Rapid inhibitory effect of progesterone on axonal transport in isolated and cultured mouse dorsal root ganglion neurons

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Objectives: Progesterone is synthesized not only in steroidogenic endocrine organs, such as the ovary, testis, and adrenal cortex, but also in the central and peripheral nervous system. Recent evidence has revealed the important role of progesterone in synaptic transmission, neuronal development, differentiation, neuronal cell survival, and neurogenesis. We examined the effects of progesterone on axonal transport, the fundamental function of neurogenesis and neurotransmission, in cultured dorsal root ganglion (DRG) neurons isolated from adult male mice.

Materials and Methods: Video-enhanced microscopic technique was used to observe, in real-time, the movement of particles in neurites of DRG neurons at 37 °C under a normal extracellular condition (Cl− concentration: 144 mM). Axonal transport was estimated by counting particles transported along the neurite in anterograde and retrograde directions.

Results: Application of 10 nM progesterone inhibited both anterograde and retrograde axonal transport within 8 minutes. The GABA A receptor agonist muscimol (100 μM) mimicked the effect of progesterone. The effects of progesterone and muscimol were both blocked by the GABA A antagonist bicuculline (100 μM) and in Cl−-free extracellular medium. Intracellular perfusion with a high concentration of Cl− (144 mM) rapidly and strongly decreased axonal transport.

Conclusion: These results suggest that progesterone produces a rapid inhibition of axonal transport in DRG neurons via activation of GABA A receptors and subsequent increase in the intracellular Cl− concentration.

Key words: progesterone, axonal transport, GABA A receptor, chloride, video-enhanced microscopy, cultured dorsal root ganglion neurons

Introduction

Progesterone is synthesized not only by steroidogenic endocrine glands, such as the ovary, testis, and adrenal cortex, but also by glial cells and some population of neurons in the central and peripheral nervous system. Evidence has revealed the important role of progesterone in neurons, such as synaptic transmission, neuronal development, neuronal survival, and neurogenesis. In the peripheral sensory system, progesterone seems to play modulatory roles for sensory reception, transmission, and plasticity, e.g., in vivo pain sensitivity is reduced by progesterone. A role for progesterone synthesized in Schwann cells has been demonstrated in the repair of myelin after lesions of the sciatic nerve in male mice and in cultures of dorsal root ganglia. Progesterone also stimulates Schwann cell proliferation and increases the number of myelinated axons in cocultures of Schwann cells and sensory neurons.

Axonal transport is an important function underlying neurotransmission, neurogenesis, and a variety of other functions in neuronal cells. Progesterone is suggested to modify sensory neuronal structure and function and is reported to restore retrograde axonal transport of cervical neurons in motoneuron disease model mice. Thus, we hypothesized that progesterone may influence axonal transport in sensory neurons. To test this hypothesis, we examined the effects of progesterone on axonal transport in cultured dorsal root ganglion (DRG) cells isolated from adult mice. Progesterone has not only a genomic action but also a rapid membrane nongenomic action as a neurosteroid. Neurosteroids including progesterone act on the GABA A receptors to increase Cl− current. Therefore, we also attempted to investigate whether or
not the action of progesterone on axonal transport is rapid; and, if so, GABA A receptors are involved in the membrane mechanism. We utilized video-enhanced microscopy to observe in real-time the axonal transport of particles within neurites. This technique makes it possible to detect the quick response of the particle movement to external stimuli.

Materials and Methods

Cell culture

The experimental protocol was approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine. Adult male c57BL/6 mice (6-8 weeks old) were euthanized with ether and the dorsal root ganglia were removed. The ganglia were immersed immediately in Ham’s F-12 medium (Life Technologies Inc., Gibco BRL Division, Grand Island, NY, USA). The ganglia were incubated for 90 minutes at 37°C in Ham’s F-12 medium containing 0.2% collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA). The ganglia were subsequently incubated for 15 minutes at 37°C with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution (g/l: KCl, 0.4; KH₂PO₄, 0.06; NaCl, 8; Na₂HPO₄/7H₂O, 0.09; D-glucose, 1; phenol red, 0.01; [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid] HEPES 3.6; NaOH, 0.3) containing 0.25% trypsin (Sigma Chemical Company, St. Louis, MO, USA). Trypsin activity was then attenuated by addition of trypsin inhibitor (0.125 mg/ml, Sigma). Following a 3-times rinse with enzyme-free medium, the ganglia were triturated using flame polished Pasteur pipettes (0.2-0.5 mm i.d.), and the suspension was plated onto polylysine (Sigma)-coated glass coverslips (30 ㎛ 40 mm, 50 ㎛ thickness). The cells were cultured in Hams’ F-12 medium containing 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 ㎍/ml) for 48 hours at 37°C under humidified conditions in 95% air and 5% CO₂ (pH 7.4). The neurites were extended from DRG cells during this culture period.

