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Research Article

Neurosteroid enhances glutamate release in rat prelimbic cortex via activation of α_1-adrenergic and σ_1 receptors

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Abstract. The present paper studied the effect and mechanism of neurosteroid pregnenolone sulfate (PREGS) on spontaneous glutamate release using electrophysiological and biochemical methods combined with a pharmacological approach. The results suggested that PREGS had a selective enhancing effect on spontaneous glutamate release in the prelimbic cortex and the hippocampus but not in the striatum. The effect of PREGS in the prelimbic cortex appeared to be via modulation of α_1-adrenergic and σ_1 receptors, but in the hippocampus it might be dependent on σ_1 receptors only. The activation of α_1-adrenergic receptors synergized σ_1 receptor activation in the prelimbic cortex. Intracellular calcium released from the endoplasmic reticulum, protein kinase C, adenylyl cyclase and protein kinase A played a key role in the effect of PREGS. Intracellular calcium, protein kinase C and adenylyl cyclase might be upstream events in the activation of protein kinase A after PREGS.

Key words. Neurosteroid; prelimbic cortex; hippocampus; striatum; excitatory synaptic transmission; whole-cell patch-clamp; α_1-adrenergic receptor; σ_1 receptor.

Neurosteroids synthesized in the brain independently of peripheral sources have been found to play important roles in the central nervous system [1–4]. Evidence is increasing that neurosteroids may be a novel kind of neuromessenger responsible for acute modulation of neurotransmission [5].

Pregnenolone sulfate (PREGS) is one of the most abundantly produced neurosteroids in the brain [6]. PREGS has been found to modulate a variety of neurotransmission processes, including cholinergic [7], dopaminergic [8], GABAergic [9] and glutamatergic neurotransmission [10]. Among these, the effect of PREGS on presynaptic glutamate release has received a great amount of attention because glutamate is the most important excitatory neurotransmitter in the brain and plays a vital role in mediating a variety of brain functions and brain diseases [11–13], but many questions remain to be answered.

The first report about the effect of PREGS on presynaptic glutamate release came from Partridge and Valenzvela [14]. They showed that when pairs of stimuli were given at short intervals, PREGS significantly increased presynaptic glutamate release in rat hippocampal slices. A subsequent study by Meyer et al. [10] confirmed this result in experiments in which the frequency of miniature excitatory postsynaptic currents (mEPSCs) were used as a measure of presynaptic glutamate release in cultured hippocampal neurons. Moreover, they found that the mechanism of the effect of PREGS was via activation of presynaptic metabotropic σ_1-like receptors. However, these studies have only observed the effect of PREGS in the hippocampus, whereas glutamatergic neurotransmission is widely distributed throughout the central nervous system [15, 16]. Whether PREGS has a selective action on presynaptic glutamate release at distinct brain regions

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is unknown. To address this question, the present study extends the observation of the effect of PREGS on presynaptic glutamate release to two other brain regions – the prelimbic cortex and the striatum. We hypothesize that the prelimbic cortex may be another important selective target for PREGS to modulate presynaptic glutamate release, because evidence has shown a correlation between the cerebral concentration of PREGS and cognitive function [17] whereas, the prelimbic cortex, as a key structure in cognitive function [18, 19], is most likely to be targeted by PREGS. We also hypothesize that PREGS may have no effects on presynaptic glutamate release in the striatum because the selective sites of the effect of PREGS on presynaptic glutamate release may be at the cognition-related brain regions, such as the prelimbic cortex and the hippocampus, rather than at the movement-related brain region, the striatum. Moreover, even for the hippocampus and the prelimbic cortex, the mechanism of the effect of PREGS is likely to be different.

To test these hypotheses, we have investigated the effect of PREGS on presynaptic glutamate release in the hippocampus, prelimbic cortex and striatum by examining the effect of PREGS on the frequency of mEPSCs with a whole-cell patch-clamp recording method in slices and further studied its mechanism.

Materials and methods

Preparation of slices

Twenty- to 30-day-old Sprague-Dawley rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneal). All experimental procedures conformed to Fudan University as well as international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering. Brain slices were prepared according to procedures described previously [20]. Briefly, following decapitation, the brains were quickly removed and submerged in ice-cold artificial cerebrospinal fluid (ACSF) containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, 10 mM sucrose, saturated with 95% O_2/5% CO_2. A block of tissue containing the prelimbic cortex, the hippocampus or the striatum was cut and placed on a layer of moistened filter paper glued to the cutting stage of a vibratome (VT1000M/E; Leica). Serial coronal slices (300 μm) were cut and transferred to an incubating chamber (30–32 °C) where they stayed for at least 1 h before recordings began.

Whole-cell recording

The slice was continuously perfused with ACSF saturated with 95% O_2/5% CO_2. Cells were visualized with an infrared-DIC microscope (Olympus BX50WI) and a CCD camera. Electrodes were pulled from glass capillaries using a Narishige micropipetter puller (model PB-7). They were filled with a solution containing 140 mM K-gluconate, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP•K_2, 0.1 mM GTP•Na_3 and 10 mM HEPES (pH 7.25) and had a resistance of 4–6 MΩ. Voltage and current signals were recorded with an Axopatch 200B amplifier (Axon) connected to a Digidata1200 interface (Axon). The data were digitized and stored on disks using pClamp (version 6; Axon). Resting membrane potential and action potentials were recorded under the current clamp mode. mEPSCs were recorded in sweeps of 2 s at a holding potential of –70 mV under the voltage clamp mode in the presence of tetrodotoxin (TTX) (1 μM) and picrotoxin (10 μM). The series resistance (Rs) was monitored by measuring the instantaneous current in response to a 5-mV voltage step command. Series resistance compensation was not used, but cells where Rs changed by >15% were discarded.

Synaptosome preparation and protein kinase A activity assay

Synaptosomes were prepared from the prelimbic cortex and the hippocampus of Sprague-Dawley rats as described previously [21]. Protein kinase A (PKA) activity was assessed with a PepTag Non-Radioactive cAMP-Dependent PKA Assay Kit from Promega according to the manufacturer's instructions. The assay was based on the changes in the net charge of the fluorescent PKA substrates before and after phosphorylation. This change in the net charge of the substrate allowed the phosphorylated and nonphosphorylated versions of the substrate to be rapidly separated on an agarose gel at neutral pH. The intensity of fluorescence of phosphorylated peptides reflected the activity of PKA [22].

Off-line data analysis

Off-line data analysis was performed using a Mini Analysis Program (Synaptosoft), SigmaPlot (Jandel Scientific) and Origin (Microcal Software). The record of mEPSCs was shown with high-time resolution, and events that did not show a typical EPSC waveform were rejected manually. The frequency, inter-event intervals, amplitude and decay of mEPSCs were measured. In most cases, >100 mEPSCs were collected under control conditions as well as for each pharmacological condition. Numerical data were expressed as mean ± SE. Statistical significance was determined using either the Kolmogorov-Smirnov test (K-S test) or Student's paired t test (unless otherwise stated). In all cases, n refers to the number of cells studied.

