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3α-Hydroxy-5β-pregnan-20-one Sulfate: A Negative Modulator of the NMDA-Induced Current in Cultured Neurons

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SUMMARY

We have shown previously that the neurosteroid pregnenolone sulfate acts as a positive allosteric modulator at the *N*-methyl-D-aspartate (NMDA) receptor while inhibiting the kainate, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), the glycine, and the γ-aminobutyric acid (GABA) responses of chick spinal cord neurons. Here, we report that 3α-hydroxy-5β-pregnan-20-one sulfate (5β3αS), a sulfated form of naturally occurring 5β3α, inhibits both the NMDA and the non-NMDA receptor-mediated responses as measured by whole cell voltage clamp recordings. 100 μM 5β3αS rapidly and reversibly inhibits the response to 30 μM NMDA by 66%, 50 μM kainate by 37%, and 25 μM AMPA by 29%. Application of 50 μM nonsulfated 5β3α does not produce any significant effect on the NMDA response, demonstrating that the sulfate moiety is important for the effect of 5β3αS on the NMDA response. The effect of 5β3αS on the NMDA response is concentration dependent, with an EC_{50} of 62 μM. 5β3αS reduces the maximum NMDA response with little effect on the NMDA EC_{50}, indicating that antagonism of the NMDA response by 5β3αS is noncompetitive. The fact that 5β3αS inhibition of the NMDA response is neither agonist nor voltage dependent demonstrates that 5β3αS does not act as an open channel blocker. Furthermore, inhibition of the NMDA response by 5β3αS is not reduced by the addition of a maximal concentration (10 μM) of glycine, indicating that 5β3αS does not act via the glycine recognition site. The inhibitory action of 5β3αS on the NMDA and non-NMDA receptors may provide a basis for inhibiting glutamate receptor-induced seizures and excitotoxic cell death.

Steroid hormones exert profound effects on brain excitability (1–3). Although the actions of many steroids appear to be mediated by genomic steroid response elements, recent studies suggest that some steroids also have direct neuromodulatory effects on excitatory glutamate (4) and inhibitory GABA (5) and glycine (6) receptors. Moreover, there is evidence for the local synthesis of certain steroids (termed "neurosteroids") in the brain (7–10).

L-Glutamate is thought to be the major excitatory neurotransmitter in the vertebrate central nervous system. It is known to activate at least three major, pharmacologically distinct classes of glutamate-gated ion channels: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors. These three ionotropic receptors are named according to their selective agonists.

NMDA receptors have attracted particular attention because of their importance in normal brain function and in pathophysiological conditions such as epilepsy and cerebral ischemia (11). The NMDA receptor appears to be essential for the induction of long-term potentiation (12), a proposed underlying mechanism for learning and memory (13), ischemic cell death, epilepsy, and other neurological diseases (14, 15). The integral channel of the NMDA receptor is permeable to Na^+, K^+, and Ca^{2+}. NMDA receptor activation thus increases intracellular Ca^{2+} in neuronal cells, and this process is thought to evoke glutamate-induced neurotoxicity (13). It seems likely that NMDA receptor antagonists may be useful therapeutic agents to protect against glutamate-induced neurotoxicity.

The NMDA receptor is subject to modulation through several pharmacologically distinct sites (16, 17), including those for glycine, Mg^{2+}, Zn^{2+}, and polyamines (18). Moreover, the dissociative anesthetics dizocilpine (MK-801), phencyclidine (PCP), and ketamine all produce a voltage-dependent and use-dependent blockade of ion channel activity (19, 20).