Experimental cell preparation

The coverslip was attached with waterproof tape under a 0.5 mm-thick stainless steel plate (50 ㎛ 80 mm) whose center was cut out hexagonally. The upper side of the steel plate was covered with another coverslip, leaving small holes on both sides to perfuse the solution. The culture medium was then replaced with HEPES-buffered saline (37°C, pH 7.4). The plate was mounted onto the temperature-controlled (37°C) stage of an inverted Zeiss Axiomat microscope (Carl Zeiss, Oberkochen, Germany) equipped with an oil-immersed Plan-Apochromat 64 × NA 1.40 objective (Carl Zeiss). The drug-containing solution was injected into one hole using a Pasteur pipette, and the solution spilling from another hole was removed using a peristaltic pump.

Solutions and drugs

The composition of HEPES-buffered saline (pH 7.4) was 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 5.5 mM D-glucose (Wako Pure Chemical, Osaka). Chloride (Cl⁻)-free solution was composed of 67.5 mM Na₂SO₄, 2.5 mM K₂SO₄, 1 mM CaSO₄, 1 mM MgSO₄, 10 mM HEPES, and 5.5 mM D-glucose (pH 7.4). Progesterone (Sigma) was dissolved in ethanol and then diluted with HEPES-buffered saline. The concentration of ethanol was 1% and had no effect on axonal transport. Each of the muscimol and (-) bicuculline methiodide (Sigma) was directly dissolved in HEPES-buffered saline. When observing the effects of progesterone under a Cl⁻-free extracellular condition, the progesterone-containing Cl⁻-free solution was used.

Video-enhanced microscopic recordings

Nomarski images obtained by inverted microscope were transformed into video images with enhanced contrast. Light transmittance was sensed by an analogue video camera (Harpon, Hamamatsu Photonics, Hamamatsu), whose contrast enhancement (gain, offset) and shading were set at the beginning of each experiment. The analogue signal was processed by a real-time digital video image enhancement system (DVS-20, Hamamatsu Photonics). Serial video images were produced by a sequence of user-defined procedures (mean background determination of 2-4 consecutive frames, recursive background subtraction, contrast enhancement). They were displayed on a video monitor (C1846, Hamamatsu Photonics) and were stored on a video recorder (PVW-2800, Sony, Tokyo).

Analysis of axonal transport

Axonal transport was estimated by counting the number of particles (diameter ≥ 50 nm) moving within neurites. A line perpendicular to the long axis of the neurite was drawn on the video monitor, and the number of particles crossing the line in each anterograde and retrograde direction was counted for 2 minutes at 3-minute intervals during periods before and after injection of the drug.

Intracellular perfusion

Whole-cell configuration was used to deliver a high concentration of Cl⁻ solution to the inside of the cell.
Patch pipettes (1-5 MΩ) filled with solution (pH 7.2) containing 150 mM KCl, 1 mM MgCl₂, 10 mM HEPES, and 1 mM ethyleneglycoltetraacetic acid (EGTA) were attached to the cell membrane. After control recordings of axonal transport, a whole-cell condition was obtained. Recordings of axonal transport were continued under such a condition. As a control experiment, cells were perfused intracellularly with solution containing 150 mM K-gluconate, 1 mM MgCl₂, 10 mM HEPES, and 1 mM EGTA. Electrical signals were obtained to gain a gigaseal and a subsequent whole-cell condition through a Patch Clamp L/M-EPC7 (List Electronics, Darmstadt, Germany) and were monitored on an oscilloscope.

Statistics
Data are expressed as the mean ± SD as a percentage of the control value that was obtained before application of the test agent. The significances of differences between values before and during application of test agents were evaluated by repeated-measures analysis of variance (ANOVA) followed by the post hoc Bonferroni-Dunn test. Statistical significance was determined as P < 0.0005.

Figure 1. Axonal transport of particles in the neurite of DRG neurons. Video-enhanced microscopic images of particles in the neurite (A, B) and their movement tracks (C) are shown. The images were taken at a 5-second interval. Arrows (C) indicate the direction and distance of the movement of individual particles for 5 seconds. Scale bar, 5 μm.

