEPES; pH 7.2. Xe-containing solutions were prepared as previously described (Dinse et al. 2005). We did not analyze concentration-response relationships for Xe as even with the maximal concentration of Xe as used here the glutamate activated currents were only blocked by ~40% (Weigt et al. 2003). The receptor agonists were applied to the cells using the L/M-SPS-8 superfusion system (List, Darmstadt, Germany). To restrict the presence of the agonists to a small volume within the dish, a combination of two perfusion systems was installed, i.e., a global bath perfusion with the inflow set at 4.5 ml/min and an outflow that removed any excess fluid, and a local inlet for the generation of a continuous stream of test solution. For a quick change between various test solutions, the local inlet consisted of the tip of an eight-barreled pipette that was positioned at a distance of 50–100 μm upstream of the measuring field. All test solutions were administered at 1 ml/min using infusion pumps (Braun, Melsungen, Germany). For the preapplication of Xe, an additional inlet, positioned 50–100 μm upstream from the measuring field, was used. The time of solution exchange was estimated from the changes in the liquid junction potential to be ~1 ms.

RESULTS AND DISCUSSION

The current responses to 5-s pulses of 50 μM glutamate and 10 μM glycine to NR1-1a/NR2A and NRNR1-1a/NR2B receptors expressed in Neuro2A cells were characterized by a fast peak followed by a slow decline which tended to a plateau at the end of agonist application (Fig. 1, *A* and *C*). In the presence of Xe both, peak and plateau currents of NR1-1a/NR2A receptors were reduced by 37.5 ± 6.2 and 38.0 ± 8.1%, respectively ($n = 8$ cells). The corresponding values for NR1-1a/NR2B were 42.0 ± 3.3 and 43.1 ± 10.7% ($n = 6$ cells). These results are in accordance with previous measurements at heterologous expressed NMDA receptors (Ogata et al. 2006; Yamakura and Harris 2000). When the A-7-C receptor mutants NR2A(A650C) and NR2B(A651C) were analyzed in the same manner, the resulting currents were predominantly small. A consistently smaller current amplitude for these mutant receptors was also described in a previous report although the EC50s for glutamate obtained for wild-type and mutant receptors were not significantly different (Yuan et al. 2005). The mutant but unmodified NMDAR channels were also blocked by Xe, the reduction of peak and plateau currents being 33.6 ± 6.7 and 36.2 ± 6.1% at NR1-1a/NR2A(A650C) and 34.9 ± 6.7 and 32.7 ± 9.5% at NR1-1a/NR2B(A651C), respectively ($n = 8$; Fig. 1, *B* and *D*).

When the NR1-1a/NR2A(A650C) and the NR1-1a/NR2B(A651C) receptor mutants were treated with MTSEA (0.4 mM) in the presence of agonists (50 μM glutamate/10 μM glycine), the current amplitude continuously increased, indicating channel modification. As these modified channels can only be closed by open channel blockers (Yuan et al. 2005), we used Mg^{2+} (1 mM) for this purpose. On washout of Mg^{2+}, the channels reopened even without application of agonists. As shown in Fig. 2, Xe blocked the mutated channel (see also Fig. 1); however, it was completely unable to block the MTSEA-modified channels. Of note, the current amplitude on washout of Mg^{2+} exceeded that obtained before in the presence of MTSEA and agonists. This increase in current amplitude reveals an additional property of MTSEA, namely to act by itself as an open-channel blocker at NMDA receptors (Jones et al. 2002).

