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Effects of neurosteroids on Ca^{2+} signaling mediated by recombinant *N*-methyl-D-aspartate receptor expressed in Chinese hamster ovary cells

Hideo Mukai[a], Shigeo Uchino[b,1], Suguru Kawato[a,*]

[a]*Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo at Komaba, Meguro, Tokyo 153-8902, Japan*

[b]*Yokohama Research Center, Mitsubishi Chemical Corporation, Aoba, Yokohama 227-8502, Japan*

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Abstract

Pregnenolone sulfate (PREGS) potentiates the *N*-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+}-signals in cultured hippocampal neurons. The NMDA receptor family has several different subunits whose expression in neurons has distinct spatial and temporal patterns. To examine subunit specificity of the PREGS action, we have investigated the effect of PREGS on recombinant GluR $\varepsilon2/\zeta1$ (NR2B/NR1) type NMDA receptors stably expressed in Chinese hamster ovary cells using heat shock promoters. PREGS enhanced the Ca^{2+} influx through the GluR $\varepsilon2/\zeta1$ receptors in a dose-dependent manner. The EC_{50} of PREGS for the GluR $\varepsilon2/\zeta1$ receptors was 8.6 μM. Other sulfated neurosteroids, dehydroepiandrosterone sulfate (DHEAS), 17β-estradiol sulfate and 3α-ol-5β-pregnan-20-one sulfate (3α5βS), inhibited the positive modulatory effect of PREGS on the GluR $\varepsilon2/\zeta1$ NMDA receptors. Ifenprodil, a specific inhibitor of GluR $\varepsilon2$ subunit, abolished the NMDA-induced Ca^{2+} influx even in the presence of PREGS. These results imply that PREGS positively modulates the Ca^{2+} influx through the GluR $\varepsilon2/\zeta1$ receptors which are expressed from the embryonic period.
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Neurosteroids are steroids synthesized de novo in the brain. Pregnenolone sulfate (PREGS) is one of the most important neurosteroids [1,11]. There is increasing evidence that neurosteroids modulate neurotransmission in the hippocampus, and that they most likely influence learning and memory [4,15]. The injection of PREGS into the hippocampus significantly enhanced memory retention in the footshock avoidance test [3]. The acute actions of neurosteroids are thought to be mediated through ion-gated channel receptors rather than through nuclear steroid receptors which promote the classic genomic actions of adrenal steroid hormones. PREGS potentiates the Ca^{2+} conductivity of the *N*-methyl-D-aspartate (NMDA) receptors in cultured rat hippocampal and cortical neurons [2,13].

The NMDA receptors play an essential role in the neuronal plasticity underlying memory, learning, and development. Recent studies have elucidated the molecular diversity of the NMDA receptors [7,9]. Functional NMDA receptors consist of two subunits of the glutamate receptor (GluR) families which are the GluR ε (NR2) and GluR ζ (NR1) subunit families. Four GluR ε subunits are distinct in distribution, properties, regulation, and physiological roles, and providing the molecular basis of the functional diversity of the NMDA receptor. NMDA receptors allow large Ca^{2+} entry which is necessary for Ca^{2+}-dependent processes in forming long-term potentiation (LTP). Therefore, the modulator of Ca^{2+} signaling through NMDA receptor is of critical importance.

Many studies have been performed to characterize the pharmacological effects of PREGS on NMDA receptors using cultured neurons [9,16]. However, because of possible heterogeneity in terms of cell type and receptor subtype, such studies are not sufficient in precise determination of the activity of PREGS. We took advantage of the recombinant NMDA receptors expressed in Chinese hamster ovary (CHO) cells.

We established CHO cell line expressing different sub-

* Corresponding author. Tel./fax: +81-3-5454-6517.

E-mail address: kawato@phys.c.u-tokyo.ac.jp (S. Kawato)

[1] Present address: Department of Neurochemistry, National Institute of Neuroscience, Kodaira, Tokyo 187-8502, Japan.

