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Inhibition by pregnenolone sulfate of nicotinic acetylcholine response in adrenal chromaffin cells

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Abstract

To evaluate whether pregnenolone sulfate, an abundant neurosteroid in the brain, modulates nicotinic receptor-mediated responses, the effect of pregnenolone sulfate on acetylcholine-induced catecholamine secretion was investigated in cultured bovine adrenal chromaffin cells. Pregnenolone sulfate inhibited acetylcholine-induced catecholamine secretion (IC_{50}: 27 μM). In addition, pregnenolone sulfate inhibited acetylcholine-induced Na^+ (IC_{50}: 12 μM) and Ca^{2+} (IC_{50}: 20 μM) influxes. However, pregnenolone sulfate did not inhibit either catecholamine secretion or Ca^{2+} influx stimulated by high K^+. Binding of [^{3}H]nicotine to nicotinic receptors was not altered by pregnenolone sulfate. The inhibitory effect on the acetylcholine-induced secretion was insurmountable by increasing acetylcholine concentrations, but was enhanced by decreasing external Na^+ concentrations. These results suggest strongly that pregnenolone sulfate noncompetitively inhibits nicotinic receptor-operated ion channels, thereby suppressing Na^+ influx through the channels and, consequently, attenuates both Ca^{2+} influx and catecholamine secretion. Our results further indicate that pregnenolone sulfate may modulate nicotinic receptor-mediated responses in the brain.

Keywords: Neurosteroid; Pregnenolone sulfate; Nicotinic acetylcholine receptor; Catecholamine secretion; Adrenal chromaffin cell

1. Introduction

Steroids are known to be synthesized de novo from cholesterol in the brain as well as in peripheral steroidogenic glands; these brain-derived steroids are termed the neurosteroids (Baulieu, 1997; Mensah-Nyagan et al., 1999). These steroids can influence brain functions, such as sleep patterns, stress, mood, anxiety, cognition and memory function. The action of steroids on neuronal functions has been shown to consist of distinct genomic and nongenomic mechanisms. In addition to the slow classical genomic mechanism via intracellular receptors, recent research has revealed that steroids modulate neuronal excitability through interaction with various neurotransmitter receptors and channels via a rapid nongenomic mechanism (Falkenstein et al., 2000).

Pregnenolone sulfate, a neurosteroid, is present in the brain in relatively high concentrations (Corpechot et al., 1983; Baulieu, 1997). The physiological roles of endogenous pregnenolone sulfate in the brain have not been still clarified, while pregnenolone sulfate has been shown to exert directly its actions on various receptors; it antagonizes responses mediated by γ-aminobutyric acid type A ($GABA_A$) receptors (Majewska and Schwartz, 1987; Wu et al., 1991; Shen et al., 1999), and it potentiates *N*-methyl-D-aspartate (NMDA) type glutamate receptor-mediated responses (Wu et al., 1991; Bowlby, 1993). It also suppresses responses of kainate type and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type glutamate receptors, and glycine receptors (Wu et al., 1991, 1997). Furthermore, pregnenolone sulfate has been reported to inhibit Ca^{2+} current in hippocampal CA1 neurons (ffrench-Mullen et al., 1994). These findings raise the possibility that pregnenolone sulfate serves as an endogenous modulator of neurotransmission. In addition, the administration of pregnenolone sulfate into the brain improves performance of various memory tasks (Flood et al., 1995; Pallares et al., 1998; Darnaudery et al., 2000). It has been reported that the degree

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of cognitive impairment observed in aged rats correlates with the concentration of pregnenolone sulfate in hippocampus, and memory deficits are reversed by intrahippocampal infusions of pregnenolone sulfate (Vallee et al., 1997).

Nicotinic acetylcholine receptors, which are diverse members of the ligand-gated ion channel superfamily of neurotransmitter receptors, are widely distributed in the central and autonomic nervous systems, and play crucial roles in the neurotransmission in their regions. A number of steroids including neurosteroids have been shown to inhibit the function of nicotinic receptors via a nongenomic mechanism (Valera et al., 1992; Ke and Lukas, 1996; Uki et al., 1999; Wagner et al., 1999). However, until now, there has been no report showing whether pregnenolone sulfate modulates nicotinic receptor function, thereby changing neurotransmitter secretion.

Bovine adrenal medullary chromaffin cells (embryologically derived from neural crest) can secrete catecholamines mainly via stimulation of nicotinic receptors by a physiological secretagogue, acetylcholine, which is released from the terminals of the splanchnic nerve. Binding of acetylcholine to nicotinic receptors leads to depolarization of the cell membrane by an influx of Na^+ through the nicotinic receptor-operated ion channels, causing an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. This Ca^{2+} influx then triggers catecholamine secretion by exocytosis (Douglas and Poisner, 1961; Wada et al., 1985). Therefore, adrenal chromaffin cells are widely used as a model system for studying receptor- and ion channel-associated catecholamine secretion in neurons, and the mechanism of catecholamine secretion is also thought to be similar to that of neurotransmitter release from the nerve terminals in the brain.

