Effects of Hyperpolarization-Activated Channel Blocker ZD7288 on Polar Excitations of Frog Sciatic Nerve

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Abstract: Previous studies have demonstrated that Ar+ laser irradiation shows a more selective blocking effect on the generation of anode-break-excitation (AE) than on cathode-make-excitation (CE), and that the effects of laser irradiation closely resemble those following the application of hyperpolarization-activated current (Ih) blocker, ZD7288. We therefore examined the effects of ZD7288 and tetrodotoxin (TTX) on polar excitations to reveal whether such a selective effect of ZD7288 on AE is specific in frog sciatic nerve. Supramaximal stimuli (10-ms pulse) were applied while for 30 min each channel blocker was applied to the stimulating sites. Analyses of chronological changes in polar excitations were performed using CEs induced by positive stimuli and AEs induced by negative stimuli, because both were generated on the same stimulating grid against the recording grids. TTX application (1 mM) decreased all types of polar excitations at 30 min after initiation of the application. When ZD7288 (1 mM) was applied, the amplitude of AE displayed a significant decrease after 30 min. When TTX or ZD7288 was applied to the middle portion between the stimulating and recording electrode grids, TTX showed the conduction block, but the latter yielded almost no effect. Western blotting analyses demonstrated expressions of the second and the third subunits of hyperpolarization-activated and cyclic-nucleotide-gated nonselective cation channels in frog sciatic nerve. Ih channels thus exist in the frog sciatic nerve, and its specific blocker ZD7288, has the potential to selectively block the generation of AE.

Key words: anode-break-excitation, cathode-make-excitation, hyperpolarization-activated current, law of polar excitation, ZD7288.

The law of polar excitation, or Pflüger’s law, states that both cathode-make-excitation (CE) and anode-break-excitation (AE) are elicited when nerves are extracellularly stimulated with a longer pulse (>3.0 ms) at suprathreshold intensity [1–3]. The underlying mechanisms of polar excitation have primarily been described from Na+-channel properties as follows [2, 3]. The Na+-channel has 3 states: resting, active, and inactive. A small depolarization changes the channel from resting to active state, and a small hyperpolarization evokes the change from inactive to resting state. During the application of a longer-pulse stimulus, the membrane potentials of individual nerve fibers on the cathode shift to depolarization, triggering an action potential (equal to CE) as an on-response, whereas membrane potentials on the anode shift to hyperpolarization, which thus shifts the channels to resting state, i.e., a ready state to fire. The terminal of the stimulation triggers another action potential (equal to AE) as an off-response, because the membrane potential shifts in the depolarizing direction by breaking the stimulation. A scant few reports have revisited the mechanisms of polar excitations [4–7], but none has suggested a contribution of different ionic channels other than Na+ and K+ channels.

The characteristics of hyperpolarization-activated current (Ih) have recently been investigated [8–19]. Ih is a voltage-gated non-selective cationic conductance activated by membrane hyperpolarization, not by depolarization. This current is blocked by cesium chloride (CsCl) or 4-ethylphenylamino-1,2-dimethyl-6-methylamino-pyrimidium chloride (ZD7288), but not by Na+-channel blocker, tetrodotoxin (TTX). Ih was first identified in cardiac sinoatrial node cells as a pacemaker current, and has now been found in many cell types in the central nervous system (CNS). Electrophysiological evidence has demonstrated that Ih contributes to the generation of spontaneous action potentials in guinea-pig primary afferent neurons [20]. Moreover, rebound action potentials reportedly appear after an application of serotonin when breaking the hyperpolarization, and Ih blockers suppress such increased activity [21, 22].

During an attempt to find a safe level of irradiation for frog sciatic nerve, we made the accidental finding that Ar+ laser irradiation could selectively suppress the generation of AE [23]. Since AE is associated with membrane hyper-
polarization, we tested an Ih blocker, ZD7288, instead of laser irradiation, and obtained almost the same result [24, 25]. Given these findings, we designed the present study to examine the effects of low to high concentrations of TTX as well as ZD7288 on polar excitations to reveal whether the selective effect of Ih blocker on AE is specific. Further, we immunocytochemically examined the expression of hyperpolarization-activated and cyclic-nucleotide-gated non-selective cation channels (HCN) on frog sciatic nerve membrane.