Drugs

Picrotoxin, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline (NBQX), pregnenolone (PREG), PREGS, haloperidol, +SKF-10047, +SCH-23390, prazosin, phe-

nylephrine (PE), ryanodine, chelerythrine, H89, MDL-12330A, K-gluconate, ATP•K_2, GTP•Na_3, forskolin, AC915, chloroethylclonidine dihydrochloride (CEC) and sulpiride were purchased from Sigma. TTX was made in the Research Institute of Aquatic Products of Hebei, P.R. China. Percoll was purchased from Amersham Biosciences Corporation. Other reagents in AR grades were products of Shanghai Chemical Plant. All drugs were dissolved in dH_2O, except for picrotoxin, PREG and PREGS, which were dissolved in DMSO. When DMSO was used as vehicle, drugs were initially dissolved in 100% DMSO and then diluted into the artificial cerebrospinal fluid at a final DMSO concentration of 0.1%. In vehicle control experiments, we confirmed that the final concentration of DMSO in the artificial cerebrospinal fluid had no detectable effects on the frequency, amplitude and decay of mEPSCs. The averaged frequency of mEPSCs before and after 0.1% DMSO was 3.70 ± 0.85 Hz and 3.29 ± 0.59 Hz, respectively ($n = 4$, $p > 0.05$); the averaged amplitude of mEPSCs before and after 0.1% DMSO was 10.81 ± 1.16 pA and 10.55 ± 1.32 pA, respectively ($n = 4$, $p > 0.05$); the averaged decay of mEPSCs before and after 0.1% DMSO was 1.75 ± 0.12 ms and 1.69 ± 0.15 ms, respectively ($n = 4$, $P > 0.05$). PREG, PREGS, haloperidol, +SCH-23390, prazosin, PE, ryanodine, chelerythrine, H89, MDL-12330A, NBQX, TTX, picrotoxin, forskolin, +SKF-10047, AC915, CEC and sulpiride were applied by bath perfusion.

Results

PREGS increases the frequency of mEPSCs in the prelimbic cortex and hippocampus, but has no effects in the striatum

Figure 1A shows the effect of PREGS (20 μM) on the frequency of mEPSCs in the prelimbic cortex. From the raw current traces (panel 1 of fig. 1A), PREGS could be seen to markedly increase the frequency of mEPSCs. Further plotting of a cumulative distribution of mEPSC intervals and statistical analysis with a K-S test showed that PREGS induced a clear shift toward shorter intervals in the cumulative curve (panel 2 of fig. 1A) and the shift was statistically significant ($p < 0.01$), indicating that, as expected from the raw data, there was indeed a substantial increase in the frequency of mEPSCs after PREGS. The effect of PREGS began to appear at 12 min after administration ($p < 0.05$) and was reversible. We repeated the experiment in five other cells and a similar effect was observed. All six cells passed the K-S test with $p < 0.01$. The mean mEPSC frequency in the pooled six cells increased from 4.65 ± 0.55 Hz before to 9.75 ± 1.65 Hz after application of PREGS (panel 4 of fig. 1A, $p < 0.05$). The averaged increase percentage during PREGS was 106.5 ± 23.3%. However, PREGS had no significant effect on the amplitude and decay of mEPSCs. The mean amplitude of mEPSCs in six pooled cells was 9.89 ± 1.19 pA before and 10.51 ± 1.52 pA after application of PREGS ($n = 6$, $p > 0.05$); the mean decay of mEPSCs in six pooled cells was 1.84 ± 0.16 ms before and 1.73 ± 0.13 ms after application of PREGS ($n = 6$, $p > 0.05$).

To check the concentration dependence of the effect of PREGS on the frequency of mEPSCs, four different concentrations of PREGS were used (10, 20, 40 and 100 μM). The result showed that 10 μM PREGS had no effect, but 20, 40 and 100 μM PREGS concentration dependently increased the frequency of mEPSCs. The averaged increasing percentage for 20, 40 and 100 μM PREGS was 106.5 ± 23.3%, 240.2 ± 105.2% and 336.4 ± 83.6%, respectively ($n = 5$–6, $p < 0.05$). We also investigated the role of the sulfation at C3 of PREGS by observing the effect of the unsulfated form of PREGS as PREG on the frequency of mEPSCs. The result showed that PREG (20 μM) had no significant effect on the frequency, amplitude and decay of mEPSCs. The averaged frequency of mEPSCs before and after PREG was 3.89 ± 0.72 Hz and 4.80 ± 1.59 Hz, respectively ($n = 5$, $p > 0.05$); the averaged amplitude of mEPSCs before and after PREG was 13.78 ± 1.02 pA and 13.06 ± 1.26 pA ($n = 5$, $p > 0.05$); the averaged decay of mEPSCs before and after PREG was 1.60 ± 0.11 ms and 1.61 ± 0.12 ms, respectively ($n = 5$, $p > 0.05$). This result suggests that the effect of PREGS requires sulfation at C-3.

We also observed the effect of PREGS (20 μM) on the frequency of mEPSCs in the hippocampus and striatum. The results showed that PREGS significantly increased the frequency of mEPSCs in the hippocampus ($n = 5$, fig. 1B) and all five cells passed the K-S test with $p < 0.01$. The averaged increase percentage during PREGS was 112.7 ± 34.5%. The effect of PREGS began to appear at 10 min after administration ($p < 0.05$) and was partially reversible. However, PREGS had no effect in the striatum ($n = 6$, fig. 1C). PREGS had no effects on the amplitude and decay of mEPSCs in both the hippocampus and the striatum. In the hippocampus, the mean amplitude of mEPSCs in five cells before and after PREGS was 14.97 ± 1.51 pA and 13.57 ± 0.93 pA, respectively ($p > 0.05$); the mean decay of mEPSCs in 5 cells before and after PREGS was 2.00 ± 0.19 ms and 1.61 ± 0.01 ms ($p > 0.05$). In the striatum, the mean amplitude of mEPSCs in six cells before and after PREGS was 13.29 ± 1.19 pA and 12.85 ± 0.99 pA ($p > 0.05$); the mean decay of mEPSCs in six cells before and after PREGS was 1.48 ± 0.13 ms and 1.55 ± 0.16 ms, respectively ($p > 0.05$).