In the present study, to evaluate whether pregnenolone sulfate modulates nicotinic acetylcholine receptor-mediated responses, we investigated the effect of pregnenolone sulfate on the secretion of catecholamines stimulated by acetylcholine in cultured bovine adrenal chromaffin cells. Since the results showed that pregnenolone sulfate inhibited the acetylcholine-induced catecholamine secretion, the mechanism of this inhibitory effect was examined. The effects of pregnenolone sulfate on voltage-dependent Na^+ and Ca^{2+} channels-mediated catecholamine secretion were also examined.

2. Materials and methods

2.1. Materials

Pregnenolone sulfate, veratridine and sodium-binding benzofuran isophthalate-acetoxymethyl ester (SBFI-AM) were obtained from Sigma (St. Louis, MO, USA); acetylcholine, nicotine, dimethyl sulfoxide and *N*-methyl-D-glucamine from Nacarai Tesque (Kyoto, Japan); Pluronic F-127 from Molecular Probes (Eugene, OR, USA); $^{45}CaCl_2$ from Amersham Bioscience (Piscataway, NJ, USA); [^{3}H]nicotine from NEN Life Science Products (Boston, MA, USA); Eagle's minimum essential medium from Nissui Seiyaku (Tokyo, Japan); Calf serum from Invitrogen (Carlsbad, CA, USA). All other chemicals were of the highest grade available from commercial sources. Tissue culture equipment was obtained from Falcon Plastics (Cockeysville, MD, USA). Pregnenolone sulfate was dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the incubation medium was 1%, which had no effect on catecholamine secretion from bovine adrenal chromaffin cells under the conditions of this study. Oxygenated Krebs–Ringer–HEPES buffer (KRH buffer) was used as the incubation medium and was composed of 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES and 5.6 mM glucose, adjusted to pH 7.4. In 56 mM KCl–KRH buffer (high K^+), the amount of NaCl was reduced to maintain isotonicity of the medium. When low-Na^+ medium was used, NaCl in KRH buffer was replaced by *N*-methyl-D-glucamine.

2.2. Isolation and primary culture of bovine adrenal chromaffin cells

Bovine adrenal glands were provided by the Center of Iwate Livestock Industry. Adrenal chromaffin cells were prepared by collagenase digestion as described previously (Tachikawa et al., 1989). The isolated cells were suspended in Eagle's minimum essential medium supplemented with 10% calf serum, antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.3 μg/ml of amphotericin B) and 3 μM cytosine arabinoside, and were plated on 35-mm diameter plastic culture dishes at a density of 2×10^6 cells/dish as a monolayer culture. For [^{3}H]nicotine binding analysis, the cells were plated on 24-well plates at a density of 5×10^5 cells/well. The cells were maintained at 37 °C in a humidified incubator under an atmosphere of 95% air and 5% CO_2 for 4 days. A total of 2×10^6 cells contained 34.6 ± 4.2 μg of catecholamines as epinephrine and norepinephrine.

2.3. Measurements of catecholamine secretion from, and $^{45}Ca^{2+}$ influx into, the cells

After 4 days in culture, the cells were rinsed twice with prewarmed KRH buffer, and preincubated in KRH buffer with or without pregnenolone sulfate for 10 min at 37 °C. They were then incubated with or without pregnenolone sulfate in the absence or presence of acetylcholine, high K^+ or veratridine for 7 min. The incubation was terminated by transferring the incubation medium to tubes in an ice-cold bath. The catecholamine secreted into the medium was extracted with 0.4 M perchloric acid and absorbed onto aluminum hydroxide (Tachikawa et al., 1989). Their amounts were estimated by the ethylenediamine condensation method (Weil-Malherbe and Bone, 1952) using a

fluorescence spectrophotometer (650-10S; Hitachi, Tokyo, Japan) at the excitation wavelength of 420 nm and the emission wavelength of 560 nm. At these wavelengths, both epinephrine and norepinephrine had the same fluorescence intensity.

After preincubation of the cells in KRH buffer with or without pregnenolone sulfate for 10 min at 37 °C, the cells were then incubated in KRH buffer containing $^{45}Ca^{2+}$ (37 kBq) with or without pregnenolone sulfate in the absence or presence of 50 μM acetylcholine, or 56 mM K^+ for 7 min. The incubation medium was removed, and the cells were immediately cooled on ice and were rinsed three times with ice-cold Ca^{2+}-free KRH buffer containing 10 mM $LaCl_3$ and 2 mM EGTA. The cells were lysed by addition of 10% Triton X-100 and then scraped off. The lysate was transferred to scintillation vials with scintillation fluid. Radioactivity was determined using a liquid scintillation counter (LSC-6100; Aloka, Tokyo, Japan).

