Exon 5 and Spermine Regulate Deactivation of NMDA Receptor Subtypes

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Rumbaugh, Gavin, Kate Prybylowski, Jian Feng Wang, and Stefano Vicini. Exon 5 and spermine regulate deactivation of NMDA receptor subtypes. J. Neurophysiol. 83: 1300–1306, 2000. Deactivation of N-methyl-D-aspartate (NMDA) channels after brief agonist exposure determines the duration of their synaptic activation during excitatory neurotransmission. We performed patch-clamp recordings of L-glutamate responses from human embryonic kidney tumor cells (HEK293) expressing NR1 subunit variants lacking exon 5 together with the NR2B subunit. These responses had deactivation components that lasted several seconds. The presence of exon 5 or spermine greatly accelerated deactivation of L-glutamate responses through alterations in desensitization. These effects were also observed at positive holding potentials and in the presence of physiological Mg2+. Thus NR1 splicing and polyamines may have profound effects on the kinetics of NMDA receptor–mediated synaptic transmission.

INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors have unique properties that distinguish them from other ligand-gated ion channels. They are blocked by magnesium at negative potentials, modulated by polyamines and hydrogen ions, and require glycine as co-agonist (Dingledine et al. 1999; McBain and Mayer 1994). NMDA receptors are composed of the NR1 subunit and at least one copy of either NR2A, NR2B, NR2C, or NR2D. The type of NR2 subunit determines agonist affinity, magnesium sensitivity, deactivation kinetics, modulation by polyamines, and channel conductance (Dingledine et al. 1999; McBain and Mayer 1994). Alternative splicing in the NR1 subunit gene has been reported for an N terminal cassette, exon 5, and two C terminal cassettes, exon 21 and exon 22 (Sugihara et al. 1992). Combinations of these cassettes can produce eight different isoforms. Exon 5 splicing results in receptors with distinct sensitivity to hydrogen ions, zinc, and polyamines, whereas the presence of C terminal cassettes affect the regulation by protein kinase C (Zukin and Benett 1995). In this study, we present the first evidence that NR1 splicing of the N terminal cassette can affect deactivation of NMDA receptor channels.

Using ultrarapid agonist applications by means of a piezoelectric translator, one can study the deactivation properties of channels in excised patches with agonist concentration and time course relevant to those occurring during synaptic transmission (Clements et al. 1992). Electrophysiological studies of recombinant NMDA receptors expressed in mammalian cells indicated that the NR2 subunit strongly influences deactivation (Monyer et al. 1994; Vicini et al. 1998). On the other hand, we have previously shown that deactivation kinetics did not change when NR1 isoforms were differentially expressed with the NR2A subunit (Vicini et al. 1998). Here we extend the study to recombinant receptors comprising NR1 subunit splice variants with the NR2B subunit, which has been recently demonstrated to enhance learning and memory in mice (Tang et al. 1999) and is widely expressed in the mammalian brain (Monyer et al. 1994; Zukin and Bennet 1995).

Polyamines such as spermine and spermidine increase peak current, decrease single-channel conductance, slow NMDA channel desensitization, and decrease affinity for glutamate (Benveniste and Mayer 1993; Lerma 1992; Rock and MacDonald 1992; Williams 1994a). Yet the action of spermine on deactivation kinetics, relevant for excitatory synaptic transmission, has not been investigated. Given the structural similarities between spermine and exon 5 (Traynelis et al. 1995), we also compared the effect of spermine on deactivation of NMDA responses from distinct recombinant receptors.

METHODS

NMDA receptor expression vector and transfection

Transfection and subcloning of NMDA receptor subunit cDNAs were performed as described in greater detail in Vicini et al. (1998). Throughout our work we use for simplicity the terminology of Sugihara et al. (1992) in defining the spliced forms of the NR1 subunits (NR1a, NR1b, NR1e, and NR1g). According to the terminology of Hollmann et al. (1993), these subunits are designated as NR1–1a, NR1–1b, NR1–1d, and NR1–1f. The NR1a cDNA was subcloned in the pRC/CMV vector (Invitrogen, Carlsbad, CA), whereas all other cDNAs were into pcDNA I/Amp (Invitrogen). The parent NR1a plasmid was a gift of Dr. Shigetada Nakanishi, Kyoto University Faculty of Medicine, Kyoto; the NR2B vector was a gift of Dr. Richard Huganir (Johns Hopkins University); and the NR1b, NR1e, and NR1g plasmids were gifts of Dr. Jim Boulter (University of California, Los Angeles).