σ_1 receptor antagonist partially blocks the effect of PREGS in the prelimbic cortex, but completely blocks it in the hippocampus

To test if the effect of PREGS in the prelimbic cortex was mediated by σ_1 receptors, we observed the influence of the σ_1 receptor antagonist haloperidol on the effect of

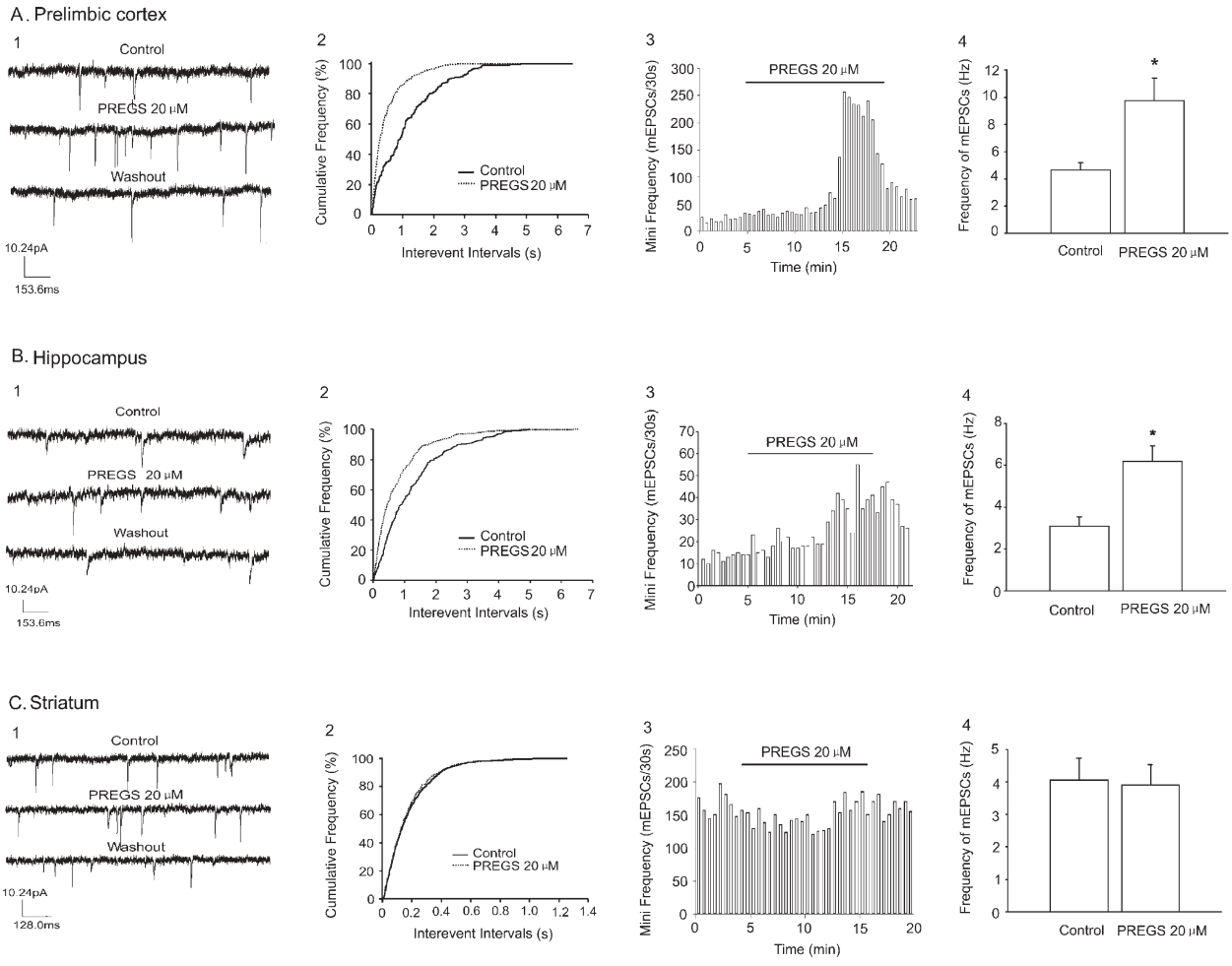

Figure 1. Effect of PREGS on the frequency of mEPSCs in the prelimbic cortex, striatum and hippocampus. Panel 1: typical records in control, PREGS and washout. Holding potential is –70 mV; panel 2: the cumulative distributions of the inter-event intervals of mEPSCs before and after PREGS; panel 3: the averaged frequency of mEPSCs before and after PREGS in a group of cells. (*A*) Effect of PREGS (20 μM) on the frequency of mEPSCs in the pyramidal cells of the layers V–VI of the prelimbic cortex. panel 2: $p < 0.01$, K-S test; Panel 3: $n = 6$, * $p < 0.05$. (*B*) Effect of PREGS (20 μM) on the frequency of mEPSCs in the pyramidal cells of the hippocampal CA1 region. Panel 2: $p < 0.01$, K-S test; panel 3: $n = 5$, * $p < 0.05$. (*C*) Effect of PREGS (20 μM) on the frequency of mEPSCs in the spiny neurons of the striatum. Panel 2: $p > 0.05$, K-S test; Panel 3: $n = 6$, $p > 0.05$.

PREGS in the prelimbic cortex. Haloperidol (0.3 μM) alone had no effects on the frequency, amplitude and decay of mEPSCs ($n = 4$, $p > 0.05$, data not shown). Panel 1 of figure 2B was the result in a typical cell, which showed that in the presence of haloperidol (0.3 μM), PREGS (20 μM) still increased the frequency of mEPSCs. The averaged frequency of mEPSCs after PREGS in the presence of haloperidol increased from 6.35 ± 0.90 Hz to 8.85 ± 0.74 Hz ($n = 6$, $p < 0.05$, panel 2 of fig. 2B). However, the percentage increase of the PREGS effect in the presence of haloperidol (50.4 ± 17.8%) was significantly lower than that without haloperidol (106.5 ± 23.3%) (panel 3 of fig. 2B, $p < 0.05$). This result suggests that the σ_1 receptor antagonist partially blocks the effect of PREGS in the prelimbic cortex, which appears to be different from that reported in the hippocampus [10]. To confirm this difference, we again observed the influence of haloperidol on the effect of PREGS in the hippocampus. The result showed that haloperidol indeed completely blocked the effect of PREGS in the hippocampus (fig. 2C, $n = 4$).

Haloperidol has been shown to bind with equal affinity to σ_1 receptors and dopamine D2 receptors [23]. To eliminate the possibility that haloperidol partially blocked the effect of PREGS by blocking D2 receptors, we observed the influence of the D2 receptor antagonist sulpiride (10 μM) on the effect of PREGS (20 μM). Sulpiride had no significant influence on the effect of PREGS. The averaged frequency of mEPSCs after PREGS in the presence of sulpiride increased from 5.34 ± 1.65 Hz to 11.25 ± 2.65 Hz ($n = 4$, $p < 0.05$). The percentage increase of the PREGS effect in the presence of sulpiride (118.5 ± 16.2%) was not significantly different ($p > 0.05$) com-

