Molecular Mechanism of Pregnenolone Sulfate Action at NR1/NR2B Receptors

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NMDA receptors are highly expressed in the CNS and are involved in excitatory synaptic transmission and synaptic plasticity as well as excitotoxicity. They have several binding sites for allosteric modulators, including neurosteroids, endogenous compounds synthesized by the nervous tissue and expected to act locally. Whole-cell patch-clamp recording from human embryonic kidney 293 cells expressing NR1-1a/NR2B receptors revealed that neurosteroid pregnenolone sulfate (PS) (300 μM), when applied to resting NMDA receptors, potentiates the amplitude of subsequent responses to 1 mM glutamate fivefold and slows their deactivation twofold. The same concentration of PS, when applied during NMDA receptor activation by 1 mM glutamate, has only a small effect. The association and dissociation rate constants of PS binding and unbinding from resting NMDA receptors are estimated to be $3.3 \pm 2.0 \text{ mM}^{-1}\text{sec}^{-1}$ and $0.12 \pm 0.02 \text{ sec}^{-1}$, respectively, corresponding to an apparent affinity K_d of 37 μM . The results of experiments indicate that the molecular mechanism of PS potentiation of NMDA receptor responses is attributable to an increase in the peak channel open probability (P_o). Responses to glutamate recorded in the continuous presence of PS exhibit marked time-dependent decline. Our results indicate that the decline is induced by a change of the NMDA receptor affinity for PS after receptor activation.

These results suggest that the PS is a modulator of NMDA receptor P_o , the effectiveness of which is lowered by glutamate binding. This modulation may have important consequences for the neuronal excitability.

Key words: neurosteroids; NMDA receptor; open probability; patch-clamp recording; recombinant receptors; glutamate

Introduction

NMDA receptors are ligand-gated ion channels that admit Ca^{2+} after binding of the neurotransmitter glutamate and are fundamental to excitatory neurotransmission and normal CNS function (for review, see Kemp and McKernan, 2002). Recent evidence indicates that NMDA receptors are heteromeric complexes comprised of NR1, NR2, and/or NR3 subunits and possess distinct recognition sites for exogenous and endogenous ligands. These include binding sites for the agonists glycine and glutamate, for open channel blockers such as MK-801((+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate), and for polyamines, protons, Mg^{2+} , Zn^{2+} , and neurosteroids (Dingledine et al., 1999).

Pregnenolone sulfate (PS), an endogenously occurring neurosteroid, has been shown to exert direct modulatory effects on several types of neurotransmitter receptors, such as GABA_A (Majewska and Schwartz, 1987; Majewska et al., 1988; Akk et al.,

2001), glycine (Wu et al., 1997), AMPA–kainate (Yaghoubi et al., 1998), and NMDA receptors (Wu et al., 1991; Bowlby, 1993). However, the molecular mechanisms by which PS affects these receptor channels are still poorly understood. Diverse mechanisms have been proposed to explain the negative modulatory effect of PS at inhibitory ligand-gated channels. Competitive inhibition has been suggested to explain inhibition of glycine receptor channels (Wu et al., 1997), whereas enhanced desensitization, slow block, and altered activation have been suggested for the inhibitory effect of PS at GABA_A receptor channels (Shen et al., 2000; Akk et al., 2001; Eisenman et al., 2003).

NMDA receptors are positively modulated by PS; however, the degree of modulation varies considerably (Wu et al., 1991; Bowlby, 1993; Ceccon et al., 2001). Although this disparity may be attributable to the fact that the degree of PS modulation is influenced by receptor subunit composition (Abdrachmanova et al., 2001; Malayev et al., 2002), other factors have to be considered, because the degree of PS-induced potentiation of responses mediated by receptors of the same subunit composition varies substantially. For example, the amplitude of responses mediated by NR1/NR2A receptors expressed in oocytes has been reported to be potentiated by 100 μM PS almost fourfold. However, no effect of PS was observed for responses mediated by the same subunit composition expressed in human embryonic kidney (HEK)293 cells (Yaghoubi et al., 1998; Ceccon et al., 2001). This may also be why distinct molecular mechanisms of PS action at NMDA receptors have been proposed, for example, the combi-

Received Feb. 18, 2004; revised Oct. 5, 2004; accepted Oct. 5, 2004.

This work was supported by the Grant Agency of the Czech Republic (309/04/1537), the Research Project of the Academy of Sciences of the Czech Republic AVOZ 5011922, and the Ministry of Education, Youth and Sports of the Czech Republic (LN008122). H.C. was supported by Research Project Z4 005 905 and the Grant Agency of Academy of Sciences of the Czech Republic (Grant IAA4055305). We thank Dr. L. Trussell for critical reading of this manuscript, Dr. R. Pohl for measurements of NMR spectra, H. Holasova for elemental analyses, and E. Stastna and M. Kuntosova for excellent technical assistance.

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DOI:10.1523/JNEUROSCI.2099-04.2004

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nation of a decrease in agonist unbinding from the NMDA receptors and decreased desensitization (Ceccon et al., 2001) and an increase in the efficacy and/or potency of glutamate and glycine binding site agonists (Bowlby, 1993; Malayev et al., 2002).

The aim of this study was to explore the effect of PS on native and recombinant NMDA receptors. Our results suggest that PS has a positive modulatory effect on NMDA receptors that is attributable to an increase in the open probability (P_o) of the channels, and furthermore that the binding and unbinding of PS from the receptor is slow and influenced by receptor activation.

