Pregnenolone Sulfate Potentiates the Inwardly Rectifying K⁺ Channel Kir2.3

Toru Kobayashi¹*, Kazuo Washiyama¹, Kazutaka Ikeda²

¹ Department of Molecular Neuropathology, Brain Research Institute, Niigata University, Chuo-ku, Niigata, Niigata, Japan, ² Division of Psychobiology, Tokyo Institute of Psychiatry, Setagaya, Tokyo, Japan

Abstract

Background: Neurosteroids have various physiological and neuropsychopharmacological effects. In addition to the genomic effects of steroids, some neurosteroids modulate several neurotransmitter receptors and channels, such as N-methyl-D-aspartate receptors, γ-aminobutyric acid type A (GABA_A) receptors, and σ₁ receptors, and voltage-gated Ca²⁺ and K⁺ channels. However, the molecular mechanisms underlying the various effects of neurosteroids have not yet been sufficiently clarified. In the nervous system, inwardly rectifying K⁺ (Kir) channels also play important roles in the control of resting membrane potential, cellular excitability and K⁺ homeostasis. Among constitutively active Kir2 channels in a major Kir subfamily, Kir2.3 channels are expressed predominantly in the forebrain, a brain area related to cognition, memory, emotion, and neuropsychiatric disorders.

Methodology/Principal Findings: The present study examined the effects of various neurosteroids on Kir2.3 channels using the Xenopus oocyte expression assay. In oocytes injected with Kir2.3 mRNA, only pregnenolone sulfate (PREGS), among nine neurosteroids tested, reversibly potentiated Kir2.3 currents. The potentiation effect was concentration-dependent in the micromolar range, and the current-voltage relationship showed inward rectification. However, the potentiation effect of PREGS was not observed when PREGS was applied intracellularly and was not affected by extracellular pH conditions. Furthermore, although Kir1.1, Kir2.1, Kir2.2, and Kir3 channels were insensitive to PREGS, in oocytes injected with Kir2.1/Kir2.3 or Kir2.2/Kir2.3 mRNA, but not Kir2.1/Kir2.2 mRNA, PREGS potentiated Kir currents. These potentiation properties in the concentration-response relationships were less potent than for Kir2.3 channels, suggesting action of PREGS on Kir2.3-containing Kir2 heteromeric channels.

Conclusions/Significance: The present results suggest that PREGS acts as a positive modulator of Kir2.3 channels. Kir2.3 channel potentiation may provide novel insights into the various effects of PREGS.

Introduction

Neurosteroids are synthesized by neurons and glial cells in the central and peripheral nervous system from cholesterol or other blood-borne steroidal precursors [1,2]. In addition to the genomic effects of steroids via intracellular steroid receptors, some steroids modulate the functions of several neurotransmitter receptors and channels, namely γ-aminobutyric acid type A (GABA_A) receptors, N-methyl-D-aspartate (NMDA) receptors, serotonin (5-hydroxytryptamine, 5-HT) subtype 3 (5-HT₃) receptors, and σ₁ receptors [3], voltage-gated Ca²⁺ and K⁺ channels [4,5], and transient receptor potential M3 channels [6], possibly leading to modulation of neuronal excitability. Steroids with these properties are referred to as neuroactive steroids independently of their origin [3]. Neurosteroids have been shown to have a variety of neuropsychopharmacological effects, such as neuroprotective, memory-enhancing, sedative, anxiolytic, sleep-modulating, antidepressant, anticonvulsant and anesthetic effects [2,3]. However, the molecular mechanisms underlying the various effects of neurosteroids have not yet been sufficiently clarified.

Inwardly rectifying K⁺ (Kir) channels play important roles in the control of resting membrane potential, cellular excitability and K⁺ homeostasis in the nervous system and various peripheral tissues [7]. Among seven Kir subfamilies, members of Kir2 channels in a major Kir subfamily are characterized by strong inward rectification and constitutive activity and are present in various cells, including neurons, glial cells, cardiac and skeletal myocytes, epithelial cells, and macrophages [8]. Four Kir2 channel members have been identified in mammals [9–13]. In the nervous system, Kir2.1 channels are expressed widely but weakly in most brain regions. Kir2.2 channels are expressed mainly in the cerebellum. Kir2.3 channels are expressed predominantly in the forebrain [14,15]. Kir2.4 channels are expressed predominantly in motorneurons of the brainstem [13]. Among Kir2 channels, Kir2.3 channels in the forebrain may be related to cognition, memory, emotion and neuropsychiatric disorders. Therefore, endogenous modulators of Kir2.3 channels may induce various physiological and neuropsychopharmacological effects. In the present study, we investigated the effects of various neurosteroids on Kir2.3 channels using the Xenopus oocyte expression assay.
Results

