Bidirectional Effect of Pregnenolone Sulfate on GluN1/GluN2A N-Methyl-D-Aspartate Receptor Gating Depending on Extracellular Calcium and Intracellular Milieu

Divyan A. Chopra, Daniel T. Monaghan, and Shashank M. Dravid

Department of Pharmacology, Creighton University, Omaha, Nebraska (D.A.C., S.M.D.); and Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska (D.T.M.)

Received June 16, 2015; accepted July 10, 2015

ABSTRACT

Pregnenolone sulfate (PS), one of the most commonly occurring neurosteroids in the central nervous system, influences the function of several receptors. PS modulates N-methyl-D-aspartate receptors (NMDARs) and has been shown to have both positive and negative modulatory effects on NMDAR currents generally in a subtype-selective manner. We assessed the gating mechanism of PS modulation of GluN1/GluN2A receptors transiently expressed in human embryonic kidney 293 cells using whole-cell and single-channel electrophysiology. Only a modest effect on the whole-cell responses was observed by PS in dialyzed (nonperforated) whole-cell recordings. Interestingly, in perforated conditions, PS was found to increase the whole-cell currents in the absence of nominal extracellular Ca\(^{2+}\), whereas PS produced an inhibition of the current responses in the presence of 0.5 mM extracellular Ca\(^{2+}\). The Ca\(^{2+}\)-binding DRPEER motif and GluN1 exon-5 were found to be critical for the Ca\(^{2+}\)-dependent bidirectional effect of PS. Single-channel cell-attached analysis demonstrated that PS primarily affected the mean open time to produce its effects: positive modulation mediated by an increase in duration of open time constants, and negative modulation mediated by a reduction in the time spent in a long-lived open state of the receptor. Further kinetic modeling of the single-channel data suggested that the positive and negative modulatory effects are mediated by different gating steps which may represent GluN2 and GluN1 subunit-selective conformational changes, respectively. Our studies provide a unique mechanism of modulation of NMDARs by an endogenous neurosteroid, which has implications for identifying state-dependent molecules.

Introduction

Excitatory neurotransmission mediated by N-methyl-D-aspartate (NMDA) receptors (NMDARs) is known to play an important role in synaptic plasticity, learning, and memory (Traynelis et al., 2010). Moreover, NMDAR dysfunction may contribute to a variety of neuropsychiatric and neurological disorders, including schizophrenia, epilepsy, stroke, and trauma (Hedegaard et al., 2012). NMDARs are tetramers composed of two obligatory glycine-binding GluN1 subunits and usually two glutamate-binding GluN2 subunits. There are four types of GluN2 subunits, GluN2A to GluN2D. The function of NMDARs is regulated by endogenous modulators such as magnesium, protons, zinc, and neurosteroids (Traynelis et al., 2010). Pregnenolone sulfate (PS) is one of the most abundant neurosteroids formed by cleavage of the cholesterol side chain in glial cells (Robel and Baulieu, 1994), and it potentiates or inhibits the NMDARs in a subtype-selective manner (Malayev et al., 2002; Horak et al., 2006).

Initial studies in spinal cord neurons suggested that PS potentiation was dependent on the agonist concentration, with the potentiation being reduced at higher agonist concentrations and almost eliminated at 1 mM NMDA (Wu et al., 1991). Based on the agonist-concentration dependent effect, it has been suggested that PS increases the agonist efficacy/potency (Malayev et al., 2002). In oocyte experiments where PS is coapplied with agonists, PS typically potentiates GluN1/GluN2A currents (Yaghoubi et al., 1998; Malayev et al., 2002). However, in fast-jump experiments in a mammalian expression system [human embryonic kidney (HEK293) cells] coapplication of PS with agonists was found to typically slow the desensitization and deactivation kinetics of GluN1/GluN2A receptors but not to increase the steady-state currents (Ceccon et al., 2001; Horak et al., 2006). In contrast, preapplication of PS followed by agonist application leads to significant increase in the peak amplitude of GluN1/GluN2A currents (Bowly, 1993; Horak et al., 2004). These studies indicate that the effect of PS is partly disuse dependent.

Overall, the effect of PS on GluN1/GluN2A receptors when coapplied with agonists differs in mammalian expression systems compared with oocyte expression systems, and this

This work was supported by the National Institutes of Health National Institute of Mental Health [Grant R01-MH060252].

dx.doi.org/10.1124/mol.115.100396

Mol Pharmacol 88:650–659, October 2015

ABBREVIATIONS: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; DOP-1105, 5-(4-bromophenyl)-3-(1,2-dihydro-6-methyl-2-oxo-4-phenyl-3-quinoxinyl)-4,5-dihydro-g-oxo-1H-pyrazole-1-butanoin acid; HEK293, human embryonic kidney 293 cells; MIL, maximum interval likelihood; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptors; PKA, protein kinase A; PS, pregnenolone sulfate; TM, transmembrane.
discrepancy has not yet been resolved, although it may involve the phosphorylation state of the receptor (Petrovic et al., 2009). Studies using site-directed mutagenesis and chimeric receptors suggest that the linker regions connect transmembrane (TM) 3 and 4 to the S2 domain of the ligand-binding domain, and part of TM3 and TM4 is a critical site of action of neurosteroids including PS (Jang et al., 2004; Horak et al., 2006). The action of PS has also been found to be mediated partly by relief of proton inhibition at GluN2A- and GluN2D-containing receptors but not GluN2B- and GluN2C-containing receptors (Kostakis et al., 2011).