Materials and Methods

Hippocampal cultures. Primary dissociated hippocampal cultures from rats were prepared in two steps (Mayer et al., 1989b). Newborn rats were killed by decapitation, and the hippocampi were removed. Trypsin digestion followed by mechanical dissociation was used to prepare a cell suspension. Single cells were plated at a density of 100,000 cells/cm² onto a confluent glial feeder layer prepared 2 weeks previously. Neuronal cultures were maintained in minimum essential medium with 10% horse serum and nutrient supplement consisting of transferrin, insulin, selenium, corticosterone, triiodothyronine, and progesterone. A metabolic inhibitor, 5-fluoro-2'-deoxyuridine, was used to suppress cell division. All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and with the approval of the Institutional Animal Care and Use Committee.

Transfection and maintenance of HEK293 cells. HEK293 cells (CRL1573; American Type Culture Collection, Manassas, VA) were cultured in Optimum I (Invitrogen, San Diego, CA) with 5% fetal bovine serum at 37°C. The day before transfection, cells were plated in 24-well plates (2×10^5 cells per well) in 0.5 ml of their normal growth medium and became confluent on the day of transfection. Equal amounts (0.3 μ g) of cDNAs for each NR1-1a (GenBank accession number U08261; generous gift from Dr. G. Westbrook, Vollum Institute, Portland, OR) (Hollmann et al., 1993), NR2B (GenBank accession number M91562; generous gift from Dr. S. Vicini, Georgetown University School of Medicine, Washington, DC) (Monyer et al., 1992), and green fluorescent protein (pQBI 25; TaKaRa, Tokyo, Japan) were mixed with 2 μ l of Lipofectamine 2000 (Invitrogen, Paisley, UK). DNA-Lipofectamine 2000 complexes were added to HEK293 cells for 5 hr. Cells were then trypsinized, resuspended in Optimum I containing 1% fetal bovine serum supplemented with 20 mM MgCl₂, 1 mM DL-APV, and 3 mM kynurenic acid, and plated on 30 mm polylysine-coated glass coverslips.

Recording, solutions, and drug application. Experiments were performed 24–48 hr after the end of HEK293 transfection and from neurons maintained in culture for 5–8 d. Whole-cell voltage-clamp recordings were made with a patch-clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA) after capacitance and series resistance (<10 M Ω) compensation of 80–90%. Agonist-induced responses were low-pass filtered at 1 kHz by an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), digitally sampled at 5 kHz, and analyzed using pClamp software version 8.1 (Axon Instruments). Patch pipettes (3–4 M Ω) pulled from borosilicate glass were filled with an intracellular solution containing the following (in mM): 125 gluconic acid, 15 CsCl, 5 EGTA, 10 HEPES, 3 MgCl₂, 0.5 CaCl₂, and 2 ATP-Mg salt, pH adjusted to 7.2 with CsOH. Extracellular solution contained the following (in mM): 160 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, and 0.2 CaCl₂, pH adjusted to 7.3 with NaOH. In some experiments, the concentration of CaCl₂ was increased to 2 mM, and 1 mM MgCl₂ was added as indicated. Glycine (10 μ M), a NMDA receptor agonist, was routinely used. TTX (0.5 μ M) and bicuculline methochloride (5 μ M) were routinely used in experiments on cultured hippocampal neurons. Neurosteroid, 20-oxopregn-5-en-3 β -yl sulfate (PS), was synthesized according to a previously published method (Arnostova et al., 1992). The purity of PS (>98%) was repeatedly tested using nuclear magnetic resonance, thin-layer chromatography, and elemental analyses. PS solutions were made from freshly prepared 100 mM stock in dimethyl sulfoxide. Control experiments were performed in extracellular solution containing dimethyl sulfoxide at the same concentration that was present in solutions containing steroids. A microprocessor-

controlled multibarrel fast-perfusion system, with a time constant of solution exchange around cells of ~10 msec, was used to apply test and control solutions (Vyklícky et al., 1990).

To study deactivation kinetics of glutamate responses and to determine P_o of NMDA receptor channels, small lifted cells were used. A brief application of glutamate was made by a stepping-motor-controlled movement of two double-barrel θ tubings. After whole-cell recording was completed, the time course of the drug application was measured by “blowing out” the cell. The currents generated by the liquid junction potential caused by a 3% dilution of the extracellular solution containing glutamate were then recorded (10–90% rise and decay times were 0.60 ± 0.16 msec; $n = 5$) (see Fig. 2, inset) (Lester and Jahr, 1992). Experiments were performed at 23–26°C at a holding potential of –60 mV unless otherwise indicated.

All drugs, unless otherwise stated, were purchased from Sigma (St. Louis, MO) or Tocris Cookson (Avonmouth, UK).

Data analysis. The apparent microscopic dissociation constant (K_d) for PS binding to the nonactivated NMDA receptor was calculated as follows:

$$K_d = k_{us}/k_{bs} \quad (1)$$

where k_{bs} and k_{us} are the association and dissociation rate constants of the neurosteroid binding and unbinding from the NMDA receptor. k_{bs} and k_{us} were calculated as follows:

$$k_{bs} = ((1/\tau_{on}) - k_{us})/[PS] \quad (2)$$

$$k_{us} = 1/\tau_{off} \quad (3)$$

where τ_{on} is the time constant of a single exponential fit of a degree of potentiation of NMDA receptor-mediated responses by a given dose of PS ([PS]) expressed as a function of duration of PS preapplication (see Fig. 3B), and τ_{off} is a time constant of a single exponential fit of a degree of diminution of maximal potentiation of NMDA receptor-mediated responses by PS expressed as a function of duration of PS washout (see Fig. 3D).

The results are presented as mean $\pm$ SD, with n equal to the number of cells studied. Statistical comparisons were made using Student's t test. Significant differences were determined at 95% confidence intervals.