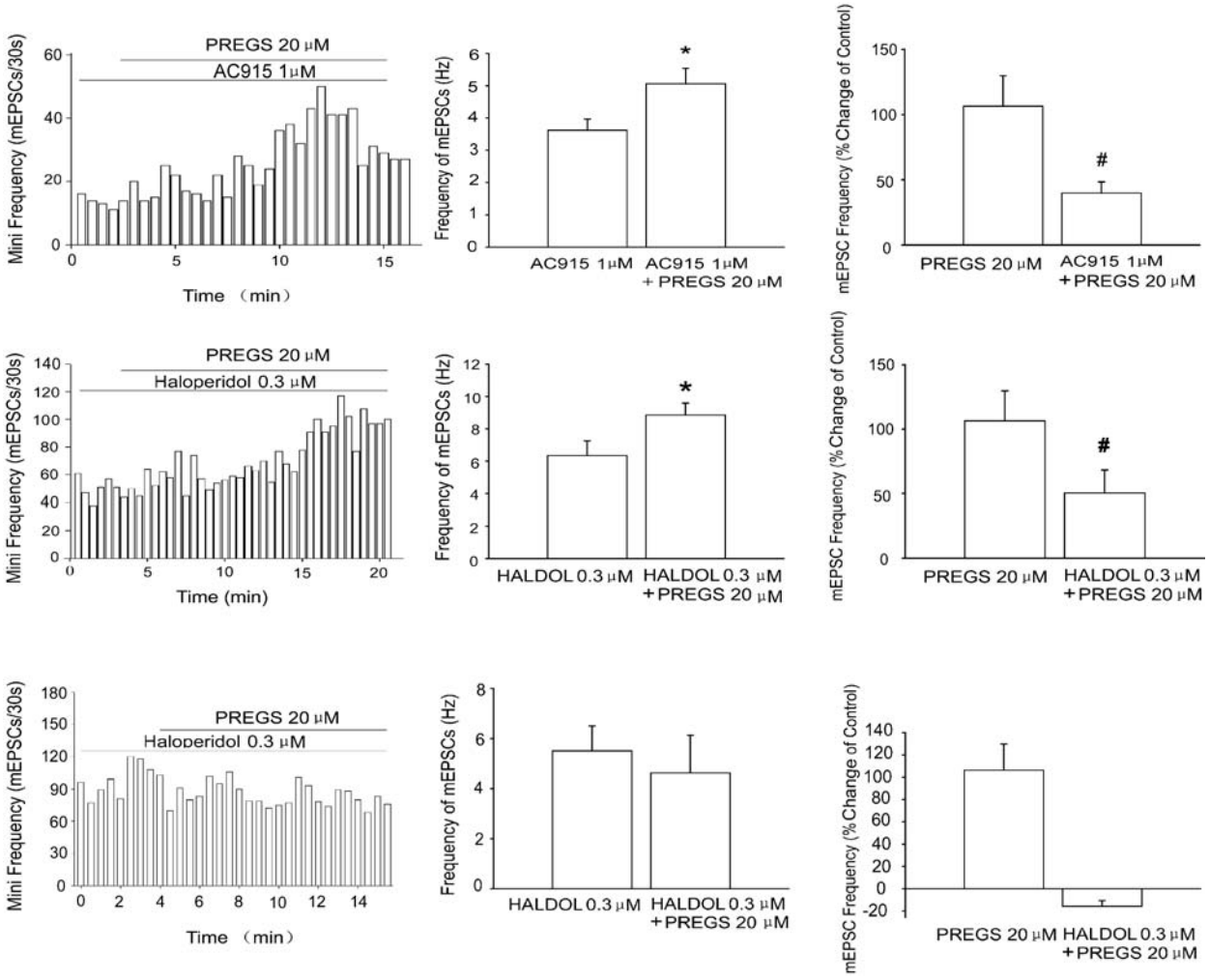

Figure 2. Influence of σ_1 receptor receptor antagonists on the effect of PREGS on the frequency of mEPSCs in the prelimbic cortex and the hippocampus. Panel 1: time course in a typical cell; panel 2: the averaged results in a group of cells; panel 3: change percentage of control. (*A*) Influence of the σ_1 receptor receptor antagonist AC915 (20 μM) on the effect of PREGS (20 μM) on the frequency of mEPSCs in the prelimbic cortex. n = 5, * $p < 0.05$, compared to AC915 group; #$p < 0.05$, compared to PREGS group. (*B*) Influence of the σ_1 receptor receptor antagonist haloperidol (0.3 μM) (HALDOL) on the effect of PREGS (20 μM) on the frequency of mEPSCs in the prelimbic cortex. n = 6, * $p < 0.05$, compared to haloperidol group; #$p < 0.05$, compared to PREGS group. (*C*) Influence of the σ_1 receptor receptor antagonist haloperidol (0.3 μM) (HALDOL) on the effect of PREGS (20 μM) on the frequency of mEPSCs in the hippocampus. n = 4, $p > 0.05$, compared to haloperidol group.

pared to that without sulpiride (106.5 ± 23.3%). In addition, to further confirm the role of σ_1 receptors in the effect of PREGS, we observed the influence of a selective σ_1 receptor antagonist AC915 [24] on the effect of PREGS. The result showed that AC915 (1 μM) partially blocked the effect of PREGS (fig. 2A) in the prelimbic cortex. The averaged frequency of mEPSCs after PREGS in the presence of AC915 increased from 3.63 ± 0.33 Hz to 5.06 ± 0.48 Hz (n = 5, $p < 0.05$), but the percentage increase of the effect of PREGS in the presence of AC915 (40.0 ± 8.4%) was significantly lower than that without AC915 (106.5 ± 23.3%) ($p < 0.05$). Interestingly, here we again demonstrated that in the hippocampus, the σ_1 receptor antagonist AC915 (1 μM) could completely block the effect of PREGS (20 μM). The averaged frequency of mEPSCs before and after PREGS in the presence of AC915 was 2.82 ± 2.00 Hz and 2.88 ± 1.81 Hz, respectively (n = 4, $p > 0.05$).

α_1-Adrenergic receptor antagonist completely blocks the effect of PREGS in the prelimbic cortex

To analyze the role of α_1-adrenergic receptors in the effect of PREGS in the prelimbic cortex, we applied the α_1 receptor antagonist prazosin prior to the application of PREGS (20 μM). Prazosin (1 μM) alone had no effects on the frequency, amplitude or decay of mEPSCs (n = 4, $p > 0.05$, data not shown). The stimulation by PREGS was completely blocked by prazosin (fig. 3A). The averaged frequency of mEPSCs before and after PREGS in the

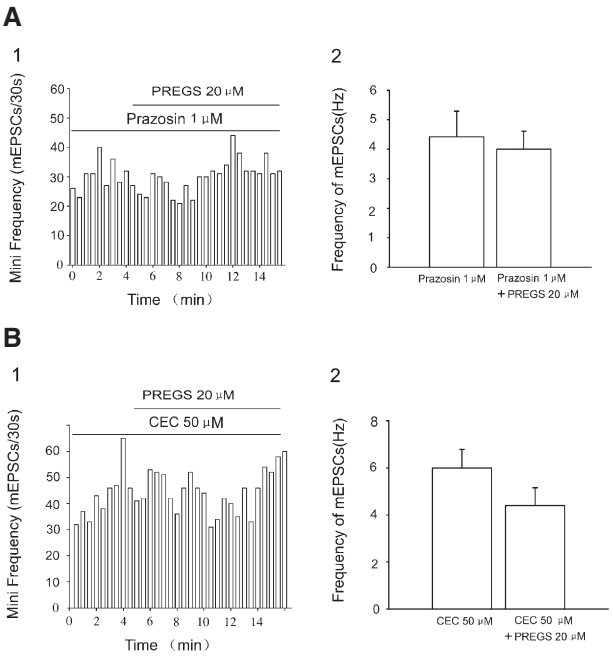

Figure 3. Influence of the α_1-adrenergic receptor antagonist prazosin and the α_{1B}-adrenergic receptor antagonist CEC on the effect of PREGS on the frequency of mEPSCs in the pyramidal cells of layers V–VI of the prelimbic cortex. Panel 1: time course in a typical cell; panel 2: the averaged results in a group of cells. (*A*) Influence of the α_1-adrenergic receptor antagonist prazosin (1 μM) on the effect of PREGS (20 μM). n = 6, p > 0.05, compared to prazosin group. (*B*) Influence of the α_{1B}-adrenergic receptor antagonist CEC (50 μM) on the effect of PREGS (20 μM). n = 6, p > 0.05, compared to CEC group.

presence of prazosin was 4.43 ± 0.86 Hz and 4.01± 0.60 Hz, respectively (panel 2 of fig. 3A, n = 6, p > 0.05), showing that the effect of PREGS completely disappeared in the presence of prazosin. This result suggests that the activation of α_1-adrenergic receptors may play a key role in the effect of PREGS in the prelimbic cortex.

