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Pregnenolone sulfate acts through a G-protein-coupled σ_1-like receptor to enhance short term facilitation in adult hippocampal neurons

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Abstract

Neurosteroids have been linked to cognitive performance, and their levels are altered in neuropsychiatric diseases. These neuromodulators are produced in the brain where they have important effects on synaptic transmission at postsynaptic γ-amino-butyric acid receptors and N-methyl-D-aspartate receptors and at presynaptic sites. We previously found, in cultured neonatal hippocampal neurons, that the neurosteroid, pregnenolone sulfate, acts presynaptically through a σ_1-like receptor to modulate basal glutamate release. The present study was designed to test whether pregnenolone sulfate acts through a similar presynaptic receptor in adult hippocampal neurons. The σ_1-receptor agonist, 2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate, enhanced paired-pulse facilitation (PPF) by a similar extent to that which we had previously reported for pregnenolone sulfate. The σ_1-receptor antagonists, 1-(4-Iodophenyl)-3-(2-adamantyl)guanidine and 1[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine, blocked the pregnenolone sulfate enhancement of PPF as did pretreatment of slices in pertussis toxin. We conclude that pregnenolone sulfate acts through a $G_{i/o}$-coupled σ_1-like receptor to enhance short-term presynaptic facilitation onto adult hippocampal CA1 neurons.

Keywords: Hippocampus; G protein; Pregnenolone sulfate; Presynaptic receptor; Sigma receptor; Steroid

1. Introduction

Neurosteroids are produced de novo in neurons and glial cells independently of peripheral adrenal and gonadal steroid synthesis (Baulieu, 1998). One of the most common neurosteroids in rodent brain, 5-pregnen-3β-ol-20-one sulfate (pregnenolone sulfate), is produced from cholesterol by the enzymes $P450_{scc}$ and sulfotransferase (Compagnone and Mellon, 2000). Pregnenolone sulfate is classified as an excitatory neurosteroid that positively modulates N-methyl-D-aspartate (NMDA) receptors (Park-Chung et al., 1997) and negatively modulates γ-amino-butyric acid ($GABA_A$) receptors (Eisenman et al., 2003). In addition to these well characterized postsynaptic effects, we have found that pregnenolone sulfate acts presynaptically to enhance glutamate neurotransmission (Meyer et al., 2002; Partridge and Valenzuela, 2001).

Endogenous neurosteroids exhibit important modulatory effects on brain function and have significant roles in mental health-related disorders. For example in preclinical studies, it has been shown that low levels of pregnenolone sulfate in rat hippocampus are associated with cognitive performance deficits that can be improved with intrahippocampal injection of pregnenolone sulfate (Vallee et al., 1997) moreover, this neurosteroid attenuates amyloid peptide-induced amnesia in mice (Maurice et al., 1998). Clinical studies indicate similar correlations between pregnenolone sulfate and neuropsychiatric disorders. For example, patients with generalized social phobia or generalized anxiety disorder have low plasma pregnenolone sulfate levels (Heydari and Le Melledo, 2002; Semeniuk et al., 2001), women being treated for anxiety-depressive disorder with fluoxetine have elevated pregnenolone sulfate levels (Bicikova et al., 2000), and there is a significant negative

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correlation between β-amyloid peptides and pregnenolone sulfate in the brains of patients with Alzheimer's Disease (Weill-Engerer et al., 2002).

Although the mechanism by which pregnenolone sulfate exerts its effects is poorly understood, numerous studies suggest that one important site of action of membrane-impermeant sulfated neurosteroids is through interaction with plasma membrane proteins. Among the membrane proteins modulated by pregnenolone sulfate are sigma$_1$ (σ_1) receptors (Maurice et al., 1999). Importantly, σ_1 receptors have been identified to be a significant site of action of therapeutic agents for neuropsychiatric disorders (Hayashi and Su, 2004) and neurosteroids have been shown to exert biological effects by interacting with these receptors (Reddy and Kulkarni, 1998; Urani et al., 1998). At the cellular level, pregnenolone sulfate acts through a σ_1 receptor to modulate NMDA-evoked norepinephrine release (Monnet et al., 1995), to $GABA_A$ mediated miniature inhibitory post-synaptic current (mIPSC) frequency (Mtchedlishvili and Kapur, 2003), and to inhibit NMDA-stimulated dopamine release (Nuwayhid and Werling, 2003).