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types of the NMDA receptors, i.e. GluR ε1/ζ1 (NR2A/NR1) and GluR ε2/ζ1 (NR2B/NR1), using heat-inducible expression system [14]. Cells were routinely maintained in eRDF-1 medium (1:1:2 mixture medium of Dulbecco's modified Eagle's medium, Ham's Nutrient Mixture F-12 and RPMI1640, without L-glutamate, glycine, and L-aspartate) supplemented with 10% fetal bovine serum (Gibco BRL), and 400 μg/ml geneticin (Sigma), 2 μg/ml blasticidin S hydrochloride (Funakoshi) and 10 μg/ml puromycin (Sigma) and 200 μM 2-amino-5-phosphonopentanoic acid (APV, a specific competitive antagonist of the NMDA receptor, from RBI) in a humidified atmosphere containing 5% CO_2 for 2 h and successive incubation at 37°C for 9 to 15 h.

For Ca^{2+} signal measurements, cells were loaded for 60 min at 37°C with 5 μM fura-2/AM (from 1 mM stock solution in DMSO, from Dojindo) in the presence of 0.02% Cremophor EL (from Sigma) in 1 ml of BSS buffer consisting of Mg^{2+}-free 10 mM Hepes buffer, pH 7.3, containing 130 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$ and 5.5 mM glucose. The cells were perfused with BSS and stimuli at the rate of 1.5 ml/min. All steroids were dissolved in DMSO. Cells were incubated for 10 min at 37°C in the presence of PREGS or other neurosteroids in BSS. We used a digital fluorescence microscope which consisted of an inverted microscope (Nikon TMD 300, Japan) equipped with a high sensitivity CCD camera (Hamamatsu Photonics C2400-77, Japan). The glass-bottom dishes were mounted on the microscope equipped with a temperature chamber which was maintained in air atmosphere at 37°C. For fura-2, we changed the excitation wavelength of 340 and 380 nm at every 2.5 s with a step motor. We measured the fluorescence above 520 nm with an excitation filter, dichroic mirror, and an emission filter.

The Ca^{2+} analysis was performed on the same cells before and after the perfusion with PREGS using the ARGUS-50 system (Hamamatsu Photonics, Japan). Intracellular free calcium concentrations ($[Ca^{2+}]_i$) were measured and calibrated for fura-2 with standard Ca^{2+}-EGTA solutions. In order to improve the signal-to-noise ratio, Ca^{2+} signals were averaged for 20–30 cells under the same microscopic field. PREGS dose-dependent curves were fitted with the logistic equation. To determine any statistical significance, the values were compared by *t*-tests. The differences were considered significant, if the *P*-values were less than 0.05.

We examined the action of PREGS on the GluR ε2/ζ1 subtype of NMDA receptors in CHO cells by observing the $[Ca^{2+}]_i$ increase upon NMDA stimulation. PREGS induced acute potentiation of the GluR ε2/ζ1 receptor-mediated Ca^{2+} signals. By preincubation of cells with 50 μM PREGS for 10 min, a significant increase in the $[Ca^{2+}]_i$ elevation was induced upon 100 μM NMDA application (Fig. 1A). In the present conditions, Mg^{2+} blockade was absent in NMDA receptors because of using Mg^{2+}-free medium. Therefore NMDA stimulation can directly open the Ca^{2+} channels of NMDA receptors independent of depolarization of membranes. This is different from voltage dependent processes which include Mg^{2+} unblocking of NMDA receptors induced by depolarization of membranes by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor-mediated Na^+/K^+ currents. It should be noted that our CHO cells did not contain voltage dependent Ca^{2+} channels as judged from no Ca^{2+} signal upon high KCl application.

