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Pregnenolone sulfate augments NMDA receptor mediated increases in intracellular Ca^{2+} in cultured rat hippocampal neurons

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The ability of the neuroactive steroid pregnenolone sulfate to alter *N*-methyl-D-aspartate (NMDA) receptor-mediated elevations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) was studied in cultured fetal rat hippocampal neurons using microspectrofluorimetry and the Ca^{2+} sensitive indicator fura-2. Pregnenolone sulfate (5-250 μM) caused a concentration-dependent and reversible potentiation of the rise (up to ~ 800%) in $[Ca^{2+}]_i$ induced by NMDA. In contrast, the steroid failed to alter basal (unstimulated) $[Ca^{2+}]_i$ or to modify the rise in $[Ca^{2+}]_i$ that occurs when hippocampal neurons are depolarized by high K^+ in the presence of the NMDA receptor antagonist CPP. These data suggest that the previously reported excitatory properties of pregnenolone sulfate may be due, in part, to an augmentation of the action of glutamic acid at the NMDA receptor.

Neuroactive steroids are natural or synthetic steroids which can rapidly alter the excitability of neurons via non-genomic mechanisms (see ref. 15 for review). To date, the best characterized neuroactive steroids are a series of 3α-hydroxy ring A reduced pregnane steroids which bind stereoselectively and with high affinity to the receptor for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS [11, 15]. Submicromolar concentrations of these steroids markedly augment GABA activated chloride (Cl^-) ion currents in a variety of neuronal and non-neuronal preparations in a manner similar to that of anesthetic barbiturates [11, 14–16]. Other steroids, notably the natural sulfate esters of pregnenolone and dehydroepiandrosterone, have been shown to have excitatory actions when applied to guinea pig septo-preoptic neurons [2] and to antagonize GABA [13] or glycine [21] activated Cl^- conductance when tested in cultured neurons or isolated synaptoneurosomes [10, 12]. There is considerable evidence that pregnenolone sulfate is synthesized de novo in brain [1] and given the recently demonstrated capacity of glial mitochondria to convert cholesterol or mevalonolactone to pregnenolone [9], the neuroactive properties of pregnenolone sulfate are of considerable interest. Recently, Wu and colleagues have reported that pregnenolone sulfate, in addition to antagonizing GABA and glycine activated Cl^- currents, is also a positive allosteric modulator of the *N*-methyl-D-aspartate (NMDA) receptor in cultured embryonic chick spinal cord neurons [20]. In the present study we confirm and extend this observation by demonstrating that pregnenolone sulfate markedly augments NMDA receptor-mediated elevations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in fetal rat hippocampal neurons.

Hippocampal neurons from 19-day-old Sprague–Dawley rat embryos were grown in primary culture as described by Segal [17]. Briefly, hippocampal tissue was triturated and the cell suspension was plated onto poly-L-lysine (Sigma, St. Louis, MO) coated glass bottom 35-mm culture dishes (MatTek, Ashland, MA) containing Modified Eagle's medium with Earles salts (Advanced Biotechnologies, Inc., Columbia, MD) supplemented with 10% fetal calf serum (Gibco Labs, Grand Island, NY), 10% horse serum (Gibco Labs, Grand Island, NY) and 2 mM glutamine (Sigma, St. Louis, MO). In addition, transferrin, insulin, selenium, corticosterone, triio-

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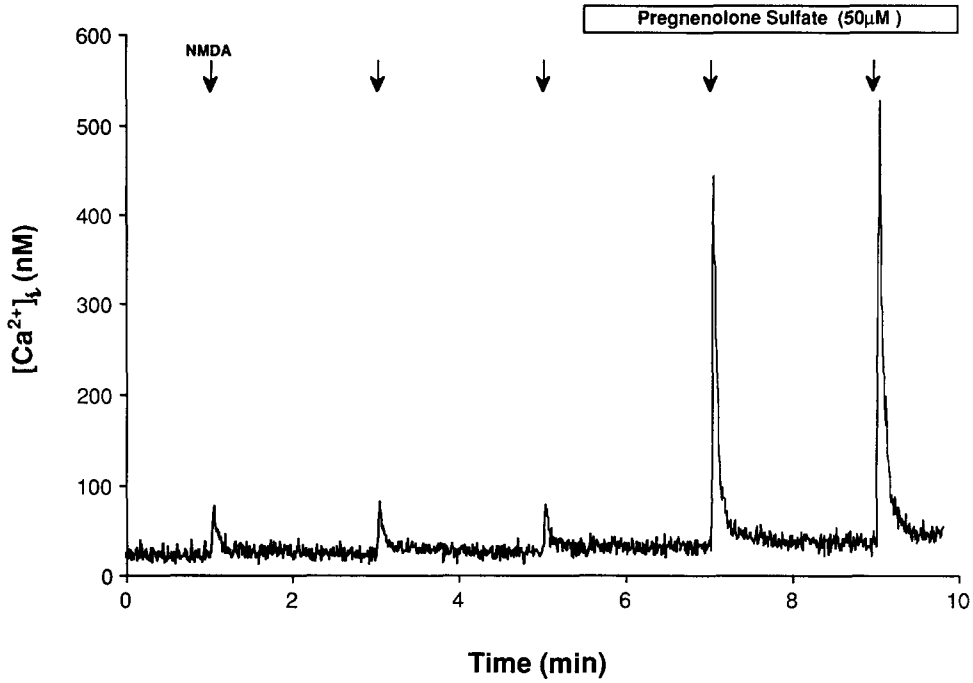