Prompted by the observation that the neurosteroid pregnenolone sulfate acts as a positive allosteric modulator at the NMDA receptor (4), we have examined a series of sulfated steroids to investigate the structural requirements for steroid modulation of NMDA receptor activity. Surprisingly, 3α-hydroxy-5β-pregnan-20-one sulfate (5β3αS) (Fig. 1), a pregnenolone sulfate analogue, inhibits the NMDA receptor-induced

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ABBREVIATIONS: CNS, central nervous system; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; EGTA, ethylene glycol bis(β-aminoethylether)-N, N,N′,N′-tetraacetic acid; GABA, γ-aminobutyric acid; HEPES, N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid; NMDA, N-methyl-D-aspartate; 5β3αS, 3α-hydroxy-5β-pregnan-20-one sulfate; DHEAS, dehydroepiandrosterone sulfate; MK-801, dizocilpine; PCP, phencyclidine.

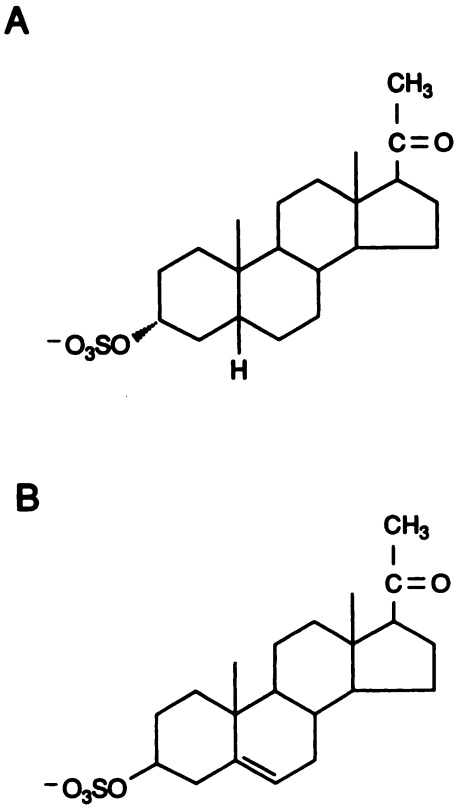

Fig. 1. Chemical structures of 3α-hydroxy-5β-pregnan-20-one sulfate (A) and pregnenolone sulfate (B).

current. Because 5β3αS is the first steroid to exhibit a robust inhibitory effect on the NMDA response, we studied the mechanism of action of 5β3αS on the NMDA response. 5β3αS inhibits the NMDA response by a voltage- and agonist-independent, noncompetitive mechanism that is different from that of open channel blockers such as MK-801. In addition, 5β3αS does not act through the glycine recognition site.

Materials and Methods

Cell cultures. Cultures of dissociated spinal cord neurons were prepared as previously described (21). Briefly, the dissociated cells from 7-day chick embryos 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 and antibiotics. Cultures were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°. Cytarabine (1 μM) was added to the culture medium after 36 hr of the initial plating to inhibit the proliferation of non-neuronal cells. One day later, this medium was replaced with a similar medium supplemented with 20.5 mM glucose, 18 mM KCl, and 2.5% chick embryo extract. Fresh medium was added twice weekly. Cultured neurons were used in experiments within 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 (22). Patch electrodes had tip openings of about 2 μm and resistances of 3–8 MΩ using an intracellular solution comprised of the following (in mM): 140 CsCl, 11 EGTA, and 10 HEPES (pH adjusted to 7.2 with CsOH). In experiments in which a high concentration of glycine was used, the intracellular solution was replaced with a low chloride (10 mM) pipette solution. This solution contained the following (in mM): 140 potassium gluconate, 10 KCl, 3 sodium gluconate, 11 EGTA, and 10 HEPES (pH adjusted to 7.2 with KOH). To prevent a marked run-down of NMDA-induced current at high NMDA concentrations, 4 mM potassium ATP was included in the intracellular solution. The bath solution contained the following (in mM): 150 NaCl, 4 KCl, 1 $CaCl_2$, and 10 HEPES (pH adjusted to 7.2 with NaOH). No glycine was added to the solutions containing NMDA, because no additional glycine was required to obtain a robust NMDA response, and there was no effect of a maximal concentration (10 μM) of glycine on 5β3αS inhibition of the NMDA response. All experiments were performed at room temperature (23–25°).

