0026-895X/93/050813-07$03.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 43:813-819

Pregnenolone Sulfate Potentiation of *N*-Methyl-D-aspartate Receptor Channels in Hippocampal Neurons

MARK R. BOWLBY

Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

Received November 25, 1992; Accepted February 6, 1993

SUMMARY

Many actions of the classical gonadal and adrenal steroid hormones are at the level of transcriptional regulation. Recent studies have shown, however, that endogenous brain metabolites of steroids exert important nongenomic modulatory effects on neuronal mechanisms. Potentiation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor by the neurosteroid pregnenolone sulfate (PS) was studied using cultured hippocampal neurons and patch-clamp techniques. The magnitude of NMDA-activated whole-cell currents was approximately doubled in the presence of 100 μM PS. The dose-response curve of PS action showed significant potentiation above 250 nM and a half-maximal effect at approximately 29 μM. Maximum potentiation was reached within 25 sec, and the potentiation was completely reversed with 60 sec of washout. The enhancement of the NMDA current is probably not due to activation of a new ionic conductance, because the reversal potential of the I-V curve did not shift in the presence of PS. Potentiation is specific for the NMDA subtype of glutamate receptor; non-NMDA currents showed only a slight inhibition (approximately 6%) in the presence of 50 μM PS. Potentiation of the NMDA current by PS occurred in the presence of saturating concentrations of NMDA and glycine, indicating that at saturating concentrations of the coagonists PS does not change the affinity between the coagonists and the NMDA receptor. The dose-response relations for NMDA and glycine were shifted slightly to the left, and the percent potentiation was significantly higher for lower concentrations of coagonists, suggesting that at low concentrations of the coagonists PS may slightly increase their affinity for the NMDA receptor. The fractional open time (nP_o) of single NMDA-activated channels was potentiated by PS in patch-clamp recordings using both the outside-out and cell-attached configurations. The potentiation of nP_o resulted from increases in the frequency of opening and in the mean channel open time. No effect was seen on single-channel conductances.

The classical view of steroid hormone action is that these molecules are synthesized by specialized glandular tissues, released into the general circulation of organisms, and carried to target tissues throughout the body via the circulatory system. At target sites, steroids pass through cell membranes by passive diffusion to combine with intracellular cytoplasmic or nuclear receptors. Activated receptor complexes, in turn, combine with specific nucleic acid recognition sequences in chromatin and, in association with other transcriptional factors, enhance or suppress gene expression (for review see Ref. 1). The necessity for activation of the transcriptional and translational machinery of cells requires that steroid hormone actions show lag times of minutes to days before the coordinated physiological consequences of their actions are observed. Similar lengthy periods of time are required for the decline of their effects.

This work was supported by National Institutes of Health Program Project Grant NS 02253-33 to E. J. Furshpan (Program Director) under Project III to E. A. Kravitz (Principal Investigator), National Institutes of Health Training Grant 5 T32 NS 07112-14, and a summer fellowship at the Marine Biological Laboratory (Woods Hole, MA), supported by The Grass Foundation.

Endogenous metabolites of steroids (termed neurosteroids) are found and synthesized *de novo* in neural tissues (for reviews see Refs. 2 and 3). One of these metabolites, pregnenolone, is formed directly from cholesterol by a mitochondrial side-chain cleavage enzyme found in glial cells (2, 4–6), whereas another, allopregnanolone, is formed by reduction of progesterone, which in turn can be formed by dehydrogenation and isomerization of pregnenolone in brain. Many metabolites also are synthesized from gonadal or adrenal steroids in the central nervous system and in peripheral tissues (for reviews see Refs. 2 and 3). In contrast to the classical genomic effects of steroids, many of these substances produce acute physiological actions on membrane proteins, with time delays ranging from seconds to minutes. These substances normally are found in neural tissues at low concentrations (nanomolar range), although their levels can be influenced strongly by stress and other conditions that normally trigger steroid hormone release (4, 7).

The 3-hydroxy neurosteroids modulate $GABA_A$ receptors in neurons, acting to either reduce (3β-hydroxysteroids) or en-

ABBREVIATIONS: GABA, γ-aminobutyric acid; PS, pregnenolone sulfate; NMDA, *N*-methyl-D-aspartate; HEPES, *N*-(2-hydroxyethyl)-1-piperazine-*N*ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PTX, pertussis toxin; G protein, guanine nucleotide-binding protein; AMPA, *DL*-α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

hance (3α-hydroxysteroids) the chloride currents generated by activation of these receptors by GABA (reviewed in Ref. 3). The stereospecificity of their actions, the protein subunit specificity of the magnitude of modulation, and the high affinity of some of these steroids for $GABA_A$ receptors suggest that these substances act through specific binding sites and not as nonspecific membrane-disordering compounds. The effects of neurosteroids appear to be at a novel binding site on the $GABA_A$ receptors, because modulation is not altered by agonists or antagonists of the benzodiazepine or barbiturate sites (8–11).

The naturally occurring sulfate ester of the 3β-hydroxysteroid pregnenolone, PS, has a particularly interesting constellation of actions on neuronal membranes; it reduces the effectiveness of GABA actions via $GABA_A$ receptors (12–14), enhances the effectiveness of glutamate actions via NMDA receptors (15, 16), and reduces voltage-activated calcium currents (17, 18). In combination, these actions could facilitate excitation of neurons at the postsynaptic level. PS also increases the magnitude of the fura-2 fluorescence signals triggered by calcium entry when NMDA is applied to hippocampal neurons in culture (15). At the single-channel level, PS reduces the channel opening frequency of $GABA_A$ receptors (14).

The present studies further define the mechanisms of action of PS on NMDA responses, using rat hippocampal neurons in culture. PS is confirmed to selectively potentiate the NMDA subtype of glutamate receptor. The kinetics of PS action are fast, with potentiation occurring within 25 sec and reversal within 60 sec. Little interaction with the NMDA and glycine binding sites can be detected at high concentrations of the coagonists, suggesting that the effects of PS on the receptor are largely independent of either site. Finally, single-channel measurements demonstrate that PS causes increases in the frequency of channel opening and in the mean channel open time but does not increase the current flow through single NMDA-activated channels.