The effects of PS on single-channel kinetics and gating of NMDARs remain poorly understood. Using whole-cell and cell-attached electrophysiology, we have identified a novel aspect of PS action. Specifically, our studies indicate extracellular Ca²⁺– and intracellular milieu–dependent actions of PS, which may provide novel insights into the positive and negative modulatory effects of PS. These novel paradigms also have important implications for our understanding of the physiologic roles of PS.

Materials and Methods

Expression of Recombinant NMDARs. HEK293 cells were maintained as previously described elsewhere (Dravid et al., 2008). The cells were transiently transfected with Viafect reagent (Promega, Madison, WI). Rat GluN1-1a (GenBank U14114, U708261; pcIneo vector; hereafter GluN1), provided by Dr. Stephen Heinemann, Salk Institute, La Jolla, CA), GluN2A (GenBank D13211, pcIneo vector, provided by Dr. Shigetada Nakanishi, Osaka Bioscience Institute, Osaka, Japan), and green fluorescent protein in the ratio of 1:2:0.5 were expressed as described elsewhere (Bhatt et al., 2013). The GluN1-1b splice variant and GluN1-1a-R663A mutant were provided by Dr. Stephen Tryanelis (Emory University, Atlanta, GA) and GluN2B (GenBank U141149, Q00960; pCDNA3.1 vector) was provided by Dr. Peter Seeburg (Max Planck Institute for Medical Research, Heidelberg, Germany). Electrophysiology experiments were performed 16–48 hours after transfection.

Electrophysiology. Electrophysiologic recordings in whole-cell and single-channel mode were obtained from transfected HEK293 cells at room temperature (22–25°C). An external solution containing (in millimolar) 150 NaCl, 3 KCl, 10 HEPES, 0.5 CaCl₂, and 6 mannitol (to adjust osmolarity) was used for the recordings unless otherwise stated. Whole-cell recordings were obtained at 25°C. An external solution containing (in millimolar) 110 cesium gluconate, 30 CsCl₂, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid], 2 Na₃ATP, and 0.3 Na₃GTP (pH 7.3). For perforated whole-cell patch-clamp recordings, 20 μg/ml of gramicidin was added to the pipette internal solution. Whole-cell configuration after gigahm seal was reached typically within 10–15 minutes. Rapid perfusion for whole-cell concentration jumps was achieved with a two-barreled T glass pipette controlled by a piezoelectric translator (Burleigh Instruments, Fishers, NY). The solution exchange times for 10–90% solution were typically ~1–2 milliseconds. Two concentration profiles were obtained: 1) 100 μM glutamate and 100 μM glycine; and 2) 100 μM glutamate, 100 μM glycine, and 100 μM PS. The cell was moved from the control solution with no drugs to a solution containing agonists ± PS. Drug application was typically for 2.5 seconds during each 15-second sweep.

Data Processing and Kinetic Modeling. Recordings containing a single active channel were idealized using QUB software (SUNY Buffalo, Buffalo, NY) as previously described elsewhere (Dravid et al., 2008; Bhatt et al., 2013). The idealized data were used for maximum interval likelihood (MIL) fitting (Qin et al., 1996). A 120-microsecond dead time was imposed using QUB. All gating steps were free and not constrained. The C5O2 model, consisting of three closed and two open states in linear configuration and two desensitized states emerging from C1 and C2, respectively (see Fig. 7), provided the best fit to the single-channel data based on the log likelihood values. Other models tested included a C4O2 model with four instead of five closed states and a model where the receptor can transition to either a fast or slow gating step as described previously (Bhatt et al., 2013). The mean open time, mean shut time, and open probability were obtained from the idealized data using ChanneLab (Synaptosoft, Decatur, GA), with an imposed dead time of 120 microseconds. Peak and steady-state responses and deactivation, and desensitization time course for whole-cell recordings were analyzed using Clampfit (pCLAMP 10.2).

Statistical Analysis. All the values are expressed as mean ± S.E.M. Data were compared using a paired t test for macroscopic current profiles and unpaired t test for the cell-attached patches. P ≤ 0.05 was considered statistically significant.