Figure 2. Effects of a transient application (20 minutes) of progesterone on axonal transport in cultured dorsal root ganglion neurons. The graph plots the percentage changes in the number of particles transported in anterograde and retrograde directions. The horizontal black bar indicates the application period.
Results

Video-enhanced microscopy displayed the movement of particles along the neurite toward terminals (anterograde) and back to the cell body (retrograde) (Figure 1). Particles included mitochondria and lysosomes and other membrane-bound vesicles such as secretory vesicles. These were identified morphologically, and also, in some experiments, by mitochondria and lysosome markers (Mitotracker and Lysotracker, Molecular Probes, Carlsbad, CA, USA). In control extracellular medium (HEPES-buffered saline, pH 7.4, 37°C), means of numbers of particles (per minute) transported in anterograde and retrograde directions were 62.3 ± 5.7 (mean ± SD; n = 52) and 61.0 ± 8.7 (n = 52), respectively.

Effects of progesterone on axonal transport

Application (20 minutes) of 10 nM progesterone promptly decreased the number of transported particles in both anterograde and retrograde directions (Figure 2). These inhibitory responses were completely reversed after the wash-out of progesterone (Figure 2). Continuous application (26 minutes) of progesterone resulted in the sustained inhibition of axonal transport (Figure 3).

Analysis of mean percent changes (n = 5) in transported particles showed that the value obtained at 5 or 8 minutes after the application was significantly lower than the control (before application). The value reached the maximum inhibition level of about 60%-70% at 14 minutes after the application and this level was sustained throughout the 26-minute application. The used progesterone concentration, 10 nM, was the minimum concentration to obtain the maximum effects. Such rapid effect of progesterone may result from its action on membrane receptors. Therefore, we performed further experiments to examine the membrane mechanisms underlying the inhibitory action of progesterone on axonal transport.

Effects of GABA\textsubscript{A} receptor agonist on axonal transport

Some rapid effects of steroids, in particular those of neurosteroids such as progesterone and its derivatives, are mediated by GABA\textsubscript{A}-gated Cl\textsuperscript{−} current. We, therefore, examined the involvement of GABA\textsubscript{A} receptors. Application of GABA\textsubscript{A} receptor agonist muscimol (100 μM) mimicked the effect of progesterone (Figure 4). The maximum inhibition was approximately 50%-60% of the baseline.
Effects of progesterone on axonal transport in the presence of GABA<sub>A</sub> receptor antagonist

We subsequently examined the effects of progesterone on axonal transport in the extracellular medium containing the GABA<sub>A</sub> receptor antagonist bicuculline. In the presence of 100 μM bicuculline, muscimol (100 μM) failed to decrease the number of particles transported in the anterograde and retrograde directions (Figure 5A). Similarly, the decreasing effect of progesterone (10 nM) on the number of particles was not detected when bicuculline (100 μM) was present in the extracellular medium (Figure 5B). These results indicate that the effect of progesterone is presumably mediated by the activation of GABA<sub>A</sub> receptors.

Effects of progesterone on axonal transport in Cl<sup>-</sup>-free extracellular medium

Subsequently, we examined the ionic mechanism for the

Figure 5. Effects of muscimol (A) and progesterone (B) on axonal transport in the presence of bicuculline, a GABA<sub>A</sub> receptor antagonist. The graph plots the percentage changes in the number of particles transported. Each point illustrates the mean (± SD) of the values obtained from 5 dorsal root ganglion neurites.

Figure 6. Effects of muscimol (A) and progesterone (B) on axonal transport in Cl<sup>-</sup>-free extracellular medium. The graph plots the percentage changes in the number of particles transported. Each point illustrates the mean (± SD) of the values obtained from 5 dorsal root ganglion neurites.
action of progesterone. It is possible to predict that the rapid effect of progesterone would be caused by Cl⁻ current through Cl⁻ channels associated with GABA_A receptors. To confirm this hypothesis, we performed experiments under a Cl⁻-free extracellular condition. In Cl⁻-free extracellular medium, muscimol (100 µM) failed to inhibit anterograde and retrograde axonal transport (Figure 6A). The inhibitory effect of progesterone (10 nM) was also abolished when Cl⁻ was absent in extracellular medium (Figure 6B). These results suggest that the effect of progesterone as well as muscimol is produced by the influx of Cl⁻ into the cells.