Materials and Methods

Cell culture. Neurons were dissociated from the hippocampi of 0–1-day-old Long-Evans or Sprague-Dawley rats using methods slightly modified from those of Furshpan and Potter (19). Hippocampi were dissected from anesthetized animals and split longitudinally through the CA3 region. Dorsal portions containing the CA1 region and part of the CA3 region were incubated at 37° for 60 min in a dissociation medium containing 0.45 mg/ml cysteine, 10 units/ml papain, 90 mM Na_2SO_4, 30 mM K_2SO_4, 16 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM HEPES, 1 mM kynurenic acid, and 0.001% phenol red, pH 7.4. The tissues next were incubated for 15 min in a dissociation solution of the same composition but containing no papain or cysteine and with 10 mg/ml ovomucoid trypsin inhibitor. They were then rinsed twice in a growth medium based upon Dulbecco's modified Eagle's medium (Sigma D5405) with the following additions: 10 mM D-glucose, 1 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 27.5 μg/ml transferrin, 8 μg/ml putrescine, 2.5 μg/ml insulin, 2.5 ng/ml sodium selenite, 20 μl/ml rat serum, and 0.1 μM progesterone. Cells were dissociated mechanically in the growth medium and plated at a density of approximately 0.5 hippocampus/8 ml of growth medium (25,000–30,000 cells/cm^2). Cultures were grown at 37° in 10% CO_2 and after 5–6 days were either irradiated (1200 rad) or exposed to 1 μM 1-β-D-arabinofuranosylcytosine for several days, to inhibit proliferation of non-neuronal cells.

Patch-clamp recording. Whole-cell voltage-clamp recordings (20) were performed, at room temperature, on cells that had been in culture for 6–21 days. Recordings were made only from pyramidal neurons, identified by their large size and the shape of their dendritic arbor, with processes visible on top of non-neuronal cells, thus allowing easy access of drugs to the cell surface. During recording sessions, cells were bathed in a modified Tyrode's solution containing (in mM) 150 NaCl, 4 KCl, 2 $CaCl_2$, and 10 HEPES. Electrodes were pulled from 100-μl Boralex micropipettes (Drummond Scientific), coated with sylgard, fire polished, and filled with a solution containing (in mM) 126 cesium methanesulfonate, 4.5 $MgCl_2$, 9 EGTA, 9 HEPES, 0.3 GTP, 4 MgATP, and 14 creatine phosphate, pH 7.4. With this solution the electrode resistance ranged from 5 to 10 MΩ. The neurosteroids and other test drugs were obtained from Sigma Chemical Co. (St. Louis, MO). These substances were added to the modified Tyrode's solution and applied to patches and cell surfaces by gravity perfusion through a linear array of eight microcapillary tubes (Drummond Microcaps, 1 μl, 32-mm length). Solutions were exchanged in <250 msec, as determined by measuring the rise time in the holding current produced by the various agonists. All test solutions delivered through the microcapillary tubes contained 0.5 μM tetrodotoxin, which effectively blocked the spontaneous synaptic activity normally present in cultures, equivalent amounts of dimethylsulfoxide (0.05–0.2%), and 10 μM glycine, unless otherwise indicated. A separate gravity-driven perfusion system bathed the culture dish at all times with modified Tyrode's solution free of drugs. The cell membrane was held at −70 mV, unless otherwise specified.

Single-channel recordings were obtained using outside-out and cell-attached membrane patches. For outside-out patches, the same internal and external solutions were used as in whole-cell recordings. For the cell-attached patch recordings, the membrane potential was brought to 0 mV using an external solution containing (in mM) 145 potassium gluconate, 5 $MgCl_2$, 10 HEPES, and 1 EGTA, plus 0.5 μM tetrodotoxin and 0.2% dimethylsulfoxide. The patch electrode was filled with modified Tyrode's solution plus the coagonists NMDA and glycine. Patches were voltage clamped at −70 mV.

In all cases, gigaohm seals onto cell membranes were formed in the modified Tyrode's solution before perfusion with test solutions. Currents were recorded using an Axopatch 200 amplifier (Axon Instruments) and were filtered at 1–2 kHz (eight-pole Bessel low-pass filter). Data were collected and analyzed using the PClamp data acquisition programs (version 5.5.1; Axon Instruments). Whole-cell currents were measured as the difference between the steady state current in the presence of an agonist and the leak conductance and are reported as the mean ± standard error. Curve fitting of dose-response data was performed using Sigmaplot 5.0 (Jandel Scientific), whereas fitting of single-channel data was performed using PClamp. A minimum of 200 μsec was used as the criterion for accepting single opening and closing events.

Results

Characteristics of the potentiation. In hippocampal cell culture preparations, consistent inward currents were seen in response to application of NMDA and glycine, with little "rundown" observed with repeated applications. Application of PS before NMDA resulted in a PS concentration-dependent enhancement of the NMDA-activated inward currents (Fig. 1A). The data were fit to the logistic equation to estimate the potency of PS for potentiating the NMDA current. The potentiation was significantly different from controls above 250 nM (analysis of variance, $p < 0.05$), with an approximate half-maximal effect at 29 μM and one-to-one binding (Fig. 1B). The dose-response curve did not fully saturate at concentrations up to 100 μM; higher concentrations could not be studied because of the limit of PS solubility in Tyrode's saline (about 100 μM).

Small, reversible, inward deflections in the holding current were often observed at PS concentrations greater than 50 μM.