Kinetic modeling. To describe the effects of PS on NMDA receptor channels, we used a five-state kinetic model of NMDA receptor function proposed originally by Lester and Jahr (1992). Simulations and fitting were performed using the Gepasi software (version 3.21) (Mendes, 1993, 1997; Mendes and Kell, 1998).

Results

PS is a disuse-dependent NMDA receptor modulator

Our previous experiments showed that the PS-induced potentiation of responses mediated by native NMDA receptors expressed in spinal cord motoneurons was restricted to specific single-channel conductance levels, indicating the role of the receptor subunit composition in the sensitivity to the neurosteroid (Abdrachmanova et al., 2001). To study the effect of PS in detail, we compared macroscopic currents recorded from HEK293 cells transfected with NR1-1a/NR2B and from cultured rat hippocampal neurons. In the presence of 10 μ M glycine, no added Mg²⁺, and low extracellular [Ca²⁺], the response to application of 1 mM glutamate on NR1-1a/NR2B receptors showed little evidence of desensitization (Fig. 1). However, after PS application, the response to glutamate exhibited complex kinetics that varied with the relative timing of the application of agonist and neurosteroid.

Figure 1A illustrates the action of 300 μ M PS, which at equilibrium induced $+38 \pm 21\%$ ($n = 5$) potentiation of a steady-state response to 1 mM glutamate when applied after the onset of the glutamate application (contemporaneous application). Termination of PS application (after 15 sec coapplication with glutamate) yielded a transient increase in response, indicating that

PS has, in addition to its positive effect, a negative allosteric effect at NR1-1a/NR2B receptors. No effect of PS on the steady-state responses to glutamate was observed at 30 and 100 μM PS (Fig. 1*E*). A robust potentiation of the glutamate response was achieved when PS was applied before NMDA receptor activation (Fig. 1*B*). After preapplication of 300 μM PS for 37 sec, the maximal responses to 1 mM glutamate recorded in the presence of 300 μM PS (preapplication and coapplication) (Fig. 1*B*) were reversibly potentiated by $271 \pm 62\%$ ($n = 9$); however, the degree of potentiation diminished in time of glutamate and PS coapplication. Termination of PS application at the end of coapplication with glutamate resulted in a transient increase in the response ($+31 \pm 5\%$).

The decline of the responses induced by the coapplication of PS and glutamate after PS preapplication was best fit by a double exponential function with mean time constant $\tau_1 = 1.0 \pm 0.3$ sec ($A_1 = 63 \pm 20\%$) and $\tau_2 = 4.1 \pm 2.5$ sec (weighted mean decay time constant $\tau_w = 2.8 \pm 1.5$ sec) ($n = 5$). The amplitude of declining response comprised $75.4 \pm 8.4\%$ ($n = 7$; calculated as 1 minus the ratio of the steady-state and peak response to the coapplication of PS and glutamate). Several mechanisms were considered to account for the decline of the potentiated responses. First, the decline was not significantly changed when the concentration of extracellular glycine was increased to 100 μM ($75.1 \pm 8.6\%$; $n = 7$; $p = 0.93$ by paired t test), indicating that the underlying mechanism is something other than a decrease in the NMDA receptor affinity for glycine and/or allosteric coupling between glycine and glutamate binding in receptor activation (Mayer et al., 1989a). Second, in experiments performed at a holding potential of +40 mV in the continuous presence of 2 mM Ca^{2+} and 1 mM Mg^{2+} , the decline was $67.3 \pm 11.5\%$ ($n = 5$), not significantly different from responses made at -60 mV in Mg^{2+} -free extracellular solution containing 0.2 mM Ca^{2+} ($p = 0.19$ by unpaired t test). These data suggest that some mechanism other than an interference of extracellular divalent ions, intracellular calcium-mediated NMDA receptor inactivation (Vyklícky, 1993), or neurosteroid-induced voltage-dependent block of NMDA receptor channels is involved in the decline of the potentiated responses (Jang et al., 2004).

Responses to glutamate (1 mM; made in the absence of PS) immediately after PS (300 μM) preapplication for 37 sec (sequential application) were strongly potentiated (Fig. 1*B*). The degree of potentiation, $+382 \pm 46\%$ ($n = 9$), was significantly higher than the responses to the coapplication of PS and glutamate after PS preapplication ($p < 0.01$ by paired t test). The decline of responses to the sequential application of glutamate and PS were best fit by a double exponential function with a mean τ_1 of 0.8 ± 0.1 sec ($A = 54 \pm 13\%$) and a τ_2 of 7.2 ± 1.1 sec ($\tau_w = 4.2 \pm 0.3$ sec) ($n = 5$). The mean values of potentiation induced by con-

Figure 1. Degree of PS-induced potentiation of NMDA receptor responses is influenced by the timing of neurosteroid and agonist application. Examples of the records obtained from HEK293 cells transfected by NR1-1a and NR2B. *A*, The current induced by 1 mM glutamate (Glu) was potentiated ($+27\%$) when 300 μM PS was coapplied (Contemporaneous application). Note a sudden increase of the response after termination of PS application (off-response) indicated by asterisk (also apparent in responses shown in *B* and *D*). *B*, Response to 1 mM glutamate together with 300 μM PS made immediately after application of 300 μM PS for 37 sec (Preapplication and coapplication) was potentiated by $+317\%$. The response to glutamate (without PS) made after 300 μM PS preapplication for 37 sec (Sequential application) was potentiated by $+559\%$ over the control response to 1 mM glutamate. *C*, Response to coapplication of 300 μM PS and 1 mM glutamate (Coapplication) made without PS preapplication was potentiated only by 15% . *D*, Response induced by 1 μM Glu was potentiated ($+97\%$) when 300 μM PS was coapplied. *E*, Bar graph summarizes the degree of potentiation of NMDA receptor responses induced by three different modes of glutamate (1 mM or 1 μM) and PS (30, 100, and 300 μM) application. *F*, Graph of the dose–response relationship of PS inhibitory effect at NR1-1a/NR2B receptors. The degree of PS inhibition, calculated as a ratio of peak response to coapplication of PS and glutamate after PS preapplication and sequential application of glutamate and PS, was concentration dependent at 30–300 μM PS. Error bars represent SD.

temporaneous application, preapplication and coapplication, and sequential application of glutamate and 30, 100, and 300 μM PS are summarized in Figure 1*E*.

