The effects of lidocaine on central respiratory neuron activity and nociceptive-related responses in the brainstem-spinal cord preparation of the newborn rat

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   - Conflicts of Interest: None

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   - Conflicts of Interest: None

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   - Attestation: Hiroshi Onimaru approved the final manuscript, and attests to the integrity of the original data and the analysis reported in this manuscript and is the archival author.
   - Conflicts of Interest: None
Name of Department(s) and Institution(s): Department of Physiology, Showa University School of Medicine

Short Title: Respiratory and anti-nociceptive effects by lidocaine

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Did a Section Editor solicit this submission? Name:

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Abstract

Background: Lidocaine is widely used in the clinical setting as a local anesthetic and anti-arrhythmia drug. Although it has been suggested that lidocaine exerts inhibitory effects on the central and peripheral neurons, there are no reports on its effects on central respiratory activity in vertebrates. In the present study, we examined the effects of lidocaine on respiratory rhythm generation and nociceptive response in brainstem-spinal cord preparations from newborn rats.

Methods: Preparations were isolated from Wistar rats (postnatal day 0-3) and superfused with artificial cerebrospinal fluid equilibrated with 95% O₂ and 5% CO₂, pH 7.4, at 25-26°C. We examined the effects of lidocaine on 4th cervical ventral root (C4)-inspiratory activity, and on the pre-inspiratory (Pre-I) and inspiratory neurons in the rostral medulla. We also examined the effects on the C4/C5 reflex responses induced by ipsilateral C7/C8 dorsal root stimulation, which are thought to be related to the nociceptive response.

Results: The application of low doses of lidocaine (10-20 µM) resulted in a slight increase of the C4 burst rate, while high doses of lidocaine (100-400 µM) decreased the burst rate in a dose-dependent manner, eventually resulting in the complete cessation of respiratory rhythm. High doses of lidocaine decreased the burst duration and negative slope conductance of Pre-I neurons, suggesting that lidocaine blocked persistent Na⁺ current. After the burst generation of the respiratory neurons ceased, depolarizing current stimulation continued to induce action potentials; however, the induction of the spike train was depressed due to strong adaptation. A low dose of lidocaine (20 µM) depressed C4/C5 spinal reflex responses.

Conclusions: Our findings indicate that lidocaine depressed nociception-related responses at lower concentrations than those that induced respiratory depression. Our report provides the
basic neuronal mechanisms to support the clinical use of lidocaine, which shows antinociceptive effects with minimal side effects on breathing.

Number of words: <298 words>
Introduction

Lidocaine, which is widely used as a local anesthetic and anti-arrhythmia drug, inhibits the voltage-gated Na channels by stabilizing their inactivation, thereby blocking neurotransmission. Lidocaine passes through the cell membrane due to its lipid solubility and binds to Na channel proteins from the inside.\(^1\) Lidocaine also crosses the blood brain barrier.\(^2,3\) Although the precise mechanisms behind the effects of lidocaine remain unclear, the systemic administration of low-dose lidocaine via continuous intravenous infusion has been shown to relieve cancer pain, chronic pain, pain due to adiposis dolorosa, and pain after surgery.\(^4-5\).

In many types of central neurons, including the cerebellar Purkinje neurons, the voltage-dependent Na\(^+\) channels, the kinetics of which differ from those of the fast Na\(^+\) channels, have been reported to produce inward currents, called persistent Na\(^+\) currents, and maintain long-lasting cellular depolarization.\(^6-7\) The persistent Na\(^+\) currents have various physiological functions, such as burst generation in many types of neurons (including respiratory burst generation).\(^8,9\) Recently, the currents have also been suggested to play an important role in Na\(^+\) channel-related diseases, such as epilepsy.\(^10-11\) Lidocaine has previously been shown to suppress persistent Na\(^+\) current.\(^12\)