In cultured neonatal neurons, pregnenolone sulfate robustly enhances miniature excitatory post-synaptic current (mEPSC) frequency, and in neonatal autapses, pregnenolone sulfate depresses presynaptic paired-pulse facilitation (PPF) (Meyer et al., 2002). A similar enhancement of mEPSC frequency occurs in CA1 neurons in the acute slice, but only before postnatal day 6 (Mameli et al., 2005). By contrast, in neurons from mature hippocampi, pregnenolone sulfate enhances facilitated glutamate release and has little effect on basal release (Partridge and Valenzuela, 2001). This developmental switch is very interesting since it implies that the role of pregnenolone sulfate in the immature brain is co-opted for a different function in the mature brain.

The goal of this study was to determine whether pregnenolone sulfate uses a similar signaling pathway in mature neurons as it does in cultured immature neurons (Meyer et al., 2002). We show here that pregnenolone sulfate indeed acts through a $G_{i/o}$-protein coupled σ_1-like receptor to enhance short-term synaptic facilitation onto adult rat hippocampal CA1 neurons. Thus, this important neurosteroid produces different effects in mature and immature neurons through a common mechanism; namely, modulation of metabotropic σ_1-like receptors.

2. Methods

2.1. Slice preparation

Coronal brain slices, containing the hippocampus, were prepared from postnatal 50–55 day old male Sprague–Dawley rats. Rats were deeply anesthetized with i.p. injection of 250 mg/kg ketamine, and slices were cut at 300 or 400 μm with a Vibratome (Pelco 101, Redding,CA) in ice-cold, low-Ca^{2+}, high-Mg^{2+} artificial cerebrospinal fluid (low-Ca^{2+} aCSF), consisting of (in mM) 124 NaCl, 5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 $MgSO_4$, 0.5 $CaCl_2$, and 10 glucose. The slices were then incubated in low-Ca^{2+} aCSF at 34 °C for 60 min and then transferred to standard aCSF, consisting of (in mM) 124 NaCl, 5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, and 10 glucose, at 25 °C for a minimum of 60 min before recording. All solutions were continuously oxygenated with 95% O_2/5% CO_2. For the pertussis toxin experiments, after incubation in low-Ca^{2+} aCSF, slices were maintained in standard aCSF plus 50 ng/ml pertussis toxin at 25 °C for a minimum of 12 h. Slices were transferred to a submerged recording chamber (Scientific Systems Design, Mercerville, NJ, for field potential recording or Warner Instrument, Hamden, CT, for patch clamping) and continuously perfused at 34 °C at a flow rate of 2 ml/min with oxygenated aCSF or aCSF to which drugs or vehicle had been added. Constant current pulses of 0.15 ms duration were delivered either singly or in pairs at a 50 ms interpulse interval to the Schaffer collaterals via a concentric bipolar electrode placed in the *stratum radiatum*. All procedures conducted on animals were previously approved by the UNM Institutional Animal Care and Use Committee. The Program of Animal Use at the UNM Health Sciences Center is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC, Int.).

2.2. Field potential recordings

Postsynaptic extracellular field potentials (fEPSPs) with population spikes were measured in CA1 *stratum pyramidale* with 3 M NaCl-filled glass microelectrodes with impedances of 1–4 MΩ using an Axoclamp-2A amplifier, Digidata 1322A interface, and pCLAMP 8 software (all from Axon Instruments, Union City, CA). Signals were digitized at 67 kHz and filtered at 2 kHz. We found sub-maximal population spikes to be a more consistent determinant of synaptic efficacy than fEPSP slope. Population spike amplitudes, measured at a stimulus strength that produced approximately 1/2 of the maximum response, were determined as the mean of both positive components minus the negative tip of the spike (see, for example, (Hsu and Smith, 2003)). PPF of population spike amplitude was determined as the percentage difference between the amplitudes of the first (pop_1) and second (pop_2) population spikes: $PPF = 100\% \times ((pop_2 - pop_1)/pop_1)$. Responses to drugs were measured only after establishing a minimum of 20 min of stable PPF.