Human embryonic kidney 293 cells (HEK293, American Type Culture Collection, Rockville, MD, ATCC No. CRL1573) were grown in minimal essential medium (GIBCO BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum. Exponentially growing cells were plated on 12-mm glass cover slips (Fisher Scientific, Pittsburgh, PA) and transfected with rat NMDA receptor subunit cDNAs using calcium phosphate precipitation. Studies on the recombinantly expressed receptors were performed within 2–3 days after transfection, and data were obtained for a given subunit combination transfected at least three different times. Cotransfection with pGreen-Lantern-1 (GIBCO BRL) allowed ready recognition of transfected cells.
Electrophysiology and rapid agonist applications

Electrophysiology and rapid agonist applications were also performed as described (Vicini et al. 1998). Briefly, transfected HEK293 cells were studied at room temperature (20–22°C). Bath solution contained (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, and 5 HEPES-NaOH (pH 7.4). Whole cell and outside-out patch recordings were performed with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) after capacitance and series resistance compensation. Intracellular (patch pipette) solutions contained (in mM) 145 Kgluconate, 5 MgCl₂, 5 bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), 5 ATP, and 10 HEPES, pH 7.2 with KOH. L-glutamate and l-cysteate (Sigma, St. Louis, MO) stock solutions in water at pH 7.4 were diluted to their final concentrations in bath solution. For fast action of agonists, we used a piezoelectric transistor (PZ 150 M Burleigh Instrument, Fishers, NY). After each patch recording, on and off rates, as well as pulse durations, were measured by “blowing out” the patch and recording currents generated by the liquid junction potential due to a 50:1 dilution of the agonist containing solution (Lester and Jahr 1992). As described (Vicini et al. 1998), responses in small lifted cells were comparable with those obtained in excised patches (see also Table 1). Spermine tetrahydrochloride (Sigma, St. Louis, MO) was added to both control and L-glutamate-containing solutions and the pH readjusted. Currents were filtered at 3 kHz with an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 10 kHz using an IBM-compatible microcomputer equipped with a Digidata 1200 data acquisition board (Axon Instruments) and PClamp 8 software (Axon Instruments). Off-line data analysis, curve fitting, and figure preparation were performed with Origin (MicroCal Software, Northampton, MA) and PClamp 8 software. Fitting of decay times of the averaged L-glutamate-activated currents was performed using a simplex algorithm based on a least-squares exponential fitting routine. Double exponential equations of the form \( I(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s) \), where \( I_f \) and \( I_s \) are the amplitudes of the fast and slow decay components, and \( \tau_f \) and \( \tau_s \) are their respective decay time constants used to fit the data. A comparison of the summed square deviation was used to estimate the quality of single versus double exponential fits. To compare decay time between different subunit combinations, we used a weighted mean decay time constant \( \tau_w = [I_f/(I_f + I_s)] \tau_f + [I_s/(I_f + I_s)] \tau_s \). Data values are expressed as means ± SE unless otherwise indicated.

RESULTS

In contrast to receptors comprising NR1 splice variants with the NR2A subunit (Vicini et al. 1998), cotransfection of the NR2B subunit with NR1 splice variants with and without exon 5 resulted in significant differences in desensitization. As shown in Fig. 1A, the deactivation of current produced by brief application of glutamate was described by a double exponential function. The weighted deactivation time constant (\( \tau_w \)) in small lifted cells expressing NR1a/NR2B (–exon5) subunits was four times larger than that in cells expressing NR1b/NR2B (+exon5) subunits (Table 1). Faster deactivation in the presence of exon 5 was also observed with NR1 splice variants that did not contain C-terminus cassettes. Similar results were obtained from excised outside-out patches (Table 1). The \( \tau_f \) in cells expressing NR1e/NR2B subunits was also larger than in cells expressing NR1g/NR2B subunits (Table 1). The persistent channel openings during l-glutamate responses in NR1a/2B transfections, as illustrated in Fig. 1B, produced a slow deactivation lasting several seconds.

The similar action of spermine and exon 5 on proton sensitivity of NMDA receptor has prompted the discovery of structural similarities between polyamines and the surface loop of exon 5 (Traynelis et al. 1995). We therefore investigated the effect of spermine on deactivation of NMDA responses from distinct recombinant receptors. As illustrated in Fig. 2, spermine significantly accelerated the decay of responses from cells transfected with NR1a/NR2B cDNAs, whereas it was ineffective on cells transfected with the NR1b variant (containing exon 5).