The degree of potentiation of responses induced by the coapplication of 300 μM PS and 1 mM glutamate after PS preapplication and the potentiation induced by sequential application of 300 μM PS and 1 mM glutamate were not affected by reducing the glutamate concentration to 1 μM . However, the steady-state responses to the simultaneous application of 300 μM PS and 1 μM glutamate were significantly more potentiated than those induced by 1 mM glutamate (Fig. 1*D,E*).

Given the approximately fourfold potentiation of responses to saturating concentration of glutamate observed in our experiments on recombinant NMDA receptors, we next determined whether native NMDA receptors are modulated to a similar extent. Cultured hippocampal neurons were used in these experiments. We have shown previously that high-affinity ifenprodil inhibition comprises 69% of NMDA receptor responses in these cells, indicating that most of the current response is mediated by NR2B-containing receptors (Turecek et al., 2004). The responses induced by the contemporaneous application of PS (300 μM) and

Figure 2. PS slows deactivation time course of NR1-1a/NR2B receptor responses. Control response to fast application of 1 mM glutamate (Glu) (left trace) and that induced after PS (300 μ M) preapplication for 37 sec (middle trace) are shown. Arrows indicate 6 msec application of glutamate. Superimposed and normalized responses with respect to their amplitude are shown on the right. Note that the deactivation of glutamate responses after PS preapplication (PS $\rightarrow$ Glu) was slowed. Inset, Average current produced by application of diluted extracellular solution.

NMDA (100 μ M) in hippocampal neurons were only slightly potentiated ($+7 \pm 8\%$; $n = 5$). However, the responses induced by the coapplication of PS and NMDA after PS preapplication, and those induced by the sequential application of PS and NMDA, were potentiated $+168 \pm 55\%$ ($n = 5$) and $+243 \pm 92\%$ ($n = 5$), respectively. Thus, the average degrees of PS (300 μ M) potentiation observed in native and recombinant receptors activated by NMDA (100 μ M; corresponding to 85% maximal response if one assumes NMDA $EC_{50} = 29.5 \mu$ M) (Vlachova et al., 1996) and glutamate (1 mM; corresponding to almost 100% maximal response if one assumes glutamate $EC_{50} = 0.76 \mu$ M) (Priestley et al., 1995) were not significantly different.

Next, experiments were performed to assess the effect of PS on the deactivation of NMDA receptor responses. The deactivation of control responses to glutamate (1 mM; applied for 6 msec) was compared with that induced after a 37 sec preapplication of PS (300 μ M). The deactivation of control responses to glutamate was best fit by a double exponential function with mean time constant $\tau_1 = 208 \pm 15$ msec ($A = 54 \pm 7\%$) and $\tau_2 = 839 \pm 61$ msec ($n = 5$). As can be seen from the responses shown in Figure 2, PS slowed the deactivation. The deactivation of the responses to glutamate after PS preapplication was fit by a double exponential function with both time constants significantly prolonged: τ_1 to 326 ± 71 msec and τ_2 to 1451 ± 177 msec ($n = 5$; $p < 0.01$ by paired t test); however, their relative distribution remained unaffected.

These results indicate that PS has dual action at NMDA receptors, positive and negative, both mediated by distinct binding sites. Differences in the kinetics of PS binding and unbinding affect the amplitude and time course of the NMDA receptor responses. The amplitude of the response to the coapplication of PS and glutamate after PS preapplication (Fig. 1B) reflects the summation of both the positive and negative effects. An enhanced off-response after the termination of PS application (Fig. 1A,B,D, asterisk) is likely to be attributable to rapid diminution of the inhibitory effect while the potentiating effect persists. The onset of the off-response (time constant, 15 ± 7 msec; $n = 8$) is likely to be limited by the rate of solution exchange around cells (see Materials and Methods) rather than rate of PS dissociation from the inhibitory binding sites. The amplitude of the peak

Figure 3. Binding and unbinding kinetics of PS with resting NR1-1a/NR2B receptors. *A*, Responses to 1 mM glutamate (Glu) were induced after varying durations (indicated in parentheses) of PS (50 μ M) application. Glutamate responses were potentiated 1.75-fold after 100 sec preapplication of 50 μ M PS. *B*, Analysis of the kinetics of PS binding to the resting NMDA receptor (raw data are shown in *A*). Graph indicates the degree of PS (50 μ M)-induced potentiation of glutamate responses expressed as a function of duration of neurosteroid preapplication. The data were fit by a single exponential function with τ indicated. *C*, The initial trace shows response to 1 mM glutamate, made immediately after PS (300 μ M) application for 37 sec. The response was potentiated 7.6-fold over the control response. Subsequent traces show responses to 1 mM glutamate after varying durations (10–50 sec) of PS washout (PS was applied at a concentration of 300 μ M for 37 sec). *D*, Analysis of the kinetics of PS unbinding from the resting NMDA receptor (raw data are shown in *B*). Graph indicates the degree of NMDA receptor potentiation expressed as a function of duration of PS wash out. The data were fit by a single exponential function with τ indicated. Using Equations 1–3, PS association rate constant ($k_{ps} = 3.3 \times 10^3 \text{ M}^{-1}\text{sec}^{-1}$), dissociation rate constant ($k_{us} = 0.11 \text{ sec}^{-1}$), and affinity ($K_d = 33 \mu$ M) were determined.

response to the sequential application of PS and glutamate reflects the degree of the potentiating effect of PS, because it is likely that it has already dissociated from the inhibitory binding sites during the initial phases of the response to glutamate.