2.3. Patch-clamp recording

Patch pipettes with impedances of 4–6 MΩ were filled with intracellular solution containing (in mM) 110 gluconic acid lactone, 110 CsOH, 0.6 EGTA, 10 HEPES, 5 NaCl, 10 TEA-Cl, 4 MgATP, pH = 7.25 (adjusted with CsOH), 270–280 milliosmoles. Whole cell currents were measured with an Axopatch 200B amplifier, a Digidata 1322A interface, and pCLAMP 9.0 software (all from Axon Instruments, Union City, CA). Signals were digitized at 200 kHz and filtered at 2 kHz. Experiments were carried out to measure the time course of decay of the NMDA component of the excitatory post-synaptic current (EPSC) caused by the use-dependent block of NMDA receptors by the open-channel blocker, (+)-5-methyl-10,11-dihydroxy-5h-dibenzo (a,d)cyclohepten-5,10-imine), MK-801, as described in (Hessler et al., 1993). Briefly, stable NMDA EPSCs, elicited in CA1 pyramidal neurons by submaximal stimulation of Schaffer collaterals at 0.1 Hz, were pharmacologically isolated in 0 Mg^{2+} aCSF

with 20 μM bicuculline and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Stimulation was then stopped and 50 μM MK-801 was added for a minimum of 3 min. Stimulation was resumed ($t=0$) and the time course of block of NMDA EPSCs was measured by plotting peak EPSC amplitude as a function of time (Fig. 4A inset). For simplicity, the time course of block was reported as a monoexponential time constant.

2.4. Chemicals

Pregnenolone sulfate was from Steraloids (Newport, RI), pertussis toxin from Calbiochem (San Diego, CA), and 1-(4-Iodophenyl)-3-(2-adamantyl)guanidine (IPAG) and 1[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine (BD-1063) from Tocris (Ellisville, MO). BD-1063, IPAG, and pregnenolone sulfate were dissolved in dimethylsulfoxide (DMSO) before dilution into external solution, and equal volumes of DMSO were added to control external solutions (vehicle control). DMSO concentrations never exceeded 0.02%. 2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate (PRE-084) and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

2.5. Statistical analysis

Statistical analyses were conducted with SPSS 12.0 (SPSS Inc., Chicago, IL) for univariate analysis of variance followed by Bonferroni post hoc tests or with Prostat 2.5–3.5 (Polysoft, Pearl River, NY) for paired Student's *t*-tests and for one-way ANOVA followed by Fischer post hoc tests. Significance was accepted at a 95% confidence level. Data are presented as mean ± S.E.M.

3. Results

While pregnenolone sulfate has well documented modulatory effects on postsynaptic receptors, which generally occur at higher concentrations (Bowlby, 1993; Eisenman et al., 2003; Yaghoubi et al., 1998), we have observed that high nM concentrations of pregnenolone sulfate cause a robust enhancement of facilitated glutamate release with very little change in basal release in adult neurons (Partridge and Valenzuela, 2001). We further found that pregnenolone sulfate has modulatory effects on neurotransmitter release in cultured neonatal neurons through presynaptic metabotropic σ_1-like receptors (Meyer et al., 2002). Therefore, we hypothesized that pregnenolone sulfate acts at presynaptic σ_1-like receptors through a $G_{i/o}$ protein second messenger system to enhance short-term facilitation of glutamate neurotransmission in the adult hippocampus.

To test this hypothesis, we compared the effect of the σ_1-receptor agonist, PRE-084, (1 μM) with that of 1 μM pregnenolone sulfate on pairs of EPSCs at 50 ms interpulse interval. Fig. 1A shows that both of these drugs caused similar increases in amplitude of the second of a pair of EPSCs up to approximately saturating stimulus strengths (see Figure legends for statistical comparisons). Thus, over a wide range of stimulus strengths, these two drugs caused a similar enhancement of PPF — a measure of presynaptic short-term facilitation (Zucker and Regehr, 2002). We next measured the effect of pregnenolone sulfate and PRE-084 on the enhancement of PPF as a function of time. Fig. 1B shows that, in the presence of either of these drugs, PPF enhancement occurred slowly reaching significance only after 15 min of drug application ($P<0.05$, one-way ANOVA with Fisher post hoc) and the enhancement was maintained for at least an additional 40 min of drug application (see Figure legends for statistical comparisons).