PREGS potentiates Kir2.3 channels

In Xenopus oocytes injected with Kir2.3 mRNA, inward currents through the expressed Kir2.3 channels were observed at a holding potential of −70 mV in an hK solution containing 96 mM K+ (Fig. 1A). Extracellular application of 30 μM pregnenolone sulfate (PREGS) reversibly potentiated Kir2.3 currents (Fig. 1A). The current responses to an additional 50 μM PREGS during application of 3 mM Ba2+, which blocks Kir channels, were not significant (1.5±1.0 nA, less than 1% of the 3 mM Ba2+-sensitive current component, n = 4; Fig. 1A). The 3 mM Ba2+-sensitive current components in oocytes expressing Kir2.3 channels (684.7±79.2 nA, n = 25) are considered to correspond to the magnitudes of Kir2.3 currents in oocytes expressing Kir2.3 channels [11]. PREGS produced no significant response in the K+ free ND98 solution containing 98 mM Na+ instead of the hK solution (5.7±2.0 nA at 50 μM, n = 4), suggesting that the PREGS-induced currents show K+ selectivity. In uninjected oocytes, 300 μM PREGS and 3 mM Ba2+ produced no significant response (less than 5 nA and 3.2±2.5 nA, respectively, n = 5; Fig. 1A) compared with oocytes injected with Kir2.3 mRNA, suggesting no significant effect of PREGS and Ba2+ on intrinsic oocyte channels. Additionally, application of dimethyl sulfoxide (DMSO), the solvent vehicle, at the highest concentration (0.3%) produced no significant response in oocytes injected with Kir2.3 mRNA (n = 4; data not shown). In contrast, 100 μM of the other neurosteroids tested: PREG, dehydroepiandrosterone (DHEA), DHEAS, progesterone, 17β-estradiol, corticosterone, 5α-pregnan-3α-ol-20-one (3α-OH-DHP, also known as allopregnanolone) and 3α, 21-dihydroxy-5α-pregnan-20-one (also known as tetrahydrodeoxycorticosterone, THDOC), produced no significant current responses in oocytes expressing Kir2.3 channels (less than 2% change of the 3 mM Ba2+-sensitive current component, with the exception of DHEAS with only 5.6±2.6% potentiation, n=4; Fig. 1B). The results suggest that Kir2.3 channels are potentiated specifically by PREGS among various neurosteroids.

Characteristics of Kir2.3 channel potentiation by PREGS

The potentiation of Kir2.3 channels by PREGS was concentration-dependent at micromolar concentrations, with a concentration of PREGS that produces 50% of the maximal effect (EC50) of 15.6±2.9 μM and a Hill coefficient (nH) of 1.43±0.03 (n = 6, Fig. 2A). The amplitudes of 30 μM PREGS-potentiated Kir2.3 currents were highly correlated with those of 3 mM Ba2+-sensitive current components (Fig. 2B), suggesting that PREGS may be associated with Kir2.3 channel expression levels.

Furthermore, instantaneous Kir2.3 currents elicited by the voltage step to −100 mV from a holding potential of 0 mV were enhanced in the presence of 30 μM PREGS applied for 3.5 min (Fig. 2C). The percentage potentiation of the steady-state Kir2.3 current at the end of the voltage step by PREGS was not significantly different from that of the instantaneous current (paired t-test, P>0.05; n = 6 at −40, −60, −80, −100 and −120 mV, respectively). These results suggest that the channels were potentiated by PREGS primarily at the holding potential of 0 mV and in a time-independent manner during each voltage pulse. Similarly to 3 mM Ba2+-sensitive currents corresponding to Kir2.3 currents, PREGS-induced currents in oocytes expressing Kir2.3 channels increased with negative membrane potentials, and the current-voltage relationships showed inward rectification (Fig. 2D), indicating a characteristic of Kir currents. Furthermore, the PREGS-induced current did not change the reversal potential. These results suggest that PREGS potentiates the function of Kir2.3 channels.

Figure 1. Effects of pregnenolone sulfate (PREGS) on Kir2.3 channels expressed in Xenopus oocytes. (A) Upper row, in an oocyte injected with Kir2.3 mRNA, current responses to 30 μM PREGS and to 50 μM PREGS in the presence of 3 mM Ba2+ are shown. Lower row, in an uninjected oocyte, no significant current responses to 300 μM PREGS and 3 mM Ba2+ are shown. Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K+. Asterisks show the zero current level. Horizontal bars show the duration of application. (B) Effects of various neurosteroids: PREG, PREGS, DHEA, DHEAS, progesterone (PROG), 17β-estradiol (E2), corticosterone (CORT), 3α-OH-DHP and THDOC, on Kir2.3 channels. The magnitudes of the effect of 100 μM neurosteroids on Kir2.3 channels were normalized to the 3 mM Ba2+ sensitive current components in oocytes expressing Kir2.3 channels (n=4 for each steroid). Data are expressed as mean±SEM. doi:10.1371/journal.pone.0006311.g001

The effects of intracellular PREGS in oocytes expressing Kir2.3 channels were also examined. The 3 mM Ba2+-sensitive current components corresponding to the magnitudes of Kir2.3 currents were not significantly affected by intracellularly applied PREGS (88.4±12.1% of untreated control, P>0.05, paired t-test, n = 4; Fig. 3A), and extracellular application of 50 μM PREGS after the injection similarly potentiated Kir currents (P>0.05, paired t-test, n = 4; Fig. 3B). These results suggest that the potentiation effect of PREGS was not caused by its intracellular action.

