Neuroscience Letters 439 (2008) 241–244

Contents lists available at ScienceDirect

Neuroscience Letters

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The effects of ouabain on resting membrane potential and hyperpolarization-activated current in neonatal rat nodose ganglion neurons

Shigeji Matsumoto*, Junichi Kitagawa, Mamoru Takeda

Department of Physiology, Nippon Dental University, School of Life Dentistry at Tokyo, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan

ARTICLE INFO

Article history:
Received 18 February 2008
Received in revised form 12 May 2008
Accepted 13 May 2008

Keywords:
Ouabain
Na^+-K^+ pump
Resting membrane potential
Hyperpolarization-activated current
Nodose ganglion

ABSTRACT

To determine whether the responses of resting membrane potential (RMP) and hyperpolarization-activated current (I_H) are altered by the application of ouabain, one of the Na^+-K^+ pump inhibitors, in neonatal rat small-diameter (<30 μm) nodose ganglion (NG) neurons, we examined the effects of 1 μM ouabain on those responses using perforated patch-clamp techniques. In current-clamp mode, the RMP was 40.2 ± 1.6 mV (n = 31). Twenty of 31 cells tested were depolarized by ouabain application, and these responses were associated with an increase in the cell input resistance. In the remaining 11 cells studied, 3 showed hyperpolarization in response to ouabain and 8 showed no effect on RMP. In voltage-clamp mode, 1 μM ouabain application enhanced the I_H in all of 10 neurons examined. These results suggest that ouabain application at 1 μM is capable of setting both the RMP level and the neuronal excitability in small-diameter NG neurons.

The majority of nodose ganglion (NG) neurons that project to the cardiovascular, pulmonary and gastrointestinal afferent inputs have unmyelinated axons (C-type neurons), and only about 10% of NG neurons have myelinated axons (A-type neurons) [1,21]. Large-and medium-sized A-type neurons are blocked by nanomolar concentrations of tetrodotoxin (TTX), but after the application of micromolar concentrations of TTX, C-type neurons (<30 μm in soma diameter) can still generate action potentials [1,21,2,5].

In most cells the coupling ratio of an electrogenic Na^+-K^+ pump is recognized as $3Na^+$:$2K^+$ [19]. Functional Na^+-K^+ ATPase is a heterodimer consisting of α (α1–α4) and β (β1–β3) subunits, and the expression patterns of these units are tissue-specific and depend upon the cell's need for active transport [12,23]. In general, the α_1 isoform of the Na^+-K^+ ATPase in rats is 10–100 times less sensitive to ouabain than either the α_2 or the α_3 Na^+-K^+ ATPase isoforms. The ouabain affinities of rat kidney cells, brain synaptosome, adipocytes and astrocytes differ greatly; the $K_{1/2}$ value of the α_1 Na^+-K^+ ATPase isoform is 30–170 μM ouabain but the ouabain concentration required for the half-inhibition of the α_2 or α_3 Na^+-K^+ ATPase isoforms is 0.1–1 μM [3,14,18,22]. However, the specific functions of the neuronal α_1 and α_3 Na^+-K^+ ATPase isoforms are not known.

The hyperpolarization-activated current (I_H) is known to be involved in shifting the resting membrane potential (RMP) to a more depolarized state [16,9]. Furthermore, the I_H is substantially active at the RMP level [16,20]. The Na^+-K^+ pump plays a functional role in maintaining Na^+-K^+ gradients [12,13,19]. From these observations, one might predict that there are interactions between the Na^+-K^+ pump and the I_H that contributes to regulation of RMP, as well as neuronal excitability. However, no such interactions have yet been addressed with reference to NG neurons.

The purpose of the present study was to examine the effects of ouabain at a low concentration (1 μM) on the changes in RMP and I_H in neonatal rat small-diameter (<30 μm in diameter) NG neurons using electrophysiological techniques.