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

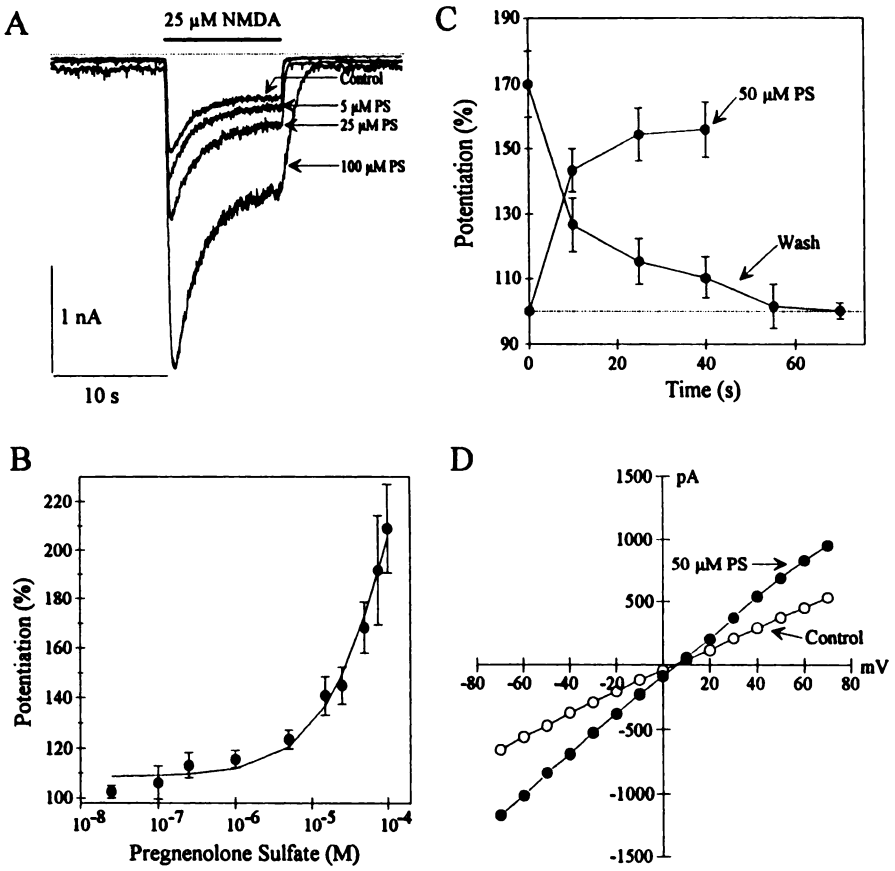

Fig. 1. NMDA-activated current is potentiated in the presence of PS. A, Current traces from a cell exposed to PS for 80 sec before addition of 25 μM NMDA. *Dotted line*, the level of 0 current. The slower return to the base-line current in the presence of 100 μM PS was due to a slower washout of NMDA, because a small amount of PS precipitate covered the neurites and thus limited access of the wash solution to the cell. B, Dose-response curve for PS. Cells were stimulated using 25 μM NMDA. Whole-cell currents were measured as the difference between the steady state current in the presence of an agonist and the leak conductance. Data were fit to a logistic equation, yielding a half-maximal effect at 29 μM and a Hill coefficient of 0.84 (n = 4–9 cells for each dose). C, The time course of potentiation and its reversal is rapid. NMDA (25 μM) was applied in the absence of PS, and then PS (50 μM) was applied continuously while NMDA was applied every 15 sec (n = 7). For washout, PS was applied for 60 sec, and then NMDA (25 μM) was applied every 15 sec while PS was washed out (n = 6). D, Current-voltage relationship measured in the presence of NMDA (25 μM) and glycine (10 μM) (after desensitization) in the absence (○) and presence (●) of 75 μM PS. Leak currents (without agonists) in the absence and presence of PS were subtracted. Membrane voltage was changed every 2 sec, in 10-mV increments. Series resistance and cell capacitance were compensated approximately 40%.

Several reasons make it unlikely that these changes could account for the potentiation of the NMDA current. First, the holding current (in 75–100 μM PS) changed little in comparison with the magnitude of the potentiated current, increasing by at most 10% of the potentiated current magnitude. In addition, data were always reported after subtraction of the base-line current, thus removing the influence of holding current. Finally, there was no evidence of single-channel openings (NMDA or other channels) induced by PS alone. What is more likely, therefore, is that the changes in holding current result from steroid partitioning into the lipid bilayer and mediating deterioration of the seals onto the cells.

The potentiation of NMDA currents by PS begins, reaches a maximum, and reverses relatively rapidly. Approximately 80% of the increase occurred within 10 sec of addition of PS, with a maximal value reached by 25 sec (Fig. 1C). The currents returned to control values by 60 sec after washout with PS-free solutions (Fig. 1C). The speed of these actions makes any genomic explanations of the mechanism of action of this neurosteroid highly unlikely.

The ionic selectivity of NMDA channels apparently did not change when the channels were exposed to PS, because the reversal potential of the NMDA current-voltage relationship did not shift significantly in the presence of 75 μM PS (Fig. 1D). These results suggest that modulation or direct activation of a second ionic conductance probably is not responsible for the PS-induced current enhancement. The potentiation was not appreciably voltage dependent at potentials ranging from −70 to +70 mV.

Specificity of PS potentiation. Recordings from hippocampal pyramidal cells in culture yielded a selective potentiation of NMDA-generated currents by PS similar to that previously reported for spinal cord neurons in culture (16). The current activated by NMDA and D-serine, in the absence of added glycine, was potentiated by 169 ± 10% in the presence of 50 μM PS, whereas the kainate- and AMPA-activated currents were slightly inhibited, by 6 ± 2% and 7 ± 3%, respectively (Fig. 2). The magnitude of potentiation of the NMDA current in the presence of D-serine as the coagonist was similar to that seen when glycine was the coagonist, indicating that modulation of glycine currents was not responsible for the observed potentiation.

Several other neuroactive steroids that are closely related in structure to PS were examined for potential modulatory effects on the NMDA current. Enhancement of current was limited to 8% with pregnenolone (10 μM), 2% with pregnenolone acetate (5 μM), 5% with progesterone (10 μM), and 10% with dehydro epiandro sterone sulfate (25 μM) (n = 6), compared with 41% enhancement by 15 μM PS (Fig. 1B), indicating that the potentiation of NMDA-activated currents may be relatively specific for PS.