Although it has been reported that the intravenous application of lidocaine induced the depression of respiratory activity,\(^13,14\) the central mechanisms are unknown. Interestingly, Onizuka at al.\(^15\) reported that lidocaine induced the excitation of respiratory pacemaker neurons in invertebrates through disinhibition. However, there are no reports concerning the effects of lidocaine on rhythm generating neurons in the respiratory center of vertebrates. We hypothesized that, at certain doses, the central application of lidocaine could induce antinociceptive effects.
without inducing respiratory depression. In the present study, we examined the effects of
lidocaine on respiratory rhythm generation in brainstem-spinal cord preparations from newborn
rats - an in vitro model which has previously been used to analyze respiratory control.\textsuperscript{16} To
assess the antinociceptive effects of drugs, it has been established that the slow ventral root
potential induced by ipsilateral dorsal root stimulation in the isolated (typically lumbar) spinal
cord of newborn rats reflects the nociceptive reflex. This in vitro experimental model is useful
for assessing the actions of analgesics.\textsuperscript{17-18} We also examined the effects of lidocaine on reflex
responses in the spinal cord, which are presumed to indicate a nociceptive response.\textsuperscript{19}
Methods

Preparation and solutions

Brainstem-spinal cord preparations from Wistar rats (postnatal day 0-3) were isolated under deep isoflurane anesthesia. The experimental protocols were approved by the Animal Research Committee of Showa University, which operates in accordance with Law No. 105 for the care and use of laboratory animals of the Japanese Government. The preparations were cut transversely at a level just rostral to the anterior inferior cerebellar artery. Preparations were superfused continuously at 2.5-3 ml/min in a 2 ml chamber with artificial cerebro-spinal fluid (ACSF), composed of (in mM) 124 NaCl, 5.0 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgCl2, 26 NaHCO3, 30 glucose, equilibrated with 95% O2 and 5% CO2 at a pH of 7.4 and maintained at a temperature of 25-26°C. Inspiratory activity corresponding to phrenic nerve activity was monitored from the fourth cervical ventral root (C4). Lidocaine (Sigma-Aldrich, Tokyo, Japan) was stocked as a 100 mM solution in dimethyl sulfoxide and was kept at 4°C. Lidocaine was dissolved with the above-described ACSF and bath-applied. To assess the effects of lidocaine on C4 activity, the burst rate (bursts/min) was calculated from the mean rate for 3-5 min.

To simultaneously evaluate the effects of lidocaine on respiratory activity and putative nociceptive responses, the ipsilateral C7/C8 dorsal root was stimulated using a glass suction electrode and the induced reflex response was recorded from C4/C5 together with rhythmic inspiratory activity through a 0.5 Hz high pass filter. The dorsal root was stimulated every 10 s with a 5-20 V, 200 µs square pulse. Dorsal root stimulation within the interburst interval induced premature C4 inspiratory bursts if the stimulus intensity was high. Therefore, in the simultaneous recordings of C4 inspiratory activity and reflex responses in the intact brainstem-spinal cord
preparation, we carefully adjusted the stimulus intensity for the induction of reflex responses such that the amplitude of the reflex responses was large enough to detect but not so high that it induced premature C4 inspiratory bursts. In some experiments, spinal cord preparations of the C4-Th2 level were isolated (thus they did not include the medulla) and used to test the above-mentioned reflex response. We measured the peak amplitude of the reflex response, which corresponded to a short latency oligosynaptic response.\textsuperscript{19} With regard to the effects of the lidocaine on the reflex response, there was no significant difference between the preparations that included and did not include the medulla. We therefore accumulated and analyzed data from both preparations. However, because it was difficult to completely exclude the effects of dorsal root stimulation on C4 inspiratory burst rate in the experiments in which the reflex responses were tested, thus the results of these experiments were excluded from the group data on the dose-dependent effects on the C4 respiratory rate.

\textit{Whole-cell patch-clamp recording and histological analysis}

The membrane potentials of the pre-inspiratory (Pre-I) and inspiratory neurons in the rostral ventrolateral medulla corresponding to the caudal part of the pFRG, in which respiratory neurons have been recorded in a number of previous studies,\textsuperscript{21,23} were recorded by a blind whole-cell patch-clamp method with a high input impedance-DC amplifier (CEZ-3100; Nihon Koden, Tokyo, Japan).\textsuperscript{20,24} The electrodes, which had an inner tip diameter of 1.2-2.0 μm and a resistance of 4-8 MΩ, were filled with the following pipette solution (mM): 130 K-gluconate, 10 EGTA, 10 HEPES, 2 Na₂-ATP, 1 CaCl₂, and 1 MgCl₂, with pH 7.2-7.3 adjusted with KOH. We analyzed the membrane potential, input resistance, burst duration, and drive potential of the Pre-I and inspiratory neurons.\textsuperscript{23} The magnitude of the drive potential was determined as the voltage
difference between the resting membrane potential in the interburst phase and the peak of the depolarization plateau during the burst phase (Table 1). In the control solution, current pulses (amplitude 10-100 pA, duration 0.5 s) were injected around the middle of the interburst period to examine the firing properties of neurons in response to a depolarizing pulse and to estimate the input resistance with a hyperpolarizing pulse.