Fig. 1. Pregnenolone sulfate augments NMDA induced elevations in $[Ca^{2+}]_i$, but fails to alter basal $[Ca^{2+}]_i$ in a fetal hippocampal neuron. NMDA (5 μM) applied for 3 s (beginning at arrows) at 120 s intervals transiently increases $[Ca^{2+}]_i$ measured using microspectrofluorimetry with the Ca^{2+}-sensitive indicator fura-2 (see text for details). When pregnenolone sulfate (50 μM) (bar) is applied, the $[Ca^{2+}]_i$ response to NMDA (5 μM) is markedly augmented. Similar results were observed in 4 additional neurons (see Fig. 3).

dothyronine, progesterone and putrescine were added to the media as described by Guthrie et al. [7]. Cells were incubated in a humidified atmosphere containing 10% CO_2 and 90% air. Culture media lacking fetal calf serum was added every 7 days after plating. Cells were used 7–14 days in culture.

Intracellular calcium ($[Ca^{2+}]_i$) was measured by microspectrofluorimetry using the Ca^{2+} sensitive indicator fura-2 [5, 6]. Neurons were illuminated on a Nikon inverted-stage microscope using a dual wavelength illumination-photometry system (SPEX-DM3000 AR-CM, SPEX Industries, Edison, NJ). Neurons were washed 3 times with a buffer containing (in mM) NaCl 145, KCl 2.5, HEPES 10, $CaCl_2$ 1, glucose 10 (adjusted to pH 7.4 with NaOH and to an osmolatity of 315–325 mOsm with sucrose). Cells were incubated with fura-2 acetoxymethyl ester (fura-2/AM) 2–5 μM for 20–30 min in the dark. Following the incubation period, cells were washed 3 times with buffer and allowed ≥20 min to complete hydrolysis of the ester. The cells were perfused at a rate of approximately 250 ml/min with buffer at 37°C. The perfusion device consisted of a six-barrel array of tubes emptying into a common tip positioned approximately 500 μm from the cell. All solutions contained 0.5 μM tetrodotoxin to eliminate voltage-sensitive Na^+ currents and 2 μM glycine to saturate the strychnine-insensitive glycine site on the NMDA receptor-channel complex. In all experiments, neurons were exposed to NMDA (5 μM) or KCl (20 mM) for 3 s intervals with or without the steroid or other drugs. A period of 1–2 min was allowed between applications.

Excitation of fura-2 was at 340 and 380 nm with emitted light monitored at 510 nm. Light reaching the photomultiplier was limited to that emitted by the cell of interest. Photon counts were digitally stored for subsequent analysis. Calibration was carried out as described by Grynkiewicz et al. [6]. R_{max} was determined in situ using a buffer at 37°C and pH 7.0 containing (in mM) KCl 130, NaCl 17, HEPES 10, glucose 10, $CaCl_2$ 1 and ionomycin 15 μM. For R_{min}, the buffer was modified so that EGTA 1 mM and EGTA acetoxymethyl ester (40 μM) was substituted for $CaCl_2$. R_{max}, R_{min} and I_{f380}/I_{b380} were found to be (mean ± S.E.M.; n=7) 11.5 ± 1.15, 0.67 ± 0.01, and 5.59 ± 0.65, respectively. An apparent K_d of 285 nM [5] was used for $[Ca^{2+}]_i$ calculations.