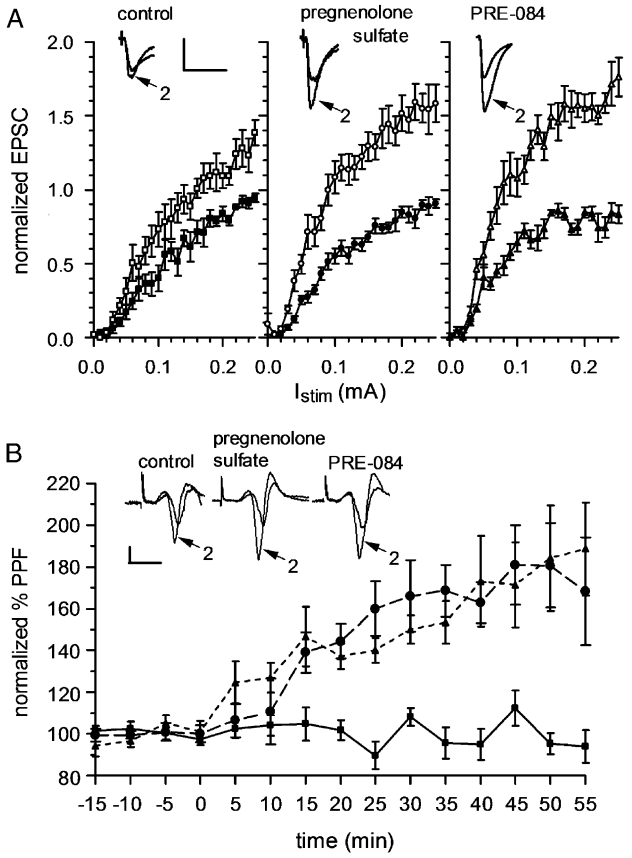

Fig. 1. Pregnenolone sulfate and a σ_1-receptor agonist produce similar enhancement of PPF. A. CA1 EPSC amplitude as a function of Schaffer collateral stimulus intensity for paired pulses at 50 ms interpulse interval normalized to the maximum of the first EPSC of the pair. Insets show superimposed typical first and second (marked in this and other Figures with "2") EPSCs measured at maximum stimulus strength (scale bars 40 ms, 200 pA). First (filled symbols) and second (open symbols) EPSC under control (■,□), 1 μM pregnenolone sulfate (●,○), or 1 μM PRE-084 (▲,△). Significant effect of drug by univariate analysis of variance ($F_{(5,1200)}=271.127$; $P<0.0005$; control $n=6$, pregnenolone sulfate $n=14$, PRE-084 $n=9$). Bonferonni post hoc tests show no significant differences for drug among the first control, pregnenolone sulfate, and PRE-084 EPSCs, but the second control EPSCs are significantly greater than the first EPSCs ($P<0.0005$) and the second pregnenolone sulfate and PRE-084 EPSCs are significantly greater than the second control EPSCs ($P<0.0005$). B. Timecourse of PPF enhancement. Each point is the average for a five minute interval (■ control, ●1 μM pregnenolone sulfate, ▲ 1 μM PRE-084). Only recordings with a first population spike within 45–55% of its respective maximum were included. For each experiment, PPF is normalized to the average PPF of recordings taken during the 20 min control interval. Significant effect of drug by univariate analysis of variance ($F_{(2,182)}=68.005$; $P<0.0005$) and a significant effect of interaction between drug and time ($F_{(22,182)}=2.223$; $P<0.005$) for 55 min of drug exposure (control $n=5$, pregnenolone sulfate $n=6$, PRE-084 $n=7$). Bonferonni post hoc tests show significant enhancement of PPF by drug treatment with pregnenolone sulfate or PRE-084 ($P<0.0005$) over control with no significant difference between pregnenolone sulfate and PRE-084 ($P>0.5$). Scale bars for inset: control 0.5 mV, 5 ms; pregnenolone sulfate 1 mV, 5 ms; PRE-084 1 mV, 5 ms.

The similarity of the effects of pregnenolone sulfate and this σ_1-receptor agonist prompted us to test whether we could block pregnenolone sulfate enhancement of PPF with σ_1-receptor antagonists.