A decreased entry and a faster recovery from desensitization, as well as decreased affinity, may explain why the presence of both exon 5 and spermine produced faster deactivating responses. Therefore we compared the entry into and recovery from desensitization between responses from cells transfected with NR1a/NR2B (n = 12) and NR1b/NR2B (n = 8) with and without spermine. As illustrated in Fig. 3, A and B, the rate of entry into desensitization was significantly slower for NR1b/NR2B receptors than NR1a/NR2B receptors. The rate of recovery was also slower for NR1a/NR2B receptors (Fig. 3, C and D). Time constants of double exponential curves used for fitting the recovery time course were 22 and 1,230 ms (40% contribution of the slower component to peak amplitude) for NR1a/NR2B responses and 35 and 950 ms (17%) for NR1b/NR2B responses. Spermine was effective in decreasing the entry into desensitization with NR1a/NR2B but not with NR1b/NR2B responses (Fig. 3B), and it accelerated the recovery from desensitization of NR1a/NR2B responses (Fig. 3D). Rate constants for recovery of NR1a/NR2B responses in the presence of spermine were 17 and 1,980 ms (20%). These results taken together indicate that the major effect exon 5 and spermine have on the NR1/NR2B NMDA receptor subtype is to accelerate deactivation by decreasing entry into desensitization and increasing recovery. This is also confirmed by the much greater contribution of the fast deactivation component to the peak response with both spermine (Fig. 2C) and exon 5 (Fig. 1 legend).

The presence of exon 5 (Durand et al. 1992) or spermine (Williams 1994a) decrease agonist affinity. Because agonist affinity affects deactivation (Lester and Jahr 1992), we also investigated the action of spermine on the much faster currents produced by rapid application of the low-affinity agonist, l-
cysteate. As originally reported (Lester and Jahr 1992), the fast deactivation for these low-affinity agonists is caused by increased unbinding rates that result in reduced entry into desensitization. Responses to brief applications of L-cysteate were slower for NR1a/NR2B than NR1b/NR2B receptors. Deactivation of currents produced in transfected cells by rapid application of L-cysteate (20 mM, 8 ms) were characterized by single exponential curves. The time constant in eight cells expressing NR1a/NR2B subunits was 43 ± 6 ms and in 10 cells expressing NR1b/NR2B subunits was 29 ± 3 ms (P < 0.05, independent t-test).

Extracellular Mg2+ produce a glycine-independent and subunit-specific potentiation of NMDA responses, and this potentiation has been proposed to occur at the same site as spermine (Paoletti et al. 1995). This raises the possibility that the action of both spermine and exon 5 may not have physiological relevance. We therefore investigated NMDA responses in cells transfected with NR1a/NR2B subunit cDNA in the presence of a physiological concentration of Mg2+ (Fig. 4. A). For these experiments, recordings were performed at positive holding potentials to remove the voltage-dependent Mg2+ blockade (McBain and Mayer 1994). As illustrated in Fig. 4, A and B, 2 mM Mg2+ failed to alter the deactivation of NMDA currents. In addition, when spermine was added to Mg2+, the weighted time constants of the responses were significantly decreased. However, when 10 mM Mg2+ was used, a small but significant shortening of the decay time of NMDA responses was observed (τw = 650 ± 62 ms in control and 430 ± 35 ms with 10 mM, Mg2+ n = 4). The difference in τw between NR1a/NR2B and NR1b/NR2B receptor was maintained at positive potentials (not shown).

DISCUSSION

We demonstrate for the first time that NMDA currents produced by receptors comprising distinct NR1 splice variants differ in deactivation. For cells expressing NR1a/NR2B receptors, persistent channel openings lasting several seconds were observed. Similar prolonged deactivation components have been observed in patch responses and in NMDA-mediated excitatory postsynaptic currents (NMDA-EPSCs) from hippocampal pyramidal neurons (Kirson and Yaari 1996; Spruston et al. 1995) implying a substantial contribution of NR1a/2B channels to native NMDA receptors.

We also observed that effect of exon 5 on deactivation of NMDA responses from NR1a/NR2B transfected cells is mimicked by spermine. Our report that spermine accelerates deactivation kinetics is relevant for excitatory synaptic transmission. In fact, NMDA-EPSCs at autaptic synapses in hippocampal neurons in culture are accelerated by histamine acting on the spermine site (Bekkers 1994). However, these findings could not be repeated for NMDA responses in hip-
pocampal slices (Bekkers et al. 1996), possibly due to the variability of action of histamine and spermine on distinct receptor subtypes. Indeed, the presence of the NR1 subunit with exon 5 as well as the presence of the NR2A subunit prevents the action of spermine on the NMDA receptor (Durand 1992; Williams 1994a). Similar observations were reported with histamine (Williams 1994b).