METHODS

All experiments were performed under appropriate conditions in accordance with the Declaration of Helsinki (revised version in 2002) and the Bioethical Standards of Animal Experiments for Soka University.

Tissue preparation. This study used 56 frogs (Xenopus laevis) weighing 55–80 g. They were anesthetized and immobilized under low temperatures (3°C–5°C), and bilateral sciatic nerves were removed from the border of the spinal cord to the knee portion in accordance with conventional methods for practical neurophysiology. An extracted nerve preparation was set on silver electrode grids in the experimental chamber, which was kept under adequate humidity using Tasaki-Ringer’s solution. All experiments were performed at room temperature (20°C–25°C).

Stimulating and recording system. A stimulator and isolator set was used for stimulating the nerve. After 0.2-ms pulses were delivered at 1 Hz to determine threshold and supramaximal intensities for compound action potential (CAP), 10-ms pulses were delivered at 0.5 Hz to examine the effects of channel blockers on polar excitations. A conventional recording system comprising biophysical oscilloscope, and a digital tape recorder were used. A MacLab system (ADInstruments, Tokyo, Japan) was used for off-line analyses. When the nerve bundle was stimulated using a longer-duration pulse (10 ms) at supramaximal intensity, both amplitudes of CE and AE before the application of agents were measured as control values and standardized as 100%.

Application of channel blockers. To reveal the effects of channel blockers on polar excitations, the following agents were used: TTX for Na⁺-channels (0.01, 0.1, and 1 mM) and ZD7288 for Ih channels (0.01, 0.1, and 1 mM). For the application of channel blockers, 2 small cotton balls saturated with blocker solution were placed on stimulating sites. In some experiments, a small cotton ball was placed on the nerve bundle between stimulating and recording electrodes to examine the effects of each blocker on CAP conduction.

Western blot analysis. For Western blot analysis, protein extracts were prepared from frog sciatic nerve, heart, gastrocnemius muscle, skin, and blood. Protein concentrations were determined using the Bradford method. Samples (30 μg protein) dissolved in 20% glycerol, 4% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue, and 10% β-mercaptoethanol were heated for 3 min at 100°C. Proteins separated using 10% polyacrylamide gel electrophoresis were transferred to nitrocellulose membranes (Invitrogen, USA). Each membrane was blocked with phosphate-buffered saline containing 5% skim milk, then incubated with anti-HCN1 to anti-HCN4 (Alpha Diagnostic International, Texas, USA) diluted at 1:1000. Secondary anti-rabbit antibodies coupled to alkaline phosphatase (Promega, Wisconsin, USA) were used at a 1:5000 dilution, and bands were visualized by incubation in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Promega).

Statistical analyses. Values are expressed as mean ± standard error of the mean (SEM). Statistical significance between CE and AE was evaluated using a 2-tailed Student’s t-test (for paired values, not assuming equal variances). The values of p < 0.01 or p < 0.05 were considered statistically significant.

RESULTS

Control experiments
We first performed a simple examination involving the law of polar excitation. Figure 1A shows responses to the positive (upper) or negative pulse (lower traces), which was delivered for 30 min with no solutions. Taking into consideration the latency shifts on switching from positive to negative stimulus, we confirmed this law: on-responses (equal to CEs) were generated from the cathode portion, whereas off-responses (equal to AEs) were generated from the anode portion. When the nerve bundle was crushed at the middle portion between the anode- and cathode-stimulating grids, AE elicited by a positive stimulus disappeared, and CE activated by a negative stimulus also disappeared (not shown). CE and AE were defined by a positive stimulus as CEp and AEp and by a negative stimulus as CEn and AEn. The analyses of chronological changes in CAPs were performed using CEp and CEn because these were generated on the same stimulating grid against the recording grids. When the initial CAP amplitudes of CEp and AEp were standardized as 100%, mean amplitudes were 105 ± 2% and 107 ± 2% by 30 min after the initiation of stimulation, respectively. When Tasaki-Ringer’s solution was applied, CEp and AEn were 98 ± 5% and 96 ± 6% by 30 min after application, respectively (Fig. 1B).