The chemical structure of PREGS shares the structural moiety of PREG and DHEAS [3]. However, 30 μM PREGS-induced Kir2.3 currents were not significantly different from those in the presence of either 100 μM PREG or 100 μM DHEAS (105.9±11.4% and 99.8±8.9% of control, respectively, P>0.05, n = 4, paired t-test; Fig. 4), suggesting that PREG and DHEAS have no significant antagonist effect on the potentiation of Kir2.3 channels by PREGS.

Kir2.3 channels are modulated by extracellular pH [16–18]. We examined whether changes in pH would alter the effects of
PREGS on Kir2.3 channels expressed in *Xenopus* oocytes. In oocytes injected with Kir2.3 mRNA, Kir2.3 currents decreased with a decrease in extracellular pH (51.9±7.9% of the 3 mM Ba\(^{2+}\)-sensitive current components at pH 7.4 for pH 6.0, n=6; and 167.6±22.1% of those at pH 7.4 for pH 9.0, n=6) as reported in previous studies [16–18]. However, the concentration-response relationships for the potentiation effects of PREGS were not significantly affected by pH 6.0, 7.4 and 9.0 (no significant pH × PREGS interaction, P>0.05, two-way ANOVA; P>0.05 at each concentration, Tukey-Kramer post hoc test; Fig. 5). These results suggest that the degree of potentiation of Kir2.3 channels by PREGS may be similar even under pathological pH conditions.

**Selective potentiation of Kir2.3 channels by PREGS**

We also examined the effects of PREGS on other Kir channels (i.e., Kir1.1, an ATP-regulated Kir channel; Kir2.1 and Kir2.2, constitutively active Kir channels; Kir3, a G protein-activated Kir channel [7]). However, in oocytes injected with mRNA for Kir1.1, Kir2.1, Kir2.2, or Kir3.1/Kir3.2 channels, 100 μM PREGS produced no significant current response (3.5±0.4, 7.2±3.7, 4.0±2.5, and 0.8±1.6% change of the 3 mM Ba\(^{2+}\)-sensitive current components: 820.7±190.8 nA for Kir1.1, 538.0±130.9 nA for Kir2.1, 1518.3±276.4 nA for Kir2.2, and 1043.5±166.0 nA for Kir3.1/Kir3.2, respectively, n=4; Fig. 6). These results suggest that PREGS selectively potentiates Kir2.3 channels.
Effects of PREGS on Kir2 heteromeric channels

Recent studies have shown that Kir2 channel subunits can form functional heteromeric channels in the *Xenopus* oocyte expression system [19,20]. We examined the effects of PREGS on Kir2 heteromeric channels. In oocytes injected with mRNA for Kir2.1/Kir2.3 or Kir2.2/Kir2.3, PREGS concentration-dependently potentiated Kir currents (respective EC50 and nH: 41.0±9.4 μM and 1.40±0.23 for Kir2.1/Kir2.3, n = 6; 27.5±5.0 μM and 1.07±0.07 for Kir2.2/Kir2.3, n = 9; Fig. 7), whereas PREGS had no significant current response in oocytes injected with Kir2.1/Kir2.2 mRNA (1.3±1.3% inhibition of the 3 mM Ba2+-sensitive current components, n = 5). The potentiation properties of PREGS in the normalized concentration-response relationships were less potent for Kir2.1/Kir2.3 and Kir2.2/Kir2.3 than for Kir2.3 channels (EC50: P<0.05, one-way ANOVA; significant difference between Kir2.1/Kir2.3 and Kir2.3, P<0.05, Tukey-Kramer post hoc test; and significant channel-type × PREGS

![Figure 3. Effect of intracellular PREGS in *Xenopus* oocytes expressing Kir2.3 channels.](image)

**(A)** Comparison of basal Kir2.3 currents before and after PREGS injection in oocytes expressing Kir2.3 channels. The amplitude of Kir2.3 currents was normalized to the amplitude of 3 mM Ba2+-sensitive current components before PREGS injection. **(B)** Comparison of 50 μM PREGS-induced Kir2.3 currents before and after PREGS injection. Data are expressed as mean±SEM.

doi:10.1371/journal.pone.0006311.g003

![Figure 4. Effects of PREG and DHEAS on PREGS-induced Kir2.3 currents.](image)

**(A)** Representative current responses to 30 μM PREGS and to 30 μM PREGS in the presence of 100 μM PREG in a *Xenopus* oocyte expressing Kir2.3 channels. Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K+. **(B)** Comparison of PREGS-induced Kir2.3 currents in the presence or absence of PREG or DHEAS. Concentrations of PREGS, PREG, and DHEAS were 30, 100, and 100 μM, respectively. Current responses to PREGS in the presence of PREG or DHEAS were normalized to the amplitude of PREGS-induced currents in the absence of PREG or DHEAS (control). Data are expressed as mean±SEM.