Results

Effect of Pregnenolone Sulfate on Macroscopic Currents Is Dependent on Intracellular Milieu and Extracellular Calcium. We tested the effect of PS on macroscopic GluN1/GluN2A whole-cell currents under dialyzed (nonperforated) conditions. PS (100 μM) was coapplied with glutamate (100 μM) and glycine (100 μM) (Fig. 1) to determine the optimum conditions for carrying out single-channel studies. PS (in the absence of nominal extracellular Ca²⁺) was found to produce a modest but statistically significant reduction in the peak response (P = 0.0285, N = 6, paired t test, Iₚ/ₚ control = 0.898 ± 0.021) with no statistically significant effect on the steady-state responses (P = 0.1037, Iₛ/Iₚ control = 0.855 ± 0.040). We further tested whether extracellular Ca²⁺ is a factor for absence of strong responses to PS. In the presence of 0.5 mM Ca²⁺, PS was found to have no statistically significant effect on the peak response (P = 0.1481, N = 5, Iₚ/Iₚ control = 1.050 ± 0.082) or steady-state responses (P = 0.0669, Iₛ/Iₚ control = 1.086 ± 0.063).

Previous studies that have evaluated the effect of coapplied PS on whole-cell GluN1/GluN2A currents in HEK293 cells have found modest or no potentiation of steady-state currents when coapplied with agonists (Cecon et al., 2001; Horak et al., 2006). Thus, our findings in whole-cell conditions are similar to the internal solution used for whole-cell recordings consisted of (in millimolar) 110 cesium gluconate, 30 CsCl₂, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid], 2 Na₃ATP, and 0.3 Na₃GTP (pH 7.3). For perforated whole-cell patch-clamp recordings, 20 μg/ml of gramicidin was added to the pipette internal solution. Whole-cell configuration after giga-ohm seal was reached typically within 10–15 minutes. Rapid perfusion for whole-cell concentration jumps was achieved with a two-barreled T glass pipette controlled by a piezoelectric translator (Burleigh Instruments, Fishers, NY). The solution exchange times for 10–90% solution were typically ~1–2 milliseconds. Two concentration profiles were obtained: 1) 100 μM glutamate and 100 μM glycine; and 2) 100 μM glutamate, 100 μM glycine, and 100 μM PS. The cell was moved from the control solution with no drugs to a solution containing agonists ± PS. Drug application was typically for 2.5 seconds during each 15-second sweep.

Data Processing and Kinetic Modeling. Recordings containing a single active channel were idealized using QUB software (SUNY Buffalo, Buffalo, NY) as previously described elsewhere (Dravid et al., 2008; Bhatt et al., 2013). The idealized data were used for maximum interval likelihood (MIL) fitting (Qin et al., 1996). A 120-microsecond dead time was imposed using QUB. All gating steps were free and not constrained. The C5O2 model, consisting of three closed and two open states in linear configuration and two desensitized states emerging from C1 and C2, respectively (see Fig. 7), provided the best fit to the single-channel data based on the log likelihood values. Other models tested included a C4O2 model with four instead of five closed states and a model where the receptor can transition to either a fast or slow gating step as described previously (Bhatt et al., 2013). The mean open time, mean shut time, and open probability were obtained from the idealized data using ChanneLab (Synaptosoft, Decatur, GA), with an imposed dead time of 120 microseconds. Peak and steady-state responses and deactivation, and desensitization time course for whole-cell recordings were analyzed using Clampfit (pCLAMP 10.2).

Statistical Analysis. All the values are expressed as mean ± S.E.M. Data were compared using a paired t test for macroscopic current profiles and unpaired t test for the cell-attached patches. P ≤ 0.05 was considered statistically significant.

Results

Effect of Pregnenolone Sulfate on Macroscopic Currents Is Dependent on Intracellular Milieu and Extracellular Calcium. We tested the effect of PS on macroscopic GluN1/GluN2A whole-cell currents under dialyzed (nonperforated) conditions. PS (100 μM) was coapplied with glutamate (100 μM) and glycine (100 μM) (Fig. 1) to determine the optimum conditions for carrying out single-channel studies. PS (in the absence of nominal extracellular Ca²⁺) was found to produce a modest but statistically significant reduction in the peak response (P = 0.0285, N = 6, paired t test, Iₚ/Iₚ control = 0.898 ± 0.021) with no statistically significant effect on the steady-state responses (P = 0.1037, Iₛ/Iₚ control = 0.855 ± 0.040). We further tested whether extracellular Ca²⁺ is a factor for absence of strong responses to PS. In the presence of 0.5 mM Ca²⁺, PS was found to have no statistically significant effect on the peak response (P = 0.1481, N = 5, Iₚ/Iₚ control = 1.050 ± 0.082) or steady-state responses (P = 0.0669, Iₛ/Iₚ control = 1.086 ± 0.063).

Previous studies that have evaluated the effect of coapplied PS on whole-cell GluN1/GluN2A currents in HEK293 cells have found modest or no potentiation of steady-state currents when coapplied with agonists (Cecon et al., 2001; Horak et al., 2006). Thus, our findings in whole-cell conditions are similar to...
these studies. In oocyte recordings, however, an increase in GluN1/GluN2A responses is consistently observed where, unlike in whole-cell recordings, the intracellular milieu is generally undisturbed.