2.4. Measurement of intracellular free Na^+ concentration

The intracellular free Na^+ concentration ($[Na^+]_i$) was measured by dual excitation fluorometry using a fluorescent Na^+ indicator, SBFI, as previously described (Tachikawa et al., 2001). The isolated cells were cultured for 4 days on 12-mm diameter coverslips cut to fit into the fluorescence spectrophotometer cuvette. The cultured cells on the coverslips were incubated in KRH buffer containing 10 μM SBFI-AM and 0.02% Pluronic F-127 for 3 hr at 37 °C. After loading, the cells were rinsed three times with KRH buffer, and the coverslips were placed in the cuvette with a special holder in the fluorescence spectrophotometer (CAF-100; Japan Spectroscopic, Tokyo, Japan). The cells were preincubated with KRH buffer at 37 °C. After a stable fluorescence signal was obtained, pregnenolone sulfate or acetylcholine was added into the cuvette. Fluorescence was simultaneously monitored at excitation wavelengths of 340 and 380 nm, respectively, and at an emission wavelength of 500 nm. The change in $[Na^+]_i$ was expressed as the ratio of the fluorescence at an excitation wavelength of 340 nm to that at a wavelength of 380 nm.

2.5. [^{3}H]Nicotine binding analysis

The binding of [^{3}H]nicotine to nicotinic receptors on intact chromaffin cells was measured according to the method described by Park et al. (1998). The cells on 24-well plate were rinsed twice with KRH buffer and preincubated in KRH buffer for 10 min at 25 °C. The cells were then incubated with 20 nM [^{3}H]nicotine and different concentrations of pregnenolone sulfate for 40 min at 25 °C. After incubation, the incubation medium was immediately removed, the cells were rinsed three times with ice-cold Ca^{2+}-free KRH buffer containing 200 μM EGTA. The cells were lysed and scraped off in 10% Triton X-100, and the radioactivity was measured by liquid scintillation counting. Nonspecific binding was determined by coincubation with 1 mM unlabeled nicotine. The specific binding of [^{3}H]nicotine was obtained by subtracting nonspecific binding from total binding.

3. Results

3.1. Effects of pregnenolone sulfate on catecholamine secretion from, and Ca^{2+} influx into, bovine adrenal chromaffin cells

We examined the effects of pregnenolone sulfate on catecholamine secretion from cultured bovine adrenal chromaffin cells stimulated by acetylcholine (50 μM), high K^+ (56 mM) or veratridine (50 μM). As shown in Fig. 1A, pregnenolone sulfate (3–100 μM) greatly inhibited acetylcholine-induced catecholamine secretion in a concentration-dependent manner with an IC_{50} value of 27 μM, whereas it had no effect on the secretion evoked by high K^+, which directly depolarizes the cell membranes without Na^+ influx and results in Ca^{2+} influx through voltage-dependent Ca^{2+} channels and, consequently, catecholamine secretion. On the other hand, the catecholamine secretion evoked by veratridine (50 μM) that directly activates voltage-dependent Na^+ channels was inhibited concentration dependently by pregnenolone sulfate (10–100 μM) with an IC_{50} value of 44 μM (Fig. 1A). Pregnenolone sulfate (100 nM–100 μM) did not affect the basal (spontaneous) catecholamine secretion from nonstimulated cells (data not shown).

The influx of Ca^{2+} into adrenal chromaffin cells is an essential step for triggering catecholamine secretion (Douglas and Poisner, 1961). As shown in Fig. 1B, pregnenolone sulfate (3–100 μM) greatly inhibited the $^{45}Ca^{2+}$ influx evoked by acetylcholine in a concentration-dependent manner with an IC_{50} value of 20 μM. On the other hand, pregnenolone sulfate at lower concentrations (100 nM–10 μM) did not alter high K^+-induced $^{45}Ca^{2+}$ influx, but at higher concentrations (30–100 μM) it enhanced the $^{45}Ca^{2+}$ influx. Pregnenolone sulfate (100 nM–100 μM) had no effect the basal $^{45}Ca^{2+}$ influx in nonstimulated cells (data not shown).

3.2. Effect of pregnenolone sulfate on acetylcholine-induced Na^+ influx

The influx of Na^+ into the adrenal chromaffin cells is a crucial first step in the process of acetylcholine-induced catecholamine secretion (Wada et al., 1985). To verify whether the Na^+ influx is inhibited by pregnenolone sulfate, its effect on the acetylcholine-induced increase in $[Na^+]_i$ was examined. As shown in Fig. 2, the stimulation of the SBFI-loaded cells with acetylcholine (50 μM) led to a large and rapid increase in the fluorescence ratio (Fig. 2A), indicating