The degree of PS (300 μ M) inhibition of the peak response to the coapplication of PS and glutamate after PS preapplication, determined as 1 minus the ratio of the peak response to sequential application of PS and glutamate, to that induced by coapplication of PS and glutamate after PS preapplication, was $33 \pm 6\%$ ($n = 5$). This value was not significantly different from the degree of PS (300 μ M) inhibition at the end of the coapplication of PS and glutamate, which was $31 \pm 5\%$ ($n = 5$). The extent of PS-induced inhibition (Fig. 1F) was significantly different at 30 and 300 μ M PS ($p = 0.011$ by unpaired t test).

Slow kinetics of PS action at NMDA receptors

Our next experiments were aimed at determining the kinetics of PS binding to resting (non-agonist-bound) NMDA receptors. As shown above, preapplication of 300 μ M PS for 37 sec resulted in 271% potentiation of glutamate responses recorded in the presence of PS; however, responses induced by the coapplication of 300 μ M PS and 1 mM glutamate made without PS preapplication were potentiated only by $26 \pm 8\%$ ($n = 5$) (Fig. 1C). The relationship between the duration of PS preapplication and the magnitude of the potentiation of NR1-1a/NR2B receptor responses was examined. At a concentration of PS of 300 μ M, the onset of potentiation was fast and difficult to assess accurately; a lower concentration (50 μ M) was therefore used. Figure 3A shows control responses to glutamate (1 mM) and responses recorded after

varying durations (1–100 sec) of PS (50 μ M) preapplication. The data points corresponding to the magnitude of PS-induced potentiation expressed as a function of time were fit by a single exponential function with a mean time constant (τ_{on}) of 3.84 ± 1.18 sec ($n = 6$). At this concentration of PS, the maximal potentiation was $79 \pm 14\%$ (Fig. 3B).

In subsequent experiments, we estimated the kinetics of PS dissociation from the receptor. Figure 3C shows that the response to glutamate (1 mM) immediately after PS (300 μ M) preapplication for 37 sec was potentiated 7.6-fold over the control glutamate response. The degree of PS-induced potentiation diminished with increasing time (10–100 sec) of washout of PS before the glutamate application. The data points corresponding to the magnitude of PS-induced potentiation expressed as a function of duration of PS washout were fit by a single exponential function with a time constant (τ_{off}) of 8.36 ± 1.29 sec ($n = 5$) (Fig. 3D). Equations 2 and 3 were used to determine the association (k_{bs}) and dissociation (k_{us}) rate constants of neurosteroid binding and unbinding from the NMDA receptor ($k_{bs} = 3.26 \pm 1.96$ $\text{mM}^{-1}\text{sec}^{-1}$ and $k_{us} = 0.12 \pm 0.02$ sec^{-1}), and Equation 1 was used to calculate the apparent microscopic dissociation constant, K_d ($K_d = 37$ μ M). These results demonstrate a very slow association and dissociation rate of PS interaction with the NMDA receptor.

PS increases open probability of NMDA receptors

Next, we determined the mechanism by which PS affects NMDA receptor responses. We used a minimal kinetic model that describes NMDA receptor states (see Fig. 5A) (Lester and Jahr, 1992) to simulate the effect of PS at NMDA receptors. If one assumes that a single rate constant is affected by PS, the results of the simulation predict that the amplitude of the responses to 1 mM glutamate is increased and the deactivation slowed if either the rate constant of channel opening (k_o) is increased or the rate constant of channel closing (k_c) is decreased. Because both microscopic rate constants k_o and k_c determine the open probability of NMDA receptor channels ($P_o = k_o/(k_o + k_c)$), it can be expected that PS exerts its macroscopic effect at NMDA receptors by altering the probability of channel opening.

Following the protocol used by Jahr (1992) and Chen et al. (1999), an open NMDA receptor channel blocker, MK-801, was used as an experimental tool to determine the P_o of NR1-1a/NR2B receptor channels. Responses to a 20 msec application of 1 mM glutamate were recorded at 20 sec intervals. When the peak amplitude became stable, a single response to glutamate was recorded in the presence of 20 μ M MK-801 in the control and agonist solution (Fig. 4A2). Subsequent applications of glutamate made in the absence of MK-801 elicited currents with smaller amplitudes but similar time courses, compared with the pre-MK-801 responses (Fig. 4A1). After washout of MK-801, the charge transfer by the response to glutamate was reduced by $\sim 85\%$ when compared with the control response made before the MK-801 application. To estimate the P_o for the peak current, we followed the analysis used by Jahr (1992) and Chen et al. (1999) and measured the fraction of the total charge transfer that occurred during a 20 msec application of glutamate in the presence of 20 μ M MK-801; this was $10.8 \pm 2.9\%$ ($n = 5$). The P_o (calculated as the product of the relative charge transfer by the response to glutamate after MK-801 application and the relative charge transfer that occurred within 20 msec after the beginning of glutamate application in the presence of MK-801) was estimated to be 0.091 ± 0.028 ($n = 5$), which is similar to that reported for the same receptor channel composition by Chen et

Figure 4. PS increases peak P_o of NR1-1a/NR2B receptors. Superimposed traces showing currents evoked by 1 mM glutamate (Glu) applied for 20 msec before (Pre-MK-801) and after (Post-MK-801) a single application of glutamate made in the presence of MK-801 in both agonist and control solutions (MK-801). Agonist-evoked response was made after a 20 sec preapplication of control solution (A1) or solution containing 100 μ M PS (B1). Glutamate-evoked current was recorded in the presence of 20 μ M MK-801 in both agonist and control solutions (A2) or control solution containing 100 μ M PS (B2). Insets in A and B illustrate the tip of the stepping-motor-controlled θ tube and the solutions used for recording: control; Glu, 1 mM glutamate; MK, 20 μ M MK-801; PS, 100 μ M PS. Responses illustrated in A and B are from different cells.

al. (1999). However, it should be noted that this method yields only an upper estimate of P_o at the peak of the glutamate response, because it assumes that all receptors that open before the peak are still open at the peak.