It has been shown that PREGS dose-dependently enhanced the $[Ca^{2+}]_i$ increase induced by 100 μM NMDA

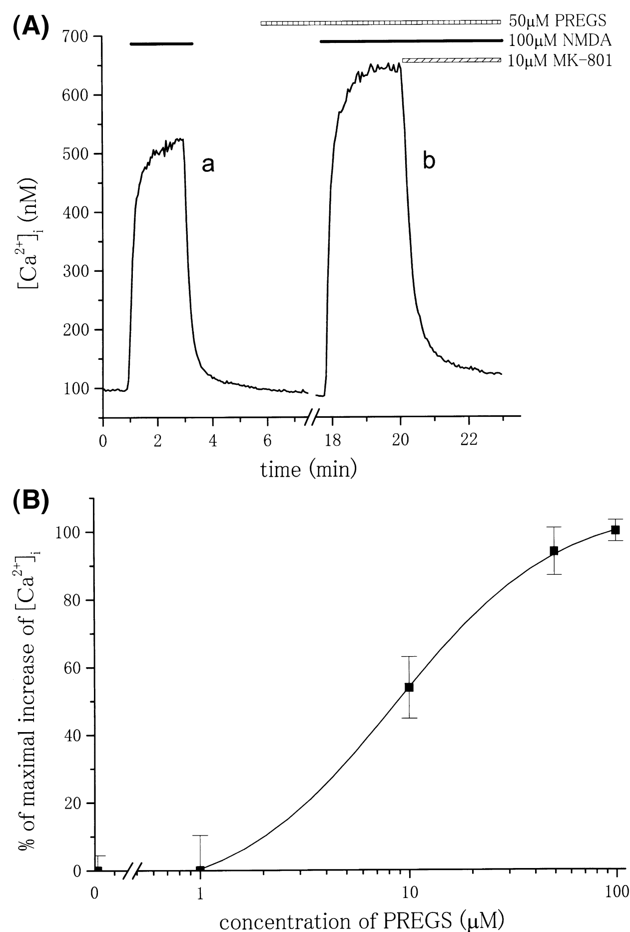

Fig. 1. (A) Effect of PREGS on the time course of NMDA-gated Ca^{2+} signals through the GluR ε2/ζ1 NMDA receptors. Curve (a) step-like Ca^{2+} elevation induced upon perfusion of only 100 μM NMDA. Curve (b) change in the $[Ca^{2+}]_i$ level induced by the successive perfusions with only 50 μM PREGS, then with 100 μM NMDA plus 50 μM PREGS, finally with 100 μM NMDA plus 50 μM PREGS plus 10 μM MK-801. The vertical scale is the $[Ca^{2+}]_i$, and the horizontal axis is the time in minutes. (B) Dose response curve for PREGS with respect to 100 μM NMDA-stimulated $[Ca^{2+}]_i$ increase in CHO cells expressing the GluR ε2/ζ1 receptors. Data are expressed as percentages of maximal effect induced by 100 μM PREGS. Values represent the mean ± SEM for 20–30 cells from three independent experiments. Solid sigmoidal dose dependence curve was obtained by calculation from the logistic equation. Vertical Axis: increase of $[Ca^{2+}]_i$ in percentage from the $[Ca^{2+}]_i$ level with only NMDA stimulation Horizontal Axis: concentration of PREGS (0, 1, 10, 50, 100 μM).

through the GluR $\varepsilon 2/\zeta 1$ receptors. When the concentration of PREGS was changed from 0 μM to 1, 10, 50, and 100 μM, the $[Ca^{2+}]_i$ was increased from 100%, to 101, 118, 132 and 141% ($n = 3$ for each concentration), where 100% stands for the $[Ca^{2+}]_i$ increase induced by only 100 μM NMDA. EC_{50} for the GluR $\varepsilon 2/\zeta 1$ receptor was calculated to be 8.6 μM, and the Hill coefficient was 1.1 (Fig. 1B).