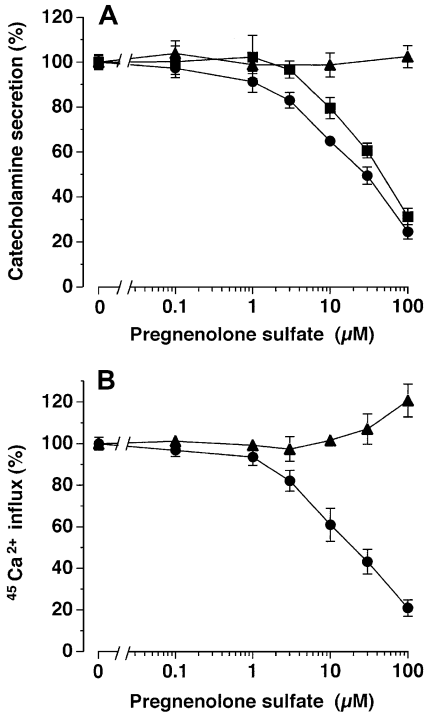

Fig. 1. Effects of pregnenolone sulfate on acetylcholine-, high K^+- or veratridine-induced catecholamine secretion (A), and Ca^{2+} influx (B) in bovine adrenal chromaffin cells. The cultured chromaffin cells were rinsed twice with prewarmed KRH buffer and preincubated in KRH buffer with or without different concentrations of pregnenolone sulfate (100 nM–100 μM) for 10 min at 37 °C. (A) Then the cells were incubated with or without pregnenolone sulfate (100 nM–100 μM) in the absence or presence of 50 μM acetylcholine (●), 56 mM K^+ (▲) or 50 μM veratridine (■) for 7 min. The amount of catecholamines secreted from the cells was determined as described in Section 2. The values for basal secretion were subtracted from the data, and acetylcholine-, high K^+- and veratridine-induced responses were assigned the value of 100%. The acetylcholine-, high K^+- or veratridine-induced catecholamine secretion were 20.0 ± 0.6%, 19.7 ± 0.4% or 13.3 ± 0.4% of the total cellular catecholamine, respectively. The basal secretion was 1.3 ± 0.1%. (B) Then the cells were incubated in KRH buffer containing 37 kBq $^{45}Ca^{2+}$ with or without pregnenolone sulfate (100 nM–100 μM) in the absence or presence of 50 μM acetylcholine (●) or 56 mM K^+ (▲) for 7 min. The amount of $^{45}Ca^{2+}$ influx into the cells was measured as described in Section 2. The values of the basal $^{45}Ca^{2+}$ influx were subtracted from the data, and acetylcholine- and 56 mM K^+-induced $^{45}Ca^{2+}$ influxes were assigned the value of 100%. The acetylcholine- and 56 mM K^+-induced $^{45}Ca^{2+}$ influxes were 11.3 ± 0.8 and 10.5 ± 0.3 nmol/2 × 10^6 cells, respectively, and the basal influx was 1.4 ± 0.1 nmol/2 × 10^6 cells. Values are means ± S.D. from at least four experiments.

that acetylcholine caused the Na^+ influx into the cells through the nicotinic receptor-operated ion channels. The acetylcholine-induced Na^+ influx was little affected by 100 nM of pregnenolone sulfate (Fig. 2B). Pregnenolone sulfate at 1 μM slightly decreased, and at 10–100 μM, greatly reduced the Na^+ influx in a concentration-dependent manner with an IC_{50} value of 12 μM (Fig. 2C–E). On the other hand, pregnenolone sulfate (100 nM–100 μM) did not affect the basal $[Na^+]_i$ in nonstimulated cells (data not shown). Thus, the concentration–inhibition curve for pregnenolone sulfate on the acetylcholine-induced catecholamine secretion was almost similar to those for the acetylcholine-induced Na^+ and Ca^{2+} influxes.

3.3. Effect of pregnenolone sulfate on [^{3}H]nicotine binding

Since the acetylcholine-induced Na^+ influx was inhibited by pregnenolone sulfate, it was possible that pregnenolone sulfate interfered with binding of acetylcholine to nicotinic receptors, thereby inhibiting the response of these receptors. Therefore, whether pregnenolone sulfate affects the binding of [^{3}H]nicotine to nicotinic receptors was examined. As shown in Fig. 3, pregnenolone sulfate did not affect the binding of [^{3}H]nicotine to nicotinic receptors at any of the concentrations tested (100 nM–100 μM).

3.4. Effects of external acetylcholine, Ca^{2+} and Na^+ concentrations on the pregnenolone sulfate inhibition of catecholamine secretion

The character of the inhibition by pregnenolone sulfate of the acetylcholine-induced secretion was examined. The cells were incubated with different concentrations of acetylcho-