doi:10.1371/journal.pone.0006311.g004

![Figure 5. Concentration-response relationships for potentiation of Kir2.3 channels by PREGS at different pH values.](image)

The magnitudes of potentiation of Kir2.3 currents by PREGS in oocytes expressing Kir2.3 channels were normalized to the 3 mM Ba2+-sensitive current components, which were 426.9±41.4 nA (pH 6.0), 554.0±79.9 nA (pH 7.4) and 729.2±36.6 nA (pH 9.0). The EC50 and nH values were 16.1±1.2 μM and 1.44±0.07 (pH 6.0, n = 10), 15.6±0.9 μM and 1.43±0.03 (pH 7.4, n = 6), and 17.1±1.5 μM and 0.70±0.03 (pH 9.0, n = 8), respectively. Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K+. Data are expressed as mean±SEM of the percentage responses.

doi:10.1371/journal.pone.0006311.g005
may also potentiate Kir2.3-containing Kir2 heteromeric channels. Tukey-Kramer

Discussion

In the present study, we demonstrated that PREGS potentiated the function of Kir2.3 channels at micromolar concentrations, whereas the eight other neurosteroids (i.e., PREG, DHEA, DHEAS, progesterone, 17β-estradiol, corticosterone, 3α-OH-DHP, and THDOC) had no significant effect on Kir2.3 channels. Kir1.1, Kir2.1, Kir2.2, and Kir3 channels expressed in Xenopus oocytes. Responses to PREGS at different concentrations were measured at a membrane potential of −70 mV. Data are expressed as mean±SEM.

doi:10.1371/journal.pone.0006311.g006

Figure 7. Comparison of the potentiation effects of PREGS on Kir2.3, Kir2.1/Kir2.3, and Kir2.2/Kir2.3 channels expressed in Xenopus oocytes. Responses to PREGS at different concentrations were normalized to the maximal response to PREGS. Current responses were measured at a membrane potential of −70 mV. Data are expressed as mean±SEM.

doi:10.1371/journal.pone.0006311.g007

applied PREGS, and potentiation of Kir2.3 channels by extracellularly applied PREGS was readily reversible with washout. PREGS exists in a negatively charged form, and it could not readily permeate the cell membrane. Therefore, the effect of PREGS was unlikely to be caused by intracellular PREGS or by interactions with intracellular molecules, including phosphatidylinositol 4,5-bisphosphate (PIP2) and long-chain fatty acids that activate Kir2.3 channels [21,22]. Additionally, PREG and DHEAS, whose structures are closely related to PREGS, had no significant effects on Kir2.3 channels or on PREGS-induced Kir2.3 potentiation, suggesting that the effect of PREGS is unlikely to be mediated by a nonspecific membrane-perturbation effect. These observations suggest that PREGS may act directly at Kir2.3 channels, and the site of action of PREGS on Kir2.3 channels may be extracellular or, at least, at a readily accessible site from the outside of the cell membrane. Moreover, the present study suggests that PREGS also potentiates Kir2 heteromeric channels containing Kir2.3 channel subunits. The Kir2.3 channel may be considered as a target site for PREGS.

Administration of PREGS has been shown to have anti-convulsant, anxiolytic, antidepressant, neurogenesis, neuroprotective, proconvulsant, and antinociceptive effects [23–29] and prevent the development of tolerance and dependence to morphine and benzodiazepines [30,31]. PREGS at micromolar concentrations has also been shown to modulate the functions of several receptors and channels, namely GABA_A receptors, glycine receptors, NMDA receptors, a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, a1 receptors, voltage-gated Ca2+ and K+ channels, and transient receptor potential M3 channels [3–6,27]. PREGS can cross the blood-brain barrier [32], and interactions of PREGS with these target sites are proposed to have important implications in the various effects of PREGS. Kir2.3 channels are highly expressed in neurons and some of oligodendroglial cells in the forebrain, such as the olfactory bulb, cerebral cortex, hippocampus and basal ganglia, and spinal cord [14,15,33,34], areas related to cognition, memory, emotion, nociception and drug addiction. Additionally, Kir2.3 channels colocalize with postsynaptic density-95 (PSD-95) in neuronal populations in the forebrain [35] and are localized at the postsynaptic membrane of excitatory synapses in the olfactory bulb [15], suggesting the existence of postsynaptic Kir2.3 channels. In the present study, PREGS potentiated Kir2.3 channels at micromolar concentrations. Because activation of Kir2.3 channels causes membrane hyperpolarization [36], PREGS may decrease excitability of neurons and glial cells in these regions. Furthermore, the distribution of Kir2.1, Kir2.2 and Kir2.3 subunits overlaps in some regions [14,33,34], suggesting the partial existence of Kir2 heteromeric channels. The present study suggests that PREGS also potentiates Kir2.3-containing Kir2 heteromeric channels. Therefore, Kir2.3 potentiation by PREGS might be involved in some of the various neuropsychopharmacological effects.