The trimethylammonium salt of pregnenolone sulfate was synthesized by the method of Dusza et al. [3] and converted to the sodium salt using the procedure of Goto et al. [4]. These salts were dissolved in dimethyl sulfoxide (final concentration <0.2%). 3-[(±)-2-Carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP) was obtained from Tocris Neuramin (Essex, England), fura-2/AM and EGTA/AM was from Molecular Probes (Eugene, OR), and ionomycin was from Calbiochem (La Jolla, CA). All

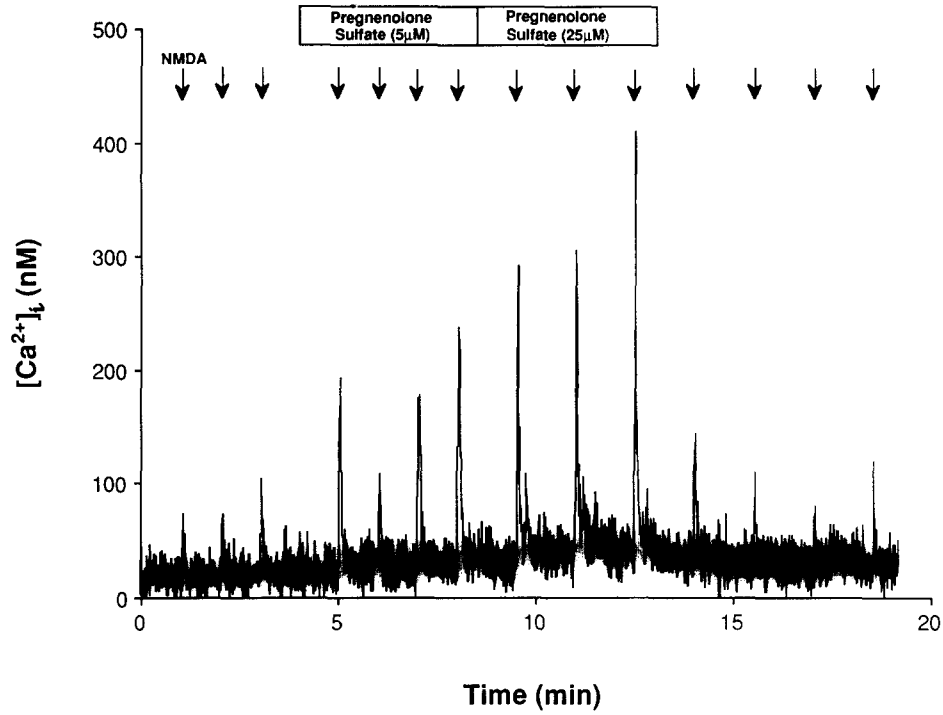

Fig. 2. Pregnenolone sulfate produces a concentration-dependent, reversible augmentation of NMDA-induced elevations in $[Ca^{2+}]_i$ in a fetal hippocampal neuron. Shown is the response of a typical neuron to pregnenolone sulfate (5 and 25 μM). Note that pregnenolone sulfate augments the rise in $[Ca^{2+}]_i$ induced by NMDA and that the response returns to baseline after discontinuing steroid application.

other drugs were obtained from Sigma (St. Louis, MO). Data are presented as the mean ± S.E.M. Statistical comparisons were made with the *t*-test. Curve fitting was performed using the computer program MKMODEL (Biosoft, Cambridge, UK)

In our experiments, we used a relatively low concentration of NMDA (5 μM) which resulted in rapid and reversible increases in $[Ca^{2+}]_i$ to 145 ± 28 nM. Pregnenolone sulfate when applied alone at concentrations $\leq$250 μM failed to alter basal $[Ca^{2+}]_i$ (Fig. 1) but markedly and reversibly enhanced NMDA-induced elevations in $[Ca^{2+}]_i$ (Figs. 1 and 2). The vehicle (DMSO) did not have a significant effect on basal $[Ca^{2+}]_i$ and failed to modify NMDA-induced elevations in $[Ca^{2+}]_i$ at concentrations up to 0.2% although there was a slight but nonsignificant potentiation at higher concentrations (data not shown). In preliminary experiments, we observed that the augmentation of NMDA-induced elevations in $[Ca^{2+}]_i$ required a preincubation time of several seconds (data not shown). Therefore, we routinely exposed cells to pregnenolone sulfate $\geq$ 60 s prior to application of NMDA. The augmentation of NMDA-induced rises in $[Ca^{2+}]_i$ was reversible so that NMDA response amplitude typically returned to control values in less than one minute (Figs. 1 and 2).