Recordings were made using an Axopatch 1B patch clamp apparatus (Axon Instruments, Burlingame, CA). Cells with series resistances greater than 10 MΩ were rejected. After partial compensation, series resistances were between 3.5 and 6 MΩ. The holding potential was maintained at −70 mV unless otherwise noted. Currents were filtered at 1 kHz using an eight-pole Bessel filter (−3 dB) and digitized (40 ms/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 pipettes. Seven-barrel pressure pipettes were positioned approximately 50 μm from the neuronal soma. Under these conditions, the drug solution in the pressure pipette rapidly and effectively replaces the solution surrounding the target neurons with less than 10% dilution (23–25). All drugs were obtained from Sigma, with the exception of AMPA hydrobromide (Research Biochemicals) and steroids (Steraloids). Stock solutions of steroids were prepared in dimethyl sulfoxide, the final concentration of which was 0.5% (v/v). To obviate the possible effects of dimethyl sulfoxide on the relevant agonist-induced currents, all other drug solutions including NMDA, kainate, AMPA and external buffer (in the pressure pipette) also contained 0.5% dimethyl sulfoxide.

The degree of modulation of the amino acid response by steroid, 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 steroid and I′ is the agonist-induced current in the presence of steroid. Throughout, results are expressed as mean ± S.E.; statistical comparison of groups was carried out using Student's *t* test.

Results

Currents elicited by NMDA, kainate, and AMPA were recorded in primary cultures of chick spinal cord neurons by the whole cell variant of the patch clamp technique. We have shown previously that pregnenolone sulfate potentiates the NMDA-induced whole cell current while inhibiting kainate- and AMPA-induced currents (4). Interestingly, 5β3αS produced an opposite modulatory effect, inhibiting the NMDA response. The effects of 100 μM 5β3αS on currents induced by NMDA, kainate, and AMPA at holding potentials of −70 mV are illustrated in Fig. 2. The response to 30 μM NMDA was inhibited (66.1 ± 2.7%, $n = 5$) when 5β3αS was applied simultaneously with NMDA. The onset and recovery of inhibition was rapid, and the inhibitory effect of 5β3αS was fully reversible after a wash period of 3–4 min. 100 μM 5β3αS also rapidly and reversibly inhibited 25 μM AMPA- (29.0 ± 3.1%, $n = 4$) and 50 μM kainate- (37.4 ± 4.7%, $n = 4$) induced currents. Application of 5β3αS alone did not produce any direct response. Table 1 shows that not all sulfated steroids inhibit the NMDA response. Dehydroepiandrosterone sulfate (DHEAS) only slightly potentiated the NMDA response (28.8 ± 8.8% potentiation, $n = 4$). Application of 50 μM nonsulfated 5β3α (which represents its maximal solubility in the external buffer) did not produce any significant effect on the 30 μM NMDA-induced current (3.6 ± 3.4% potentiation, $n = 4$) (Fig. 3), indicating that the sulfate