Experiments were approved by the Animal Use and Care Committee of Nippon Dental University, and we carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

The acute dissociation of neonatal rat nodose ganglion (NG) neurons was performed by using the same technique as described in previous studies [7,15,28]. In brief, neonatal Wistar rats (6–11 days old, 14–26 g) were deeply anesthetized with pentobarbital sodium (50–60/mg kg, i.p.). Their nodose ganglia were quickly removed and immersed in an ice-cold buffer equilibrated in 100% O_2. The buffer contained (in mM): 120 NaCl, 5 KCl, 0.1 $CaCl_2$, 1 $MgCl_2$, 20 PIPES, 0.1 ascorbic acid and 15 glucose (pH 7.3). The dissected nodose ganglia were incubated for 20–30 min at 35 °C in a PIPES buffer containing collaginase type XI (12 mg/ml, Sigma–Aldrich, St. Loius, MO) and type I (12 mg/ml, Sigma–Aldrich). Single cells were obtained by triturating the suspension through a wide-pore Pasteur pipette and were subsequently plated on the poly-L-lysine pretreated glass

* Corresponding author. Tel.: +81 3 3261 8706; fax: +81 3 3261 8740.
E-mail address: matsu-s@tky.ndu.ac.jp (S. Matsumoto).

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doi:10.1016/j.neulet.2008.05.032

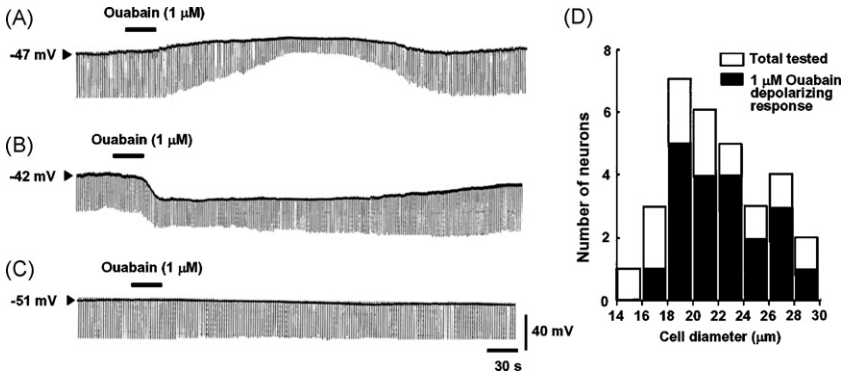

Fig. 1. Effects of ouabain (1 μM) on the resting membrane potential and input resistance. (A) Depolarization with decreased input resistance, (B) Hyperpolarization with decreased input resistance, and (C) No significant effect. (D) Occurrence of ouabain (1 μM)-induced membrane depolarization in small-diameter NG neurons.

cover slips on a 35 mm dish. The plating medium contained Leibovitz's L-15 solution (Invitrogen Corp) supplemented with 10% newborn calf serum, 50 U/ml penicillin–streptomycin (Invitrogen Corp), 26 mM $NaHCO_3$ and 30 mM glucose. The cells were maintained in 5% CO_2 at 37 °C and used for recording between 2 and 10 h after plating. After incubation, the coverslips were transferred to the recording chamber in a standard external solution containing (in mM): 155 NaCl, 3 KCl, 1 CaCl, 1 $MgCl_2$, 10 HEPES and 20 glucose (pH 7.3). The recording chamber (volume = 0.5 ml) was mounted onto an inverted microscope (Nikon, Tokyo, Japan) equipped with phase-contrast filters, video camera, and two micromanipulators. The chamber was perfused under gravity with a standard external solution at approximately 0.5 ml/min.

Electrophysiological recordings were performed using the rapid perforated-patch techniques described previously [24,25] at room temperature (22–25 °C). Fine-polished patch-pipettes (2–5 MΩ) were filled with 120 mM potassium methanesulphonate, 20 mM HEPES and 2 mM EGTA (pH 7.3), containing amphotericin B (100 μg/ml).