The same protocol was used to estimate the P_o of NR1-1a/NR2B receptor channels affected by PS preapplication. Responses to a 20 msec application of 1 mM glutamate immediately after PS (100 μ M) preapplication for 20 sec (sequential application) were recorded before and after (Fig. 4B1) a single response to a sequential application of PS and glutamate made in the presence of 20 μ M MK-801 in the control and agonist solution (Fig. 4B2). The analysis showed that after washout of MK-801, the charge transfer by the response to a sequential application of PS and glutamate was reduced by $\sim 87\%$ when compared with the response made before the MK-801 application. In the presence of 20 μ M MK-801, $22.4 \pm 3.8\%$ ($n = 4$) of the charge transfer occurred within 20 msec after the beginning of the glutamate application that followed the PS preapplication (Fig. 4B1). The estimated value of the P_o of the NR1-1a/NR2B receptor channels after PS (100 μ M) preapplication was 0.195 ± 0.029 ($n = 4$), significantly different from the control P_o ($p = 0.001$ by $p < 0.01$ by paired t test). An independent set of experiments was performed to determine the degree of potentiation of responses to a 20 msec application of 1 mM glutamate immediately after PS preapplication (sequential application) in lifted HEK293 cells. The peak amplitude of responses was potentiated 2.2-fold (± 0.2 ; $n = 6$) after 100 μ M PS preapplication, similar to the magnitude

of the relative increase in the peak P_o , which was increased 2.1-fold by the same concentration of PS.

Discussion

The studies presented here show that PS, a relatively abundant neurosteroid (Corpechot et al., 1983), modulates the activity of NR1-1a/NR2B receptors. Using different modes of PS and glutamate application with fast solution exchange enables us to reveal positive and negative modulatory effects of this neurosteroid.

Molecular mechanism of PS-induced potentiation

Kinetic experiments (Fig. 3) allowed us to determine the association ($k_{bs} = 3.3 \text{ mM}^{-1}\text{sec}^{-1}$) and dissociation ($k_{us} = 0.12 \text{ sec}^{-1}$) rate constants of PS binding and unbinding and the apparent dissociation constant of PS binding to resting NR1-1a/NR2B receptors ($K_d = 37 \text{ }\mu\text{M}$). Our estimates of the PS K_d value are in a good agreement with $\text{EC}_{50} = 33 \text{ }\mu\text{M}$ determined for recombinant NR1-1a/NR2B receptors expressed in oocytes and activated by a low concentration of NMDA (Malayev et al., 2002), as well as with the values 29 and $57 \text{ }\mu\text{M}$ determined for native NMDA receptors in cultured hippocampal and spinal cord neurons, respectively (Wu et al., 1991; Bowlby, 1993). Analysis of the PS effect at responses induced in NR1-1a/NR2B receptors by a saturating concentration of glutamate indicates that the maximal potentiation induced by a saturating concentration of PS approaches 6.4-fold. Surprisingly, only a negligible effect of PS was observed on the amplitude of responses induced by a saturating concentration of glutamate in HEK293 cells and oocytes transfected by NR1-1a/NR2B (Ceccon et al., 2001; Malayev et al., 2002). The reason(s) for this are not clear, but the insufficient duration of PS preapplication (Fig. 1, compare B, C) and the slow rate of solution exchange in large cells such as oocytes must be considered.

An apparent increase in the potency of glutamate and glycine induced by stabilization of the “active” state of the receptor (Malayev et al., 2002) and a decrease in glutamate unbinding and decreased desensitization (Wu et al., 1991; Ceccon et al., 2001; Malayev et al., 2002) have been proposed to explain the effects of PS at NR1/NR2B receptors. The results of simulations we made using a minimal kinetic model of NMDA receptor states (Fig. 5) indicate that a decrease in the value of the rate constant of agonist unbinding (k_u) and receptor resensitization (k_r) or an increase in the rate constant of receptor desensitization (k_d) decelerates the deactivation of responses to a saturating concentration of glutamate. This agrees with the experimental data (Abdrachmanova et al., 2001; Ceccon et al., 2001). However, in contrast to our experimental findings of a several-fold potentiation of NR1-1a/NR2B receptor responses, the simulation indicates that a decrease in the value of k_u and k_r and/or increase in the value of k_d either has no effect or decreases the amplitude of the glutamate responses. This result makes it unlikely that the modulation of glutamate potency and receptor desensitization underlies the molecular mechanism of PS action at NMDA receptors. The results of simulation show that the increase in amplitude and deceleration of deactivation of responses to glutamate induced by PS can be explained by the altered open probability of NMDA receptor channels (Figs. 1, 2). This hypothesis was confirmed experimentally by direct estimates of P_o by a protocol used previously (Jahr, 1992; Chen et al., 1999). Results of single NMDA receptor channel analysis indicate that PS increases the frequency of single-channel openings rather than mean open time (Bowlby, 1993; Abdrachmanova et al., 2001), favoring a PS-induced increase in k_o rather than a change in k_c .