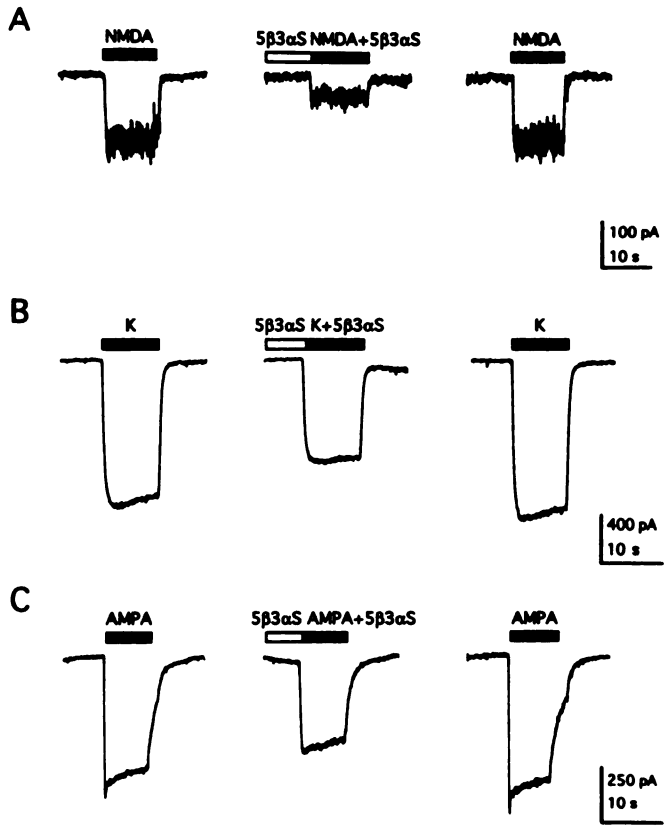

Fig. 2. 5β3αS inhibits rapidly and reversibly the NMDA, kainate and AMPA responses. V_H = -70 mV A, 5β3αS (100 μM) inhibits the current induced by 30 μM NMDA. B, 5β3αS (100 μM) inhibits the current induced by 50 μM kainate (K). C, 5β3αS (100 μM) inhibits the current induced by 25 μM AMPA. *Horizontal bar above* each trace, period of drug application.

TABLE 1

Effects of steroids on the 30 μM NMDA-induced response

Holding potential is −70 mV. Steroids are applied as described under Materials and Methods. Values are means ± S.E. Number of cells is indicated in parentheses.

Steroid	Change of response (%)
5β3αS (100 μM)	−66.1 ± 2.7 (5)
5β3α (10 μM)	−3.4 ± 3.5 (4)
(50 μM)	+3.6 ± 3.4 (4)
Androsterone sulfate (100 μM)	−18.1 ± 2.6 (3)
DHEAS (100 μM)	+28.8 ± 8.8 (4)
β-Estradiol benzoate (20 μM)	+24.3 ± 18 (3)

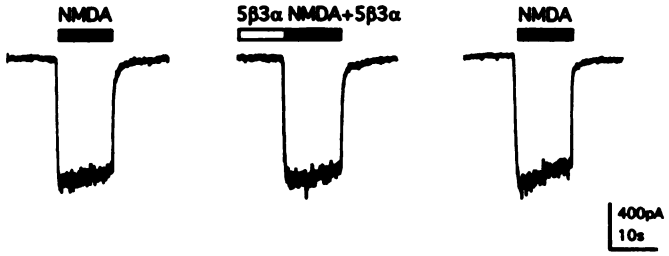

Fig. 3. 5β3α does not have an inhibitory effect on the NMDA response. Application of 50 μM 5β3α does not produce a significant effect on the 30 μM NMDA-induced current.

moiety is important for the effect of 5β3αS on the NMDA response.

To quantitatively evaluate the potency and efficacy of 5β3αS for NMDA receptors, pooled data were used to construct the concentration-response curve for inhibition of the 30 μM NMDA response by 5β3αS. As shown in Fig. 4, 5β3αS produced a concentration-dependent blockade of the current induced by 30 μM NMDA, and curve-fit analysis revealed an EC_{50} of 62.1 μM and maximal inhibition of 101.1%.

To investigate the mechanism of inhibition by 5β3αS, pooled data were used to construct a concentration-response curve for NMDA in the presence and absence of 50 μM 5β3αS. To obviate cell-to-cell variability with respect to the maximal current induced by NMDA, all responses were normalized to the peak current induced by 100 μM NMDA. As shown in Fig. 5, 5β3αS markedly reduced the NMDA maximal response with little effect on the NMDA EC_{50}. (In the absence of 5β3αS, EC_{50} = 155.6 μM, I_{max}= 2.56, and n_H = 1.01. In the presence of 5β3αS, EC_{50} = 105.7 μM, I_{max}= 1.10, and n_H = 1.46.) Our NMDA EC_{50} values are close to the EC_{50} reported for cultured chick motor neurons (26). This result suggests that the action of 5β3αS on the NMDA response is noncompetitive in nature and that 5β3αS acts through a site distinct from the NMDA recognition site.