It has previously been shown that NMDAR responses and their modulation by endogenous or synthetic molecules is affected by phosphorylation and dephosphorylation pathways (Petrovic et al., 2009; Acker et al., 2011). In a typical whole-cell recording, dialyzing the intracellular components might affect the phosphorylation/dephosphorylation machinery of the cell. Hence, we performed perforated whole-cell recordings using gramicidin to test whether keeping intracellular milieu intact would affect PS modulatory actions.

Under perforated whole-cell conditions and in the absence of extracellular Ca^{2+}, PS statistically significantly increased the peak response ($P = 0.00104, N = 7, I_{PS}/I_{control} = 1.814 \pm 0.073$) and the steady-state response ($P = 0.0019, I_{PS}/I_{control} = 1.818 \pm 0.097$). In the presence of 0.5 mM extracellular Ca^{2+}, PS significantly reduced the peak response ($P = 0.0083, N = 7, I_{PS}/I_{control} = 0.60 \pm 0.036$) and the steady-state response ($P = 0.0124, I_{PS}/I_{control} = 0.582 \pm 0.025$). PS did not significantly affect the desensitization or deactivation time constants under any of the conditions tested (data not shown). A transient rise in current was observed in the whole-cell recordings when the solution containing PS was washed out (Fig. 1). This feature is similar to that demonstrated previously when PS and agonists are coapplied (Horak et al., 2004).

**Molecular Determinants of Ca^{2+}-Dependent Inhibition by Pregnenolone Sulfate.** We further assessed the potential molecular determinants of extracellular Ca^{2+}-dependent inhibition by PS. We first replicated the observation of inhibition of GluN1/GluN2A currents by PS in the presence of 0.5 mM external Ca^{2+} under perforated whole-cell recording conditions (Fig. 2A). One of the sites where Ca^{2+} binds in the extracellular vestibule is the DRPEER motif (Watanabe et al., 2002; Karakas and Furukawa, 2014). We tested the effect of PS on GluN1R663A/GluN2A receptors in perforated whole-cell patch-clamp recordings. This mutant was chosen based on its most exterior positioning, which may prevent it from having
basal effects (as indicated in Watanabe et al., 2002) yet may allow for testing the importance of this region in the modulatory effect of PS.

We found that PS potentiated the peak current ($P = 0.0389$, $N = 5$, $I_{PS}/I_{control} = 1.654 \pm 0.191$) as well as the steady-state current responses ($P = 0.0264$, $N = 5$, $I_{PS}/I_{control} = 1.543 \pm 0.138$) from GluN1R663A/GluN2A receptors in the presence of 0.5 mM Ca$^{2+}$ (Fig. 2B). The degree of potentiation was comparable to the potentiation by PS in the absence of nominal Ca$^{2+}$. This finding demonstrates a critical role of the DRPEER motif in the bidirectional effect of PS depending on extracellular Ca$^{2+}$.

We further tested the effect of exon-5 insert (present in GluN1-1b), which is a key molecular determinant for proton and zinc inhibition (Traynelis et al., 2010) as well as proton-dependent differential efficacy of PS at GluN1/GluN2A versus GluN1/GluN2B receptors (Kostakis et al., 2011). The whole-cell responses at GluN1-1b/GluN2A receptors were statistically significantly increased by PS in the presence of 0.5 mM Ca$^{2+}$ (Fig. 2C), showing an increase in the peak current ($P = 0.0357$, $N = 7$, $I_{PS}/I_{control} = 1.525 \pm 0.105$) as well as steady-state current responses ($P = 0.0199$, $N = 7$, $I_{PS}/I_{control} = 1.360 \pm 0.11$). PS was also found to increase the deactivation kinetics of the GluN1-1b/GluN2A receptors (data not shown).

Together these data demonstrate that conformational changes induced by presence of exon-5 can mask the inhibitory effect of PS produced due to its allosteric interaction with Ca$^{2+}$ binding at the DRPEER motif. However, it should be noted that we cannot rule out other possibilities, such as a change in proton-sensitivity of the receptor leading to changes in the mechanism of action of PS.

Finally, we tested whether the Ca$^{2+}$-dependent inhibition is specific for GluN2A-containing receptors. In contrast to GluN1/GluN2A receptors, no statistically significant inhibition by PS was observed in the presence of Ca$^{2+}$ at the GluN1/GluN2B receptors ($N = 5$; Fig. 2D), although no potentiation was observed either. However, PS did statistically significantly increase the decay kinetics of the GluN1/GluN2B receptors (data not shown).

**Pregnenolone Sulfate Affects Mean Open Time of GluN1/GluN2A Receptors.** After we had identified the conditions where the potentiating and inhibiting effects of PS are robust, we assessed the single-channel effects of PS under these conditions. We obtained cell-attached patches with one active channel for evaluating the effect of PS on GluN1/GluN2A gating (Fig. 3).