Site of action of the neurosteroid. PS (50 μM) potentiated the NMDA-activated current at all concentrations of NMDA and glycine, although the percentage potentiation was greater at lower concentrations (Fig. 3). NMDA and glycine dose-response curves, even at saturating concentrations, were shifted upward in the presence of PS (Fig. 3, B and E). This suggests that at saturating concentrations of the coagonists PS does not change the affinity between the coagonists and the NMDA receptor and there is no competition between PS and the

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

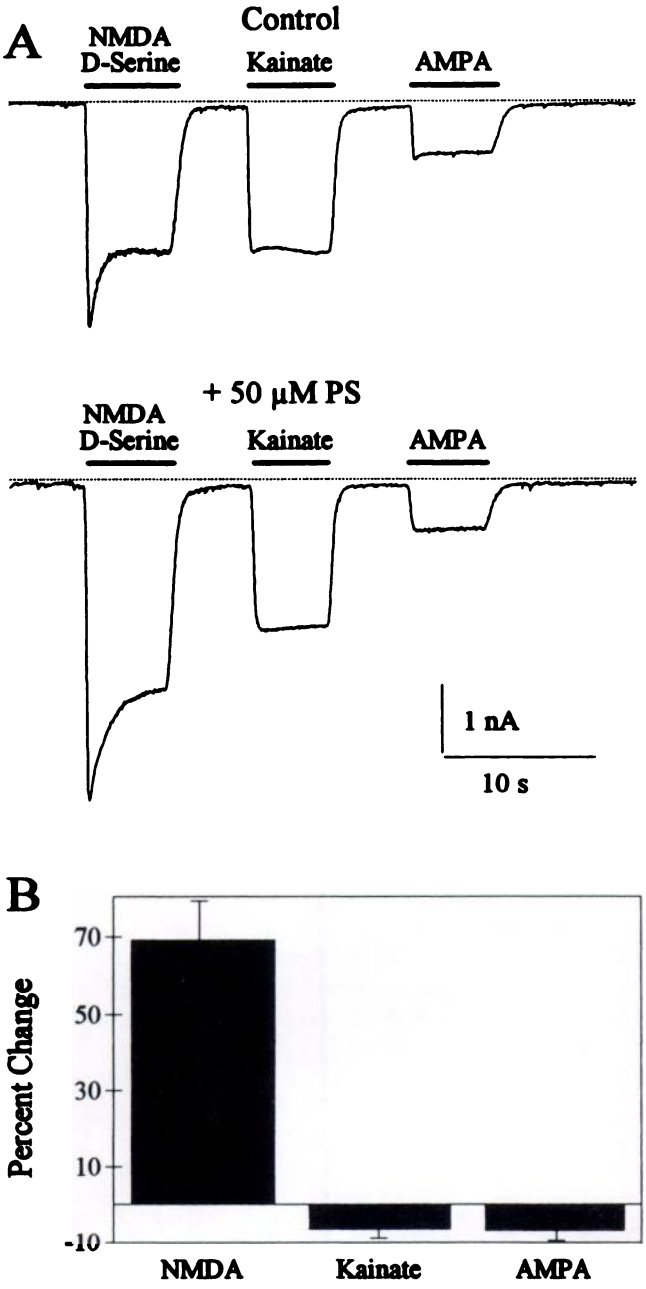

Fig. 2. Non-NMDA-activated glutamate currents are not potentiated by PS. Agonists were applied to cells ($n = 6$) for 5 sec, both in the absence (control) and in the presence of 50 μM PS. A, Control and PS-exposed current traces from one cell exposed first to 25 μM NMDA and 25 μM D-serine, then to 40 μM kainate, and finally to 20 μM AMPA. PS was applied 60 sec before the second current trace. B, Mean percent change in the NMDA, kainate, and AMPA currents ($n = 8$, 6, and 6, respectively) in response to 50 μM PS. Agonist concentrations were as in A.

coagonists for receptor activation. The EC_{50} values for both NMDA and glycine did shift slightly with addition of PS, although the shifts were not statistically significant because the 95% confidence limits overlap. The percentage potentiation was significantly greater at lower concentrations of the coagonists (analysis of variance, $p < 0.05$; Fig. 3, C and F), and an increase in the rate of desensitization with 10 nM glycine often occurred (Fig. 3D), suggesting that at low concentrations of the coagonists PS may be slightly affecting their binding to the receptor.

The effect of PS on cells treated for 24–48 hr with 200–500 ng/ml PTX was examined to determine whether PTX-sensitive G proteins are involved in the mechanism of PS action. In PTX-treated cells, 50 μM PS produced a 64 ± 7% increase in the NMDA current, compared with a 77 ± 12% increase in the control cells ($n = 4$). PTX did not significantly (t test, $p > 0.05$) block the PS-mediated potentiation of the NMDA-activated whole-cell current, and thus PTX-sensitive G proteins are probably not involved in the mechanism of PS action.

Single-channel measurements. The effect of PS on NMDA receptors was investigated at the single-channel level using excised outside-out membrane patches. The time course of PS action was similar to that in the whole-cell configuration, with a maximal effect being observed within 30 sec of PS application. In the patch illustrated in Fig. 4, the fractional open time in the presence of 5 μM NMDA was increased from 3.5% in the control solution (Fig. 4A) to 6.8% in the presence of 50 μM PS (Fig. 4D) (mean increase, 197 ± 32%; $n = 3$). In 55 sec of recording time in each solution, the number of openings increased from 839 in the control solution to 1589 in the presence of PS (mean increase, 180 ± 46%). In some patches, there was also a small PS-induced increase in mean channel open time ($\langle t_o \rangle$; mean increase, 130 ± 12%; two of three patches). In many patches two channel substates or types were observed, i.e., small conductance and large conductance forms. These two types of openings were similar to the substates of the NMDA channel described previously (21, 22). The frequency of both small and large openings was increased with PS. PS did not alter the conductance of either type of opening (Fig. 4, C and F).