Bulk concentrations of PREGS in brain tissues have been estimated to be in the nanomolar range [37,38], or even lower [39,40]. However, the PREG synthase cytochrome P450 side-chain cleavage enzyme and hydroxysteroid sulfoxtransferase, which converts PREG to PREGS, are expressed in the brain [2], and these enzymes have been shown to colocalize in hippocampal neurons [38], suggesting local synthesis of PREGS. Furthermore, Mameli et al. [41] reported that a PREG-like neurosteroid released from depolarized postsynaptic CA1 neurons increased the frequency of AMPA-mediated miniature excitatory postsynaptic currents via modulation of presynaptic NMDA receptors, with a magnitude equivalent to that caused by exogenously applied PREGS at 17 μM. This effect was blocked by anti-PREGS antibodies. These findings suggest that local concentrations of released PREGS around these neurons might be in the micromolar range.
Moreover, brain levels of neurosteroids, including PREGS, have been shown to be elevated under several pathological conditions, such as cerebral ischemia, epilepsy, stress, and drug addiction [3,42–46]. Altogether, Kir2.3 channels in the forebrain, including the hippocampus, might be potentiated by PREGS via paracrine and autocrine mechanisms under such conditions. Additionally, although PREG is structurally related to PREGS, PREG had no significant effect on Kir2.3 channels (Figs. 1B,4). Steroid sulfatase, which converts PREGS to PREG, has been identified in various brain regions [47–49]. Conversion between PREGS and PREG by sulfotransferase and steroid sulfatase might regulate the effect of PREGS on Kir2.3 channels in the brain.

Finally, Kir2.3 channels are also expressed in Schwann cells near the nodes of Ranvier in sciatic nerves, cardiomyocytes, and renal cortical collecting duct principal cells [50–52]. Typical plasma concentrations of PREGS have been reported to be 0.2 to 0.4 μM, although plasma concentrations of PREGS in some healthy subjects have been reported to be approximately 1 μM [53]. However, plasma PREGS levels can reach micromolar levels during pregnancy and in patients with 21-hydroxylase deficiency [54,55]. Elevated PREGS levels overlapped with the concentrations that were effective in potentiating Kir2.3 channels in the present study. Additionally, elevated plasma PREGS levels have been observed in patients with anxiety-depressive disorder, alcohol addiction, or hyperthyroidism [56–58]. Kir2.3 channels in the peripheral tissues may be potentiated by elevated PREGS concentrations in certain conditions. Potentiation of Kir2.3 channels by PREGS might affect the regulation of K+ buffering in peripheral nerves, the control of cardiomyocyte excitability and K+ homeostasis in the kidney. Interestingly, PREGS has been identified in sciatic nerves [59], and PREG has been identified in Schwann cells [60]. In sciatic nerves, Kir2.3 channels may also be potentiated by locally released PREGS. Further studies using local administration of PREGS and in vivo preparations, such as culture cells and brain slices, may advance our understanding of the physiological and pharmacological effects of PREGS on Kir2.3 channels in the nervous system, heart, and kidney. Kir2.3 channel potentiation may provide novel insights into the various effects of PREGS.

Materials and Methods

Compounds

PREG, PREGS, DHEA, DHEAS, progesterone, 17β-estradiol, corticosterone, 3α-OH-DHP, and THDOC were purchased from Sigma-Aldrich (St. Louis, MO, USA); PREGS was dissolved in distilled water or DMSO, and the others were dissolved in DMSO. The stock solution of each compound was stored at −30°C until use. Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

Preparation of specific mRNA

Plasmids containing the entire coding sequences for the mouse Kir2.2 (GenBank accession number: AB035889), Kir2.3, Kir3.1, and Kir3.2 channel subunits were obtained as described previously [61–63]. The sequence of amino acids deduced from the nucleotide sequence of C57BL/6Njcl mouse Kir2.2 in the plasmid pSP35T revealed seven amino acid substitutions compared with BALB/c mouse Kir2.2 [12]. However, the substitutions were identical to those of rat Kir2.2, with the exception of a change from Phe to Leu at codon 255 [10]. Additionally, cDNAs for rat Kir1.1 in pSPORT and mouse Kir2.1 in pcDNA1 were generously provided by Drs. Steven C. Hebert and Lily Y. Jan, respectively [9,64]. The plasmid pSKKir2.2 was linearized by digestion with SacI, and the others were digested with the appropriate enzyme as described previously [9,62–64]. The specific mRNAs were synthesized in vitro using the mMESSAGE mMACHINE<sup>™</sup> In Vitro Transcription Kit (Ambion, Austin, TX, USA).