Three subtypes of α_1-adrenergic receptors have been found in the central nervous system: α_{1A}, α_{1B}, α_{1D} [25]. Among them, α_{1B} receptors are highly expressed in the cerebral cortex [25–27], so the α_{1B} receptor subtype may play a key role in the effect of PREGS in the prelimbic cortex. To test this hypothesis, we observed the influence of the α_{1B} receptor antagonist CEC (50 μM) on the effect of PREGS (20 μM). Panel 1 of figure 3B shows the result in a typical cell: in the presence of CEC, the effect of PREGS disappeared. The averaged frequency of mEPSCs repeated in six cells before and after PREGS in the presence of CEC was 5.98 ± 0.78 Hz and 4.40 ± 0.76 Hz, respectively (panel 2 of fig. 3B, p > 0.05), showing that the effect of PREGS disappeared in the presence of CEC. This result suggests that the activation of the α_{1B}-adrenergic receptor subtype may play a key role in the effect of PREGS in the prelimbic cortex.

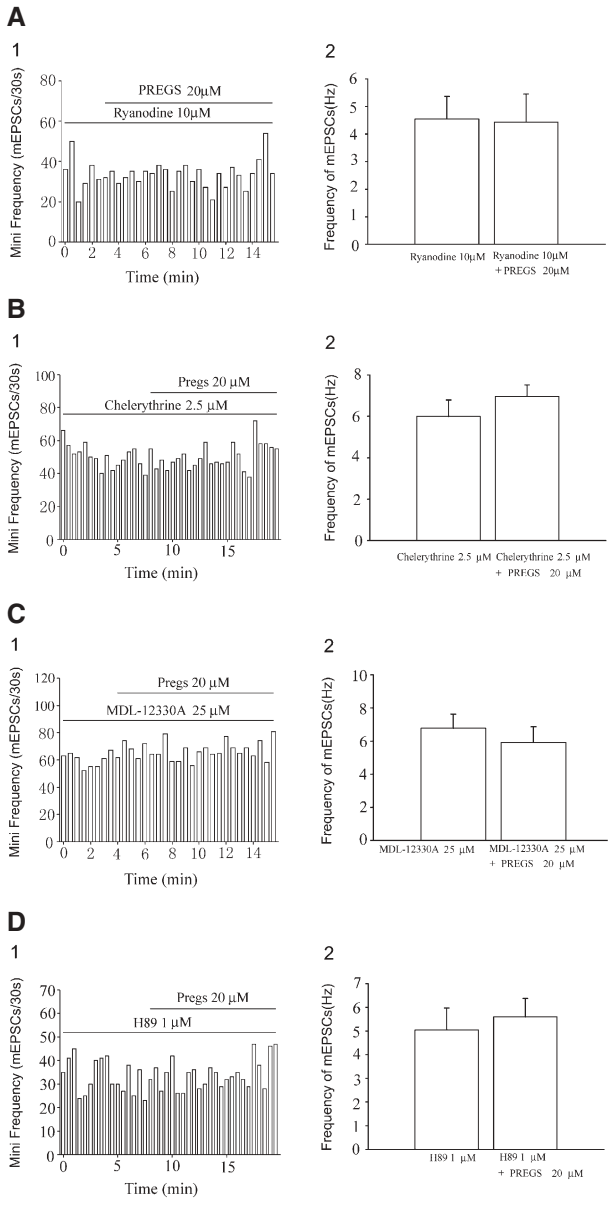

Figure 4. Influence of the endoplasmic reticulum calcium release channel blocker, the PKC inhibitor, the AC inhibitor and the PKA inhibitor on the effect of PREGS on the frequency of mEPSCs in the pyramidal cells of layers V–VI of the prelimbic cortex. Panel 1: time course in a typical cell; panel 2: the averaged results in a group of cells. (*A*) Influence of the blockade of the calcium release channels (ryanodine receptors) in the endoplasmic reticulum with high concentrations of ryanodine (10 μM) on the effect of PREGS (20 μM). n = 6, p > 0.05, compared to the ryanodine group. (*B*) Influence of the PKC inhibitor chelerythrine (2.5 μM) on the effect of PREGS (20 μM). Right panel: n = 6, p > 0.05, compared to chelerythrine group. (*C*) Influence of the AC inhibitor MDL-12330A (25 μM) on the effect of PREGS (20 μM). Right panel: n = 6, p > 0.05, compared to MDL-12330A group. (*D*) Influence of the PKA inhibitor H89 (1 μM) on the effect of PREGS (20 μM). Right panel: n = 6, p > 0.05, compared to H89 group.

Intracellular calcium, protein kinase C, adenylyl cyclase and PKA play a key role in the effect of PREGS

To test the possible involvement of the intracellular calcium released from the endoplasmic reticulum, protein kinase C (PKC), adenylyl cyclase (AC) and PKA in the effect of PREGS, we observed the influence of the endoplasmic reticulum calcium release channel blocker ryanodine (10 μM) [28], the PKC inhibitor chelerythrine (2.5 μM) [29], the AC inhibitor MDL-12330A (25 μM) [30] and the PKA inhibitor H89 (1 μM) [31] on the effect of PREGS (20 μM). All these inhibitors alone had no effects on the frequency, amplitude or decay of mEPSCs ($n = 4–6$, $p > 0.05$, data not shown), but they could block the effect of PREGS (fig. 4A–D, $p > 0.05$, $n = 6$), suggesting that the intracellular calcium released from the endoplasmic reticulum, PKC, AC and PKA might all play an important role in the effect of PREGS.

PREGS enhances the activity of PKA in both the prelimbic cortex and hippocampus, but the influence of an α_1-adrenergic receptor antagonist and σ_1-likereceptor antagonist on the effect of PREGS in these two regions is different

As shown in figure 5A, an addition of PREGS (20 μM) in the milieu resulted in a significant increase in PKA activity in both the prelimbic cortex and hippocampus. PKA activity was enhanced to 22.5 ± 15.3% in the prelimbic cortex (panel 1 of fig. 5A, $n = 6$, $p < 0.05$) and 18.8 ± 3.0% in the hippocampus (panel 2 of fig. 5A, $n = 6$, $p < 0.05$), respectively, relative to the control. Interestingly, consistent with our electrophysiological results, the increase in PKA activity was partially blocked by the σ_1 receptor antagonist haloperidol (0.3 μM) in the prelimbic cortex (panel 1 of fig. 5B, $n = 6$), but in the hippocampus it was completely blocked (panel 2 of fig. 5B, $n = 6$). In addition, in the prelimbic cortex, the α_1 receptor antagonist prazosin (1 μM) could completely block the effect of PREGS (panel 1 of fig. 5C, $n = 6$), but in the hippocampus, it had no significant influence on the effect of PREGS (panel 2 of fig. 5C, $n = 6$).