When PS was applied to the outside of cells during recording from cell-attached patches, similar results and time courses were seen as with the outside-out patches. In the patch illustrated in Fig. 5, the fractional open time increased from 0.2% in control recordings to 0.7% in the presence of 50 μM PS (mean increase, 223 ± 58%; $n = 4$). The frequency of openings increased from 169 (control) to 317 (PS) openings in 110 sec of recording time (mean increase, 156 ± 18%). In this type of recording situation, in contrast to the outside-out recordings, the effects on $\langle t_o \rangle$ were much more significant. The $\langle t_o \rangle$ increased from 1.1 ± 0.1 msec in the control to 2.5 ± 0.1 msec with PS (mean increase, 211 ± 71%). The record shown in Fig. 5 shows two long openings with flickery behavior in the presence of PS. These flickery openings were not a specific action of PS, because similar openings were seen in control records. They were, however, more obvious with PS due to the increase in $\langle t_o \rangle$.

Discussion

The studies presented here, using hippocampal neurons in culture, confirm that the NMDA subtype of glutamate receptor produces a greater macroscopic current in the presence of PS. These observations expand on studies carried out using spinal cord neurons in culture (16) and derive from experiments demonstrating an excitatory action of PS and related compounds on central nervous system neurons in intact tissues (23). The present experiments add the following details on the mechanism of action of the steroid: 1) PS potentiates NMDA-activated currents equally across the normal physiological range of holding potentials; 2) similarly to an earlier study (16), the non-NMDA subtypes of glutamate currents are inhibited

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

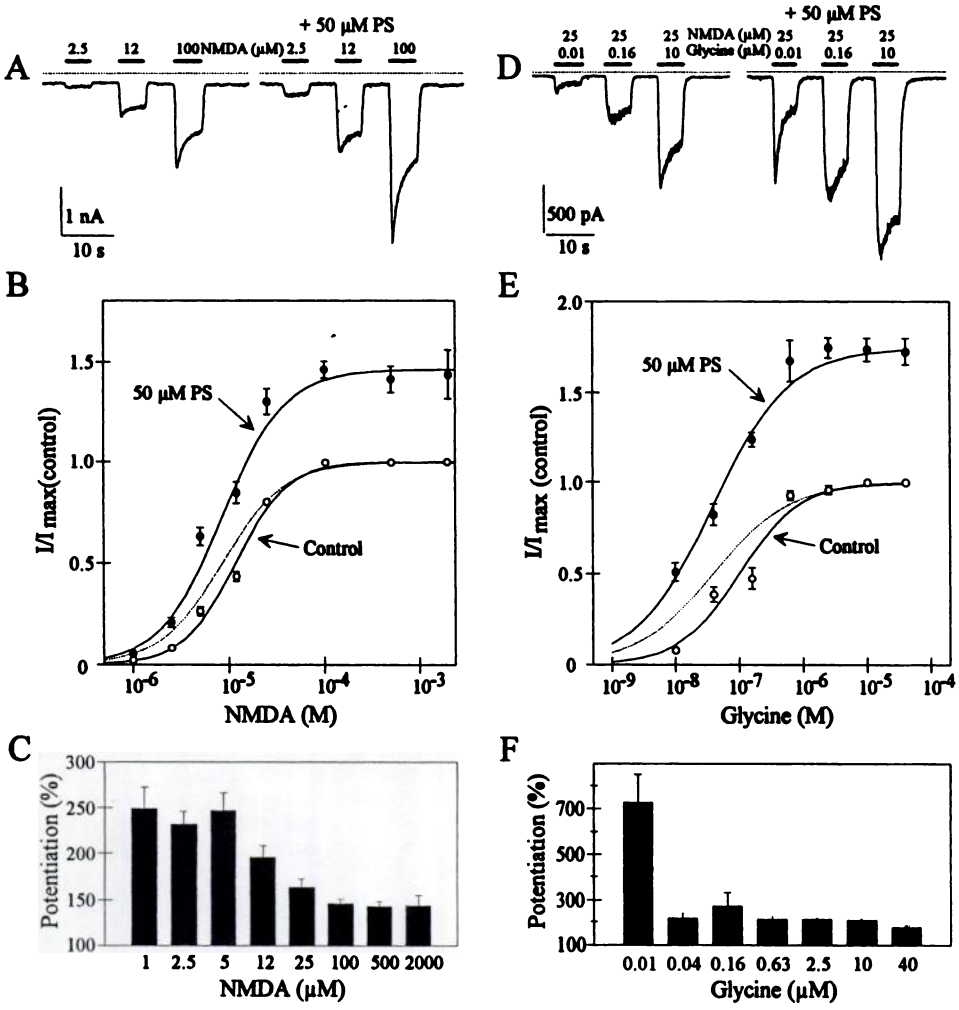

Fig. 3. Dose-response curves for NMDA and glycine are shifted upward in the presence of PS. A, Whole-cell current was measured in response to three concentrations of NMDA, PS (50 μM) was then applied for 60 sec, and the doses of NMDA were repeated in the presence of PS (n = 4–7). *Dotted line*, the 0 current level. B, Dose-response curves for NMDA in the absence and presence of 50 μM PS. Current was calculated as a fraction of the current produced by a saturating concentration of NMDA in the absence of PS. The data points were fit to the logistic equation, which predicted an EC_{50} of 11.8 ± 0.4 μM (95% confidence interval, 8.7–15.9 μM) and a Hill coefficient of 1.6 for the control and an EC_{50} of 8.6 ± 0.4 μM (95% confidence interval, 6.6–11.1 μM) and a Hill coefficient of 1.4 with PS. *Dotted line*, potentiated dose-response curve scaled to the control level. C, Percent potentiation for each concentration of NMDA. D, Whole-cell current measured in response to 25 μM NMDA and three concentrations of glycine; agonists and PS were applied as in A (n = 6–12). E, Dose-response curves for glycine in the absence and presence of 50 μM PS. Current was calculated as a fraction of the current produced by a saturating concentration of glycine in the absence of hormone. The data points were fit to the logistic equation, which predicted an EC_{50} of 0.10 ± 0.010 μM (95% confidence interval, 0.025–0.41 μM) and a Hill coefficient of 0.92 for the control and an EC_{50} of 0.039 ± 0.0052 μM (95% confidence interval, 0.008–0.19 μM) and a Hill coefficient of 0.73 with PS. *Dotted line*, potentiated dose-response curve scaled to the control level. F, Percent potentiation for each concentration of glycine.

slightly by PS; 3) when complete dose-response curves for glycine and NMDA are constructed, no change in affinity of the coagonists for the receptor is observed, except at low concentrations of the coagonists; and 4) the single NMDA receptor channel opening frequency and mean open time are increased by PS, whereas the single-channel conductance remains unchanged.