Since TTX is a well-known Na⁺-channel blocker, the effects on polar excitations were examined with changing concentration. Figure 1C shows a typical example of polar excitations at 0, 10, 20, and 30 min after TTX application (0.1 mM). CAP amplitudes for all types of polar excitations gradually decreased: CEp and AEn amplitudes be-
Effects of ZD7288 on Polar Excitations

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came 35 ± 9% and 33 ± 7% by 30 min after application, respectively (Fig. 1D). The application of 1 mM TTX abolished excitations completely after 30 min (Fig. 1E).

Effects of Ih channel blocker, ZD7288

The most effective example of 1 mM ZD7288 application is shown in Fig. 2A. At both positive and negative stimuli, the amplitude of CE was almost unchanged, but that of AE gradually attenuated. In this case, CEp and AEp were 82% and 41% after 30 min, and CEn and AEn were 91% and 36%, respectively.

Figure 2B shows an alteration of mean amplitudes in CEp and AEn following 30 min of 0.01 mM ZD7288 application. After 30 min, the amplitude of CEp was 106 ± 10%, greater than that of AEn (80 ± 13%). However, no significant difference was found between CEp and AEn at 30 min (p = 0.10558). A higher concentration of ZD7288 was associated with greater effect on AE (Fig. 2, C and D).
D). In regard to a 0.1 mM application, the amplitudes of CEp and AEn were respectively 105 ± 6% and 77 ± 6% (p < 0.05) after 10 min, 105 ± 6% and 78 ± 6% (p < 0.05) after 20 min, and 104 ± 7% and 73 ± 4% (p < 0.05) after 30 min. Between the amplitudes at 0 and 30 min, CEp showed no significant difference (p = 0.32499), but AEn displayed a significant difference (p < 0.05). For the group that received 1 mM ZD7288 application, the amplitudes of CEp and AEn were respectively 95 ± 7% and 57 ± 14% at 10 min (p < 0.05), 90 ± 7% and 47 ± 15% at 20 min (p < 0.01), and 81 ± 12% and 40 ± 16% at 30 min (p < 0.01). From 0 min to 30 min, CEp showed no significant difference (p = 0.08300), but AEn displayed a significant difference (p < 0.01).

Effects of TTX and ZD7288 on CAP conduction

Because the mechanism underlying CAP conduction along nerves is known to resemble that of CE generation, we examined the effects of TTX and ZD7288 on CAP conduction, not generation. These agents were applied to the middle portion between stimulating and recording electrodes.

Figure 3A shows TTX application (1 mM). Traces show that all types of polar excitation gradually decreased and disappeared after 30 min. For the group that received 1 mM TTX application, amplitudes of CEp and AEn were respectively 78 ± 14% and 66 ± 8% at 10 min (p = 0.19098), 29 ± 14% and 11 ± 5% at 20 min (p = 0.22181), and 11 ± 4% and 2 ± 2% at 30 min (p = 0.15453). Conversely, ZD7288 (1 mM) application had no influence on CAP conduction, as observed in traces and averaged graphs (Fig. 3B). Even amplitudes of CEp and AEn at 30 min after application were 92 ± 6% and 84 ± 16%, respectively, and no significant differences between values were identified (p = 0.31470). No significant differences between amplitudes at 0 and 30 min were identified for each type (p = 0.07231, 0.17161, respectively).

Immunohistochemical study of HCN1-4 expression

An immunohistochemical study was performed to demonstrate the existence of Ih channel proteins on the nerve bundle. We first examined HCN2 expression because of the widespread distribution throughout the brain [17], but the immunohistochemical studies related to HCN expressions have not yet been examined in the peripheral nervous system (PNS). Figure 4A shows a Western blot for HCN2 protein. An approximately 97-kDa band corresponding to HCN2 was clearly apparent in sciatic nerve and heart samples. HCN3 expression was also observed in sciatic nerve, heart, and gastrocnemius samples (Fig. 4B). As to HCN1 and HCN4, the same procedures were performed, resulting in no expressions, or less prominent ones, at least in sciatic nerve samples (not shown).

DISCUSSION

The present study demonstrated the expression of at least two (HCN2 and HCN3) of the 4 subtypes of HCN channels in frog sciatic nerve bundle, and clarified the contribution of those channels to the generation of AE, though
the former evidence identified only the presence of HCN2 or HCN3 protein on the axolemma.