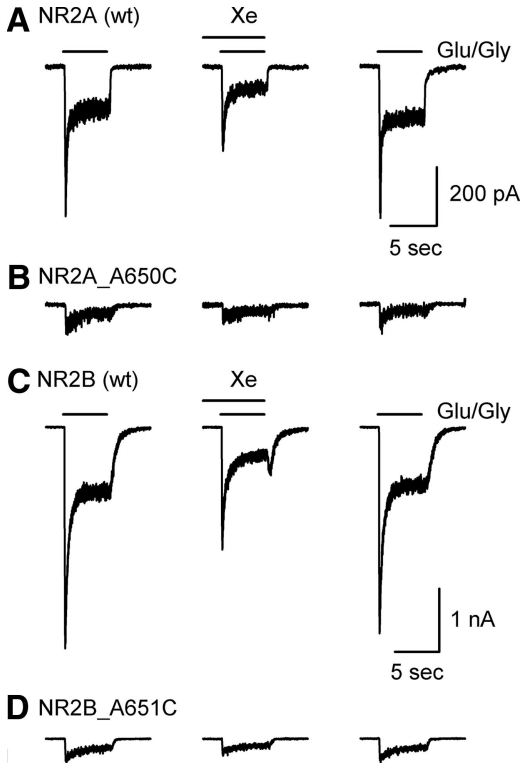

FIG. 1. Inhibition of glutamate-evoked membrane currents by xenon (Xe). Representative current traces on application of glutamate (50 μM) and glycine (10 μM) to Neuro2A cells transfected with cDNA encoding for wild-type *N*-methyl-D-aspartate (NMDA) receptors composed of NR1-1a/NR2A (*A*) or NR1-1a/NR2B subunits (*C*) are shown. The current responses for the corresponding cysteine (C) substitution of alanine (A) at position 7 (A-7-C) mutants are illustrated in *B*, NR2A(A650C), and *D*, NR2B(A651C). Agonists were applied for 5 s in the absence (controls *left* and *right*) and presence of Xe (3.5 mM). Xe was preapplied for 20 s (*middle*). Applications of glutamate/glycine and Xe are denoted by horizontal bars. Cells were voltage-clamped at −80 mV.

In a previous report, Yuan and colleagues compared the inhibitory potency of MK-801 and Mg^{2+} to block the mutated and modified channels in the absence and presence of agonists (Yuan et al. 2005). Whereas Mg^{2+} ions were equally effective under both conditions, MK-801 preferentially blocked the modified channels in the absence but not in the presence of the agonists. To test whether the latter attitude might also apply to Xe, we compared the inhibitory capacity of Xe in the presence and absence of the agonists. It turned out, that Xe is unable to block the mutated and modified ion channels irrespective whether the agonists are present or not (Fig. 3). As the current of the NR2A(A650C) mutant unlike that of the NR2B(A651C) mutant had a tendency to decline in the absence of agonists, we extended the time for Xe application from 10 s (see Fig. 2, *right*) to 30 s to not miss a slow occurring effect of Xe. Also under these conditions, we could not detect an inhibitory effect of Xe as the decline was the same in the presence and absence of Xe.

To analyze whether preceding observations are specific for position 7, we established additional cysteine substitutions at position 6 and 8 (L-6-C and A-8-C) of the NR2A and the NR2B subunits. As the current amplitudes of the L-6-C mutants on either subunit were unresolvable, we did not include these mutants in our study. The current amplitudes of the

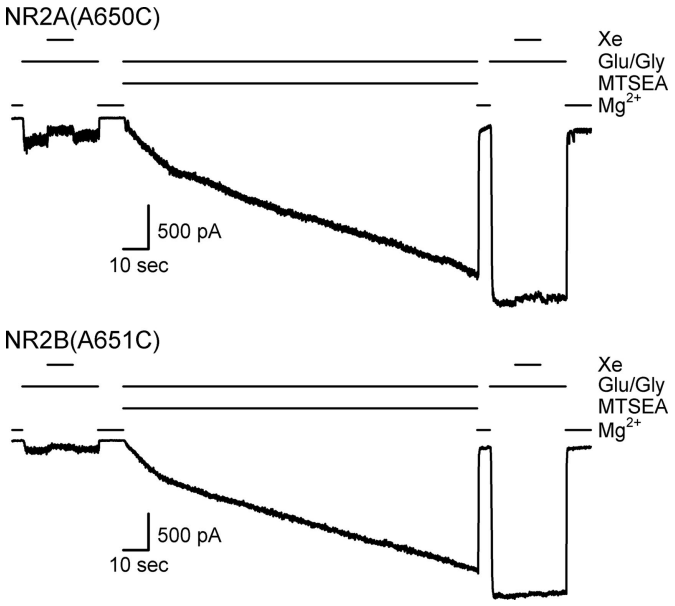

FIG. 2. Xe-sensitivity of A-7-C-mutated NMDA receptors before and after methane thiosulfonate etyhlammonium (MTSEA) treatment. The unmodified NMDA receptor mutants NR1-1a/NR2A(A650C) and NR1-1a/NR2B(A651C) are still blocked by Xe (*left*). During incubation with MTSEA (0.4 mM) in the presence of agonists (50 μM glutamate/10 μM glycine) current amplitudes increase in a time-dependent manner, reflecting channel modification (*middle*). After MTSEA-modification, Xe sensitivity is abolished (*right*). Drug applications are denoted by horizontal bars. Cells were voltage-clamped at −80 mV.