Oocyte electrophysiology

*Xenopus laevis* oocytes (Stages V and VI) were isolated from adult female frogs (Copacetic, Soma, Aomori, Japan) that were anesthetized by immersion in water containing 0.15% tricaine (Sigma-Aldrich) as described previously [65]. All procedures for the care and treatment of animals were approved by Niigata University Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines. Oocytes were injected with mRNA for Kir1.1 (1 ng), Kir2.1 (0.5 ng), Kir2.2 (0.5 ng), Kir2.3 (1 ng), Kir2.1/Kir2.2, Kir2.1/Kir2.3, Kir2.2/Kir2.3 (each 0.5 ng), or Kir3.1/Kir3.2 combinations (each 0.3 ng) for brain-type Kir3 channels. Oocytes were incubated at 19°C in Barth’s solution after treatment with 0.9 mg/ml collagenase and manually defolliculated. Whole-cell currents of the oocytes were recorded from 2 to 7 days after the injection with a conventional two-electrode voltage clamp [62,66]. The membrane potential was held at −70 mV, unless otherwise specified. Microelectrodes were filled with 3 M KCl. The oocytes were placed in a 0.05 ml narrow chamber and superfused continuously with a high-K+ (hK) solution (composition in mM: NaCl 2, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.5 and HEPES 5, pH 7.4 with KOH) or a K+-free high-Na+ (ND98) solution (composition in mM: NaCl 98, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.5 and HEPES 5, pH 7.4 with NaOH). In the hK solution, the K<sup>+</sup> equilibrium potential was close to 0 mV, and the inward K<sup>+</sup> current flow through the Kir channels was observed at negative holding potentials [9,11,12,62,64]. For examining the effects of intracellular PREGS, 13.8 nl of 10 mM PREGS dissolved in distilled water containing 5% DMSO was injected into an oocyte using a Nanoliter injector (World Precision Instruments, Sarasota, FL, USA) as described previously [67]. The oocyte currents were then continuously recorded for approximately 30–40 min. Because the volume of the oocyte was approximately 1 μl, the intracellular concentration of PREGS was presumed to be approximately 136 μM. Furthermore, injection of the same volume of the solvent vehicle had no significant effect on Kir2.3 currents (n = 4). For analysis of concentration-response relationships, data were fitted to a standard logistic equation using KaleidaGraph (Synergy Software, Reading, PA, USA). EC<sub>50</sub> and n<sub>H</sub> values were obtained from the concentration-response relationships.

Data analyses

Data are expressed as mean±SEM, and n is the number of oocytes tested. Statistical analysis of differences between groups was performed using paired t-test, one-way analysis of variance (ANOVA), or two-way ANOVA followed by Tukey-Kramer post hoc test. Values of P<0.05 were considered statistically significant.

Acknowledgments

We are grateful to Dr. Kansaku Baba for his cooperation and Kazuo Kobayashi (Niigata University) for his assistance. We also thank Dr. Steven C. Hebert (Yale University) and Dr. Lily Y. Jan (University of California, San Francisco) for generously providing Kir1.1 cDNA and Kir2.1 cDNA, respectively.

Author Contributions

Conceived and designed the experiments: TK. Performed the experiments: TK. Analyzed the data: TK KW KI. Contributed reagents/materials/analysis tools: TK KW KI. Wrote the paper: TK KI.
References