The augmentation of NMDA-induced elevations in $[Ca^{2+}]_i$ by pregnenolone sulfate was concentration-dependent. We observed a significant augmentation of NMDA responses in many (but not all) neurons by steroid concentrations as low as 5 μM (Fig. 2) while consistent and robust ($\geq$150%) increases in $[Ca^{2+}]_i$ were observed at concentrations of 10–250 μM (Fig. 3). The estimated maximal (E_{max}) value for augmentation of the NMDA response by pregnenolone sulfate was 789% with an EC_{50} of 33 μM and a Hill coefficient of 2.4.

To investigate whether the augmentation of NMDA-induced elevations in $[Ca^{2+}]_i$ by pregnenolone sulfate was specific for NMDA receptor-operated Ca^{2+} conductance, we tested whether pregnenolone sulfate would also alter the rise in $[Ca^{2+}]_i$ induced by depolarizing concentrations of K^+. Using a relatively low depolarizing concentration of K^+ (20 mM) (which resulted in an average rise in $[Ca^{2+}]_i$ of 362 ± 65 nM, n=12) coapplication of pregnenolone sulfate (50 μM) resulted in a large augmentation of the K^+-induced rise in $[Ca^{2+}]_i$ (Fig. 4A). However, the augmentation of the K^+-induced rise in $[Ca^{2+}]_i$ by pregnenolone sulfate was blocked by CPP (30 μM), a selective NMDA receptor antagonist [8] (Fig. 4B). Thus, it is likely that depolarization of hippocampal neurons by high K^+ results in the release of endogenous glutamate and secondary activation of NMDA receptors. In the presence of the NMDA blocker, no potentiation was observed, demonstrating that pregnenolone sulfate selectively augments Ca^{2+} entry via the NMDA receptor com-

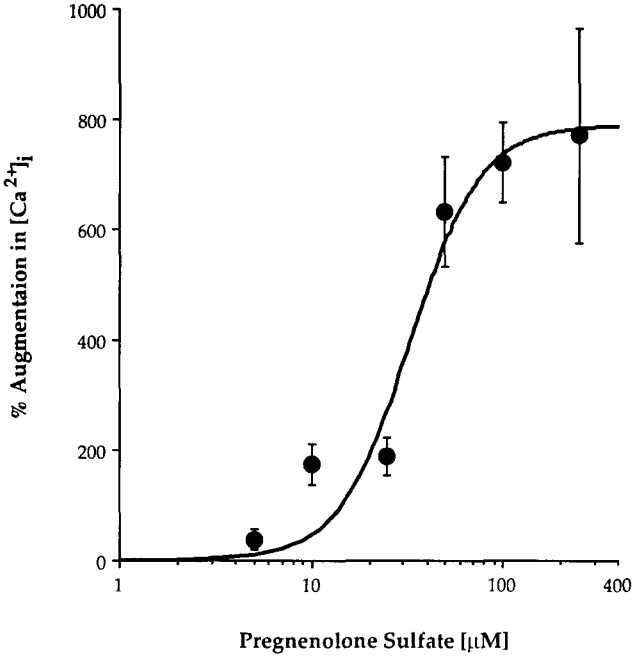

Fig. 3. Concentration-response curve for pregnenolone sulfate enhancement of the NMDA-induced elevation of $[Ca^{2+}]_i$ in rat hippocampal neurons. Data represent the percent augmentation of the NMDA (5 μM for 3 s) induced rise in $[Ca^{2+}]_i$. Each point represents the mean ± S.E.M. of experiments with 3–5 neurons. In a few experiments the trimethylammonium salt of pregnenolone sulfate was used and gave similar results to the sodium salt. The data were fit according to the equation,

$$\%\ \text{Augmentation in } [Ca^{2+}]_i = \frac{E_{max}[PS]^n}{EC_{50}{}^n + [PS]^n}$$

where [PS] is the concentration of pregnenolone sulfate, E_{max} = 789%, EC_{50} = 33 μM and n=2.4.