A-8-C mutants were rather small and inhibition by Xe was 34.5 ± 4.7% for NR2A(A651C) and 41.8 ± 8.7% for NR2B(A652C; n = 4 to 7 cells). In contrast to the A-7-C mutants, the A-8-C mutants did not undergo modification and Xe sensitivity was the same before and after MTSEA application (not shown).

As all NMDA receptors contain several extracellular cysteines (Jones et al. 2002), we also investigated the effect of MTSEA in the wild-type receptors. Co-application of MTSEA and agonists did not evoke a continuous increase of the current amplitude, indicating that the wild-type receptor was not modified in the same manner as the mutant receptor. However, the inhibitory potency of MTSEA was evident as can be deduced from the diminished current amplitude obtained by co-application of MTSEA and agonists versus application of agonists alone (Fig. 4). These results are in agreement with a previous report demonstrating a reversible inhibition of NMDA wild-type receptors by MTSEA (Jones et al. 2002). However, in contrast to the mutant receptor, the Xe sensitivity was the same before and after application of MTSEA (Fig. 4). Thus even when MTSEA modified other cysteines within NMDA wild-type receptors, it did not abolish the sensitivity to Xe, indicating that the introduced and modified cysteines at position 7 of the SYTANLAAF motif are crucial for the abolition of the Xe sensitivity.

Because the inhibitory effect of Xe is completely abolished at the A-7-C mutants after MTSEA treatment, we weren't able to compare the pharmacological profile of Xe with other open channel blockers before and after MTSEA modification. Alternatively, we analyzed Xe for subunit-specific interactions, taking into account that many open channel blockers of the NMDA receptor reveal a subunit-specific profile, which is not unexpected as the channel pore is formed by the NR1 subunit in combination with one of the NR2(A, B, C, D) subunits. So far, Mg^{2+} ions or MK-801 have been reported to have a weaker effect on NR2D containing receptors whereas memantine preferentially interacts with the NR2C and NR2D subunits