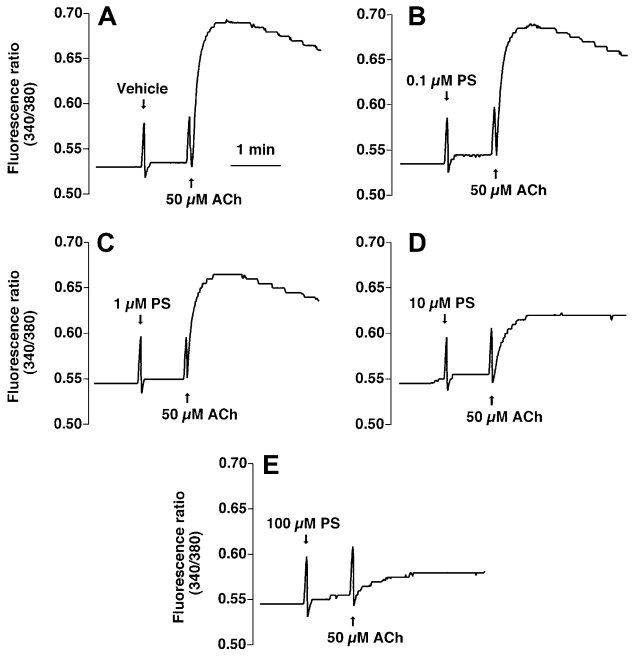

Fig. 2. Effect of pregnenolone sulfate on acetylcholine-induced increase in $[Na^+]_i$. The SBFI-loaded cells in a cuvette were preincubated with KRH buffer in the fluorescence meter at 37 °C. After the cells were incubated with or without different concentrations of pregnenolone sulfate (100 nM–100 μM) for 1 min, acetylcholine (50 μM) was added to the cuvette in the fluorescence meter. Fluorescence was recorded before and after the addition of the test agents. The change in $[Na^+]_i$ was expressed as the ratio of the fluorescence at an excitation wavelength of 340 nm to that at 380 nm. Data are from a representative sample of three experiments. PS, pregnenolone sulfate; ACh, acetylcholine.

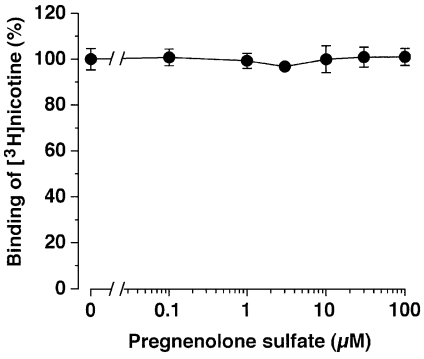

Fig. 3. Effect of pregnenolone sulfate on [^{3}H]nicotine binding. The cells were rinsed twice with KRH buffer and preincubated in KRH buffer for 10 min at 25 °C. The cells were then incubated with or without different concentrations of pregnenolone sulfate (100 nM–100 μM) in the presence of 20 nM [^{3}H]nicotine for 40 min at 25 °C. The amount of [^{3}H]nicotine bound to the cells was determined as described in Section 2. The nonspecific binding, which was determined in the presence of 1 mM unlabeled nicotine, was subtracted from the total binding. The specific binding of [^{3}H]nicotine was determined and was assigned the value of 100%. Values are means ± S.D. from at least three experiments.

line (300 nM–300 μM) in the absence or presence of pregnenolone sulfate at three different concentrations (10, 30 and 100 μM). As shown in Fig. 4, acetylcholine concentration dependently caused catecholamine secretion with an EC_{50} value of 12 μM. Pregnenolone sulfate attenuated the acetylcholine-induced maximum catecholamine secretion without altering the EC_{50} value of acetylcholine.

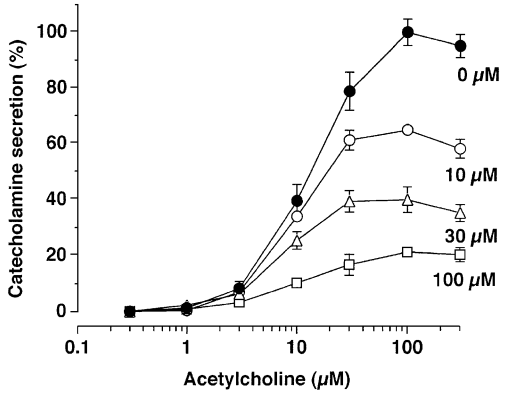

Fig. 4. Effect of pregnenolone sulfate on catecholamine secretion evoked by different concentrations of acetylcholine. The cells were rinsed twice with prewarmed KRH buffer and preincubated in KRH buffer with or without various concentrations of pregnenolone sulfate (10, 30 and 100 μM) for 10 min at 37 °C. The cells were then incubated without (●) or with pregnenolone sulfate (10 μM, ○; 30 μM, △; and 100 μM, □) in the absence or presence of different concentrations of acetylcholine (0.3–300 μM) for 7 min. The amount of catecholamines secreted from the cells was determined as described in Section 2. The values for basal secretion were subtracted from the data, and the acetylcholine-induced maximal response was assigned the value of 100%. The maximal catecholamine secretion induced by 100 μM acetylcholine was 22.1 ± 0.7% of total cellular catecholamine. The basal secretion was 1.5 ± 0.2%. Values are means ± S.D. from at least four experiments.

The EC_{50} values were 10, 9 and 11 μM at 10, 30 and 100 μM of pregnenolone sulfate, respectively. Thus, the inhibitory effect of pregnenolone sulfate was insurmountable by increasing acetylcholine concentrations.

