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ACCELERATED COMMUNICATION

Pregnenolone Sulfate: A Positive Allosteric Modulator at the *N*-Methyl-D-aspartate Receptor

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SUMMARY

The *N*-methyl-D-aspartate (NMDA) receptor is believed to play a major role in learning and in excitotoxic neuronal damage associated with stroke and epilepsy. Pregnenolone sulfate, a neurosteroid, specifically enhances NMDA-gated currents in spinal cord neurons, while inhibiting receptors for the inhibitory amino acids glycine and γ-aminobutyric acid, as well as non-NMDA glutamate receptors. This observation is consistent with the hypothesis that neurosteroids such as pregnenolone sulfate are involved in regulating the balance between excitation and inhibition in the central nervous system.

L-Glutamate is thought to be the major excitatory neurotransmitter in the CNS. Two major pharmacological classes of glutamate-gated ion channels have been identified. NMDA receptors respond preferentially to the synthetic aspartate analog NMDA, whereas non-NMDA receptors respond preferentially to kainate and AMPA. NMDA receptors have attracted particular attention by virtue of their proposed role in long term potentiation (1), learning (2), hypoxic neuronal damage, and epilepsy (3).

Although steroid effects mediated by genomic steroid response elements have been studied extensively, it is now evident that many steroids also have direct neuromodulatory effects on a variety of neurotransmitter receptors (4, 5). Moreover, there is evidence for the local synthesis of certain steroids (termed "neurosteroids") in the brain (6–9). In particular, the neurosteroid PS (Fig. 1) has been proposed as an endogenous negative modulator of the $GABA_A$ receptor in brain (10). We have shown previously that PS is also a negative modulator of the glycine receptor (5). We now show that PS specifically potentiates the response of the NMDA receptor, while inhibiting GABA, glycine, and non-NMDA receptors.

Materials and Methods

Cell cultures. Cultures of dissociated spinal cord neurons were prepared from 7-day chick embryos (5). Briefly, dissociated cells were plated on collagen-coated 35-mm tissue culture dishes, in Eagle's minimum essential medium supplemented with 2.4 mM glutamine, 10% (v/v) heat-inactivated horse serum, 5% (v/v) chick embryo extract, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°. Cytosine arabinoside (1 μM) was added after 36 hr to control the proliferation of nonneuronal cells. This medium was removed 1 day later and replaced with a similar medium supplemented with 20.5 mM glucose and 18 mM KCl and containing 2.5% chick embryo extract. Fresh medium was added twice weekly. Cultured neurons were used in experiments 2–4 weeks after plating.

Electrophysiology. Experiments were carried out in 35-mm tissue culture dishes on the stage of an inverted phase-contrast microscope. Whole-cell currents were recorded by the whole-cell variant of the patch-clamp technique (11). Patch electrodes were fabricated with a double pull from thin-wall borosilicate glass microcapillary pipets (Fischer) on a David Kopf vertical pipet puller (model 700D). Electrode resistance was 5.2 ± 0.12 MΩ (n = 65) when filled with intracellular solution. The electrode solution usually contained (in mM) 10 KCl, 3 sodium gluconate, 140 potassium gluconate, 11 EGTA, and 10 HEPES (pH adjusted to 7.2 with KOH). For GABA experiments, a high Cl^- intracellular solution (in mM, 140 KCl, 3 NaCl, 1 $MgCl_2$, 11 EGTA, and 10 HEPES, pH adjusted to 7.2 with KOH) was used. The bath solution contained (in mM) 150 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH adjusted to 7.2 with NaOH). Because NMDA-induced currents are subject to voltage-dependent block by extracellular Mg^{2+}, no magnesium salts were added to the bath solution for NMDA experiments. All experiments were performed at room temperature (23–25°).

Recordings were made using a Yale MK V amplifier. After formation of a tight seal (typically 1–10 GΩ), capacitative transients were minimized. The patch of membrane under the pipet tip was then ruptured by gentle suction, to obtain the whole-cell configuration. Cells with

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ABBREVIATIONS: CNS, central nervous system; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GABA, γ-aminobutyric acid; HEPES, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; PS, pregnenolone sulfate.

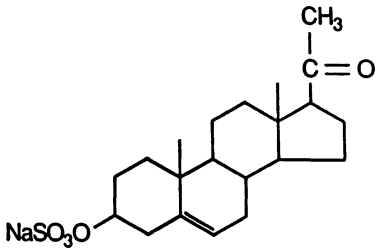

Fig. 1. Chemical structure of PS sodium.

series resistance greater than 10 MΩ were rejected. Series resistance, which initially measured 7.3 ± 0.14 MΩ (n = 65), was compensated (>60%). Only cells with resting membrane potential more negative than −55 mV and input resistance in excess of 150 MΩ were used. All recordings were made with the cell membrane potential clamped at −70 mV. Currents were filtered at 1 kHz using an eight-pole Bessel filter and digitized (40 msec/point) using an on-line data acquisition system (pClamp; Axon Instruments).