To assure that the PREGS-induced modulation of the Ca^{2+} signal is mediated by the NMDA receptors, we simultaneously applied channel blockers MK-801 or ketamine with NMDA. They completely abolished the Ca^{2+} signals in the PREGS-treated cells. The effect of PREGS was also completely abolished by perfusion with the BSS. The preperfusion of thapsigargin at 200 nM did not affect the PREGS effect on NMDA receptor-mediated Ca^{2+} influx, excluding the contribution of the intracellular Ca^{2+} stores, the endoplasmic reticulum.

Because sulfation of pregnenolone is essential for its neuroactivity, we examined the effect of other sulfated neurosteroids to understand the possible role of the sulfate group of PREGS. We investigated the competitive effect of DHEAS, 17β-estradiol sulfate, 3α-ol-5β-pregnan-20-one sulfate (3α5βS) on the enhancement effect of PREGS for the GluR $\varepsilon 2/\zeta 1$ receptors (Fig. 2). DHEAS and 17β-estradiol sulfate at 100 μM decreased the elevation of $[Ca^{2+}]_i$ by 50 μM PREGS from 132 to 103% (DHEAS) and 94% (17β-estradiol sulfate). Both DHEAS and 17β-estradiol sulfate completely abolished the positive modulatory effect of PREGS. 3α5βS at 100 μM not only abolished the effect of PREGS but also considerably inhibited the Ca^{2+} signaling by NMDA on the GluR $\varepsilon 2/\zeta 1$ receptors down to 52%. It should be noted that both DHEAS and 17β-estradiol sulfate

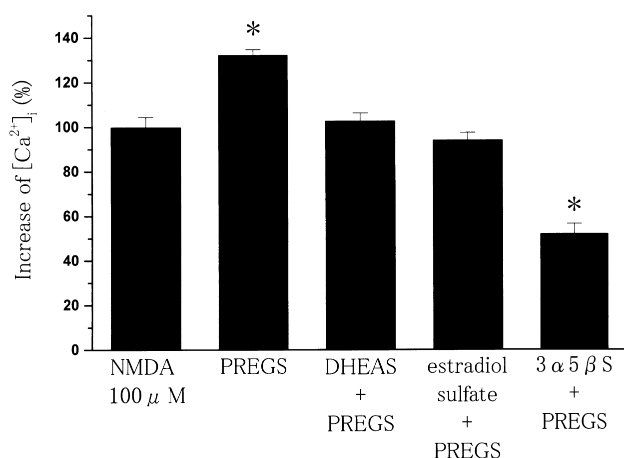

Fig. 2. Competitive effect of sulfated steroids on the PREGS-potentiated $[Ca^{2+}]_i$ elevation through GluR $\varepsilon 2/\zeta 1$ receptors. Vertical Axis: Increase of $[Ca^{2+}]_i$ in percentage for NMDA-gated Ca^{2+} signals. Values represent the mean ± SEM for 20–30 cells from three independent experiments. Error bars, standard errors of the means. *Indicates a statistically significant ($P < 0.05$) change. Horizontal Axis: 100 μM NMDA, +50 μM PREGS, +100 μM DHEAS + 50 μM PREGS, +100 μM estradiol sulfate +50 μM PREGS, +100 μM 3α5βS +50 μM PREGS.

only slightly modulated NMDA-gating Ca^{2+} signals with the $[Ca^{2+}]_i$ of 88–103% in the absence of PREGS. 3α5βS markedly reduced NMDA-gating Ca^{2+} signals to 45% in the absence of PREGS. 3α5βS was shown to be an inhibitor for the GluR $\varepsilon 1/\zeta 1$ receptors and hippocampal NMDA receptors [5]. The observed potentiation effect of PREGS should not be non-specific due to the membrane-solvation of steroids or membrane-disordering with steroids, because the addition of other sulfated neurosteroids abolished the potentiation effect of PREGS although the total concentration of the steroids was increased.