In the first set of recordings, CaCl$_2$ was absent from the pipette internal solution. The mean open time ($\pm$ S.E.M.) in the control patches was found to be $1.52 \pm 0.17$ milliseconds (114,675 events; $n = 9$). In the presence of PS, the mean open time was statistically significantly higher: $3.11 \pm 0.24$ milliseconds (93,505 events; $n = 5$, $P = 0.00017$, unpaired $t$ test). The mean shut time was not affected by PS: $17.3 \pm 1.8$ milliseconds in control patches (115,032 events) and $15.3 \pm 2.9$ milliseconds in PS patches (93,840 events, $P = 0.528$). The open probability, measured over the entire length of the
recordings, was found to increase from 0.082 ± 0.007 in control patches to 0.186 ± 0.034 in PS patches (P < 0.0022). The amplitude of openings was unaffected by PS: 5.01 ± 0.18 pA for control patches and 5.09 ± 0.16 pA for PS.

Thus, it appears that the major effect of PS is on the mean open time of the GluN1/GluN2A receptors, which leads to higher open probability in the presence of PS. Compared with previous studies the overall open probability of GluN1/GluN2A was found to be lower in our cell-attached patches. This may be due to differences in the recording solutions or mode of recording or a difference in the modal gating of the receptor. However, it should be noted that under our recording conditions the mean open time and open probability for GluN1/GluN2A were higher compared with GluN1/GluN2B (Bhatt et al., 2013), with a similar order of magnitude as previously described elsewhere in outside-out patches (Erreger et al., 2005).

After we included 0.5 mM Ca\(^{2+}\) in the extracellular solution, PS was found to reduce the mean open time from 1.53 ± 0.18 milliseconds (57,406 events; n = 5; Fig. 4) in control patches to 0.72 ± 0.07 milliseconds (85,892 events; n = 6; P = 0.0012, unpaired t test). No statistically significant change in the mean shut time was observed in control patches: 19.3 ± 2.0 milliseconds (57,824 events) versus PS patches: 19.4 ± 3.9 milliseconds (85,903 events) (P = 0.981). The overall open probability was found to be statistically significantly reduced by PS in the presence of 0.5 mM Ca\(^{2+}\). The open probability in control patches was 0.076 ± 0.012, which was statistically significantly reduced in the presence of PS to 0.041 ± 0.006 (P = 0.023). The amplitude of openings was unaffected by PS in the presence of extracellular Ca\(^{2+}\) (control = 5.24 ± 0.28 pA; PS = 5.27 ± 0.28 pA). Because no change in the mean shut time was seen, the reduction in open probability was primarily due to the shorter mean open time. No statistically significant differences in single-channel properties were observed in control patches obtained under conditions of the absence or presence of extracellular 0.5 mM Ca\(^{2+}\). Thus, extracellular Ca\(^{2+}\) bidirectionally modulates PS responses similar to the results obtained in perforated whole-cell recordings.

Pregnenolone Sulfate Produces Unique Effects on Open and Shut Time Constants That Underlie Potentiation and Inhibition of GluN1/GluN2A Receptors. We further evaluated the effect of PS on the open and shut time characteristics. We fitted the single-channel data from individual patches using MIL to a model consisting of two open states and five shut states, of which two were the longer desensitized states as previously described elsewhere (Dravid et al., 2008). Both control and PS patches produced reasonably good fits with this scheme, suggesting that this model provides a reasonable description of the receptor gating (assuming that in the presence of 100 μM PS the receptor binding sites for PS are close to 100% occupied).

We first compared the patches obtained in the absence of extracellular Ca\(^{2+}\), which showed ~2-fold potentiation in the open probability. The global fit for all events in the absence of extracellular Ca\(^{2+}\) is presented in Fig. 5, and the time
constants and area of individual patches are presented in Table 1.

A major effect of PS as evident from this analysis is an increase in time constants for the open states \([t_1 = 0.31 \pm 0.04, t_2 = 1.76 \pm 0.26, \text{PS}, t_1 = 0.86 \pm 0.27 (P = 0.0179), t_2 = 3.62 \pm 0.37 (P = 0.0013)]\). The shift in time constants is consistent with an increase in the mean open time observed in the presence of PS (Fig. 3). No

fig. 4. Effect of pregnenolone sulfate on the GluN1/GluN2A receptor single-channel properties depends on extracellular Ca\(^{2+}\). Cell-attached recordings were obtained with addition of 0.5 mM Ca\(^{2+}\) to the pipette internal solution. The representative steady-state, single-channel control recording is from patches containing one active GluN1/GluN2A receptor with the absence or the presence of PS (100 \(\mu\)M). PS \((n = 6)\) reduced the mean open time of the receptor compared with control patches \((n = 5)\). PS did not have any significant effect on the mean shut time of the receptor \((P = 0.981)\). The probability of opening was significantly reduced by PS \((P = 0.0013)\). Data are compared with unpaired \(t\) test. \(*P < 0.05; **P < 0.01.\)