Fig. 5 shows the inhibitory effect of pregnenolone sulfate on catecholamine secretion at different concentrations of external Ca^{2+} and Na^+. In the normal medium (2.6 mM Ca^{2+}, 125 mM Na^+), pregnenolone sulfate (30 μM) inhibited acetylcholine-induced catecholamine secretion by 52%. The inhibition was 51% and 54% at 5.2 and 7.8 mM Ca^{2+}, respectively (Fig. 5A). When low-Na^+ medium was used, the inhibition was 67% and 74% at 63 and 32 mM

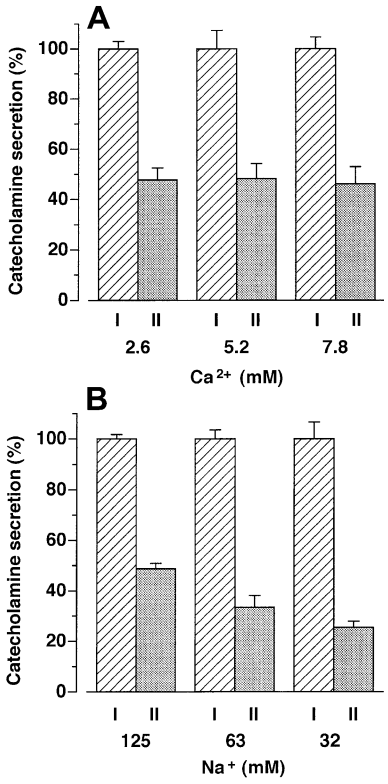

Fig. 5. Effects of external Ca^{2+} and Na^+ concentrations on the inhibition by pregnenolone sulfate of catecholamine secretion. The cells were rinsed twice with prewarmed KRH buffer and were preincubated in KRH buffer with (II) or without 30 μM pregnenolone sulfate (I) for 10 min at 37 °C. The cells then incubated with (II) or without 30 μM pregnenolone sulfate (I) in the absence or presence of 50 μM acetylcholine in KRH buffer under various concentrations of (A) Ca^{2+} (2.6, 5.2 and 7.8 mM) or (B) Na^+ (125, 63 and 32 mM) for 7 min. The amount of catecholamines secreted from the cells was determined as described in Section 2. The values for basal secretion were subtracted from the data, and acetylcholine-induced responses at each concentration of Ca^{2+} or Na^+ were assigned the value of 100%. (A) The catecholamine secretions induced by acetylcholine at various concentrations of external Ca^{2+} (2.6, 5.2 and 7.8 mM) were 20.2 ± 0.6%, 22.1 ± 1.5% and 25.6 ± 1.2% of total cellular catecholamine, respectively. The basal secretions were 1.6 ± 0.2%, 1.7 ± 0.2% and 1.6 ± 0.3%, respectively. (B) The secretions induced by acetylcholine at various concentrations of external Na^+ (125, 63 and 32 mM) were 19.6 ± 0.3%, 13.2 ± 0.4% and 9.8 ± 0.5%, respectively. The basal secretions were 1.6 ± 0.2%, 1.5 ± 0.3% and 1.6 ± 0.2%, respectively. Values are means ± S.D. from at least four experiments.

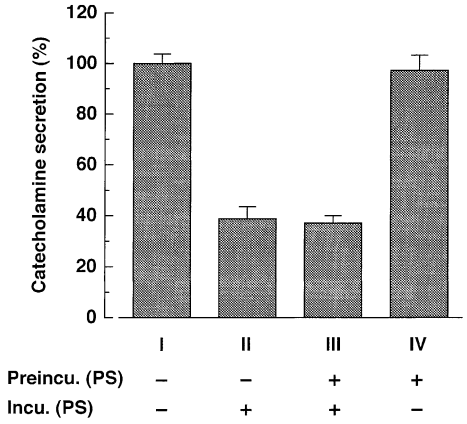

Fig. 6. Reversibility of the inhibitory effect of pregnenolone sulfate on catecholamine secretion. The cells were rinsed twice with prewarmed KRH buffer and were preincubated in KRH buffer with (III and IV) or without 100 μM pregnenolone sulfate (I and II) for 10 min at 37 °C (Preincu.). The cells were rinsed three times with KRH buffer and then incubated with (II and III) or without 100 μM pregnenolone sulfate (I and IV) in the absence or presence of 50 μM acetylcholine for 7 min (Incu.). The amount of catecholamines secreted from the cells was determined as described in Section 2. The values for basal secretion were subtracted from the data, and the acetylcholine-induced response was assigned the value of 100%. The acetylcholine-induced catecholamine secretion was 19.5 ± 0.7% of total cellular catecholamine. The basal secretion was 1.4 ± 0.1%. Values are means ± S.D. from four experiments. Preincu., preincubation; Incu., incubation; PS, pregnenolone sulfate.

Na^+, respectively (Fig. 5B). Thus, the inhibitory effect of pregnenolone sulfate was enhanced by decreasing Na^+ concentrations, but increasing Ca^{2+} concentrations did not alter the inhibition.