Under the blockade of the potassium and calcium channels, the detection of negative slope conductance is thought to be an indicator of the presence of persistent Na+ current.23,26-27 In some experiments, we analyzed negative slope conductance in response to depolarizing voltage-ramp stimulation under voltage clamp conditions using a continuous voltage-clamp amplifier (current-voltage converter type) (Axopatch 1D, Axon Inc., Foster City, CA, USA). In this experiment, electrodes were filled with the following (potassium channel blockade) pipette solution (mM): 100 CsCl, 20 TEA-Cl, 11 K-BAPTA, 4 Na2-ATP, 1CaCl2, 2 MgCl2, 10 Hepes, and 0.5% Lucifer Yellow (lithium salt), with a pH of 7.2-7.3 adjusted with NaOH.28 After the establishment of whole cell recordings, we added 0.1 mM CdCl2 into the external solution to block the calcuim channels. C4 activity disappeared within 10 min. The cell was then clamped at -70 mV. To detect negative slope current, we tested the slope of ramp stimulation in the range of 10-50 mV/s. Na+ current contamination, i.e. transient, unclamped action potential-generating Na+ current which appeared as downward spikes in the current trace (see Results)26 was observed in most cases under all of the tested slope conditions (under our experimental conditions). This was presumably because the space clamp was insufficient for the large dendritic field of these respiratory neurons.9 In most cases, we used 46.7 mV/s-ramp stimulation because the negative slope component was clearly detectable, despite the contamination of the fast Na+ current.
For the histological analysis of the recorded cells, the electrode tips were filled with 0.5% Lucifer Yellow (lithium salt). After the experiments, preparations were fixed overnight at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS), transferred into 18% sucrose/PBS and cut into 50 μm-thick transverse sections. Neurons were visualized with Lucifer-Yellow staining under a fluorescence microscope (BX60, Olympus Optical, Tokyo, Japan), and photographed. We confirmed that most of the neurons in the intracellular-recordings were located in the caudal part of the pFRG, which corresponded to the level within ±100 μM rostro-caudal to the caudal end of the facial nucleus.23

Data analysis

The sample size was determined based on general consensus of in vitro studies for basic science in which statistical significance could be achieved with the sample size more than 5 in every experiment19,21,24, but all efforts were made to minimize the number of animals. All of the data analyses were performed using the LabChart 7 Pro software program (ADInstruments, Castle Hill, Australia). Data are presented as the mean and standard deviation (SD) for all preparations. The significance of the values was analyzed by paired t-test for effects on C4 burst rate, membrane parameters and the peak current in the negative slope (considering statistically significant with a 2-tailed P value <0.05) or a one-way ANOVA followed by a Tukey-Kramer multiple comparisons test for spinal reflex response (GraphPad InStat; GraphPad Software Inc., La Jolla, CA, USA) at a confidence level of P < 0.05.
Results

The effects of lidocaine on C4 inspiratory activity

We first examined the effects of lidocaine (2-400 μM, n=58) on the C4 burst rate. The 15 min bath application of lidocaine (100-400 μM) induced a dose-dependent decrease in the C4 burst rate (Fig. 1A-D). C4 bursts were completely blocked by the 15 min application of 400 μM of lidocaine (Fig. 1G). In contrast, the application of lower concentrations of lidocaine (10-20 μM) tended to increase the C4 burst rate (Fig. 1E-G). After lidocaine washout (100-200 μM) in 63% preparation, the burst rate showed a partial recovery and the bursts changed into an episodic pattern which consisted of multiple (3-6) short discharges (Fig. 2).