PS has a wide range of effects on membrane channels, but the effects are selective and differ for each channel type. Thus, at concentrations in the high nanomolar to micromolar range, PS potentiates NMDA currents (15, 16, present study), reduces the magnitude of GABA and glycine currents (12–14, 24), and inhibits voltage-activated Ca^{2+} channels in hippocampal neurons (17, 18). The diversity and specificity of the actions make it unlikely that PS acts on membrane proteins via a nonspecific membrane-disordering mechanism, like that proposed for barbiturates and short-chain alcohols (25, 26). It seems likely that specific sites of action for PS and related substances will be found on subunits of the NMDA receptor, as has been shown for neurosteroid action on $GABA_A$ receptors.

Several experimental results suggest that potentiation of the NMDA receptor by PS may result from a direct action of the steroid on the receptor. First, the potentiation by PS begins within seconds of its application and reverses in a similar time period on washout of the steroid. Second, PTX does not block the effect of PS, indicating that PTX-sensitive G proteins are not directly involved in the mechanism of action. Third, potentiation of NMDA channels in excised outside-out membrane patches also is consistent with a direct action. The potentiation that occurs in the cell-attached patch configuration, however, suggests that either 1) a diffusible second messenger pathway is involved or 2) the lipophilic steroid is entering the cell or cell membrane and reaching the channels in the patch by diffusion. This second possibility may be more likely, due to the lipophilic nature of steroid hormones. Further experiments with NMDA receptor channels in a cell-free system would probably resolve this issue. The present results do, however, make highly unlikely the classical mechanisms of steroid hormone action involving transcriptional mechanisms.

Potentiation of the NMDA current by PS occurs in the presence of saturating concentrations of NMDA and glycine, indicating that at saturating concentrations of the coagonists 1) PS does not change the affinity of the coagonists for the receptor and 2) there is no competition between PS and these coagonists for receptor activation. In addition, PS does not appear to recruit an additional group of NMDA receptors, because the saturation point in the dose-response curve remains unchanged. Several modulatory sites have been described on the NMDA receptor, and PS may function by binding to a previously described site or to a novel site to produce allosteric