We thus tested the effect of σ_1-receptor antagonists IPAG and BD-1063 on the pregnenolone sulfate-induced enhancement of PPF. After recording a stable baseline PPF in vehicle control, the slices were exposed to 50 nM IPAG or 1 μM BD-1063 for 20 min and PPF was recorded. There was no significant difference in PPF between vehicle control and BD-1063 (paired t-test $P>0.1$ $n=7$) or IPAG (paired t-test $P>0.1$ $n=6$). Slices were then maintained for an additional 25 to 30 min in 1 μM pregnenolone sulfate in the continued presence of IPAG or BD-1063 before recording PPF again. There was no significant difference in PPF between vehicle control and pregnenolone sulfate+BD-1063 (Fig. 2A, $P>0.1$) or pregnenolone sulfate+IPAG (Fig. 2B, $P>0.5$). Taken together, our data with σ_1-receptor agonists and antagonists are consistent with pregnenolone sulfate acting at a presynaptic σ_1-like receptor to enhance short-term facilitation.

Since plasma membrane σ_1 receptors have been shown to act through $G_{i/o}$ proteins (Monnet et al., 1995; Tokuyama et al., 1999; Ueda et al., 2001), we next assessed the involvement of $G_{i/o}$ protein in the action of pregnenolone sulfate in our system. We used pertussis toxin, which prevents $G_{i/o}$ protein activation through ADP-ribosylation. In brain slices incubated with 50 ng/ml pertussis toxin for a minimum of 12 h, there was no enhancement of PPF following the addition of 1 μM pregnenolone sulfate (Fig. 3A, $P>0.5$). By contrast, pregnenolone sulfate produced approximately the same percentage enhancement of PPF in slices that had been maintained in aCSF without pertussis toxin for at least 12 h (Fig. 3B) as it did in slices that were used less than 8 h after being dissected (enhancement of PPF=76.0±17.9%; $P<0.01$ (paired t-test); $n=5$). Furthermore, slices that were maintained for 12 h or more in pertussis toxin exhibited PPF (58.6±3.8%, $n=8$) that did not differ from that of both <12 h control slices (60.7±5.7%, $n=8$) and >12 h control slices (61.2±5.4%, $n=12$). Thus pertussis toxin treatment does not appear to affect other second messenger systems involved in short-term facilitation. Taken together, these data support our hypothesis that pregnenolone sulfate affects short-term facilitation through a $G_{i/o}$-coupled σ_1-like receptor.

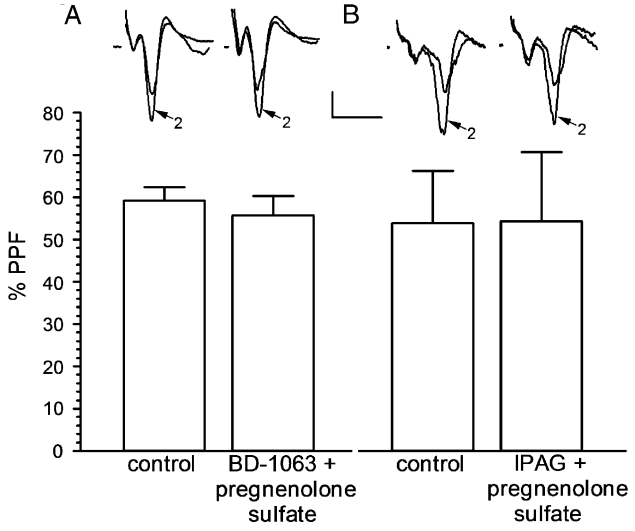

Fig. 2. σ_1-receptor antagonists block pregnenolone sulfate enhancement of PPF. Pregnenolone sulfate-induced enhancement of PPF is blocked by the σ_1-receptor antagonists, IPAG and BD-1063. A. 1 μM BD-1063 blocks enhancement of PPF by 1 μM pregnenolone sulfate (paired t-test $P>0.1$ $n=7$). B. 50 nM IPAG blocks enhancement of PPF by 1 μM pregnenolone sulfate (paired t-test $P>0.5$, $n=6$). Scale bars for inset: A: 1 mV, 5 ms; B: 0.2 mV, 5 ms.