Fig. 5. Pregnenolone sulfate–mediated potentiation of GluN1/GluN2A receptors involves a shift in open states to longer durations and a reduction in the occupancy of long-lived shut states. The single-channel currents from cell-attached patches in the absence of nominal Ca\(^{2+}\) with one active GluN1/GluN2A receptor were idealized for each patch and summed to generate global dwell time histograms. The open time histogram was fitted by a sum of two exponential components: control, 114,675 open events \((n = 9); \text{PS}, 93,505\) events \((n = 5)\). The time constants and percentage area are shown in the inset. The dwell times from each patch were individually fitted and are presented in Table 1. PS was found to significantly increase \(t_1\) and \(t_2\) time constants but not the area (Table 1). The composite shut time histogram was fitted by a sum of five exponential functions: control, 115,032 closed periods \((n = 9); \text{PS}, 93,840\) closed periods \((n = 5)\). PS was found to significantly reduce \(t_2\) and \(t_3\) time constants and increase \(t_5\) (Table 1). Only the area of \(t_5\) was significantly reduced by PS. SQRT, square root.
Inhibitory effect of pregnenolone sulfate on GluN1/GluN2A receptors is primarily due to reduced dwell time in a longer open state. Global dwell-time histograms were generated by summation of idealized data from individual cell-attached patches in the presence of 0.5 mM extracellular Ca\(^{2+}\) (Fig. 6; Table 1). SQRT, square root.

<table>
<thead>
<tr>
<th>Time Constants and Areas</th>
<th>0 Calcium</th>
<th></th>
<th>0.5 Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PS</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>((n = 5))</td>
<td>((n = 5))</td>
<td>((n = 5))</td>
</tr>
<tr>
<td>Open time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\tau_1) (ms)</td>
<td>0.31 ± 0.04</td>
<td>0.86 ± 0.27*</td>
<td>0.39 ± 0.09</td>
</tr>
<tr>
<td>(\tau_2) (ms)</td>
<td>1.76 ± 0.26</td>
<td>3.62 ± 0.37**</td>
<td>1.70 ± 0.21</td>
</tr>
<tr>
<td>a1 (%)</td>
<td>22 ± 4</td>
<td>25 ± 8</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>a2 (%)</td>
<td>78 ± 4</td>
<td>75 ± 8</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>Shut time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\tau_1) (ms)</td>
<td>0.63 ± 0.04</td>
<td>0.63 ± 0.16</td>
<td>0.69 ± 0.12</td>
</tr>
<tr>
<td>(\tau_2) (ms)</td>
<td>5.80 ± 0.43</td>
<td>2.90 ± 0.53**</td>
<td>5.50 ± 1.08</td>
</tr>
<tr>
<td>(\tau_3) (ms)</td>
<td>19.3 ± 1.2</td>
<td>12.4 ± 1.3**</td>
<td>15.7 ± 3.5</td>
</tr>
<tr>
<td>(\tau_4) (ms)</td>
<td>73.6 ± 8.8</td>
<td>50.4 ± 10.1</td>
<td>90.0 ± 34.6</td>
</tr>
<tr>
<td>(\tau_5) (ms)</td>
<td>1117 ± 198</td>
<td>3249 ± 759*</td>
<td>1406 ± 227</td>
</tr>
<tr>
<td>a1 (%)</td>
<td>30 ± 4</td>
<td>33 ± 7</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>a2 (%)</td>
<td>27 ± 1</td>
<td>31 ± 4</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>a3 (%)</td>
<td>37 ± 2</td>
<td>32 ± 5</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>a4 (%)</td>
<td>4 ± 1</td>
<td>4 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>a5 (%)</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.04*</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\).

Analysis of shut time constants revealed that the time constants \(\tau_2\), \(\tau_3\), and \(\tau_5\) were statistically significantly different (Table 1). The \(\tau_2\) (\(P = 0.0014\)) and \(\tau_3\) (\(P = 0.0038\)) were reduced in the presence of PS, and \(\tau_5\) (\(P = 0.0044\)) was statistically significantly increased. No change in the area of shut time constants was observed except that of \(\tau_5\), which was reduced in the presence of PS (\(P = 0.0351\)).

We next evaluated the effect of PS on open and shut times in the presence of 0.5 mM extracellular Ca\(^{2+}\) (Fig. 6; Table 1). As clearly evident, a statistically significant reduction in the areas of the longer time constant \(\tau_2\) (\(P = 0.00002\)) and an increase in areas of \(\tau_1\) (\(P = 0.00002\)) were found in the presence of PS; however, no change of the time constants themselves was observed (control, \(\tau_1 = 0.39 ± 0.09\) (22% ± 7%); \(\tau_2 = 1.70 ± 0.21\) (78% ± 7%); PS, \(\tau_1 = 0.50 ± 0.04\) (87% ± 5%); \(\tau_2 = 2.30 ± 0.61\) (13% ± 5%). These results suggest that the primary inhibitory effect of PS is via reduction in the dwell time in the longer open state. No statistically significant differences were observed in the shut time constants except \(\tau_1\), which was statistically significantly increased in the presence of PS (\(P = 0.0417\)) (Table 1).