**Existence of Ih channels in the peripheral nerve bundle**

Ih channels were first identified in the rabbit sinoatrial node and were assumed to play a role in the pace-making activity of myocytes [8]. Intensive and widespread investigations have since been conducted on the electrophysiological characteristics and immunohistochemical distributions of Ih channels [8–19].

Notomi and Shigemoto clearly reported on the immunohistochemical localization of Ih channel subunits HCN1-4 in the rat brain [19]. Immunoreactivity for HCN1 showed predominantly cortical distribution, with intense signals in the neocortex, hippocampus, superior colliculus, and cerebellum. HCN3 and HCN4 exhibited subcortical distribution mainly concentrated in the hypothalamus and thalamus, respectively. Immunoreactivity for HCN2 displayed a widespread distribution throughout the brain. Moreover, HCNs were localized not only in somatodendritic compartments, but also in axonal compartments of neurons.

No immunohistochemical studies have examined the distributions of Ih channels in the PNS. However, Ih channels have been recorded in dorsal root ganglion (DRG) neurons, and Ih modulation in DRG neurons by serotonin or clonidine has also been reported [21, 22, 26]. Scroggs et al. demonstrated that Ih is infrequently expressed by C- and Aδ-type DRG neurons, but it is prominent in most Aβ- and Aβ1-type neurons [21]. Although this electrophysiological evidence was obtained from DRG somata, some reports have suggested the existence of Ih in the axon terminals of the PNS, and also in CNS neurons. For example, the presynaptic Ih channel has been postulated to enhance transmitter release in the crayfish neuromuscular junction [27] and also to be necessary for hippocampal mossy fiber long-term potentiation [28].

Using frog sciatic nerve bundle preparations, we demonstrated differences in effect on polar excitations between Ih-channel blocker ZD7288 and Na-channel blocker TTX. We also revealed HCN2 and HCN3 expressions in the nerve bundle sample. These findings strongly suggest that Ih channels may be distributed not only in somatic compartments, but also in axonal bundles and in terminal compartments in the PNS.

**Ih channels and polar excitations**

The law of polar excitation is a basic physiological phenomenon of the nerve bundle as performed in practical experiments. The underlying mechanism of polar excitations has been mainly described from the properties of Na channels [1–3]. Although recent studies revisiting the mechanisms of polar excitations have proposed alternative models to the one initially described by Hodgkin and Huxley [4–7], none have suggested a contribution of ionic channels other than Na+ or K+ channels to the generation of polar excitation.

We have demonstrated that a specific Ih channel blocker, ZD7288, can selectively suppress the generation of AE, but not CE, and also the conduction of both types of CAP. Cardenas et al. reported that 5-HT-induced inward current and fast current are blocked by another Ih-channel blocker Cs+, but not Ba2+ in rat medium- and large-diameter DRG neurons, and that 5-HT facilitates action potential generation by anode-break excitation [22]. Therefore not only Na+ channels, but also Ih channels are quite likely to contribute to the generation of AE in the sciatic nerve bundle.

**Function of Ih channels in the nerve bundle**

The question remains whether such polar excitations occur under physiological conditions. In particular, AE generation seems to be an extremely rare event. We thus need to make some comments on Ih functions in the nerve bundle other than the contribution to AE generation.

Ih has been proposed to play a role in the generation of spontaneous action potentials in the primary afferent neuron [20] and in the thalamic relay neuron [10], and that the modulation of Ih activation results in profound influences on background cell firings of hippocampal CA1 interneurons [13]. Moreover, Ih is thought to provide a mechanism for limiting excessive hyperpolarization on negative shifts of membrane potential [29] and to contribute to resting
membrane potential [30]. Another noteworthy characteristic of Ih is that activation can be modulated by first or second messengers, including 5-HT, monoamines, clonidine, and cAMP [22, 31–33].

When we apply the assumed functions regarding somadendritic Ih channels described above to the axonal functions, some biphasic functions can be presumed. Ih diminishes the effect of excitatory inputs to stabilize membrane potential toward resting potential, whereas it helps to set the level of resting potential toward action potential firing threshold [34]. Switching between such inhibitory and excitatory functions may occur under the effect of several types of modulators, though how to spontaneously generate a large hyperpolarization that activates Ih channels in the nerve bundle remains unclear. The excitatory function of Ih might elucidate mechanisms underlying sensory or motor neurophysiopathies in the PNS.

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