The effects of lidocaine on the membrane potentials

We examined the effects of lidocaine (100-400 μM) on the membrane potentials and burst activity of 8 Pre-I and 14 inspiratory neurons in the caudal pFRG (Table 1). The burst duration of the Pre-I and Post-I phases of Pre-I neurons decreased in a dose-dependent manner after the application of 100-400 μM lidocaine (Fig. 3, Table 1). The burst activity vanished irreversibly after the application of 400 μM lidocaine (Fig. 3). The burst duration of inspiratory neurons tended to decrease after the application of lidocaine (>100 μM); however, these changes did not reach statistical significance due to both the large degree of variation and the small sample size (Table 1). The application of 400 μM lidocaine resulted in the irreversible cessation of inspiratory burst generation (Fig. 4). The driving potentials of both neurons also tended to decrease in a dose-dependent manner, but the change did not reach statistical significance (Table...
We did not find significant change in the membrane potentials and the input resistances of neurons in response to lidocaine treatment.

After the cessation of C4, Pre-I (n=5) and inspiratory (n=8) neuron burst activities in response to the application of 400 μM of lidocaine, action potentials continued to be induced during membrane depolarization by current pulse injection (500 ms). However, the number of induced action potentials was decreased in comparison to control, as the induction of action potentials was limited to the initial part of the depolarization by the stimulation pulse (Fig. 5A).

Since lidocaine reduced the burst duration of Pre-I neurons (as shown by the above results), we examined the effects of lidocaine on negative slope conductance under voltage clamp conditions in Pre-I neurons (n=5). A representative trace from a Pre-I neuron is illustrated in Fig. 5B. The activation of persistent Na+ current was reflected by negative slope conductance on the current-voltage plot. The average peak inward current, -43.8 ± 11.4 pA (n=5), was obtained by subtracting the linear leak current from the total current. The application of lidocaine (100 μM) for 15 min resulted in a considerable reduction of the inward deflection in comparison to control (51.1 ± 11.0%, P = 0.00074 by paired t-test), indicating that the lidocaine-sensitivity of the Na+ current might underlie the conductance.

The effects of lidocaine on spinal reflex responses

The dorsal root stimulation of C7/C8 induced reflex responses in the C4/C5 ventral root, which typically lasted for 0.5-1 s and which were presumed to be oligosynaptic responses (Fig. 6).19 The effects of lidocaine on C4/C5 reflex responses that were induced by ipsilateral C7/C8 dorsal root stimulation were examined in 11 preparations at concentrations of 5 or 20 μM. The
amplitude of the C4/C5 inspiratory nerve activity did not change in response to the application of
lidocaine at these ranges of concentration. However, as shown in Fig. 6, the amplitude of the
C4/C5 reflex responses decreased after the application of 20 μM lidocaine. The depressing
effects partially recovered 20-30 min after washout (Table 2).

Discussion

Low doses of lidocaine (10-20 μM) tended to increase the C4 burst rate, while high doses of
lidocaine (100-400 μM) decreased the C4 burst rate in a dose-dependent manner and then
blocked the burst activity completely. The burst duration of Pre-I neurons decreased in a dose-
dependent manner after the application of high doses of lidocaine (100-400 μM). After the
cessation of Pre-I and inspiratory neuron burst generation was induced by a high dose of
lidocaine, depolarizing current stimulation continued to induce action potentials, whereas the
induction of the spike train was depressed due to strong adaptation. The application of lidocaine
(100 μM) reduced the negative slope conductance, suggesting the partial blockade of persistent
Na⁺ current. After the washout of lidocaine, the C4 inspiratory burst structure gradually
transformed into an episodic burst pattern in which one burst was composed of multiple short
discharges. A low dose of lidocaine (20 μM) had inhibitory effects on the C4/C5 spinal reflex
response induced by the ipsilateral dorsal root stimulation of C7/C8, which was presumed to
reflect, at least in part, a nociceptive response.

Our findings suggest that the respiratory depression that is induced by high doses of
lidocaine is due to the inhibitory effect on the burst generation of Pre-I and inspiratory neurons.
In contrast, the detailed mechanisms behind the slight facilitation of respiratory rhythm by low
doses of lidocaine are not clear. Onizuka et al. reported that lidocaine facilitated molluscan
respiratory rhythm through the inhibition of the GABA system. Lidocaine has also been reported to cause the inhibition of the mammalian central inhibitory system. This mechanism might partially explain the facilitatory effects that were induced by low doses of lidocaine in the present study.