α_1-Adrenergic receptor activation synergizes σ_1 receptor activation in the prelimbic cortex

To study the interaction between the activation of the α_1 and σ_1 receptors after PREGS, we observed the effect of the application of each agonist alone and the influence of the α_1 receptor agonist on the effect of the σ_1 receptor agonist. The result showed that the α_1-adrenergic receptor agonist PE (1 μM) applied alone increased the frequency of mEPSCs in the prelimbic cortex (fig. 6A, $n = 6$, $p < 0.05$), but the application of the σ_1 receptor agonist +SKF-10047 (10 μM) alone had no significant effect (fig. 6B, $n = 6$, $p > 0.05$). Interestingly, when +SKF-10047 was added during the plateau of the PE effect, it could significantly increase the frequency of mEPSCs (fig. 6C, $n = 6$, $p < 0.05$, compared to PE group). This result suggests that α_1-adrenergic receptor activation may synergize the activation of σ_1 receptors. To confirm this conclusion, we used the PKA activity as a measure to observe if the α_1-adrenergic receptor activation could synergize the activation of σ_1 receptors. The result was

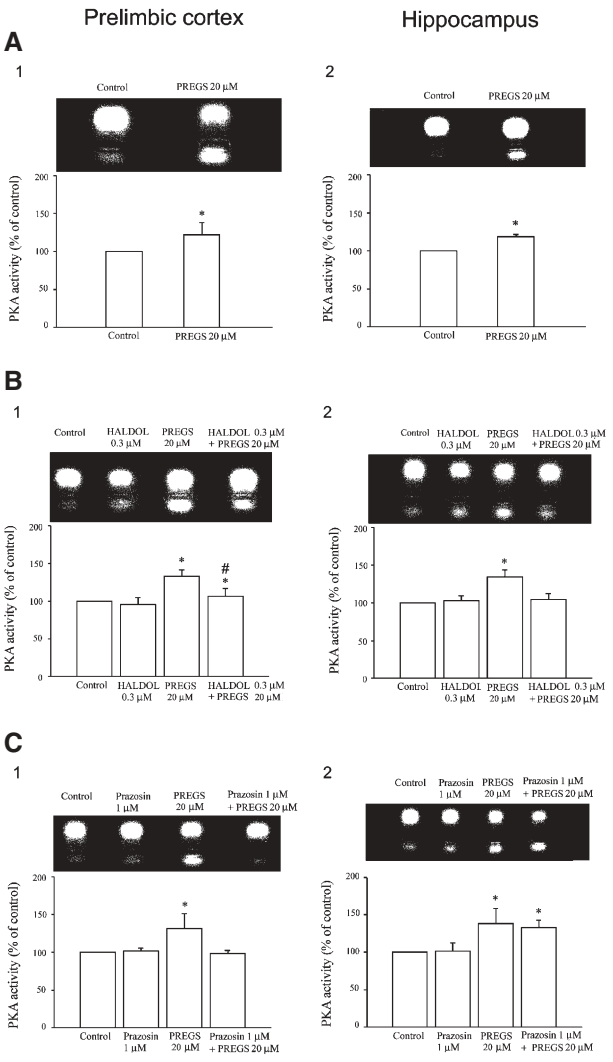

Figure 5. Effect of PREGS on the activity of PKA and influence of the σ_1 receptor antagonist haloperidol (HALDOL) and the α_1 receptor antagonist prazosin on the effect of PREGS in synaptosomes of the prelimbic cortex and hippocampus. Top panel: representative gel electrophoresis from PKA activity assay; bottom panel: the averaged results in a group of cells. (*A*) Effect of PREGS (20 μM) on the activity of PKA in the prelimbic cortex (panel 1) and the hippocampus (panel 2). $n = 6$, $^*p < 0.05$, compared to control group, Student's paired t test. (*B*) Influence of the σ_1 receptor antagonist haloperidol (0.3 μM) (HALDOL) on the effect of PREGS (20 μM) on the activity of PKA in the prelimbic cortex (panel 1) and hippocampus (panel 2). $n = 6$, $^*p < 0.05$, compared to control group, $^{\#}p < 0.05$, compared to PREGS group, Student's paired t test. (*C*) Influence of the α_1 receptor antagonist prazosin (1 μM) on the effect of PREGS (20 μM) on the activity of PKA in the prelimbic cortex (panel 1) and hippocampus (panel 2). $n = 6$, $^*p < 0.05$, compared to control group.

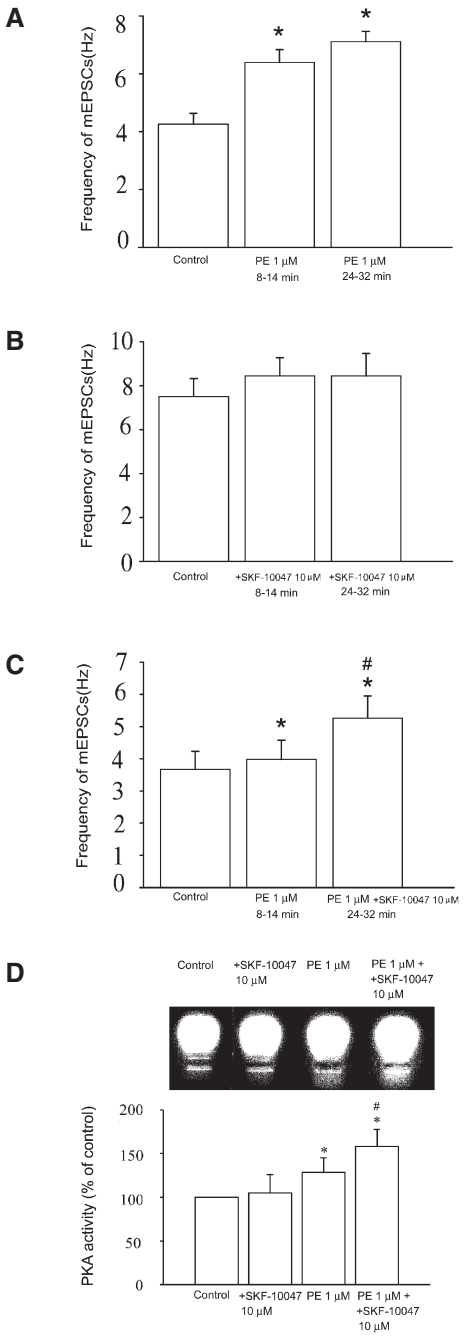

Figure 6. Effect of the α_1-adrenergic receptor agonist PE and the σ_1 receptor agonist +SKF-10047 on the frequency of mEPSCs, and influence of PE on the effect of +SKF-10047 in the pyramidal cells of the layers V–VI of the prelimbic cortex. (*A*) Effect of PE (1 μM) on the frequency of mEPSCs. n = 6, *p < 0.05, compared to control group. (*B*) Effect of +SKF-10047 (10 μM) on the frequency of mEPSCs. n = 6, p > 0.05, compared to control group. (*C*) Influence of PE (1 μM) on the effect of +SKF-10047 (10 μM). n = 6, *p < 0.05, compared to control group, #p < 0.05, compared to PE group. (*D*) Effect of the α_1-adrenergic receptor agonist PE (1 μM) and the σ_1 receptor agonist +SKF-10047 (10 μM) on the activity of PKA and influence of PE on the effect of +SKF-10047 in synaptosomes of the prelimbic cortex. Top panel: representative gel electrophoresis from PKA activity assay; bottom panel: the averaged results in a group of cells. n = 6, *p < 0.05, compared to control group, #p < 0.05, compared to PE group.