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

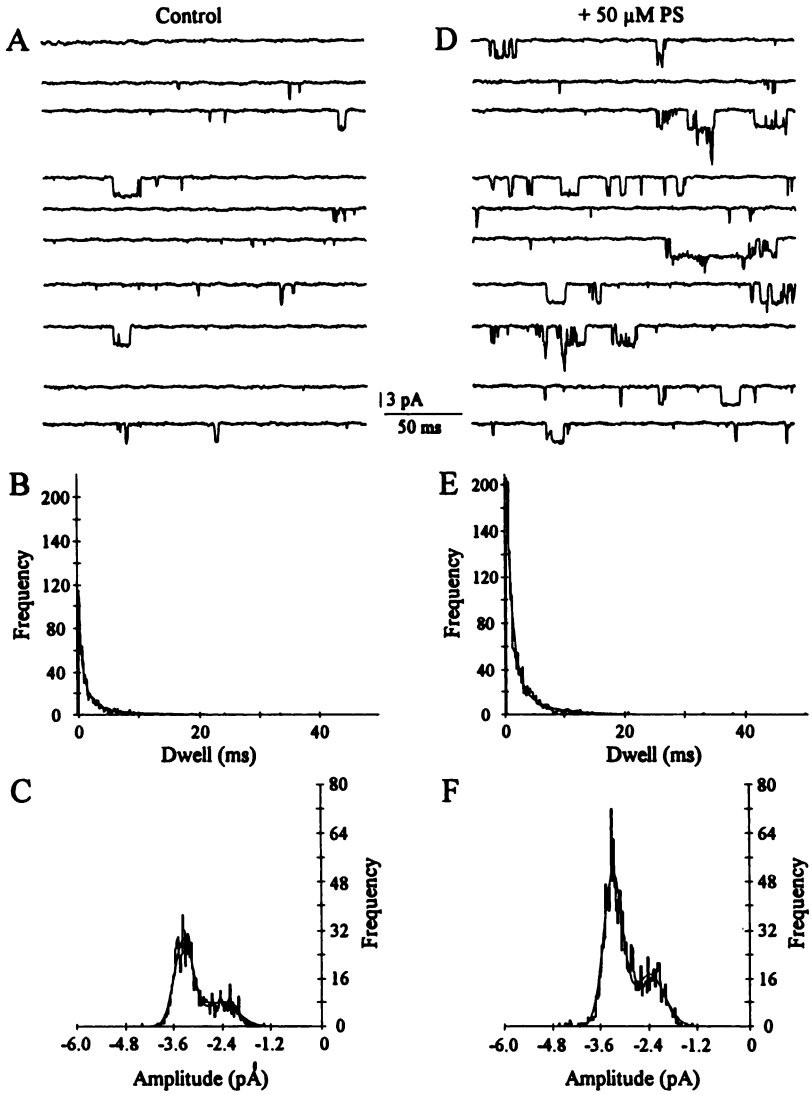

Fig. 4. Frequency of single-channel openings increases in the presence of PS. Single channels were recorded in an excised outside-out patch, held at −70 mV, in the presence of 5 μM NMDA and 1 μM glycine, with either 0.2% dimethylsulfoxide (control) or 50 μM PS (in the presence of 0.2% dimethylsulfoxide). No channel openings were observed in the absence of the coagonists, either with or without PS. A, Sequential traces in the absence of PS. Two channel substates or types were observed, similar to those described previously (21, 22). B, Open-time histogram in the absence of PS was fit by the sum of three exponential functions, with time constants of 0.3, 1.3, and 6.8 msec. A minimum of 200 μsec was used as the criterion for accepting single opening and closing events. C, Amplitude histogram in the absence of PS showed two peaks, corresponding to the two channel types observed in A. The histogram was fit by the sum of two gaussian functions, with means of −2.52 ± 0.05 pA and −3.45 ± 0.01 pA. D, Sequential traces in the presence of PS. The same channel substates as in A were observed. E, Open-time histogram in the presence of PS showed an increased frequency of openings. The histogram was fit by the sum of three exponential functions, with time constants of 0.9, 4.0, and 5.2 msec. F, Amplitude histogram in the presence of PS was similar to that in C and was fit by the sum of two gaussian functions, with means of −2.46 ± 0.03 pA and −3.37 ± 0.01 pA.

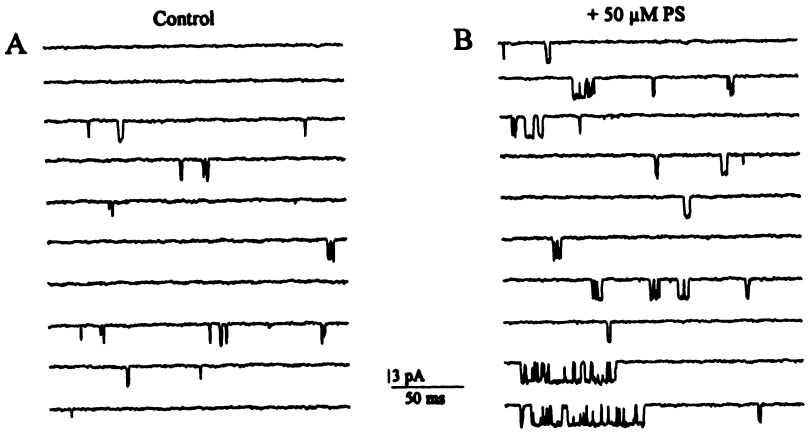

Fig. 5. The frequency and mean channel open time of channels recorded in the cell-attached configuration increase with PS. A continuous record with the coagonists NMDA (25 μM) and glycine (1 μM) included in the pipette is shown. The 145 mM potassium gluconate solution, containing 0.2% dimethylsulfoxide (control) (A) or 50 μM PS (B), was applied externally to zero the cell membrane potential. The patch was held at −70 mV.

changes in receptor conformation. Such a mechanism has been proposed to explain the actions of neurosteroids on $GABA_A$ receptors and may be the general mechanism of action of these compounds (12).

The dose-response relations for NMDA and glycine showed small and statistically insignificant leftward shifts in their EC_{50} values in the presence of PS. The percent potentiation of responses by PS, however, was significantly greater at lower concentrations of the two coagonists. These results suggest that at low concentrations of the coagonists PS may slightly increase the affinity of the coagonists for the NMDA receptor.

The PS-induced increases in the frequency of opening and mean open time for NMDA-activated channels are similar to the reported actions of other neurosteroids on the $GABA_A$ channel. Several progesterone metabolites cause increases in the mean open time and burst duration of $GABA_A$ channels (9,

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

27), whereas PS decreases the channel opening frequency of similar populations of $GABA_A$ channels (14). Apparently PS does not directly gate NMDA channels in the absence of NMDA and glycine, because no channel openings were observed with PS alone. Small increases in membrane conductance, which were sometimes observed at high concentrations of PS, appear to result from interference with the membrane seal by this lipophilic substance. An interesting difference seen when recordings of PS actions on outside-out patches are compared with actions on cell-attached patches is that the effects on mean channel open time are seen much more readily under the latter experimental conditions. This may be caused by the loss of other intracellular modulators in the excised patch configuration, analogous to the "rundown" of current that is sometimes observed.

In considering the physiological significance of PS, it is important to note that pregnenolone, and its sulfate and ester metabolites, are synthesized from cholesterol and normally found both in intact brain and in glial cultures (4–6). PS levels in anterior parts of the rat brain are about 16 ng/g of total protein (4). These levels probably can produce small potentiations of NMDA currents. Unfortunately, it is not known whether higher local concentrations of these substances exist in the extracellular space around neurons, whether the substances normally are synthesized and released by glial cells, and, if they are released, whether this takes place in a continuous fashion or in response to physiological cues. It has been reported, however, that the levels of pregnenolone and PS found in the brain are independent of adrenal steroidogenesis (4). The observations that PS increases NMDA-activated excitatory current in neurons (15, 16, present study), decreases $GABA_A$- and glycine-activated inhibitory currents (12–14, 24), and decreases calcium channel currents (17, 18) raise many interesting possibilities about functional roles for these substances. Such an enhancement of excitatory input to neurons, along with a reduction of calcium entry, could be important in facilitating information transfer along selected pathways in the nervous system, although neurons susceptible to NMDA-mediated excitotoxicity may be more likely to experience cell death. Recently, it was demonstrated that intracerebroventricular injections of PS enhanced memory retention for footshock active avoidance training (28). Whether endogenous PS and related neurosteroids normally are involved in processes like memory training or other kinds of synaptic plasticity remain exciting prospects for future investigations of this interesting class of compounds.

Acknowledgments

Thanks go to E. Kravitz, B. Bean, and K. Swartz for advice, encouragement, and comments on the manuscript.