The hyperpolarization-activated current (I_H) was defined in response to Cs^+ application, and was produced by the application of the step pulses between −120 and −40 mV from the holding potential (HP) of −60 mV in 10 mV increments. The amplitudes and the rates of increase in the absolute current increased along with depolarization. To evaluate changes in the cell membrane resistance during recordings in the current-clamp mode, negative current pulses (50–600 pA, 250 ms, 0.2 Hz) were injected through the patch pipette. Access resistance did not change significantly throughout the experiments. Voltage-clamp recordings were conducted using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA, USA). The signals were low-pass-filtered at 1–5 kHz and digitized at 10 kHz. The data were stored on a computer disk for off-line analysis. Data were analyzed using one-way ANOVA followed by Duncan's new multiple range test. P values less than 0.05 were considered statistically significant.

Ouabain was obtained from Funakoshi Co. Ltd. (Tokyo, Japan). One hundred milligram of ouabain was dissolved in the external solution and stored at −20 °C.

To determine how ouabain application altered the activity of NG neurons, we examined changes in the resting membrane potential (RMP) of 31 small-diameter (25.7 ± 2.1 μm) NG neurons in response to 1 μM ouabain application. The RMP recorded in the whole-cell current-clamp mode was 40.2 ± 1.2 mV (n = 31). As shown in Fig. 1A–C, three different RMP responses, such as depolarization (A), hyperpolarization (B) and no effect (C), were observed after 1 μM ouabain application. Approximately, 64.5% of the 31 NG neurons examined were depolarized after 1 μM ouabain application. In those neurons, the input resistance decreased by 36.8 ± 17.4% (P < 0.05, vs. control), In 3 of the NG neurons (9.7%, 3/31) showing hyperpolarization after 1 μM ouabain application, the input resistance decreased by 22.0 ± 1.0% (P < 0.05, vs. control) and RMP decreased by 22.3 ± 9.4 mV (P < 0.05, vs. control). The remaining 8 NG neurons (25.8%) showed no significant changes in the RMP in response to ouabain application at 1 μM. The histograms in Fig. 1D show the distribution of NG neurons that were depolarized by 1 μM ouabain application. We chose to further examine small-diameter NG neurons.

The inward currents evoked by the hyperpolarizing step pulses consisted of two components: an instantanenous inward current (I_{inst}) and a slow-activating inward current. The I_H was determined by substracting I_{inst} from the total current measured at the end of the pulses (Fig. 2A). This current was observed in the10 neurons tested. By 2 min after 1 μM ouabain application, an increase in the holding current, as expected a depolarizing effect of ouabain, was seen in the instantaneous current jump and I_H substantially increased (Fig. 2B). The peak amplitude of I_H at −120 mV was 229.5 ± 44.7 pA under control conditions

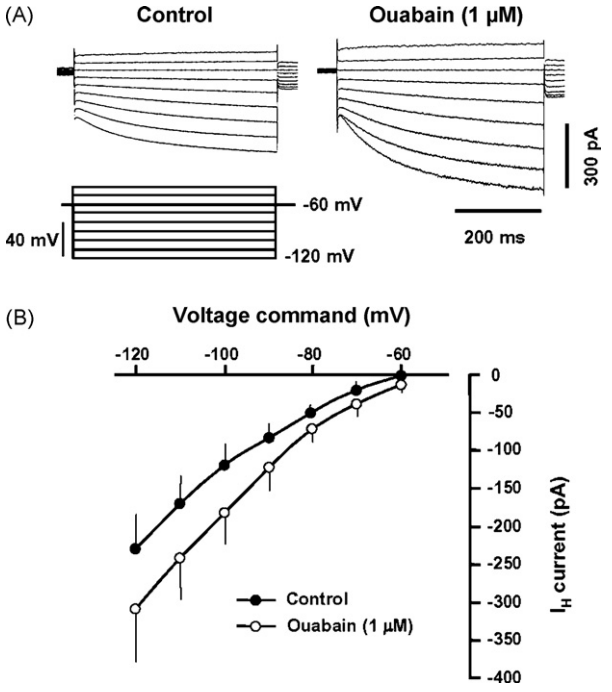

Fig. 2. Effect of ouabain (1 μM) on I_H in small-diameter NG neurons. (A) Example of excitatory effect of ouabain (1 μM). (B) Current–voltage relationship for I_H recorded before (●) and after (○) the application of 1 μM ouabain (n = 10).

and after 1 μM ouabain application, its amplitude increased to 307.7 ± 69.8 pA ($P < 0.05$, vs. control, $n = 10$). The magnitude of I_H was 30.4 ± 3.8% higher following 1 μM ouabain application. Ouabain application had no effect on the I_H of the single remaining neuron.