consistent with that of the electrophysiological experiments. Application of the α_1-adrenergic receptor agonist PE (1 μM) alone increased the activity of PKA (fig. 6D, n = 6, p < 0.05, compared to control); the σ_1 receptor agonist +SKF-10047 (10 μM) alone had no effect (fig. 6D, n = 6, p > 0.05, compared to control), but in the presence of PE, +SKF-10047 could significantly increase the activity of PKA (fig. 6D, n = 6, p < 0.05, compared to PE group).

Intracellular calcium, PKC and AC are upstream events of the activation of PKA after PREGS in both the prelimbic cortex and hippocampus

To study the upstream events of the activation of PKA after PREGS, we observed the influence of the endoplasmic reticulum calcium release channel blocker ryanodine (10 μM), the PKC inhibitor chelerythrine (2.5 μM) and the AC inhibitor MDL-12330A (25 μM) on the effect of PREGS (20 μM) on the activity of PKA. The results showed that ryanodine, chelerythrine and MDL-12330A all blocked the effect of PREGS on the activity of PKA both in the prelimbic cortex and hippocampus (fig. 7, n = 4–6), suggesting that although the receptors activated by PREGS were different in the prelimbic cortex and hippocampus, but the downstream signal transduction pathway of the activation of the receptors by PREGS was very similar in these two regions.

Discussion

Using cultured hippocampal neurons from immature rats [10] or facilitated glutamate release as a measure [14], previous studies demonstrated that PREGS could increase presynaptic glutamate release in the hippocampus. The present study confirmed this conclusion by using spontaneous glutamate release as a measure in slices. In addition, we extended the observation to the prelimbic cortex and the striatum and found that PREGS also had this effect in the prelimbic cortex, but not in the striatum. This finding suggests that PREGS may have a selective action on presynaptic glutamate release in different brain regions.

The mechanism of the effect of PREGS on presynaptic glutamate release in the hippocampus has been reported to be via activation of presynaptic σ_1 receptors [10]. This conclusion was supported by our similar experiments here, but, interestingly, in the prelimbic cortex, the σ_1 receptor antagonists only partially blocked the effect of PREGS, suggesting that in the prelimbic cortex, the mechanism of the effect of PREGS on presynaptic glutamate release was more complicated.

In addition to σ_1 receptors, other approaches for the effect of PREGS in the prelimbic cortex may involve many aspects. In the present study, we tested the role of

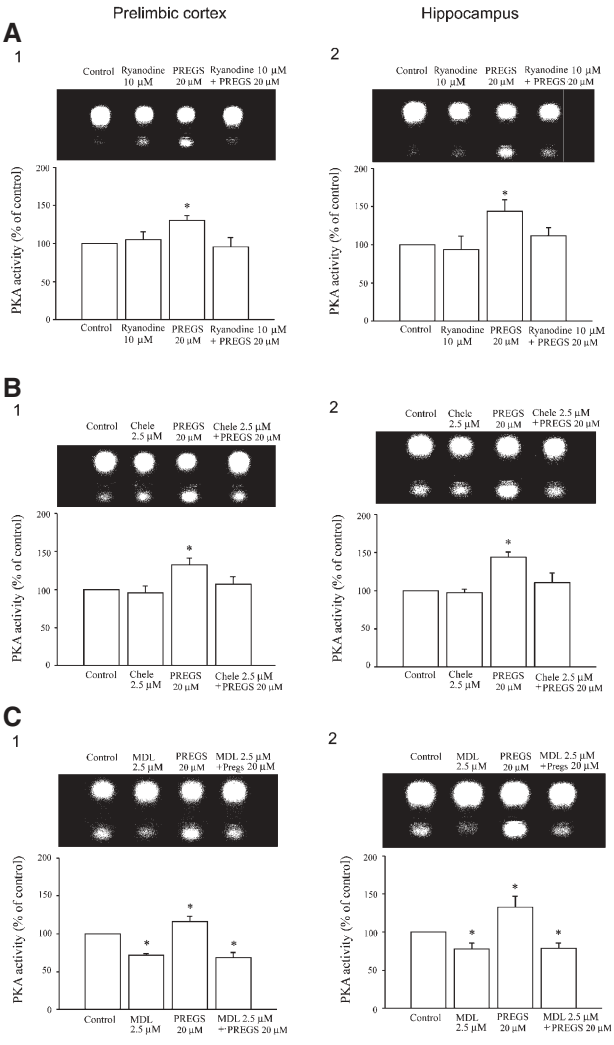

Figure 7. Influence of the endoplasmic reticulum calcium release channel blocker, the PKC inhibitor and the AC inhibitor on the effect of PREGS on the activity of PKA in synaptosomes of the prelimbic cortex and the hippocampus. Top panel: representative gel electrophoresis from PKA activity assay; Bottom panel: the averaged results in a group of cells. (*A*) Influence of the endoplasmic reticulum calcium release channel blocker ryanodine (10 μM) on the effect of PREGS (20 μM) on the activity of PKA in the prelimbic cortex (panel 1) and hippocampus (panel 2). Bottom panel: n = 6, *p < 0.05, compared to control group. (*B*) Influence of the PKC inhibitor chelerythrine (Chele) (2.5 μM) on the effect of PREGS (20 μM) on the activity of PKA in the prelimbic cortex (panel 1) and the hippocampus (panel 2). Bottom panel: n = 6, *p < 0.05, compared to control group. (*C*) Influence of the AC inhibitor MDL-12330A (MDL) (25 μM) on the effect of PREGS (20 μM) on the activity of PKA in the prelimbic cortex (panel 1) and the hippocampus (panel 2). Bottom panel: n = 4, *p < 0.05, compared to control group.

dopaminergic D1 receptors and adrenergic α_1 receptors on the effect of PREGS because if these receptors are activated, the release of presynaptic glutamate in the prelimbic cortex can increase significantly [32, 33]. The results showed that the D1 receptor antagonist SCH23390 (10 μM) had no significant influence on the PREGS

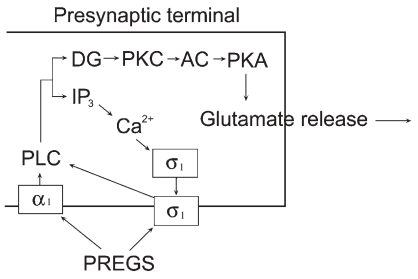

Figure 8. Schematic representation of the possible mechanism underlying the effect of PREGS on the frequency of mEPSCs in pyramidal cells of layers of V–VI of the prelimbic cortex.

effect (data not shown), but the α_1 receptor antagonist prazosin could completely block the effect of PREGS. This result suggests that the activation of α_1 receptors plays a key role in the effect of PREGS. To our knowledge, this is the first report that PREGS can activate α_1 receptors.