References

1. Evans, R. M. The steroid and thyroid hormone receptor superfamily. *Science (Washington D. C.)* **240**:889–895 (1988).
2. Baulieu, E. E. Neurosteroids: a new function in the brain. *Biol. Cell.* **71**:3–10 (1991).
3. Paul, S. M., and R. H. Purdy. Neuroactive steroids. *FASEB J.* **6**:2311–2322 (1992).
4. Corpéchot, C., M. Synguelakis, S. Talha, M. Axelson, J. Sjövall, R. Vihko, E. E. Baulieu, and P. Robel. Pregnenolone and its sulfate ester in the rat brain. *Brain Res.* **270**:119–125 (1983).
5. Hu, Z. Y., E. Bourreau, I. Jung-Testas, P. Robel, and E. E. Baulieu. Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone. *Proc. Natl. Acad. Sci. USA* **84**:8215–8219 (1987).
6. Jung-Testas, I., Z. Y. Hu, E. E. Baulieu, and P. Robel. Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology* **125**:2083–2091 (1989).
7. Purdy, R. H., A. L. Morrow, P. H. Moore, and S. M. Paul. Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc. Natl. Acad. Sci. USA* **88**:4553–4557 (1991).
8. Gee, K. W., W. C. Chang, R. E. Brinton, and B. S. McEwen. GABA-dependent modulation of the Cl^- ionophore by steroids in rat brain. *Eur. J. Pharmacol.* **136**:419–423 (1987).
9. Lambert, J. J., J. A. Peters, and G. A. Cottrell. Actions of synthetic and endogenous steroids on the $GABA_A$ receptor. *Trends Pharmacol. Sci.* **8**:224–227 (1987).
10. Lambert, J. J., C. Hill-Venning, J. A. Peters, N. C. Sturgess, and T. G. Hales. The actions of anesthetic steroids on inhibitory and excitatory amino acid receptors, in Fidia Research Foundation Symposium Series (E. Costa and E. Barnard, eds.), Vol. 6. Thieme Medical Publishers, New York, 219–236 (1991).
11. Morrow, A. L., J. R. Pace, R. H. Purdy, and S. M. Paul. Characterization of steroid interactions with γ-aminobutyric acid receptor-gated chloride ion channels: evidence for multiple steroid recognition sites. *Mol. Pharmacol.* **37**:263–270 (1990).
12. Majewska, M. D., S. Demirgoren, C. E. Spivak, and E. D. London. The neurosteroid dehydroepiandrosterone sulfate is an allosteric antagonist of the $GABA_A$ receptor. *Brain Res.* **526**:143–146 (1990).
13. Majewska, M. D., and R. D. Schwartz. Pregnenolone sulfate: an endogenous antagonist of the γ-aminobutyric acid receptor complex in brain? *Brain Res.* **404**:355–360 (1987).
14. Mienville, J. M., and S. Vicini. Pregnenolone sulfate antagonizes $GABA_A$ receptor-mediated currents via a reduction of channel opening frequency. *Brain Res.* **489**:190–194 (1989).
15. Irwin, R. P., N. J. Maragakis, M. A. Rogawski, R. H. Purdy, D. H. Farb, and S. M. Paul. Pregnenolone sulfate augments NMDA receptor mediated increases in intracellular Ca^{2+} in cultured rat hippocampal neurons. *Neurosci. Lett.* **141**:30–34 (1992).
16. Wu, F. S., T. T. Gibbs, and D. H. Farb. Pregnenolone sulfate: a positive allosteric modulator at the *N*-methyl-D-aspartate receptor. *Mol. Pharmacol.* **40**:333–336 (1991).
17. ffrench-Mullen, J. M. H., and K. T. Spence. Neurosteroids block Ca^{2+} channel current in freshly isolated hippocampal CA1 neurons. *Eur. J. Pharmacol.* **202**:269–272 (1991).
18. Spence, K. T., C. R. Plata-Salaman, and J. M. H. ffrench-Mullen. The neurosteroids pregnenolone and pregnenolone-sulfate, but not progesterone, block Ca^{2+} currents in acutely isolated hippocampal CA1 neurons. *Life Sci.* **49**:PL235–PL239 (1991).
19. Furshpan, E. J., and D. D. Potter. Seizure-like activity and cellular damage in rat hippocampal neurons in cell culture. *Neuron* **3**:199–207 (1989).
20. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
21. Ascher, P., P. Bregestovski, and L. Nowak. *N*-Methyl-D-aspartate-activated channels of mouse central neurones in magnesium-free solutions. *J. Physiol. (Lond.)* **399**:207–226 (1988).
22. Gibb, A. J., and D. Colquhoun. Activation of *N*-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus. *J. Physiol. (Lond.)* **456**:143–179 (1992).
23. Carette, B., and P. Poulain. Excitatory effect of dehydroepiandrosterone, its sulphate ester and pregnenolone sulphate, applied by iontophoresis and pressure, on single neurones in the septo-preoptic area of the guinea pig. *Neurosci. Lett.* **45**:205–210 (1984).
24. Wu, F. S., T. T. Gibbs, and D. H. Farb. Inverse modulation of γ-aminobutyric acid- and glycine-induced currents by progesterone. *Mol. Pharmacol.* **37**:597–602 (1990).
25. Lawrence, D. K., and E. W. Gill. Structurally specific effects of some steroid anesthetics on spin-labeled liposomes. *Mol. Pharmacol.* **11**:280–286 (1975).
26. Lovinger, D. M., G. White, and F. F. Weight. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science (Washington D. C.)* **243**:1721–1724 (1989).
27. Callachan, H., G. A. Cottrell, N. Y. Hather, J. J. Lambert, J. M. Nooney, and J. A. Peters. Modulation of the $GABA_A$ receptor by progesterone metabolites. *Proc. R. Soc. Lond. B Biol. Sci.* **231**:359–369 (1987).
28. Flood, J. F., J. E. Morley, and E. Roberts. Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. *Proc. Natl. Acad. Sci. USA* **89**:1567–1571 (1992).

Send reprint requests to: Mark R. Bowlby, Department of Biochemistry, Brandeis University, 415 South St., Waltham, MA 02254.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012