Drug solutions were applied to single neurons by pressure ejection (15 psi) from seven-barrel pipets (12, 13). Pipets were pulled from Omega dot tubing to a tip diameter of about 1 μm/barrel and were broken back to about 3–5 μm/barrel after filling. Seven-barrel pressure pipets were positioned approximately 50 μm from the neuronal soma. Under these conditions, the drug solution in the pressure pipet rapidly and effectively replaces the solution surrounding the target neuron, with <10% dilution (12). All drugs were obtained from Sigma, with the exception of AMPA hydrobromide (Research Biochemicals) and PS sodium (Steraloids). Stock solutions of steroids were prepared in dimethyl sulfoxide (final concentration, 0.5%, v/v). To obviate the possible effect of dimethyl sulfoxide on the relevant agonist-induced currents, all other drug solutions, including NMDA, kainate, AMPA, GABA, glycine, and external buffer (in the pressure pipet), also contained 0.5% dimethyl sulfoxide. In all experiments, neurons received a 10-sec prepulse of either external buffer or drug solution, followed by a 10-sec application of agonist, followed by a 10–20-sec pulse of external buffer solution. A period of 2–3 min was allowed between successive applications of agonist.

The degree of modulation of the amino acid response by PS, the percent change, is expressed as $(I'/I - 1) \times 100\%$, where I is the average of control responses obtained from the same cell before application and after washout of PS and I' is the agonist-induced current in the presence of PS. In all cases, complete or nearly complete reversal of the steroid effect was obtained after washout. Throughout, results are expressed as mean ± standard error; statistical comparison of groups was carried out using Student's t test.

Results and Discussion

Currents elicited by NMDA, kainate, AMPA, or GABA were recorded in primary cultures of chick spinal cord neurons by the whole-cell variant of the patch-clamp technique (11). At −70 mV, responses to 30 μM NMDA were increased approximately 3-fold by PS (100 μM). Enhancement of the NMDA response by PS was lower at 100 μM NMDA (86 ± 7.5%; n = 4) and virtually eliminated at 1 mM NMDA (1.3 ± 1.3%; n = 2), suggesting that PS increases the potency of NMDA, rather than the maximum response. In contrast, responses elicited by kainate or AMPA were slightly inhibited by PS (Fig. 2; Table 1). The observation that AMPA- and kainate-induced currents are inhibited to the same extent by PS is consistent with the view that these two compounds activate the same receptor (14). Not all steroids enhance the response to NMDA. Hydrocortisone (100 μM) failed to enhance the NMDA-induced current, producing instead a slight (15 ± 2.4%; n = 6) inhibition of the NMDA response.