3.5. Reversibility of the inhibitory effect of pregnenolone sulfate on catecholamine secretion

To confirm whether the inhibitory effect of pregnenolone sulfate is reversible, we examined the reversibility of the inhibition on the acetylcholine-induced secretion. The cells were preincubated with or without pregnenolone sulfate (100 μM), and rinsed three times with KRH buffer, and then incubated with or without pregnenolone sulfate (100 μM) in the presence of 50 μM acetylcholine. As shown in Fig. 6 (column IV), the cells which were preincubated with pregnenolone sulfate, rinsed with KRH buffer, then incubated without pregnenolone sulfate in the presence of acetylcholine, completely regained the ability to secrete catecholamine. This result indicates that the inhibitory effect on the secretion is reversible.

4. Discussion

The effect of pregnenolone sulfate on acetylcholine-induced catecholamine secretion was investigated in cultured bovine adrenal chromaffin cells. In this study, we demonstrated that pregnenolone sulfate inhibits nicotinic acetylcholine receptor-mediated responses. Pregnenolone sulfate inhibited catecholamine secretion from the cells stimulated by acetylcholine (IC_{50}: 27 μM) (Fig. 1A). In addition, pregnenolone sulfate inhibited both acetylcholine-induced Na^+ (IC_{50}: 12 μM) and Ca^{2+} (IC_{50}: 20 μM) influxes (Figs. 2 and 1B), and the extent of the inhibition was almost similar to that of the acetylcholine-induced secretion. However, pregnenolone sulfate did not inhibit either catecholamine secretion or Ca^{2+} influx stimulated by high K^+ (Fig. 1), which directly activates voltage-dependent Ca^{2+} channels, suggesting that pregnenolone sulfate does not suppress either the voltage-dependent Ca^{2+} channels or the Ca^{2+}-dependent exocytotic process involved in catecholamine secretion. Furthermore, the inhibitory effect was not overcome by increasing external Ca^{2+} concentrations (Fig. 5A), indicating that the inhibition is distinct from that of blockers of the L-type voltage-dependent Ca^{2+} channels, which are competitive with external Ca^{2+} concentrations, such as diltiazem (Wada et al., 1983). These results indicate that the inhibition may occur at the level of nicotinic receptors.

Consistent with our result that pregnenolone sulfate did not inhibit the high K^+-induced catecholamine secretion, dehydroepiandrosterone sulfate has also been reported to have no effect on the high K^+-induced catecholamine secretion from bovine adrenal chromaffin cells (Liu et al., 1996). On the other hand, progesterone (Dar and Zinder, 1997) and 17α-estradiol (Lopez et al., 1991) have been demonstrated to inhibit the high K^+-induced secretion. Since both pregnenolone sulfate and dehydroepiandrosterone sulfate are sulfate esters of 3β-hydroxy-Δ5-steroids (i.e. pregnenolone and dehydroepiandrosterone), the hydrophobicity of these steroids due to the sulfate moiety would be much lower than that of progesterone or 17α-estradiol. The hydrophobicity and/or the chemical structure of steroids might be involved in steroidal modulation of voltage-dependent Ca^{2+} channel function.

In the present study, pregnenolone sulfate at higher concentrations (30–100 μM) increased the high K^+-induced Ca^{2+} influx (Fig. 1B). This result concurs with the report that pregnenolone sulfate enhances 20 mM K^+-induced increase in intracellular free Ca^{2+} concentration in rat hippocampal neurons. The enhancement is produced by an increase of Ca^{2+} influx via NMDA receptors that are activated by glutamate, whose secretion from the neurons is evoked by 20 mM K^+, and that are then potentiated by pregnenolone sulfate (Irwin et al., 1992). Further study is needed to verify whether the increase of Ca^{2+} influx is due to activated NMDA receptors in the adrenal chromaffin cells.

The present study showed that pregnenolone sulfate suppresses catecholamine secretion stimulated by veratridine, which directly activates voltage-dependent Na^+ channels (Fig. 1A). Since pregnenolone sulfate did not suppress the exocytotic process involved in catecholamine secretion, this result suggests that pregnenolone sulfate inhibits volt-

age-dependent Na^+ channels. It has been reported that some endogenous and synthetic steroids inhibit the influx of the organic cation, [^{14}C]guanidinium, evoked by veratridine in N1E-115 mouse neuroblastoma cells (Barann et al., 1999). Inhibition by progesterone of veratridine-induced catecholamine secretion has also been reported for bovine adrenal chromaffin cells (Dar and Zinder, 1997). Although little is available on the effects of steroids on voltage-dependent Na^+ channels, these Na^+ channels may also be a target for steroids. However, it is known that, in adrenal chromaffin cells, the contribution of voltage-dependent Na^+ channels to the physiological secretion of catecholamines evoked by nicotinic receptor agonist such as acetylcholine is small (Wada et al., 1985).