Effects of intracellular perfusion of Cl⁻
We finally investigated the intracellular perfusion of a high concentration of Cl⁻ solution. Using pipettes containing K-Cl (144 mM Cl⁻) or K-gluconate, changes in axonal transport were observed during the period from a cell-attached condition to a whole-cell condition. In K-Cl perfused cells, the number of particles was rapidly decreased to a relatively low level. In contrast, K-gluconate-perfused cells showed a slight increase in the number of particles. These results suggest that axonal transport in cultured DRG neurons is inhibited by Cl⁻ accumulation inside the cell.

Discussion
The present results obtained by using the video-enhanced microscopic technique indicated that progesterone rapidly (within 8 minutes) inhibits axonal transport in cultured mouse DRG neurons. The inhibitory response to progesterone was sustained during application of progesterone but reversed when progesterone was removed. Evidence shows that progesterone acts on the cell membrane to rapidly modulate some neuronal cell functions such as synaptic transmission and in vitro LHRH (luteinizing hormone-releasing hormone) release from the rat hypothalamus. Other nongenomic effects of progesterone include anesthetic action and the control of mating behavior. To our knowledge, our findings are the first that show axonal transport in cultured DRG neurons is inhibited by Cl⁻ accumulation inside the cell.

The extracellular concentration of progesterone fluctuates during the estrous cycle or during pregnancy. In addition to this classical recognition, progesterone has been known to be ubiquitously synthesized in glial cells and in some populations of neurons in the central and peripheral nervous system. Therefore, the mechanisms of the modulatory effect of progesterone on axonal transport may operate under physiological conditions, and circulating levels of progesterone may modulate axonal transport of neurons.

Previous studies clarified the membrane mechanisms for rapid effects of progesterone. For example, analgesic effects of progesterone are mediated through the GABA_A receptor complex. A progesterone receptor has been identified in peripheral and central glial cells, and neurosteroids including progesterone can modify GABA responses through direct interactions with the GABA_A-
receptor complex. The rapid effects of progesterone on membrane potentials and behavioral activities are mediated by GABA_A-gated Cl^- current. These effects may involve a specific binding site for the steroids on the GABA_A receptors. We also studied membrane mechanisms for the modulation of axonal transport induced by progesterone, because the response to progesterone was rapid, which is predicted to be a nongenomic action. Our results indicate that the GABA_A receptor agonist muscimol mimicked the effect of progesterone. The inhibitory effects of progesterone as well as muscimol on axonal transport were completely blocked by the GABA_A receptor antagonist bicuculline. Furthermore, the inhibitory effect of progesterone was not observed in Cl^-free extracellular medium. Intracellular perfusion of a high concentration of Cl^- solution immediately and strongly inhibited axonal transport. These results suggest that the effect of progesterone on axonal transport is mediated by the influx of Cl^- through GABA_A receptor activation and that an increase in intracellular Cl^- concentration may be the critical factor.

Although GABA_A receptor activation is well known to be inhibitory, the previous electrophysiological studies indicate that peripheral neurons, such as DRG neurons, sympathetic neurons, and juvenile central neurons, are depolarized (excited) by GABA and GABA_A receptor agonists. This is explained by the theory that in these cells intracellular Cl^- concentration is high because of the activity of inward Cl^- transporter (Na^+-K^+-2Cl^- cotransporter), and then the equilibrium potential of GABA, E_GABA, which is equal to Ec^+, is more positive than the resting potentials. According to this theory, GABA_A receptor activation should result in Cl^- efflux in these cells including DRG neurons. And if so, the present results indicating the involvement of Cl^- influx through GABA_A receptor activation seem inconsistent. However, recent evidence has shown that GABA_A receptors allow not only Cl^- but also other anions such as bicarbonate to pass through. Thus, it has been proposed that the depolarization induced by GABA_A receptor activation results from the efflux of bicarbonate associated with the influx of Cl^- through GABA_A receptors. The equilibrium potential of bicarbonate is much more positive than the resting potential, and then the cell is depolarized. This theory indicates that GABA_A receptor activation results in the influx of Cl^- in spite of the cell depolarization and supports the present results. Moreover, a recent intracellular Cl^- imaging study has shown that muscimol induces intracellular Cl^- accumulation in cell line P19 cells, though these cells are depolarized by GABA.

Therefore, it is possible that in DRG neurons, as well, GABA_A receptor activation causes Cl^- influx while the cells are depolarized.

In conclusion, we have demonstrated that progesterone rapidly inhibits axonal transport of cultured mouse DRG neurites. The increase in intracellular Cl^- concentration appears to be a process underlying expression of the effects of progesterone. This mechanism may operate in response to the release of progesterone from endocrine organs as well as the nervous system. These results suggest that progesterone may be a factor that physiologically modulates axonal transport.

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References