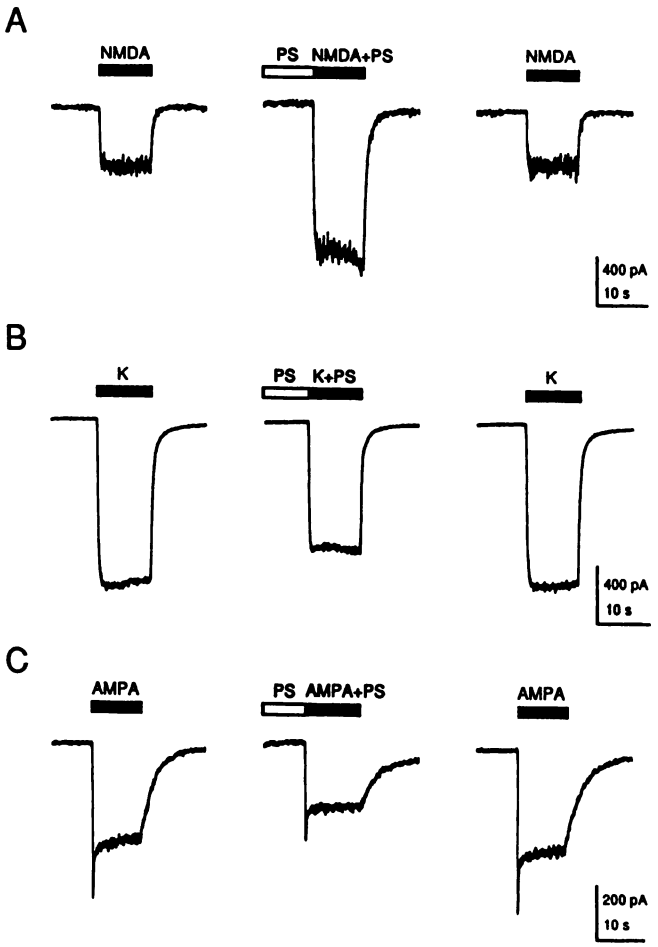

Fig. 2. PS potentiates the NMDA response and inhibits kainate and AMPA responses. A, 100 μM PS dramatically potentiates the current induced by 30 μM NMDA. B, PS (100 μM) inhibits slightly the current induced by 50 μM kainate (*K*). C, PS (100 μM) inhibits slightly the current induced by 25 μM AMPA. *Horizontal bar above each trace*, period of drug application.

TABLE 1

Effects of 100 μM PS on excitatory amino acid responses

The degree of modulation by PS of the amino acid response is expressed as the percentage of change in the amino acid response in the presence of PS, relative to control responses obtained from the same cell before application and after washout of PS. Values are means ± standard errors. The number of cells tested is given in parentheses.

	Change in responses
	%
NMDA (30 μM)	+197 ± 10.6 (6)
Kainate (50 μM)	−25 ± 2.3 (6)
AMPA (25 μM)	−29 ± 3.8 (6)

The response of the NMDA receptor is positively modulated by glycine (15), and glycine may be an absolute requirement for receptor function (16). Because the glycine site associated with the NMDA receptor is of high affinity, it was important to verify that the enhancement produced by PS was not due to glycine contamination of the PS solution. For this reason, we examined the effect of PS on NMDA responses in the presence of a maximal concentration (10 μM) of glycine. Potentiation of the NMDA response by PS did not differ significantly from that measured without added glycine, indicating that glycine contamination cannot account for the observed effect (Fig. 3A).

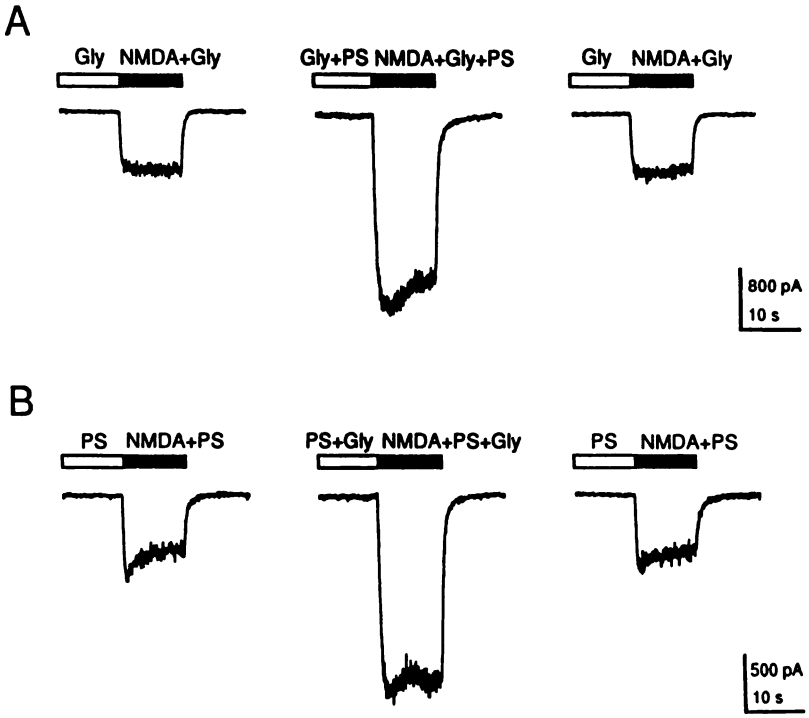

Fig. 3. PS and glycine potentiate the NMDA response by independent mechanisms. A, In the presence of a maximal concentration (10 μM) of glycine (*Gly*), PS (100 μM) markedly enhances (179 ± 17.1%; n = 4) the current activated by 30 μM NMDA. B, In the presence of a near-maximal concentration (100 μM) of PS, 10 μM glycine reversibly potentiates (210 ± 36.5%; n = 4) the peak NMDA response. *Horizontal bar above each trace*, period of drug application.

The observation that PS potentiation of the NMDA response is unchanged in the presence of maximal glycine argues that PS cannot act via the glycine modulatory site on the NMDA receptor, unless it does so with much greater efficacy than glycine itself. In the latter case, glycine should not be able to further enhance the NMDA response in the presence of a high concentration of PS. Fig. 3B shows that potentiation of the NMDA response by glycine (10 μM) was still evident in the presence of a near-maximal concentration (100 μM) of PS, indicating that the steroid modulatory site is distinct from the glycine modulatory site.