Because the noncompetitive NMDA antagonists such as MK-801 act on the ion channel in a use- and voltage-dependent manner (19), we tested the voltage dependence of the effect of 5β3αS on the NMDA-induced current (Fig. 6). The average inhibition produced by 100 μM 5β3αS at +50 mV (62.0 ± 2.3%, n = 5) was not significantly different from that at −70 mV (63.2 ± 2.7%, n = 5) ($p > 0.05$, paired t test). This result indicates that the blockade by 5β3αS of the NMDA response is voltage independent. We have not seen any use dependence on the time scale of these experiments; the first application of 5β3αS in the presence of NMDA elicited an immediate blockade of both peak and plateau responses, and the recovery of the NMDA response after washout of 5β3αS was rapid.

The response of the NMDA receptor is positively modulated by glycine, and glycine may be an absolute requirement for

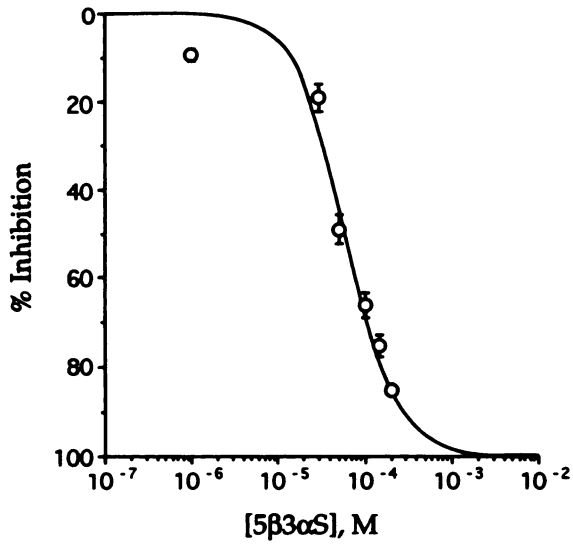

Fig. 4. Concentration-response curve for inhibition of the NMDA response by 5β3αS. *Data points*, percentage change in peak current in the presence of 5β3αS (mean of four to eight experiments). *Error bars*, standard errors. 5β3αS concentration-response curve is fitted with the logistic equation (30). (% inhibition)/(% inhibition)$_{max}$ = $[5\beta3\alpha S]^{nH}$/($[5\beta3\alpha S]^{nH}$ + EC_{50}^{nH}), where [5β3αS] is the concentration of 5β3αS, and n_H is the Hill coefficient. 5β3αS inhibits the current induced by 30 μM NMDA and curve-fit analysis reveals an EC_{50} of 62.1 μM, maximal inhibition of 101.13%, and n_H of 1.47.