1. Baulieu EE, Robel P, Schumacher M (2001) Neurosteroids: beginning of the story. Int Rev Neurobiol 46: 1–32.
2. Mellon SH, Griffin LD (2002) Neurosteroids: biochemistry and clinical significance. Trends Endocrinol Metabolism 13: 33–43.
3. Rapeepat R, Holbover B (1999) Neuronal activity: mechanisms of action and neurophysiological perspectives. Trends Neurosci 22: 410–416.
4. Brench-Muller JMM, Danks P, Spence KT (1994) Neurosteroids modulate calcium currents in hippocampal CA1 neurons via a pertussis-toxin-sensitive G-protein coupled mechanism. J Neurosci 14: 1963–1977.
5. Wang Q, Wang L, Wardwell-Sawan (1998) Modulation of cloned human neuronal voltage-gated potassium channels (hKv1.1 and hKv2.1) by neurosteroids. J Pharmacol Exp Ther 280: 47: 49–55.
6. Wagner TFJ, Loch S, Lambert S, Straubl A, Mannsche S, et al. (2006) Transient receptor potential M3 channels are ionotropic steroids in pancreatic β cells. Nat Cell Biol 10: 1421–1430.
7. Reimann F, Ashcroft FM (1999) Inwardly rectifying potassium channels. Curr Opin Cell Biol 11: 503–508.
8. Doupnik CA, Davidson N, Lester HA (1995) The inward rectifier potassium channel family. Curr Opin Neurobiol 5: 269–277.
9. Kubo Y, Baldwin TJ, Jan YN, Jan LY (1995) Primary structure and functional expression of a mouse inward rectifier potassium channel. Nature 362: 127–133.
10. Koyama H, Morishige KI, Takahashi N, Zanelli JS, Fass DN, et al. (1994) Molecular cloning, functional expression and localization of a novel inward rectifier potassium channel in the rat brain. FASEB J 341: 303–307.
11. Peier KL, Radeke CM, VanDaelen CA (1994) Primary structure and characterization of a small-conductance inwardly rectifying potassium channel from human hippocampus. Proc Natl Acad Sci USA 91: 6240–6244.
12. Takahashi N, Morishige KL, Jahangir A, Yamada M, Findlay I, et al. (1994) Molecular cloning and functional expression of cDNA encoding a second class of inward rectifier potassium channels in the mouse brain. J Biol Chem 269: 23274–23279.
13. Topper C, Doring F, Wischmeyr E, Karschin C, Brockhaus J, et al. (1998) Kir2.4: a novel inward rectifier channel associated with motoneurons of the cranial nerve nuclei. J Neurosci 18: 4096–4105.
14. Karschin C, Dilmann E, Stahmer W, Karschin A (1996) IRK1-3 and GIRK1-4 inwardly rectifying K+ channel mRNAs are differentially expressed in the adult rat brain. J Neurosci 16: 3559–3570.
15. Iraci CE, Fine R, Pichichero M, Ino M, Tomokiyo H, Nagada K, et al. (2002) Inward rectifier K+ channel Kir2.5 is localized at the postsynaptic membrane of excitatory synapses. Am J Physiol 282: C1396–1403.
16. Couder KL, Peier KL, Radeke CM, VanDaelen CA (1995) Identification and molecular localization of a pH-sensing domain for the inward rectifier potassium channel KirH. Neuron 15: 1137–1148.
17. Mutuo T, Vaidyanathan R, Tolkacheva EG, Dhamou AS, Taffet SM, et al. (2007) Kir2.3 isoform confers pH sensitivity to heteromeric Kir2.1/Kir2.3 channels in HEK293 cells. Heart Rhythm 4: 1079–1086.
18. Ucheh GN, Balazs R, Ureche L, Struz-Soboham N, Lang F, et al. (2008) Novel insights into the structural basis of pH-sensitivity in inward rectifier K+ channels. Cell Physiol Biochem 21: 347–356.
19. Preiss-Muller R, Schlichtholz G, Goergen T, Heinen S, Bruggemann A, et al. (2004) Characterization of Kir2.3 potassium channel subunits contributes to the phenotype of Andersen’s syndrome. Proc Natl Acad Sci USA 99: 7774–7779.
20. Schram G, Pourrier M, Wang Z, White M, Nattel S (2003) Barium block of Kir2.3 and human cardiac inward rectifier currents: evidence for subunit-heteromeric contribution to native currents. Cardiovasc Res 59: 328–338.
21. Zhang H, Yan X, Minshall R, Logothetis DE (2004) Characterization of the PREGS Potentiates Kir2.3 channel HIR. Neuron 15: 1157–1168.
22. Liu Y, Liu D, Printzenhoff D, Coghlan MJ, Harris R, et al. (2002) Tenidap, a novel anti-inflammatory agent, is an opener of the inwardly rectifying Kir channel hKir2.3. Eur J Pharmacol 433: 153–160.
23. Coe J, Lij diary D, Millich P,钤 fow J, et al. (2003) Pregnenolone and its sulfate in the rat brain. Brain Res 270: 119–125.
24. Kimoto T, Tsurugizawa T, Ohta Y, Makino J, Tamura H, et al. (2001) Neurosteroid synthesis by cytochrome P450-containing systems localized in the rat brain hippocampal neurons. Neurochem Res 142: 3573–3589.
25. Higashi T, Sugita H, Yagi T, Shimaoda K (2003) Studies on neurosteroids: XVI. Levels of pregnenolone sulfate in rat brains determined by enzymelinked immunosorbent assay not requiring solubilization. Biol Pharm Bull 26: 709–711.
26. Liu S, Sjovall J, Griffiths WJ (2003) Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography-electrospray mass spectrometry. Anal Chem 75: 8085–8086.
27. Massel M, Garta M, Partrige L, Vaquero-Zapata CE (2005) Neurosteroid-induced plasticity of immature synapses via retrograde modulation of presynaptic NMDA receptors. J Neurosci 25: 2285–2294.