Several lines of evidence suggest that the inhibitory effect of steroids on nicotinic receptor function occurs due to steroid interactions at the membrane lipid–protein interface or the extracellular site on nicotinic receptors distinct from nicotinic agonist or competitive antagonist binding sites (Ke and Lukas, 1996; Arias, 1998). Pregnenolone sulfate attenuated the acetylcholine-induced maximum secretion without altering the EC_{50} value (Fig. 4), and did not alter the binding of [^{3}H]nicotine to nicotinic receptors (Fig. 3). These findings indicate that the inhibition is exerted via action sites distinct from the binding sites of agonist or competitive antagonist. Moreover, reducing external Na^+ concentrations enhanced the inhibitory effect (Fig. 5B), and the acetylcholine-induced Na^+ influx was reduced by pregnenolone sulfate (Fig. 2), suggesting that the ability of the nicotinic receptor-operated ion channels is directly implicated in the inhibitory effect. The nicotinic receptor-operated ion channels have been shown to be influenced by changes in membrane lipid environment (Zanello et al., 1996). However, it is unlikely that the inhibition is exerted through a perturbation of membrane lipids, since measurement of anisotropy in the cells by the diphenylhexatriene fluorescence polarization technique that we used earlier (Tachikawa et al., 2001) indicates that pregnenolone sulfate does not alter membrane fluidity (data not shown). These results suggest that nicotinic receptor function is directly inhibited by pregnenolone sulfate. Furthermore, both the reversibility and the rapid onset of the inhibitory effect strongly suggest that this effect is attributable to a nongenomic mechanism. The inhibitory pattern of pregnenolone sulfate is consistent with the finding that steroids inhibit noncompetitively nicotinic receptor function through a nongenomic mechanism (Valera et al., 1992; Ke and Lukas, 1996; Uki et al., 1999; Wagner et al., 1999). Based on these results, it is highly probable that pregnenolone sulfate noncompetitively and reversibly inhibits the nicotinic receptor-operated ion channels through a nongenomic mechanism, thereby suppresses Na^+ influx through the channels, and consequently attenuates both Ca^{2+} influx and catecholamine secretion. These findings suggest that pregnenolone sulfate modulates nicotinic receptor function, thereby changing neurotransmitter release in the brain. Further study of the action of pregnenolone sulfate on nicotinic receptor-operated ion channels is now in progress, using oocytes expressing nicotinic receptors.

The tissue concentrations of endogenous pregnenolone sulfate in brain have been reported to be in the range of about 10–30 ng/g tissue (Corpechot et al., 1983, 1997; Baulieu, 1997). These whole tissue concentrations are much lower than the minimum pregnenolone sulfate concentrations that would be expected to inhibit the nicotinic receptors and/or the voltage-dependent Na^+ channels. However, there is considerable evidence that neurosteroids can be synthesized in certain neurons and glial cells in the brain (Baulieu, 1997; Ukena et al., 1998; Kimoto et al., 2001). These synthesized neurosteroids appear to serve a paracrine (possibly also autocrine) role through local release of neurosteroids (Kimoto et al., 2001; Plassart-Schiess and Baulieu, 2001). In addition, it has been reported that the concentrations of neurosteroids increase in response to changes in the physiological state, such as stress (Lanthier and Patwardhan, 1987; Purdy et al., 1991; Barbaccia et al., 1996). Therefore, these considerations suggest that the concentration of pregnenolone sulfate may reach levels sufficient to produce the modulations around neurons through the local release and/or the facilitation of neurosteroid synthesis.

Pregnenolone sulfate administration into the brain has been reported to induce an increase in acetylcholine release (Pallares et al., 1998; Darnaudery et al., 2000), which is postulated to be one of the reasons for the memory-enhancing property of pregnenolone sulfate, and in dopamine release (Barrot et al., 1999), which is involved in mood and motivation. Assuming that in vitro data are relevant to the in vivo condition, these increases have been speculated to be due to an excitatory effect of pregnenolone sulfate as a negative modulator of $GABA_A$ receptors and/or as a positive modulator of NMDA receptors. The potencies of pregnenolone sulfate on nicotinic receptors (IC_{50}: 12–27 μM) and voltage-dependent Na^+ channels (IC_{50}: 44 μM) are in roughly same range as its potency on $GABA_A$ (IC_{50}: ~5–20 μM; Majewska and Schwartz, 1987; Wu et al., 1991; Shen et al., 1999) and NMDA (EC_{50}: ~20–50 μM; Wu et al., 1991; Bowlby, 1993) receptors. These findings indicate that the increase of neurotransmitter release might result from the coordination of the negative modulation by pregnenolone sulfate of nicotinic receptors and/or voltage-dependent Na^+ channels and the modulations of $GABA_A$ and NMDA receptors. Pregnenolone sulfate might contribute to regulation of the balance between excitation and inhibition in the brain.

In conclusion, pregnenolone sulfate noncompetitively and reversibly inhibits nicotinic receptor function in a nongenomic manner, thereby attenuating nicotinic receptor-mediated responses, and also suppresses voltage-dependent Na^+ channels. These findings indicate that pregnenolone sulfate may modulate neuronal excitability and/or neurotransmitter release via nicotinic receptors and/or voltage-dependent Na^+ channels in the brain.

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