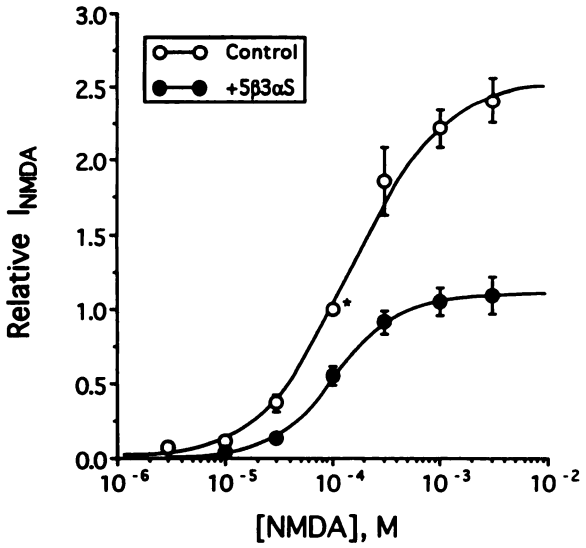

Fig. 5. Antagonism of the NMDA response by 5β3αS is noncompetitive. Concentration-response curves for NMDA in the presence and absence of 50 μM 5β3αS. All responses are normalized to the peak current (*) induced by 100 μM NMDA. *Data Points*, normalized peak currents (mean of 4 to 9 experiments). *Error bars*, standard errors. Error bars are not indicated when smaller than the size of the circle. Each set of data points is fitted by non-linear regression to the logistic equation (30): $I/I_{max} = [NMDA]^{nH}/([NMDA]^{nH} + EC_{50}^{nH})$, where I_{max} is the maximal normalized current, [NMDA] is the concentration of NMDA, and n_H is the Hill coefficient. In the absence of 5β3αS, EC_{50} = 155.6 μM, I_{max} = 2.56, and n_H = 1.01. In the presence of 5β3αS, EC_{50} = 105.7 μM, I_{max} = 1.10, and n_H = 1.46.

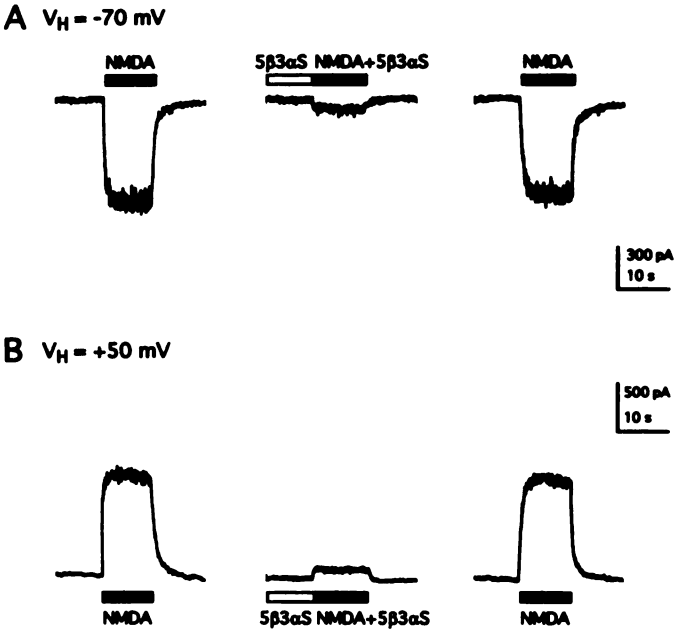

Fig. 6. Inhibition of the NMDA response by 5β3αS is not voltage-dependent. A, V_H = -70 mV 5β3αS (100 μM) inhibits the current induced by 30 μM NMDA. B, V_H = +50 mV 5β3αS also inhibits the 30 μM NMDA response.

receptor function (27). Some inhibitors of the NMDA response, such as 7-chlorokynurenic acid, act by blocking the glycine site of the NMDA receptor (17). To determine whether 5β3αS acts as a competitive antagonist at the glycine recognition site, we examined the effect of 5β3αS on the NMDA responses in the presence of a saturating concentration (10 μM) of glycine. As

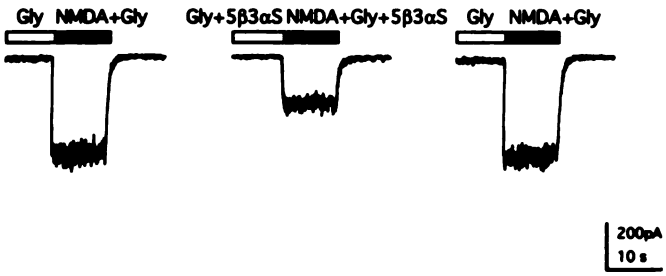

Fig. 7. 5β3αS does not act via the glycine recognition site. 5β3αS (50 μM) still inhibits the current induced by 30 μM NMDA in the presence of a saturating concentration of glycine (Gly).

shown in Fig. 7, 50 μM 5β3αS still reduced the current induced by 30 μM NMDA with added glycine. Moreover, the average inhibition by 5β3αS (51.3 ± 3.5%, n = 4) was not significantly different from that in the absence of glycine (49.1 ± 3.4%, n = 4) ($p > 0.5$, unpaired t test). This result is consistent with the view that the effect of 5β3αS on the NMDA-induced current is not mediated by the glycine recognition site.