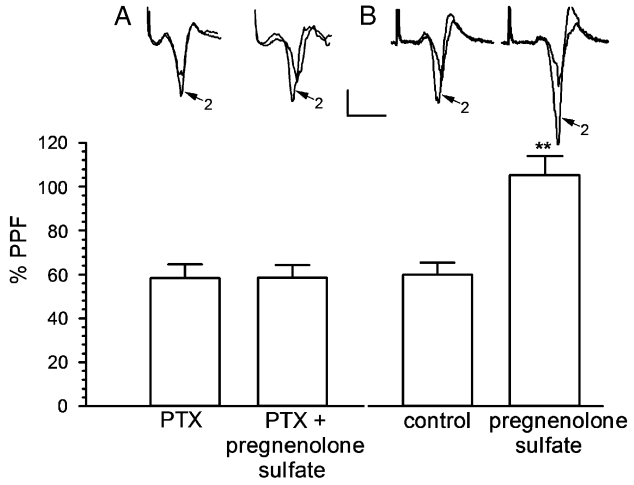

Fig. 3. Blocking $G_{i/o}$ activation prevents pregnenolone sulfate enhancement of PPF. Effect of pregnenolone sulfate on PPF in slices maintained for a minimum of 12 h compared to PPF in sister slices maintained under the same conditions. Recordings were made between 30 and 48 min after switching to pregnenolone sulfate+pertussis toxin or to pregnenolone sulfate. A. Response to 1 μM pregnenolone sulfate in sister slices maintained in 50 ng/ml pertussis toxin (paired t-test $P>0.5$ $n=5$). B. Response to 1 μM pregnenolone sulfate in sister slices maintained in aCSF (paired t-test $P<0.005$, $n=5$). Scale bars for inset: 1 mV, 5 ms.

Under many conditions, the amount of PPF in the second of a pair of responses is inversely proportional to the size of the first response (e.g. Bark et al., 2004; Zucker and Regehr, 2002). We have, however, observed enhancement of short-term facilitation by pregnenolone sulfate that is independent of an effect on the first response (Partridge and Valenzuela, 2001), suggesting that this neurosteroid enhances facilitated glutamate release by a mechanism that is independent of changes in basal release. To more directly test this possibility, we measured the timecourse of block of evoked NMDA currents by the open-channel blocker MK-801 (Hessler et al., 1993). Fig. 4A shows that the block of NMDA currents follows an equivalent exponential timecourse in aCSF, 1 μM pregnenolone sulfate, and 1 μM PRE-084. We measured time constants for each individual experiment and these are summarized in Fig. 4B. Neither pregnenolone sulfate nor PRE-084 affected the time constant of MK-801 block from that observed in aCSF (control vs. pregnenolone sulfate $P>0.5$, control vs. PRE-084 $P>0.5$) indicating that neither the neurosteroid nor the σ_1 agonist altered basal glutamate release. As shown in Fig. 4C, however, 2 μM Cd^{2+}, which is known to decrease presynaptic glutamate release (Hessler et al., 1993), significantly increased the time constant of MK-801 block (control vs. Cd^{2+} $P<0.05$).

4. Discussion

Our data indicate that pregnenolone sulfate enhances short-term facilitation by acting as a positive modulator of presynaptic σ_1-like $G_{i/o}$-protein-coupled receptors. Pregne-