Fig. 4 shows that enhancement of the NMDA response by PS was evident over roughly the same concentration range as inhibition of the GABA response, although PS was somewhat less potent at the NMDA receptor (EC_{50} = 57 μM) than at the GABA receptor (EC_{50} = 7 μM). In both cases, Hill slopes were close to 1, consistent with a single class of noninteracting sites. Although a full dose-response curve was not constructed for inhibition of the glycine response by PS, the response to 50 μM glycine was inhibited 61 ± 2% (n = 6) by 5 μM PS and 91 ± 2% (n = 6) by 100 μM PS, suggesting that the potency of PS for inhibition of the glycine response is slightly greater than for inhibition of the GABA response.

The highest reported tissue concentrations of endogenous PS in the CNS are <1 μM (6, 9), below the concentration range at which neuromodulation is observed. This may indicate that PS is not the best candidate for being an endogenous neurosteroid modulator of $GABA_A$, glycine, and NMDA receptors. In the case of the $GABA_A$ receptor, other neurosteroid modulators have been identified with higher potency than PS (4), and we are now searching for more potent steroid modulators of glutamate receptors. On the other hand, because neurosteroids such as PS may be synthesized locally in brain from cholesterol (8, 17), high local or transient PS concentrations could occur. There is evidence that neurosteroid levels can vary in response

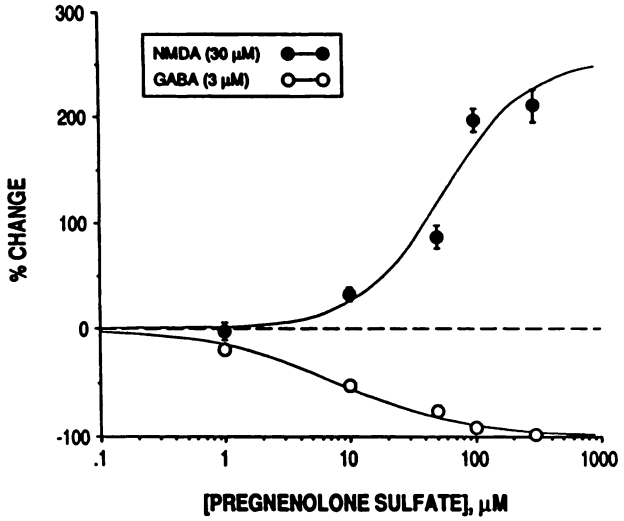

Fig. 4. Dose-response curves for modulation of NMDA- and GABA-induced currents by PS. *Data points*, percentage change in peak current in the presence of PS (mean of four to seven experiments). *Error bars*, standard errors. PS dose-response curves were fitted with the logistic equation (22)

$$\frac{\%\ \text{Change}}{(\%\ \text{Change})_{max}} = \frac{[PS]^{n_H}}{[PS]^{n_H} + EC_{50}^{n_H}}$$

where n_H is the Hill coefficient. For enhancement of the NMDA response, EC_{50} = 57.4 μM, n_H = 1.26, and the maximal potentiation = 256%. For inhibition of the GABA response, EC_{50} = 7.2 μM, n_H = 0.85, and the maximal inhibition = 100%.

to environmental changes. Increases in brain neurosteroid levels have been observed in rat brain after stress (18) and after exposure of male rats to females (19). Moreover, the effects of PS on $GABA_A$, glycine, and NMDA receptors would tend to synergize. By enhancing activation of excitatory NMDA receptors by glutamate, while suppressing activation of inhibitory

receptors by GABA and glycine, even low concentrations of PS might shift the balance of excitation/inhibition in the excitatory direction. This tendency may be restrained somewhat by the inhibitory effect of PS on non-NMDA receptors; however, it is notable that, whereas the NMDA response is tripled and the GABA and glycine responses are virtually eliminated by 100 μM PS, the kainate and AMPA responses are reduced by only about 25% (Table 1).

Even if PS does not normally function as an endogenous neuromodulator in the CNS, it may play a role in CNS pathology. For example, it has been suggested that inhibition of GABA-mediated inhibitory neurotransmission by PS could contribute to seizures (20). This speculation is reinforced by the finding that PS also inhibits the action of glycine, the other major inhibitory neurotransmitter in the CNS, while enhancing the action of NMDA. Similarly, it is possible that PS may contribute to excitotoxic cell death in the CNS, which is thought to be mediated in large part by NMDA receptors (21).

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