Previous studies suggested that the shut time components consisting of \(\tau_2\) and \(\tau_3\) likely represent a conformational change in the GluN2 subunit and that in \(\tau_1\) may represent...
produce bidirectional effects depending on extracellular Ca^{2+}. When PS was coapplied with agonists in the absence of Ca^{2+}, PS led to an increase in the open probability whereas in the presence of 0.5 mM Ca^{2+} PS led to a reduction in the open probability of GluN1/GluN2A receptors.

**Mechanism for Positive and Negative Modulatory Effects of Pregnenolone Sulfate.** It has been shown that the action of PS on NMDARs is phosphorylation dependent, so intracellular milieu may affect PS-mediated modulation. Previous studies have reported PS modulation to be mediated partly by protein kinase A (PKA) (Petrovic et al., 2009). The potentiating effect of PS in outside-out patches is lost after 2 minutes of obtaining the patch, and this effect can be reversed by addition of PKA (Petrovic et al., 2009). Thus, one possibility is that the potentiating effect on steady-state currents is lost in dialyzed whole-cell mode due to inhibition of intracellular PKA or other changes that affect NMDAR posttranslational modifications.

This phenomenon of intracellular milieu-dependent effects may also be relevant to other agents. Recent studies have demonstrated that the IC50 of a subtype-selective negative allosteric modulator DQP-1105 [5-(4-bromophenyl)-3-(1,2-dihydro-6-methyl-2-oxo-4-phenyl-3-quinolinyl)-4,5-dihydro-g-oxo-1H-pyrazole-1-butanolic acid] is dependent on whole-cell configuration. It was found that the inhibitory action of DQP-1105 was reduced at GluN1/GluN2A receptors and a greater magnitude of subtype-selectivity between GluN1/GluN2D and GluN1/GluN2A receptors was observed in perforated mode of recording using gramicidin compared with under dialyzed condition (Acker et al., 2011). Also of note, the whole-cell dialyzed configuration is known to increase the desensitization of the NMDAR (Sather et al., 1990). Because GluN2A-containing receptors have greater desensitization while GluN2D-containing receptors appear to lack desensitization (Traynelis et al., 2010), it is likely that the difference in open probability in the two-preparations for the two subtypes may also underlie the differential effect of DQP-1105. This may also be relevant to the differential effect of PS in dialyzed and perforated conditions in our present study.

Our data suggest that 0.5 mM Ca^{2+} is sufficient to switch PS modulation from positive to negative effect under perforated whole-cell mode and in cell-attached recordings (Figs. 1–4). Thus, the potential site of Ca^{2+} binding or action may provide understanding of the inhibitory site of PS. Two Ca^{2+} binding sites have been identified in the NMDARs. One is present in the pore of a functional NMDAR (Jahr and Stevens, 1993). The other Ca^{2+} binding site is present in the DRPEER region located in the linker region between the ligand-binding domain and transmembrane domain of the GluN1 subunit (Watanabe et al., 2002). Both sites have been shown to increase the Ca^{2+}-block of the channel and reduce the channel conductance (Jahr and Stevens, 1993; Watanabe et al., 2002). However, we did not find a significant change in single-channel amplitude under control 0.5 mM Ca^{2+} conditions or with PS, which only reduced the mean open time. Thus, the inhibitory mechanism of external Ca^{2+} alone appears to be different from the inhibitory effect of PS.

Using GluN2A and GluN2C chimeras and site-directed mutagenesis, it has been shown that PS does not act by binding to the amino terminal domain, ligand-binding domain, or intracellular carboxyl-terminal domain of GluN2A receptors (Horak et al., 2006; Cameron et al.,
2012). It exerts its effect by acting on the TM3–TM4 loop of the NMDARs (Park-Chung et al., 1997; Horak et al., 2006; Borovska et al., 2012). Additionally, in oocyte studies the potentiating effect of PS on GluN1/GluN2A receptors is influenced by the presence or absence of exon-5 in the GluN1 subunit. In the absence of exon-5 (GluN1-1a, which we have used) the potentiation by PS is lower compared with when exon-5 is present (GluN1-1b) (Kostakis et al., 2011). This finding is relevant to the location of DRPEER Ca$^{2+}$-binding site on the GluN1 subunit.

Thus, the Ca$^{2+}$-dependent effects of PS may arise due to an allosteric interaction between Ca$^{2+}$-binding to the DRPEER site and PS binding to an “inhibitory site” influenced by GluN1 subunit, which together reduce the stability of long-lived open times. In contrast, the absence of Ca$^{2+}$ prevents this allosteric interaction and inhibitory action of PS. Our results with the GluN1R663A mutant in the DRPEER motif and exon-5 insert are in agreement with this hypothesis because PS instead of inhibiting responses in the presence of 0.5 mM Ca$^{2+}$ led to potentiation of the receptor to a similar extent as in the absence of nominal Ca$^{2+}$.