28. Barbaccia ML, Roccutti G, Trabacchi M, Montolio MC, Concas A, et al. (1994) Time-dependent changes in rat brain neuroactive steroid concentrations and GARα1 receptor function after acute stress. Neuroendocrinology 63: 166–172.
29. Torres JM, Ortega E (2003) DHEA, PREG and their sulfate derivatives on plasma and brain after CRH and ACTH administration. Neurochem Res 28: 1107–1119.
30. Nguyen PN, Van EB, Castillo-Meledes M, Walker DW, Hirst JF (2004) Increased allopregnenolone levels in the fetal sheep brain following umbilical cord occlusion. J Physiol 560: 593–605.
31. Caldeira JC, Wu Y, Mameli M, Purdy RH, Li PK, et al. (2004) Fetal alcohol exposure alters neurosteroid levels in the developing rat brain. J Neurochem 90: 1380–1389.
32. van Cuijk G, Hou FN (2004) Effects of morphine dependence and withdrawal on levels of neurosteroids in rat brain. Acta Pharmacol Sin 25: 1285–1291.
33. Iwamori M, Moser HW, Kishimoto Y (1976) Steroid sulfatase in brain: comparison of sulfohydrolase activities for various steroid sulfates in normal and pathological brains, including the various forms of metachromatic leukodystrophy. J Neurochem 27: 1393–1395.
34. Hill M, Parizek A, Klak J, Hampl R, Sulcová J, et al. (2002) Neuroactive substances of the bovine brain. J Physiol 547: 231–250.
35. Stockelbroeck S, Nassen A, Ugele B, Ludwig M, Watzka M, et al. (2004) Steroid sulfatase (STS) expression in the human temporal lobe: enzyme activity, mRNA expression and immunohistochemistry study. J Neurochem 89: 403–417.
36. Fradkin J, Tjeerdsen TT, Jones M, Ellman MH, Schwarcz T (1996) Inwardly rectifying K+ channels that may participate in Keffering are localized in microvilli of Schwann cells. J Neurosci 16: 4096–4105.
37. Seve P, David M, Morel Y, de Peretti E, Forest MG, Loras B, et al. (1986) Usefulness of plasma pregnenolone sulfate in testing pituitary-adrenal function in children. Acta Endocrinol Suppl 279: H1123–1133.
38. Mehnig P, Zhang L, Shirer A, Nattel S (2002) Differential distribution of Kir2.1 and Kir2.3 subunits in cardiac atrium and ventricle. Am J Physiol 283: H1123–1133.
39. Havlicekova H, Hill M, Rampa R, Starka L (2002) Sex- and age-related changes in postpartum in maternal and umbilical blood: 3.3 hydroxysteroids. J Clin Endocrinol Metab 87: 2286–2291.
40. de Peretti E, Forest MG, Loras B, Morel Y, David M, et al. (1986) Usefulness of plasma pregnenolone sulfate in testing pituitary-adrenal function in children. Acta Endocrinol Suppl 279: 259–263.
41. Misra M, Parizak A, Klak J, Hampsch R, Sulcová J, et al. (2002) Neuroactive steroids, their precursors and polar conjugates during parturition and postpartum in maternal and umbilical blood: 3.3b-hydroxy-5-ene steroids. J Steroid Biochem Mol Biol 82: 241–250.
56. Bicková M, Tallová J, Hill M, Krausová Z, Hampl R (2000) Serum concentrations of some neuroactive steroids in women suffering from mixed anxiety-depressive disorder. Neurochem Res 25: 1623–1627.
57. Hill M, Papov P, Havíková H, Kancheva L, Vrbíková J, et al. (2005) Altered profiles of serum neuroactive steroids in premenopausal women treated for alcohol addiction. Steroids 70: 515–524.
58. Tagawa N, Tamanaka J, Fujimami A, Kobayashi Y, Takano T, et al. (2000) Serum dehydroepiandrosterone, dehydroepiandrosterone sulfate and pregnenolone sulfate concentrations in patients with hyperthyroidism and hypothyroidism. Clin Chem 46: 523–528.
59. Morfin R, Young J, Corpéchot C, Egestad B, Sjövall J, et al. (1992) Neurosteroids: pregnenolone in human sciatic nerves. Proc Natl Acad Sci USA 89: 6790–6793.
60. Akwa Y, Schumacher M, Jung-Testas I, Baulieu EE (1993) Neurosteroids in rat sciatic nerves and Schwann cells. C R Acad Sci III 316: 410–414.
61. Kobayashi T, Ikeda K, Ichikawa T, Abe S, Togashi S, et al. (1995) Molecular cloning of a mouse G-protein-activated K⁺ channel (mGIRK1) and distinct distributions of three GIRK (GIRK1, 2 and 3) mRNAs in mouse brain. Biochem Biophys Res Commun 208: 1166–1173.
62. Kobayashi T, Ikeda K, Kojima H, Niki H, Yano R, et al. (1999) Ethanol opens G-protein-activated inwardly rectifying K⁺ channels. Nat Neurosci 2: 1091–1097.
63. Kobayashi T, Ikeda K, Kumanishi T (2000) Inhibition by various antipsychotic drugs of the G-protein-activated inwardly rectifying K⁺ (GIRK) channels expressed in Xenopus oocytes. Br J Pharmacol 128: 1716–1722.
64. Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, et al. (1995) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. Nature 362: 31–38.
65. Kobayashi T, Ikeda K, Kumanishi T (2002) Functional characterization of an endogenous Xenopus oocyte adenosine receptor. Br J Pharmacol 135: 313–322.
66. Ikeda K, Yoshii M, Sora I, Kobayashi T (2003) Opioid receptor coupling to GIRK channels. In vitro studies using a Xenopus oocyte expression system and in vivo studies on weaver mutant mice. Methods Mol Med 84: 53–64.
67. Kobayashi T, Washiyama K, Ikeda K (2003) Inhibition of G protein-activated inwardly rectifying K⁺ channels by fluoxetine (Prozac). Br J Pharmacol 138: 1119–1126.