Lidocaine blocks the voltage-gated Na⁺ channels. It is thought that lidocaine penetrates the cell membrane and then binds to the cytoplasmic side of the channel. Studies in dorsal root ganglia have suggested that persistent Na⁺ current is blocked by a low concentration of lidocaine (10 μM) and that transient Na⁺ current is blocked by high concentrations of lidocaine (5 mM). In the present study, we confirmed that lidocaine (100 μM) depressed persistent Na⁺ current and decreased the burst duration of Pre-I neurons. These inhibitory effects may be the main reason for the inhibition of respiratory rhythm by lidocaine.

After the washout of bath-applied lidocaine (100 – 200 μM), we found that the structure of C4 inspiratory bursts gradually transformed into an episodic burst pattern in which one burst was composed of 3-6 short discharges (Fig. 3). A similar cluster-type burst pattern was also induced by treatment with riluzole. Lidocaine and riluzole both decreased the repetitive firing of action potentials during depolarizing stimulation in Pre-I and inspiratory neurons. We hypothesize that the depression of repetitive firing by lidocaine may cause the induction of the cluster-type burst pattern, whereas it is not clear how blockade of persistent Na⁺ current contributes to the alteration of the C4 burst pattern and the blockade of repetitive firing. It is known that the effects of lidocaine are short-lived under in vivo condition. The slow recovery from the effects of lidocaine on respiratory rhythm and reflex responses after washout that were observed in the present study may imply that the removal of intracellular lidocaine is difficult under the present experimental conditions.
The clinical use of systemically-administered lidocaine for pain treatment (post-operative pain relief) was first introduced in 1961. Since then, the systemic administration of lidocaine has been increasingly reported in the management of neuropathic pain. Clinical and experimental evidence indicates that the effective plasma concentration of lidocaine for the management of chronic pain is 1-2 µg/ml, which is equivalent to 3.5-7.0 µM /l. Lidocaine produces analgesia through the blockade of the peripheral and central Na⁺ channels. Studies in animal preparations have suggested a link between spontaneous ectopic discharges of the injured nerve and the peripheral mechanisms of neuropathic pain, and indicate that such spontaneous discharges can be suppressed by the intravenous administration of lidocaine at concentrations well below those that are necessary to produce conduction blockade in nerves. Our findings indicated that the concentration of lidocaine that was required to depress nociceptive-related responses (20 µM) was lower than that which induced respiratory depression. This concentration was slightly higher than the plasma concentration required for the effective management of chronic pain (see above). Although the results from experiments with in vitro preparations cannot simply be expanded to understand the effects of drugs in human clinical use or in adult in vivo preparations, our findings provide the basic neuronal mechanisms that support the clinical use of lidocaine, which shows antinociceptive effects with minimal side effects on breathing.
References


23. Ballanyi K, Ruangkittisakul A, Onimaru H. Opioids prolong and anoxia shortens delay between onset of preinspiratory (pFRG) and inspiratory (preBotC) network bursting in newborn rat brainstems. Pflugers Arch 2009;458:571-87.


Tables

Table 1. The effects of lidocaine on pre-inspiratory (Pre-I) (A) and inspiratory (Insp) neurons (B)

(A)

<table>
<thead>
<tr>
<th>Pre-I</th>
<th>Control (n=8)</th>
<th>Lidocaine 100 µM (n=5)</th>
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<tbody>
<tr>
<td>Pre-I phase duration (sec) (a)</td>
<td>0.412 ± 0.188</td>
<td>0.330 ± 0.165</td>
</tr>
<tr>
<td>Post-I phase duration (sec) (b)</td>
<td>2.677 ± 0.983</td>
<td>1.243 ± 1.292</td>
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<tr>
<td>Pre-I + Post-I phase (sec) (a+b)</td>
<td>3.089 ± 0.993</td>
<td>1.573 ± 1.402</td>
</tr>
<tr>
<td>Drive potential (mV) (c)</td>
<td>9.9 ± 7.6</td>
<td>7.7 ± 4.7</td>
</tr>
<tr>
<td>Inspiratory inhibition (mV) (d)</td>
<td>-0.9 ± 6.8</td>
<td>-3.0 ± 5.9</td>
</tr>
<tr>
<td>Membrane potential (mV) (V)</td>
<td>-46.8 ± 3.6</td>
<td>-46.7 ± 4.7</td>
</tr>
<tr>
<td>Input resistance (MΩ) (R)</td>
<td>422 ± 146</td>
<td>479 ± 235</td>
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(B)