Ifenprodil is a specific inhibitor of the GluR $\varepsilon 2$ subunit. The IC_{50} of ifenprodil on the receptors was 0.34 μM [17]. The presence of 50 μM PREGS could not antagonize the inhibitory effect of 4 μM ifenprodil on GluR $\varepsilon 2/\zeta 1$ receptor-mediated Ca^{2+} signals, implying that the site of the PREGS action is on the GluR $\varepsilon 2/\zeta 1$ receptors.

PREGS at 50 μM also enhanced the GluR $\varepsilon 1/\zeta 1$ receptor-mediated Ca^{2+} influx to 126% which is slightly smaller than 132% for the GluR $\varepsilon 2/\zeta 1$ receptors. Before the present report about the enhancement of $[Ca^{2+}]_i$ through the GluR $\varepsilon 2/\zeta 1$ receptors with PREGS, the potentiation of the NMDA-induced rise of Ca^{2+} influx through recombinant NMDA receptors was reported only for the GluR $\varepsilon 1/\zeta 1$ receptors expressed in *Xenopus oocytes* with the electrophysiological studies [10]. The co-presence of 3α5βS and PREGS reduced the PREGS-induced potentiation also on the GluR $\varepsilon 1/\zeta 1$ receptors. The GluR $\varepsilon 1/\zeta 1$ and GluR $\varepsilon 2/\zeta 1$ have several common physiological and pharmacological features as NMDA-gated Ca^{2+} channels, that is, sensitivity to Mg^{2+} ions, enhancement of NMDA-induced Ca^{2+} conductivity in the presence of glycine, and channel blocking with MK-801, except that only GluR $\varepsilon 2$ is inhibited by ifenprodil [17]. The GluR $\varepsilon 2$ subunit is expressed in a limited area of the brain such as the cortex and the hippocampus, while the GluR $\varepsilon 1$ subunit is expressed over the entire region of the brain including cerebellum. The GluR $\varepsilon 2$ subunit was expressed not only in the adult brain but also in the embryonic and early postnatal brain, whereas the expression of the GluR $\varepsilon 1$ subunit was detected only postnatally and rarely observed in embryonic period [16]. Because neurosteroidogenesis including PREGS synthesis is very active especially in embryonic and early postnatal period with significant neural network development [1], it is of importance to examine the action of PREGS on GluR $\varepsilon 2/\zeta 1$ receptors which were demonstrated to be the major functional NMDA receptors expressed in embryonic and early postnatal neural tissue [7,9]. The GluR $\varepsilon 2$ knock-out mice died shortly after birth [8] and the GluR $\varepsilon 1$ knock-out mice had a lower efficiency of LTP formation [12].

Because the incubation time of 10 min with PREGS was too short for its genomic effects to appear via intracellular nuclear steroid receptors, the present effect should be due to the direct effect of PREGS on the GluR $\varepsilon 2/\zeta 1$ receptors.

When compared with the action of PREGS, neuronal synthesis of PREGS has not been well elucidated. The diffi-

culty was due to the small amount of steroidogenic proteins and also due to concentration of the investigations on glial cells. We have recently discovered the steroidogenic systems in hippocampal neurons where PREGS synthesis was significantly enhanced by Ca^{2+} influx through the NMDA receptors [6]. The positive amplification mechanism is indicated as follows: NMDA-gating Ca^{2+} influx triggers steroidogenesis by cytochrome P450scc-containing steroidogenic systems resulting in an increase in the production of PREGS, which in turn potentiates the NMDA receptor-mediated Ca^{2+} influx. Relatively high concentration of PREGS of 50 μM employed in this study may be physiological, because the local concentration of PREGS synthesized in the hippocampus was estimated to be 8–80 μM [6]. These results and current observation add significant reality to the physiological importance of modulatory effect of PREGS in neuronal communications.

Our findings also suggest that the allosteric action of PREGS on the GluR ε2/ζ1 receptors in the rat brain play a role in the development of neurons via Ca^{2+} signals induced by glutamate as well as in the adult brain.

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