Figure 5. Kinetic modeling of PS effect on NMDA receptors predicts that decrease in the closing and/or increase in the opening rate constant can result in potentiation of the amplitude and slowing deactivation of responses. *A*, Diagram of NMDA receptor states; R, O, and D represent conformational states of the NMDA receptor with closed ion channel, open ion channel, and desensitized, respectively; A represents the agonist. *B*, Simulations show responses of a five-state model to 1 mM glutamate for 2 sec. The rate constants used for simulation of the control response (continuous line) were as follows: $k_b = 5 \text{ }\mu\text{M}^{-1}\text{sec}^{-1}$ and $k_u = 5 \text{ sec}^{-1}$ (see Clements and Westbrook, 1991); $k_d = 0.19 \text{ sec}^{-1}$ and $k_r = 0.66 \text{ sec}^{-1}$ determined experimentally by a fit of the control glutamate responses to the five-state kinetic model; $k_o = 11 \text{ sec}^{-1}$ and $k_c = 99 \text{ sec}^{-1}$ were chosen arbitrarily to provide $P_o = 10\%$ (see Chen et al., 1999). Decrease of the unbinding rate constant ($<k_u$; dashed line) to 1 sec^{-1} had no effect on the amplitude of the response; however, the deactivation time course was slowed. *C*, Increase of the desensitization rate constant ($>k_d$) to 1.9 sec^{-1} resulted in diminution of the response; however, the deactivation time course was decelerated. The control response (continuous line) and response with altered desensitization rate constant (dashed line) are shown superimposed in inset after scaling to better illustrate the differences in the deactivation. *D*, Increase in the opening rate constant ($>k_o$) to 99 sec^{-1} and decrease of the closing rate constant ($<k_c$) result in potentiation of the responses and deceleration of the deactivation time courses. The control response (continuous line) and response with altered opening rate constant (dashed line) are shown superimposed after scaling to better illustrate the differences in deactivation.

Model for PS disuse-dependent action

A striking feature of the PS-dependent modulation of NMDA receptors is an order-of-magnitude difference in the degree of potentiation, depending on whether PS was interacting with resting or activated receptors. This indicates that the affinity of the NMDA receptor for PS is decreased after receptor activation. This hypothesis is supported by the differences in the time constant of diminution of the responses to sequential application of PS and glutamate ($\tau = 2.8 \text{ sec}$, likely reflecting dissociation of PS from the agonist-activated receptor) (Fig. 1) and the time constant of PS dissociation from the resting receptor ($\tau_{\text{off}} = 8.4 \text{ sec}$) (Fig. 3). Other mechanisms that may account for the time-dependent diminution of responses to the coapplication of PS and glutamate after PS preapplication were also considered. Because the degree of time-dependent diminution was not appreciably affected by a 1000-fold decrease in the concentration of glutamate (from 1 mM to $1 \text{ }\mu\text{M}$), a change in the membrane potential, and a 10-fold increase in the extracellular glycine NMDA receptor desensitization, inactivation and alteration of allosteric coupling between glutamate and glycine binding sites are unlikely to be the molecular basis for the response diminution (Mayer et al., 1989a; Vyklicky et al., 1990; Lester and Jahr, 1992; Sather et al., 1992).

Figure 6, A1 and A2, shows our working model of PS action at

Figure 6. Use-dependent reduction of PS-induced potentiation of NMDA receptor responses. *A1*, *A2*, Kinetic scheme of NMDA receptor states in the presence of PS; R, O, and D represent conformational states of the NMDA receptor with closed ion channel, open ion channel, and desensitized, respectively; A, the agonist [glutamate (Glu)]; S, the neurosteroid (PS). *B1*, Response to 1 mM glutamate (duration is indicated by a filled bar; left trace) was potentiated 3.5-fold when recorded in the presence of 300 μ M PS (indicated by open bar) made immediately after application of 300 μ M PS for 37 sec (middle trace). Response to glutamate recorded in the presence of PS is shown on the right in an expanded time scale (represented by dots). The transient was fitted to a 10-state kinetic model shown in *A1* to determine the rate of PS binding ($k_{(RAA)bs}$) and unbinding ($k_{(RAA)us}$) from NMDA receptors that have bound two molecules of glutamate; however, their channels are in a closed state (RAA). The following rate constants were used: k_b and $k'_b = 5 \mu\text{M}^{-1}\text{sec}^{-1}$; k_u and $k'_u = 5 \text{ sec}^{-1}$; k_d and $k'_d = 0.19 \text{ sec}^{-1}$; k_i and $k'_i = 0.66 \text{ sec}^{-1}$; $k_o = 11 \text{ sec}^{-1}$; $k'_o = 133 \text{ sec}^{-1}$; k_c and $k'_c = 99 \text{ sec}^{-1}$; $k_{bs} = 3.26 \text{ mM}^{-1}\text{sec}^{-1}$; $k_{us} = 0.12 \text{ sec}^{-1}$. The optimally fitted transient from the kinetic model is superimposed (line). The $k_{(RAA)bs}$ was $0.44 \text{ mM}^{-1}\text{sec}^{-1}$ and $k_{(RAA)us} = 1.38 \text{ sec}^{-1}$, corresponding to a $K_d = 3.14 \text{ mM}$ for the activated state of the receptor. *B2*, Response to sequential application of 300 μ M PS and 1 mM glutamate (left trace) was potentiated 5.4-fold over the control response to glutamate (shown in *B1*). Response to sequential application of PS and glutamate is shown on the right in an expanded time scale (represented by dots). The transient was fitted to a 10-state kinetic model shown in *A1* to determine the rate of PS unbinding ($k_{(RAA)us}$) from NMDA receptors. The optimally fitted transient from the kinetic model is superimposed (line). The $k_{(RAA)us} = 1.14 \text{ sec}^{-1}$. *C*, The simulations show responses of a model shown in *A1* to contemporaneous application of 300 μ M PS and 1 mM glutamate (left) or 1 μ M glutamate (right). The rate constants used for this simulation were $k_{(RAA)bs} = 0.80 \text{ mM}^{-1}\text{sec}^{-1}$ and $k_{(RAA)us} = 1.05 \text{ sec}^{-1}$, and the remaining rate constants were the same as those listed above. For the inhibitory effect of 300 μ M PS, 29% inhibition with fast on and off kinetics was assumed. The duration of PS and glutamate is indicated by an open and filled bar, respectively. The amplitude bar indicates the fraction of NMDA receptor channels that are in an open state.