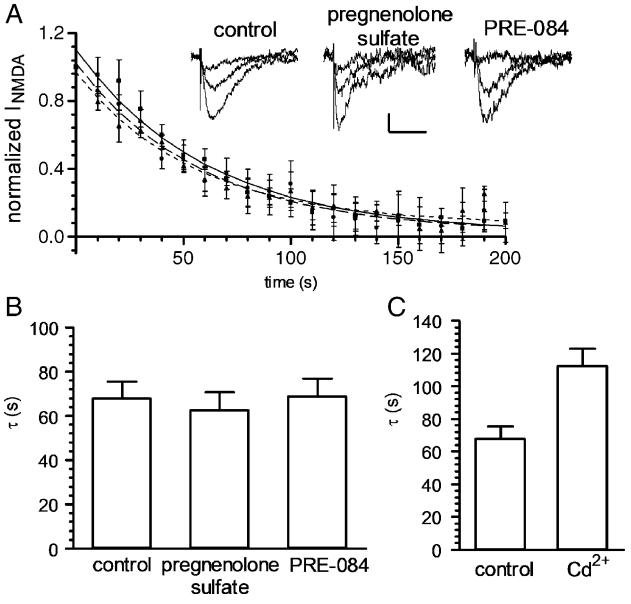

Fig. 4. σ_1-receptor activation does not alter basal glutamate release. Presynaptic release measured by block of NMDA currents in 50 μM MK-801. Slices were bathed in 1 μM PRE-084 for a minimum of 25 min (average 109 min) or 1 μM pregnenolone sulfate for a minimum of 40 min (average 54 min) before cells were patched. A. Exponential fits to the average of normalized data from individual experiments. Control (■, solid line), pregnenolone sulfate (●, dashed line), or PRE-084 (▲, dotted line). Inset shows representative NMDA current traces taken at three times during MK-801 block. Scale bars: 20/10/10 pA, 50 ms. B. Decay time constants, fit by least squares regression to a single exponential, were determined from peak current traces in each experiment (control $n=7$, pregnenolone sulfate $n=4$, PRE-084 $n=5$). One-way ANOVA with Fisher post hoc test: control vs. pregnenolone sulfate $P>0.5$, control vs. PRE-084 $P>0.5$. C. Similarly determined time constants in 2 μM Cd^{2+} were significantly increased from control (one-way ANOVA with Fisher post hoc test: control ($n=7$) vs. Cd^{2+} ($n=4$) $P<0.05$).

nolone sulfate and PRE-084 caused similar enhancement of the second, facilitated EPSC over a wide range of stimulus intensities (Fig. 1A) minimizing the possibility that these two drugs affect PPF by differently altering the synaptic input–output relationship. The slow timecourse of action of both pregnenolone sulfate and PRE-084 (Fig. 1B) is consistent with an effect through a second messenger system, which is further consistent with our finding that pertussis toxin blocks the effect of pregnenolone sulfate (Fig. 3). Two different σ_1-receptor antagonists were effective at blocking the ability of pregnenolone sulfate to enhance PPF (Fig. 2). Finally, since neither pregnenolone sulfate nor PRE-084 affects basal glutamate release (Fig. 4), neither of these agents appears to enhance facilitation indirectly through a reduction in basal release.

In this study, we have found that pregnenolone sulfate enhances PPF in the Schaffer collateral input to adult hippocampal CA1 neurons through a G-protein-dependent mechanism that is independent of an effect on basal evoked glutamate release. Similar alterations in facilitated release, without a change in the basal probability of release, have been observed, for instance, during development in mossy fiber terminals (Mori-Kawakami et al., 2003), in synaptotagmin knockout mice (Ferguson et al., 2004), and following transfection of neuronal calcium sensor-1 into cultured hippocampal neurons (Sippy et al., 2003). Our observation that pregnenolone sulfate does not affect basal glutamate release in adult hippocampal neurons is consistent with other reports that found no change in fEPSP slope or input–output curves with low mM concentrations of pregnenolone sulfate (Sliwinski et al., 2004). Interestingly, similar concentrations of pregnenolone sulfate enhance long-term potentiation at these synapses (Shibuya et al., 2003; Sliwinski et al., 2004) without affecting post tetanic potentiation (Sliwinski et al., 2004). This is an important observation since it demonstrates that neurosteroids can act selectively on presynaptic short-term synaptic facilitation, which is thought to depend on a Ca^{2+}-binding site that is independent from that for either phasic release or for potentiation and augmentation (Zucker and Regehr, 2002).

Early indication of neurosteroid action at a σ_1-binding site came from binding studies (Su et al., 1988) and since that time, a wealth of preclinical and clinical studies have associated the action of neurosteroids with σ_1 receptors (for review, see Maurice et al., 1999). In addition to our earlier observation of the involvement of σ_1-like receptors in pregnenolone sulfate-dependent modulation of glutamate release in cultured neonatal neurons (Meyer et al., 2002), pregnenolone sulfate has been shown to act presynaptically through a σ_1-like receptor to reduce the frequency of GABAergic spontaneous inhibitory postsynaptic currents and mIPSCs (Mtchedlishvili and Kapur, 2003) and to act on calcium channels through a $G_{i/o}$-coupled receptor (ffrench-Mullen et al., 1994). We show here, for the first time, the role of presynaptic σ_1-like G-protein-coupled receptors as a site of action for neurosteroids in modulating facilitated glutamate release in mature hippocampal neurons.