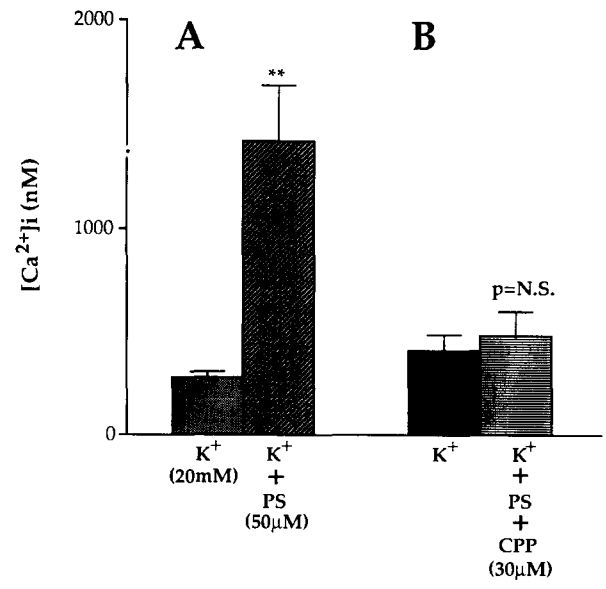

Fig. 4. The augmentation of K^+-induced elevation of $[Ca^{2+}]_i$ by pregnenolone sulfate (PS) is blocked by CPP in rat hippocampal neurons. A: mean ± S.E.M. change in $[Ca^{2+}]_i$ induced by a 3 s exposure to K^+ (20 mM) compared to K^+ plus pregnenolone sulfate (50 μM) (n=6; paired (2-tail) t-test, $^{**}P<0.01$). B: same as in A, but with CPP (30 μM) applied with pregnenolone sulfate (n=9; P=n.s.).

plex and not that due to activation of voltage-gated Ca^{2+} channels.

The ability of pregnenolone sulfate (50 μM) to augment NMDA-induced rises in hippocampal neuron $[Ca^{2+}]_i$ was also examined using higher concentrations of NMDA (500 μM). The latter induced a rise in $[Ca^{2+}]_i$ of 1329 ± 521 nM, and coapplication of pregnenolone sulfate (50 μM) resulted in an insignificant augmentation of this response to 1540 ± 347 nM (n=4, P=n.s.). These data are compatible with pregnenolone sulfate allosterically increasing the affinity of NMDA for its receptor and (or) modifying receptor-gated channels kinetics. However, they suggest that pregnenolone sulfate is not capable of promoting the activation of normally quiescent channels.

We have observed that pregnenolone sulfate at concentrations between 5 and 250 μM markedly augments NMDA receptor-mediated responses in cultured rat hippocampal neurons. Our findings confirm and extend those of Wu and coworkers [20] carried out in chick spinal cord neurons using the whole cell patch clamp technique which demonstrated an augmentation of NMDA current by pregnenolone sulfate. In the present study, we measured NMDA-induced elevations in $[Ca^{2+}]_i$ in rat hippocampal neurons using microspectrofluorimetry and the Ca^{2+}-sensitive indicator fura-2 and observed a robust positive modulatory effect of pregnenolone sulfate on NMDA responses with little or no effect of the steroid on basal (unstimulated) $[Ca^{2+}]_i$. We observed a similar EC_{50} to that obtained by Wu et al. [20]; the larger E_{max} value obtained in the present study is likely related to the lower concentration of NMDA we used. In addition, it is conceivable that the large E_{max} observed is, in part, related to Ca^{2+}-induced Ca^{2+} release from intracellular stores. The failure of pregnenolone sulfate to alter resting $[Ca^{2+}]_i$ suggests that, even at relatively high concentrations, pregnenolone sulfate does not act nonspecifically to increase Ca^{2+} entry or its release from intracellular organelles. Although a marked and robust augmentation of NMDA-induced $[Ca^{2+}]_i$ is observed at steroid concentrations of 50–250 μM, concentrations as low as 5–10 μM were also effective in many neurons. Nevertheless, these concentrations may exceed the 'total' brain concentration of pregnenolone sulfate reported by Baulieu and Robel [1]. However, it is conceivable that considerably higher concentrations of this steroid may be achieved locally given the capacity of glial mitochondria

to convert cholesterol to pregnenolone [9]. Recently, Spence et al. [18] has reported that low micromolar concentrations of pregnenolone and pregnenolone sulfate block a specific Ca^{2+} current in acutely isolated CA1 hippocampal neurons. We did not observe a significant attenuation of the K^+ induced rise in $[Ca^{2+}]_i$ by the coapplication of pregnenolone sulfate and CPP (Fig. 4B) possibly because other Ca^{2+} currents contribute to the bulk of the Ca^{2+} influx under these conditions.

At present, the mechanism responsible for pregnenolone sulfate's modulatory effect on NMDA receptors is unknown. It will be of interest to determine if this neuroactive steroid interacts with a known modulatory site on the NMDA receptor or whether, as has been demonstrated for the $GABA_A$ receptor [15, 19], there is a unique steroid recognition site. Since our experiments were carried out in saturating glycine concentrations, it is unlikely that pregnenolone sulfate acts at the glycine coagonist site on the NMDA receptor, although it could affect the NMDA recognition site or the polyamine modulatory site. In any case, whatever its site of action, our present results support the concept that pregnenolone sulfate may be an endogenous modulator of NMDA receptor responses.

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