The Na^+-K^+ pump exports 3 Na^+ for every 2 K^+ imported into the cell [19] and therefore produces an electrogenic outward-hyperpolarizing current that contributes to setting the RMP. In this study, we used a low concentration (1 μM) of ouabain to modulate the Na^+-K^+ pump function. However, ouabain application evoked depolarization in 64.5% (20/31) of the tested NG neurons and the response was associated with a decrease in the cell input resistance, resulting from a depolarizing effect of ouabain. Higashi et al. [6] have demonstrated that ouabain (≥1 μM) application often produced a hyperpolarization accompanied by reduced cell input resistance in rabbit NG neurons and that the afterhyperpolrization elicited by intracellular calcium injection was prolonged by either ouabain (1 μM) or caffeine (10 nM). These results indicate that ouabain application at a concentration of 1 μM can increase calcium-influx via voltage-dependent Ca^{2+} channels during the action potential. The difference between the studies of Higashi et al. [6] and ours may involve that of species. However, in a small population of cells (9.7%, 3/31), we identified ouabain-induced hyperpolarization. This response probably implies that the hyperpolarization seen after 1 μM ouabain application is due to an increase in K^+ conductance, which can reverse in polarity near at the K^+ equilibrium potential. The fact that ouabain application had no significant effect on the RMP in some NG neurons (25.8%, 8/31) leads us to suggest that they may not possess the α_3 Na^+-K^+ ATPase isoform. Because the α_1 Na^+-K^+ ATPase is known to play a major role in the housekeeping process of the cell and because the ouabain concentrations needed for the half-inhibitions of the α_1 and α_3 ATP isoforms are different [3,14,18,22], it is difficult to speculate that ouabain at a low concentration (1 μM) acts predominantly on the α_1 Na^+-K^+ ATPase isoform.

The I_H is known to be active near the RMP level (approximately −60 mV) [16]. It has been reported that time-dependent rectifications of the I_H are seen only in A-type NG neurons, but not in C-type NG neurons [17,21,27]. Doan and Kunze [4], however, found that I_H contributed to setting both the RMP and the neuronal excitability in NG neurons of all sizes. Hyperpolarization of RMP may remove the inactivation of the voltage-gated Na^+, Ca^{2+}, and K^+ channels and as a result, increased the number of channels available for activation during a depolarizing stimulus [4]. Indeed, there is evidence that the I_H was significantly larger than in TG neurons of rats with a chronic constriction nerve injury model of the infraorbital nerve or after transection of the inferior alveolar nerve, as compared with that of naïve rats [11,26]. Furthermore, the amplitude of I_H in adult NG neurons is increased after applications of prostaglandin E_2 (PGE_2) and cAMP analogues [8]. In this study, we found that the application of 1 μM ouabain caused a significant increase in the I_H in all of the neurons examined. Kang et al. [10] have demonstrated bidirectional interactions between Na^+-K^+ pumps and H-channels in a presumed Na^+ microdomain of primary sensory neurons in mesencephalic trigeminal nucleus (MTN). This was based on the fact that hyperpolarization activated and cyclic nucleotide modulated subunits (HCN1/2) and α_3 Na^+-K^+ ATPase isoforms were colocalized in plasma membrane of MTN neurons having numerous spines. However, there is no evidence showing the existence of a Na^+ microdomain, which may be created in numerous spines, on the surface of NG neurons. Further studies are needed to elucidate the possibility as suggested by Kang et al. [10] in MTN neurons. From instantaneous current jumps the holding current of I_H was increased by 1 μM ouabain application, and I_H activated slowly, as seen by the time-course of the slow component of I_H. The former effect is due to a decrease in the cell input resistance. The latter effect reflects faithfully a cation influx at the holding membrane potential. Accordingly, it is possible to speculate that there are some interactions between the Na^+-K^+ pump and I_H to regulate both the RMP level and the neuronal excitability in small-diameter NG neurons.

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