Currently, the only identified σ_1 receptor in the brain is a 25 kDa σ_1-binding protein (Hanner et al., 1996; Seth et al., 1998) with a putative two-membrane spanning structure (Aydar et al., 2002). It is unclear whether this σ_1-binding protein could be the G-protein-coupled receptor identified in our studies because it does not adhere to the accepted 7 membrane-spanning structure of known G-protein-coupled receptors. On the other hand, σ_1-receptor activation has been shown to be coupled to G-protein signaling in a number of systems (e.g. Hayashi et al., 2000; Meyer et al., 2002; Monnet et al., 1995; Morin-Surun et al., 1999; Mtchedlishvili and Kapur, 2003; Soriani et al., 1999; Ueda et al., 2001). Thus there may be an additional σ_1-like receptor that is 7 membrane-spanning and G-protein coupled (e.g. Zhu et al., 2003). Another possibility is that the 25 kDa σ_1-binding protein acts in a dimer complex with a 7 membrane-spanning G-protein-coupled receptor similar to the interaction of RAMP1 with the metabotropic calcitonin receptor (Morfis et al., 2003), especially since

the 25 kDa σ_1-binding protein interacts with voltage-gated potassium channels (Aydar et al., 2002).

An additional important question raised by this study is the nature of the target within the presynaptic terminal for the G-protein second messenger system activated by pregnenolone sulfate. Short-term presynaptic facilitation results from a transient increase in the probability of neurotransmitter release most likely as the result of residual Ca^{2+} in the presynaptic terminal (Wu and Saggau, 1994; Zucker and Regehr, 2002). Modulation of PPF might reflect changes in the size or decay kinetics of this Ca^{2+} pool or in the affinity of the site at which residual Ca^{2+} acts to facilitate neurotransmitter release (Atluri and Regehr, 1996). Although there is some controversy about whether presynaptic Ca^{2+} stores and Ca^{2+}-induced Ca^{2+} release play a role in short-term presynaptic plasticity (Carter et al., 2002; Emptage et al., 2001), these stores could be an additional target for second messenger modulation of facilitated release. Further possible targets for the observed second messenger action include, endogenous fast Ca^{2+} buffer systems (Blatow et al., 2003) or Ca^{2+}-sensitive proteins downstream from residual Ca^{2+} (Kamiya et al., 2002). Our results do not distinguish among these potential targets and this remains an important subject for future research.

Pregnenolone sulfate, which is one of the most abundant neurosteroids (Corpechot et al., 1993), acts presynaptically to modulate neurotransmitter release. In neonatal neurons, this neurosteroid is released in a depolarization-dependent manner to act as a retrograde messenger on presynaptic sites (Mameli et al., 2005) with an effect that is primarily on basal glutamate release. A dramatic switch occurs by postnatal day 6 (Mameli et al., 2005) so that in mature neurons, the presynaptic effect is specifically targeted to facilitated glutamate release. Interestingly, there is an elevation in pregnenolone sulfate levels at partuition (Bicikova et al., 2002; Klak et al., 2003).

We have previously demonstrated an EC_{50} for the action of pregnenolone sulfate on PPF of just less than 1 μM (Partridge and Valenzuela, 2001); however, hippocampal homogenates from adult male rats contain about an order of magnitude less pregnenolone sulfate (Kimoto et al., 2001; Vallee et al., 1997). We recently found that inhibition of 3β-hydroxysteroid sulfatase, which converts pregnenolone sulfate to pregnenolone, can produce the same effects on synaptic plasticity as 1 μM exogenously applied pregnenolone sulfate (Thomas et al., 2005). Because this neurosteroid can be released locally at synaptic sites (Mameli et al., 2005), its concentration is expected to be considerably higher in the perisynaptic microdomain. Therefore, we conclude that the 1 μM concentration of pregnenolone sulfate used in these studies approximates the physiological range at its presynaptic site of action.

Endogenous neurosteroids have important roles in mental health-related disorders and drugs that modify the activity of neurosteroids have great potential in neuropsychopharmacological therapy (Debonnel and de Montigny, 1996; Rupprecht and Holsboer, 1999). The σ_1 receptor has been increasingly accepted as an important site of action for the cognitive effects of neurosteroids and the therapeutic potential for intervention at this site is becoming apparent (Maurice et al., 1999, 2001). The data presented here provide a new insight into mechanisms that may be involved in such therapeutic intervention.

Acknowledgements

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