Slow deactivation of NMDA receptors is determined by entry and exit from desensitized states (Lester and Jahr 1992). Given the reported decreased entry into desensitization with polyamines such as spermine and spermidine (Benveniste and Mayer 1993; Lerma 1992; Williams 1994a), one would predict deactivation to be accelerated. We confirmed this prediction and propose that a decreased entry and a faster recovery from desensitization may

![Diagram](http://jn.physiology.org/)

**FIG. 2.** Spermine accelerates deactivation of currents in cells expressing NR1a/NR2B heteromers. A and B: average of 3–5 consecutive responses produced by l-glutamate (1 mM, 4 ms) applications are compared between cells transfected with NR1a/NR2B (A) and NR1b/NR2B (B) cDNAs. \( t_f \), \( t_s \), and \( \% f \) are also indicated. Currents produced by l-glutamate applications in the presence of spermine are also shown to the right of control responses and superimposed after scaling at bottom of A. C: variation from control of the kinetic parameters characterizing responses recorded from cells transfected with combinations of NR2B together with NR1a (n = 12) and NR1b (n = 8) cDNAs in the presence of 500 \( \mu \)M spermine. * \( P < 0.05 \) paired 2-tailed \( t \)-test.
explain in part why the presence of both exon 5 and spermine produced faster deactivating responses. The fast component of deactivation is mainly related to unbinding and is determined by agonist affinity (Lester and Jahr 1992). A contribution to faster deactivation could also be expected from the reported decrease in agonist affinity with exon 5 (Durand et al. 1992) or spermine (Williams 1994a). Indeed, we observed that the fast decay components of L-glutamate and L-cysteate responses were shorter with exon 5. Additionally, the fast decay component of L-glutamate responses in NR1a/NR2B cells also became shorter with spermine.

The action of both spermine and exon 5 in the presence of physiological Mg$^{2+}$ confers physiological relevance to our findings. In support of this observation, Williams et al. (1994a) failed to observed competition between Mg$^{2+}$ and spermine with 1 mM Mg$^{2+}$. It is possible, however, that Mg$^{2+}$ and spermine may have similar actions at nonphysiological Mg$^{2+}$ concentrations (10 mM) as observed by Paolelli et al. (1995). Our finding that spermine decreases deactivation at both positive and negative holding voltages also indicates that the observed effect is not related to the reported voltage-dependent channel blocking mechanisms (Rock and MacDonald 1992). In the presence of Mg$^{2+}$, the faster voltage-dependent block at negative holding potential masks the action of spermine. However, as the membrane depolarizes the action of spermine should be felt even in physiological Mg$^{2+}$.

A decrease in NMDA-EPSC decay during development has been proposed to underlie a reduction in plasticity of excitatory synapses relevant to the so-called “critical period” (Carmignoto and Vicini 1992; Crair and Malenka 1995; Hestrin 1992). Evidence is growing that the developmental increase in the NR2A subunit may play a major role in these changes (Flint et al. 1997; Stocca and Vicini 1998; Tovar and Westbrook 1999). Our results imply that when the NR2B subunit is expressed, NR1 splicing can also play an important role in developmental changes in NMDA-EPSC kinetics. Indeed, developmental regulation and cell-specific expression of NR1 mRNA splice variants has been shown by in situ hybridization (Laurie and Seeburg 1994; Paupard et al. 1997). Specifically, the NR1a spliced forms were widely and abundantly distributed through-
out the brain, whereas the NR1b variants were located in similar patterns in fewer areas. A clear developmental increase of NR1b forms occurs in areas such as the thalamus and in cerebellar granule neurons (Laurie and Seeburg 1994; Paupard et al. 1997). One can speculate that in these areas a developmental change of NMDA-EPSC kinetics would parallel the increased expression of exon 5 in cell populations expressing the NR2B subunit. A rapid turnover of the NR1 subunit has recently been demonstrated in mammalian neurons (Huh and Wenthold 1992). One could also speculate that an activity-dependent alteration of alternative splicing patterns in the rat brain. Eur. J. Neurosci. 11: 788–802, 1999.


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FIG. 4. Spermine accelerates deactivation of currents in cells expressing NR1a/NR2B heteromers in the presence of physiological Mg+2. A: average of 3–5 consecutive responses produced by L-glutamate (1 mM, 4 ms) applications are illustrated at positive and negative holding potentials in cells transfected with NR1a/NR2B cDNAs. Currents produced in the same cell by L-glutamate applications are compared in control, the presence of 2 mM Mg+2 (Mg) and 2 mM Mg+2 with 300 μM spermine (Mg + Sp). Traces are also shown superimposed after scaling at bottom of A. B: summary of the average weighted time constant of deactivation of NMDA responses in 6 cells expressing NR1a/NR2B receptors in control, the presence of 2 mM Mg+2 (Mg) and 2 mM Mg+2 with 300 μM spermine (Mg + Sp) at +60 mV holding potential. * P < 0.05 paired 2-tailed t-test.

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