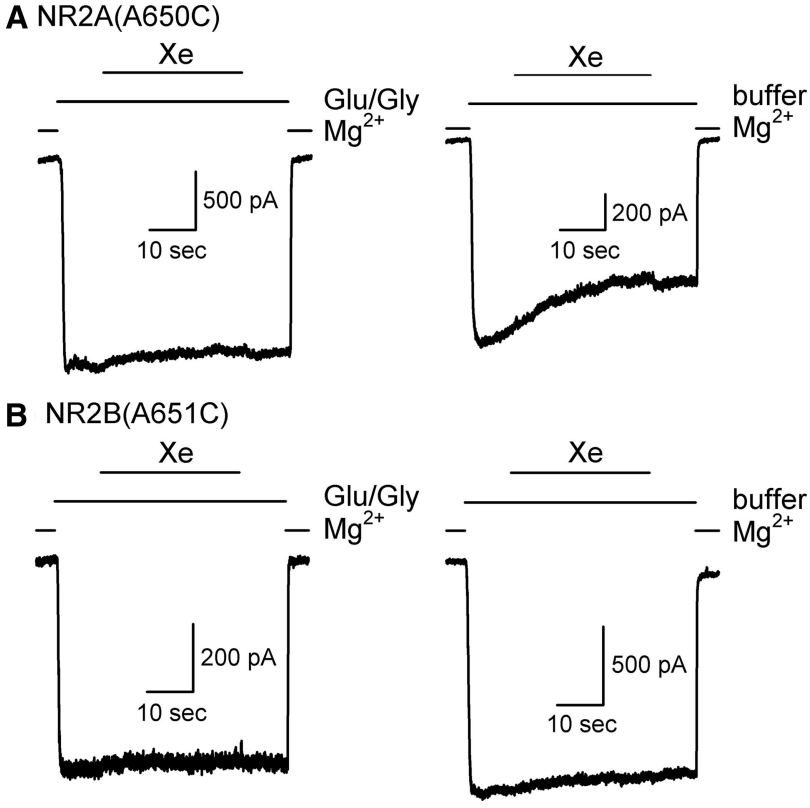

FIG. 3. Xe-sensitivity of A-7-C-mutated and -modified NMDA receptors in the presence and absence of agonists. Experiments were performed as shown in Fig. 2 with a prolonged application of Xe (30 s instead of 10 s). The graph illustrates the last part of the current traces after MTSEA modification. As shown in Fig. 2, MTSEA-modified channels are closed by Mg^{2+} ions and reopend in the presence of agonists (50 μM glutamate +10 μM glycine; *left*) or in the absence of agonists (buffer, *right*). Xe sensitivity is abolished independent from the presence or absence of agonists. Note, although the channel of the NR2A(A650C) mutant reopens on removal of Mg^{2+} ions, the current amplitude consistently declined, a behavior not observed for the NR2B(A651C) mutant. Drug applications are denoted by horizontal bars. Cells were voltage-clamped at −80 mV.

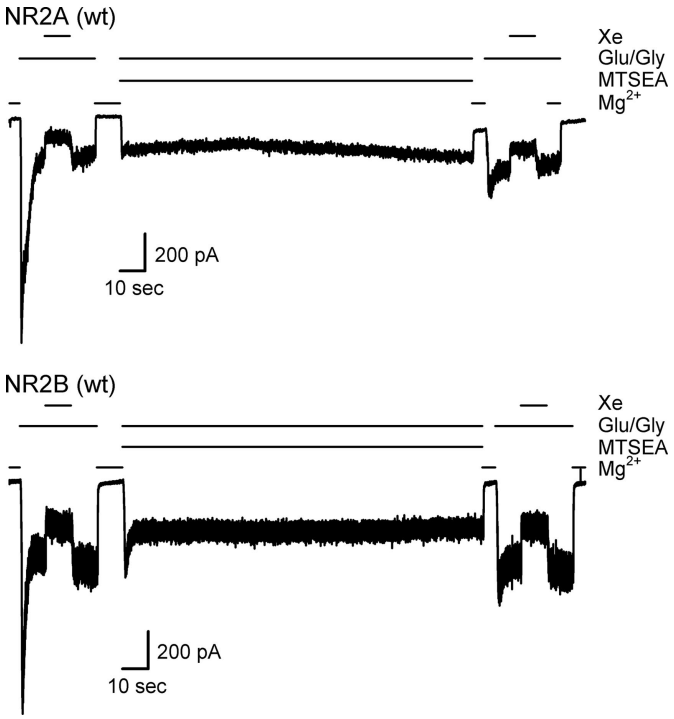

FIG. 4. MTSEA does not abolish xenon sensitivity of NMDA wild-type receptors. In NR1-1a/NR2A and NR-1a/NR2B wild-type receptors, Xe sensitivity is the same before and after treatment with MTSEA (0.4 mM) in the continuous presence of agonists (50 μM glutamate/10 μM glycine). Compared with mutant receptors, MTSEA does not increase current amplitude but inhibits agonist-induced currents. Drug applications are denoted by horizontal bars. Cells were voltage-clamped at −80 mV.

(Bresink et al. 1996; Monyer et al. 1994; Parsons et al. 1999). When we tested the effect of Xe on NR2C- and NR2D-containing NMDA receptors, we found that NR2C containing receptors were inhibited by 41.3 ± 2.7% (n = 9) and those with the NR2D subunit by 41.4 ± 3.7% (n = 9), Fig. 5. Altogether, our data, including those from the NR2A and NR2B subunits indicate that the inhibitory potency of Xe is the same on either NR2 subunit. Thus also in this respect Xe does not resemble the pharmacological profile of known open channel blockers.

Here, for the first time, we present a NMDA receptor mutant that, after modification by MTSEA, is completely insensitive to Xe. As MK-801 and Mg^{2+}, two well-known open-channel blockers of the NMDAR, are still capable of blocking these channels whereas Xe is not, it is rather unlikely that Xe operates as a classical open channel blocker. However, Kashiwagi, who studied the effects of mutations in and around the transmembrane and pore forming regions of the NMDA receptor on different channel blockers, concluded that there are different binding sites within the ion channel and suggested that some of the mutated residues might either alter the access of the blocker to its binding site or disrupt the binding site by allosteric interactions (Kashiwagi et al. 2002). Therefore as our mutation is located in the extracellular vestibule, a well-known target for channel blockers (Sobolevsky et al. 2002a), we cannot exclude a possible allosteric interaction of Xe with the ion pore.