NR1-1a/NR2B receptors, which is based on a scheme previously suggested for NMDA receptor activation (Lester and Jahr, 1992); it assumes mirror conformational states of the NMDA receptor with PS bound at one-to-one stoichiometry. One-to-one stoichiometry was sufficient to reproduce the experimental data (see below); however, this does not exclude the possibility that more molecules of PS bind to the receptor with already one molecule sufficient to exert the full potentiating effect. The results of our experiments show that PS can bind and unbind from the resting, agonist-free receptor; transition $R \leftrightarrow SR$ (where R is the NMDA receptor and S the neurosteroid) was therefore added to the kinetic scheme. Because PS may also bind and unbind from agonist (A)-induced conformational states of the NMDA receptor channel [RA, RAA, with open ion channel (O), and desensitized (D)], each of the following transitions was sequentially included in the kinetic scheme: $RA \leftrightarrow SRA$, $RAA \leftrightarrow SRAA$, $O \leftrightarrow SO$, and $D \leftrightarrow SD$. The model parameters were then adjusted to give the best fit to the data. A model with neither $RA \leftrightarrow SRA$ nor $D \leftrightarrow SD$ transitions allowed a good fit. This was mainly attributable to the relatively small fraction of receptors that occupy these states and from which PS could dissociate.

A model with either $RAA \leftrightarrow SRAA$ (Fig. 6*A1*) or $O \leftrightarrow SO$ (Fig. 6*A2*) transitions provided a good fit to the experimental data. Figure 6*B1* (right trace) shows the response to the coapplication of PS (300 μ M) and 1 mM glutamate after PS preapplication fitted with a model with transition $RAA \leftrightarrow SRAA$ (scheme in Fig. 6*A1*) and in which rate constants ($k_{(RAA)bs}$ and $k_{(RAA)us}$) were allowed to vary. In all six cells, this model (Fig. 6*A1*) provided reasonable fit to the experimental data. The mean values of the rate constants determined from the fit were $k_{(RAA)bs} = 0.80 \pm 0.62 \text{ mM}^{-1}\text{sec}^{-1}$ and $k_{(RAA)us} = 1.05 \pm 0.22 \text{ sec}^{-1}$, corresponding to the $K_d = 2.06 \pm 1.39 \text{ mM}$ of PS binding to the activated state of the NMDA receptor. Figure 6*B2* (right trace) shows the response to sequential application of PS (300 μ M) and 1 mM glutamate fitted with a model with transition $RAA \leftrightarrow SRAA$ (scheme in Fig. 6*A1*), and in which the rate constant $k_{(RAA)bs}$ was set to zero and $k_{(RAA)us}$ allowed to vary. The mean value of the rate constant determined from the fit was $k_{(RAA)us} = 0.97 \pm 0.47 \text{ sec}^{-1}$ ($n = 6$). The simulations illustrated in Figure 6*C* show responses to contemporaneous application of PS and glutamate. Traces generated using the model shown in Figure 6*A1* reproduce well the records induced by contemporaneous application of PS and glutamate, as shown in Figure 1, *A* and *D*.

The responses to the coapplication of PS and glutamate after PS preapplication were also well fit with a model in which transition $O \leftrightarrow SO$ (Fig. 6*A2*) was allowed. The mean values of the rate constants determined from this fit were $k_{(O)bs} = 6.38 \pm 1.74 \text{ mM}^{-1}\text{sec}^{-1}$ and $k_{(O)us} = 0.91 \pm 0.36 \text{ sec}^{-1}$, corresponding to the $K_d = 153 \pm 74 \mu\text{M}$ of PS binding to the activated state of the NMDA receptor. Responses to sequential application of PS (300 μ M) and 1 mM glutamate were also well fitted with this model (Fig. 6*A2*), in which rate constant $k_{(O)bs}$ was set to zero and $k_{(O)us}$ allowed to vary. The mean value of the rate constant determined from the fit was $k_{(O)us} = 1.06 \pm 0.50 \text{ sec}^{-1}$ ($n = 6$). These data indicate that after glutamate binding, the receptor undergoes conformational changes associated with agonist binding (RAA) and/or channel opening (O) that result in a lower affinity for PS of 5- to 50-fold. These data agree well with a recently proposed detailed model of NMDA receptor activation that indicates that the conformational changes of the receptor take place before channel opening (Banke and Traynelis, 2003).

Our results show the powerful potentiating effect of an endogenously occurring neurosteroid at native and recombinant

NMDA receptors. They may have significant implications for the design of therapeutic agents with potential clinical uses as cognitive enhancers and in the treatment of psychiatric disorders in which glutamatergic transmission is reduced or defective (Zorumski et al., 2000; Kemp and McKernan, 2002; Schumacher et al., 2003).

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