Three major subtypes of α_1 receptors, termed α_{1A}, α_{1B} and α_{1D}, have been found in the central nervous system [27], but their distribution in the central nervous system is not even. For example, the α_{1A} and α_{1D} subtypes are found to be heavily expressed in both the cerebral cortex and the hippocampus, but are extremely low in the striatum; α_{1B} receptors are highly expressed in the cerebral cortex, but little expression is found in either the hippocampus or striatum [27–29]. Therefore, the subtype targeted by PREGS is most likely α_{1B} receptors. To test this hypothesis, we observed the influence of the α_{1B} receptor antagonist on the effect of PREGS. The result showed that the α_{1B} receptor antagonist CEC can completely cancel the effect of PREGS, suggesting that α_{1B} receptors play a key role in the effect of PREGS in the prelimbic cortex.

The downstream signal transduction pathways of the activation of α_1 receptors by PREGS appears to involve two aspects: one may be mediated by the α_1 receptor signal transduction pathway itself and the other may be mediated through synergizing the activation of the σ_1 receptor signal transduction pathway by α_1 receptors (fig. 8). This hypothesis is supported by the present observations: (i) the σ_1 receptor antagonist only partially inhibited the effect of PREGS because it could not inhibit the effect of PREGS mediated by α_1 receptors; (ii) the α_1 receptor antagonist could completely inhibit the effect of PREGS because the activation of α_1 receptors is a key step in the effect of PREGS and only activation of this receptor can initiate the α_1 receptor signal pathways and synergize the activation of σ_1 receptors; (iii) σ_1 receptor agonist alone has no effect on presynaptic glutamate release and PKA activity, but in the presence of α_1 receptor agonist, it can produce significant effects because the activation of the σ_1 receptor requires a synergizing effect from the activation of α_1 receptors.

How the activation of α_1 receptors synergizes the activation of σ_1 receptors remains to be studied. The σ_1 receptor is one major subtype of the σ receptors which represent a unique class of receptors distinct from any other known receptors [34]. There is evidence suggesting that σ_1 receptors exist in neurons [35]. Biochemical experiments have shown that the σ_1 receptor is an intracellular protein anchored on the endoplasmic reticulum and the translocation of σ_1 receptor from the cytoplasm to the membrane requires a calcium efflux from the endoplasmic reticulum [36]. Therefore, we speculate that after the activation of α_1 receptors by PREGS, it induces an efflux of calcium from the endoplasmic reticulum, which, on the one hand, induces a release of glutamate and, on the other, causes σ_1 receptors to translocate from the cytoplasm to the membrane where they receive the activation by PREGS that further promotes the release of glutamate (fig. 8). The evidence supporting this hypothesis was that the activation of α_1 receptors could induce the release of calcium from the endoplasmic reticulum [37] and the blockade of calcium release from the endoplasmic reticulum with a high concentration of ryanodine could block the effect of the α_1 receptor agonist and the synergizing effect of the α_1 receptor agonist on the σ_1 receptor agonist (data not shown).

An increase in intracellular calcium is known to be coupled with the activation of PKC [37]. PKC is likely therefore to play an important role in the mediation of the effect of PREGS. This point is confirmed by the present result that the PKC inhibitor can inhibit the effect of PREGS. In addition, the present study also shows that AC and PKA play an important role in the effect of PREGS. Moreover, the relationship among these enzyme activations by PREGS is apparently in series, rather than in parallel, because inhibition of any of them can completely block the effect of PREGS. However, the sequence of the activation of these enzymes by PREGS is unclear. A study by Yoshimura and Cooper [38] demonstrated that PKC activation could lead to the activation of AC, suggesting that a sequence of first activating PKC and then activating AC was possible. In addition, the downstream kinase of AC, which was activated by cAMP, was already known to be PKA [39]. So the activation of PKC and AC might be upstream events of the activation of PKA (fig. 8). This hypothesis was confirmed by the present results: if PKC and AC were blocked, the activating effect of PREGS on PKA was completely blocked.

Another interesting phenomenon we observed was that although the downstream signal transduction pathways of the activation of the receptors by PREGS are very similar in the prelimbic cortex and hippocampus, the receptors activated by PREGS in these two brain regions are different, that is, in the prelimbic cortex, PREGS produces effects via activation of α_{1B}-adrenergic and σ_1 receptors, but in the hippocampus only via the activation of σ_1 receptors. In addition, the present study also shows that PREGS has no effect in the striatum. The reason for these differences is most likely related to the different expression of α_{1B} and σ_1 receptors in these brain regions. High levels of expression of σ_1 receptors were found in the hippocampus [40, 41], which might account for the observation that PREGS could produce its effect via activation of σ_1 receptors in the hippocampus; little σ_1 receptor expression was found in the striatum [40, 41], which might account for the observation that PREGS had no effect in the striatum; a unique σ_1 receptor expression with punctae of various sizes dispersed between unstained neuronal cell bodies was found in the deep layers of the cerebral cortex [40, 41], which might account for the observation that the effect of PREGS via σ_1 receptors in the prelimbic cortex required a synergistic effect from the activation of α_{1B} receptors; a high expression of α_{1B} receptors was found in the cerebral cortex, but little expression in either the hippocampus or striatum [27–29], which might account for the observation that PREGS produced its effect via activation of α_{1B} receptors in the prelimbic cortex.

PREGS is a C-3 sulfated derivative of pregnenolone. Both PREGS and pregnenolone have been known to be synthesized and present at a high concentration in the brain [3, 42–45], but the actions of these two neurosteroids may not be the same because the sulfation at C-3 of pregnenolone may add some novel activities to pregnenolone. This point is confirmed by the present results that unsulfated pregnenolone has no effects on the frequency of mEPSCs, but the sulfated derivative at C-3 of pregnenolone can produce significant effects.

The prelimbic cortex is an important brain region involved in cognitive function. The functional activity of the prelimbic cortex is dictated by many factors. Among them, the excitatory synaptic transmission in the pyramidal cells of the layers V–VI is very important [31]. Therefore, the present finding that PREGS could significantly increase presynaptic glutamate release in pyramidal cells of the layers V–VI of the prelimbic cortex provides experimental evidence for understanding the mechanism of action of PREGS on cognitive function.

More interestingly, the present study shows that the receptors activated by PREGS in the prelimbic cortex and hippocampus are different, which not only provides experimental evidence for understanding the mechanism of action of PREGS in the prelimbic cortex and hippocampus, but also provides clues for designing drugs to selectively modulate the PREGS action in these two regions.

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