Thus our results enforce the question whether position 7 of the SYTANLAAF motif, located within transmembrane region 3 (TM3), represents a specific binding site for the noble gas Xe. Although none of the tested mutations per se had a prominent effect on Xe sensitivity, it cannot totally be excluded that neither position 7 nor position 8 of the SYTANLAAF motif are important interaction sites for Xe with the NMDA receptor. The replacement of one alanine by cysteine might not be strong enough to eliminate this interaction in which probably several amino acids are involved. Altogether, only the A-7-C mutants that undergo a strong modification by MTESA, loose their Xe sensitivity, whereas the other C substituted mutants which are not modified by MTSEA retain their Xe sensitivity.

Previously, other point mutations located within either TM3 of NR1 (F639A) or TM4 of NR2A (A825W) have also been described to partly reduce the sensitivity to Xe (Ogata et al. 2006).These authors reasoned that either both subunits might contain complementary binding pockets for anesthetics, or as an alternative, that at least one of these sites might be involved in "transduction" rather than in binding of Xe (Ogata et al. 2006). Both suggestions may also apply to our observations. First, the point mutations investigated here might affect transduction as they reside within the highly conserved SYTANLAAF motif that is assumed to be an important element of the NMDA receptor transducing ligand binding to channel opening (Wollmuth and Sobolevsky 2004). Second, anesthetic binding sites within amphiphilic cavities formed within the core of transmembrane domains have been postulated for $GABA_AR$ and glycine receptors (Bertaccini et al. 2005; Jenkins et al. 2001). Very recently, the existence of a hydrophobic and flexible gas cavity as a possible binding site for Xe located within transmembrane domains of the NMDA receptor was deduced from crystallographic data of annexin V (Colloc'h et al. 2007), considered to be a prototype for the NMDA receptor (Berendes et al. 1993; Demange et al. 1994). According to this model, the binding of Xe within such a cavity would result in a volume expansion of the cavity thereby altering the flexibility of the pore and affecting receptor function (Colloc'h et al. 2007). In this regard, the modification of NR2A(A650C) or NR2B(A651C) by means of MTSEA, resulting in the introduction of a larger residue, might either directly hinder Xe to bind

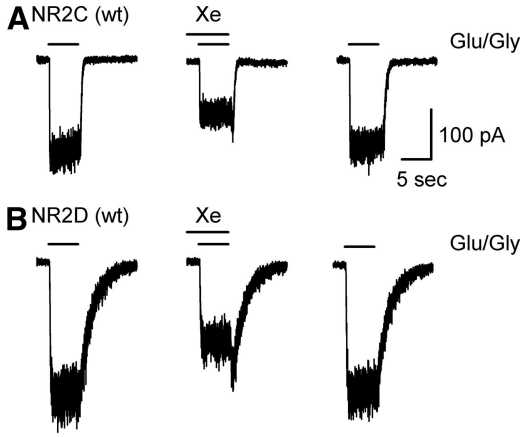

FIG. 5. Xe-sensitivity of NMDA receptors containing the NR2C and NR2D subunit. Representative current traces on application of glutamate (50 μM) and glycine (10 μM) to Neuro2A cells transfected with cDNA encoding for wild-type NMDA receptors composed of NR1-1a/NR2C (*A*) or NR1-1a/NR2D subunits (*B*) are shown. Agonists were applied for 5 s in the absence (controls *left* and *right*) and presence of Xe (3.5 mM). Xe was preapplied for 20 s (*middle*). Applications of glutamate/glycine and Xe are denoted by horizontal bars. Cells were voltage-clamped at −80 mV.

or to access its binding site. Or, provided that the site of modification is close to the putative binding cavity, it might also prevent volume expansion of the cavity.

In conclusion, the receptor modification described here is appropriate to differentiate the inhibitory action of Xe from the actions of MK-801 and Mg^{2+}. Overall, Xe seems not to act as a classical open-channel blocker at the NMDA receptor.

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