**Bidirectional Actions of Pregnenolone Sulfate Are Produced by Mechanistically Distinct Gating Steps.** Our single-channel data demonstrate a significant effect of PS on mean open time in producing both inhibition and potentiation of the receptor. This is most evident in the free-energy plots where effects on open time are most predominant (Fig. 7). The properties of PS-induced inhibition are quite peculiar in that the longer open state is almost completely abolished. This correlates with a substantial reduction in the potency of the receptor. This is most evident in the free-energy plots where effects on open time are most prominent (Fig. 7). The properties of PS-induced inhibition are quite peculiar in that the longer open state is almost completely abolished. This correlates with a substantial reduction in the potency of the receptor. Scale bar: 1 kBT. *P < 0.05; **P < 0.01; ***P < 0.001.

![Kinetic mechanism describing the effects of pregnenolone sulfate on GluN1/GluN2A receptor activation.](image)

**Fig. 7.** Kinetic mechanism describing the effects of pregnenolone sulfate on GluN1/GluN2A receptor activation. MIL fit of single-channel data to understand the kinetic mechanism of GluN1/GluN2A receptor modulation by PS is shown. All rates are in s$^{-1}$. Bold numbers with asterisks denote the rates that were significantly different from glutamate/glycine control patches. Data were analyzed using unpaired t test. Free-energy trajectories for the kinetic states in the different models are presented. Control with 0.5 mM Ca$^{2+}$ or without nominal Ca$^{2+}$ produced similar profiles. The free energies of the active open states are most dramatically affected by PS during either inhibition or potentiation of the receptor. Scale bar: 1 kBT. *P < 0.05; **P < 0.01; ***P < 0.001.

Based on our data, PS interaction with Ca$^{2+}$ obstructs the dilation of pore to a more stable conformational representing the longer open time. Removal of nominal Ca$^{2+}$ or presumably preventing Ca$^{2+}$ interaction with DRPEER site or masking the effect of Ca$^{2+}$ with the exon-5 insert (Figs. 1 and 2) unravels the potentiating mechanism of PS. The potentiating mechanism engages molecular determinants close to the TM3 and TM4 regions linker, forming the external vestibule as demonstrated previously elsewhere (Kostakis et al., 2011). Indeed, restricting linkers in TM3–TM4 or using the reducing agent dithiothreitol, which acts on GluN1 cysteines close to the TM3–TM4 linker (Sullivan et al., 1994), affects multiple gating steps not restricted to transitions to open states, similar to our kinetic analysis of effect of PS in the absence of nominal Ca$^{2+}$ (Talukder and Wollmuth, 2011). This difference in PS inhibition mainly affecting fast-gating steps transitioning to open states while PS potentiation affects slow-gating steps in addition to its effect on mean open time. In contrast, the inhibition of the receptor by PS in the presence of 0.5 mM Ca$^{2+}$ may involve modification to the GluN1-gating step because it led to a specific change in r1. This hypothesis is also supported by our results in the DRPEER mutant and GluN1-1b splice variant where the PS inhibition is eliminated.

**Conclusion and Remaining Questions.** In most of the previous studies in neurons and mammalian expression systems, a potentiating effect is observed with preapplication of PS. In fact at GluN1/GluN2C receptors while coapplication leads to PS-induced inhibition, preapplication of PS followed by agonist-alone application leads to potentiation of currents (Horak et al., 2006). The effect of preapplication is likely conformational change while a faster gating step may represent a GluN1 subunit conformational change (Banke and Traynelis, 2003; Erreger et al., 2005; Murthy et al., 2012).

Thus, based on our data, it is possible that the potentiation of the receptor is mediated by modification of the slower putative GluN2-gating step because we observed changes in longer shut time constants r2 and r3 and slower gating steps in translation to its effect on mean open time. In contrast, the inhibition of the receptor by PS in the presence of 0.5 mM Ca$^{2+}$ may involve modification to the GluN1-gating step because it led to a specific change in r1. This hypothesis is also supported by our results in the DRPEER mutant and GluN1-1b splice variant where the PS inhibition is eliminated.
a more relevant phenomenon to the physiology of the normal central nervous system because PS appears to be present under basal conditions (Robel and Baulieu, 1994). Thus future experiments to address the differences in whole-cell responses in dialyzed and perforated conditions upon preapplication of PS may reveal interesting results that may be relevant to neurosteroid physiology in the central nervous system.

Interestingly, PS is also being evaluated for its efficacy for treating cognitive and behavioral impairments in mental disorders (see, for example, Marx et al., 2014; and Wong et al., 2015). Our data suggest that there is a need to better understand the pharmacologic basis of actions of PS and to assess the ability of newly discovered allosteric modulators of NMDAR to serve as intracellular cell state–dependent agents.

**Authorship Contributions**

**Participated in research design:** Chapra, Monaghan, Dravid.

**Conducted experiments:** Chapra.

**Wrote or contributed to the writing of the manuscript:** Chapra, Dravid.

**References**


**Address correspondence to:** Dr. Shashank M. Dravid, Department of Pharmacology, Creighton University, School of Medicine, 2500 California Plaza, Omaha, NE 68178. E-mail: shashankdravid@creighton.edu