Discussion

In this study, we have shown that 5β3αS, a sulfated form of naturally occurring 5β3α (28), inhibits both NMDA and non-NMDA receptor-mediated responses. The highly lipophilic nature of the steroids and the evidence that phospholipids are capable of binding steroids with high specificity (3) raise the possibility that the effect of 5β3αS is mediated by interactions with the membrane lipids surrounding the NMDA receptor protein. However, we have shown that another pregnenolone sulfate analogue DHEAS has only a little effect (28.8% potentiation) on the NMDA response in comparison with the effect of pregnenolone sulfate, which potentiates the NMDA response by 197% (4). This finding argues that antagonism of the NMDA-induced current by 5β3αS is a specific effect.

There are a number of potential sites at which 5β3αS could exert its blocking action including: 1) competitive inhibition at the NMDA binding site, 2) blockade of the NMDA receptor-associated channel, or 3) noncompetitive inhibition or allosteric modulation at a distinct site.

First, competition of 5β3αS for the NMDA binding site cannot account for the observed result. Our results (Fig. 5) show that the antagonism by 5β3αS of the NMDA-induced current is not attenuated by increasing concentrations of NMDA.

Second, blockade of a cation channel by a negatively charged molecule such as 5β3αS seems unlikely on theoretical grounds. The cloned NMDA receptor has a putative second transmembrane domain which is thought to be involved in lining the ion channel and is flanked by negatively charged amino acid residues (29). This would deter entry of a sulfated molecule into the channel. The lack of voltage and use dependence (Fig. 6) also suggests that the interaction of this compound with the inner channel wall is unlikely.

The NMDA receptor is thought to have a glycine recognition site (27) in addition to the agonist binding site. The inhibitory action of 5β3αS on the NMDA-induced currents cannot be surmounted by a saturating concentration of glycine. This experiment suggests that the steroid and glycine do not act at a common site.

Even though it is known that steroids have profound effects

on brain excitability (1–3), the structure-activity relationship of steroids as modulators of the NMDA response remains to be determined. We have shown previously that pregnenolone sulfate acts as a positive allosteric modulator at the NMDA receptor. Surprisingly, a few modifications of pregnenolone sulfate structure (reducing the C-5 position double bond, 3β-hydroxy to 3α-hydroxy) changes the modulation of the NMDA receptor mediated response from a positive to a negative direction. Interestingly, 5β3αS produces opposite effects to pregnenolone sulfate only at the NMDA response, whereas it elicits inhibitory effects similar to pregnenolone sulfate at the AMPA and kainate response. This result provides evidence that the structural requirements for modulation of NMDA and non-NMDA receptors by steroids might be different. Also, the ineffectiveness of non-sulfated 5β3α on the NMDA response demonstrates that sulfation converts an inactive steroid to an active steroid inhibitor, suggesting that steroid sulfotransferase could be an important enzyme in regulating NMDA receptor activity in the CNS.

This study not only reveals another mechanism of non-competitive blockade of the NMDA-induced current but gives a basis for understanding the structural requirements of steroids for NMDA receptor activation. Our results suggest that the steroid 5β3αS, an analogue of pregnenolone sulfate, may represent a novel class of broad spectrum antagonists of excitatory amino acid receptors. It will be important to determine whether such sulfated steroids may be useful as anticonvulsant or anti-excitotoxic therapeutic agents.

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