<table>
<thead>
<tr>
<th>Insp</th>
<th>Control (n=14)</th>
<th>Lidocaine 100 µM (n=4)</th>
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<tr>
<td>Insp phase duration (sec) (e)</td>
<td>1.465 ± 0.772</td>
<td>0.718 ± 0.159</td>
</tr>
<tr>
<td>Drive potential (mV) (f)</td>
<td>14.528 ± 3.725</td>
<td>14.435 ± 3.892</td>
</tr>
<tr>
<td>Membrane potential (mV) (V)</td>
<td>-46.4 ± 4.9</td>
<td>-46.0 ± 5.3</td>
</tr>
<tr>
<td>Input resistance (MΩ) (R)</td>
<td>407 ± 189</td>
<td>486 ± 125</td>
</tr>
</tbody>
</table>

In some of the preparations, a second application of lidocaine was tested at different concentrations (from 100 to 400 µM or from 200 to 400 µM) following a recovery period of 15-20 min after the first application. * P<0.05; compared with the control values by paired t-test.
(P=0.0015 in “a”, P=0.0056 in “b”, P=0.0046 in “a+b”, P=0.0197 in “e”, P=0.057 in “c”, P=0.059 in “f” at 400 μM).
Table 2. The effects of lidocaine on the spinal reflex response

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Lidocaine (15 min)</th>
<th>Washout (20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µM (n=5)</td>
<td>74.2 ± 20.3%</td>
<td>110.3 ± 36.8%</td>
</tr>
<tr>
<td>20 µM (n=6)</td>
<td>59.3 ± 18.5% (P = 0.0415)</td>
<td>77.4 ± 25.0%</td>
</tr>
</tbody>
</table>

The peak amplitude of the response is given as a percentage of the control value.

Adjusted P-values were calculated by a Tukey-Kramer multiple comparisons test after a one-way ANOVA (F(2, 5) = 5.526, P = 0.0242). Normality test P values were > 0.10.
Figures and Illustrations
Figure Legends

Figure 1. The effects of lidocaine on C4 inspiratory activity.

A, The changes in the C4 trace in response to the application of 200 and 300 μM lidocaine. B, A faster sweep representation of C4 activity in control. C, The activity after the 15 min application of 200 μM of lidocaine. D, The activity after the 15 min application of 300 μM of lidocaine. E, The effects of 20 μM lidocaine on C4 activity. F, The change in the C4 inspiratory burst rate in response to the application of 20 μM of lidocaine, calculated from the data in E. Note the increase in the C4 rate. G, The dose-dependency of the effects of lidocaine on C4 burst rate. Each bar represents the % values after 15 min application (in comparison to control). Results were made from a total of 58 preparations (5-15 in each condition). Note that the application of low-concentration lidocaine facilitated respiratory rhythm, while high-concentration lidocaine reduced respiratory rhythm. *P<0.05 and ***P<0.001; compared with the control values by paired t-test [P=0.167 at 2 μM (n=5), P=0.077 at 10 μM (n=6), P=0.020 at 20 μM (n=9), P=0.093 at 50 μM (n=8), P=0.013 at 100 μM (n=15), P=0.00014 at 200 μM (n=9), P=0.00016 at 400 μM (n=6)].

Figure 2. The change of the C4 burst pattern after lidocaine treatment. A, Control. B, After the 15 min application of 200 μM lidocaine. C, The C4 burst pattern after 15 min of washout following the 15 min application of 200 μM of lidocaine. Note the appearance of a cluster-type burst pattern that consists of four or five repetitive short discharges.

Figure 3. The effects of lidocaine on a Pre-I neuron in the rostral ventrolateral medulla corresponding to the caudal parafacial region. A, The slower sweep representations of the membrane potential (Vm) and C4 activity in response to the application of 200 μM of lidocaine and the subsequent reapplication of 400 μM of lidocaine. B, The faster sweep representations of the membrane potential trajectory in control. C, The activity after the 15 min application of 200 μM lidocaine. Note the significant decrease in the burst duration of the Pre-I neurons.
Figure 4. The effects of lidocaine on an inspiratory neuron in the rostral ventrolateral medulla corresponding to the caudal parafacial region. A, The slower sweep representations of the membrane potential (Vm) and C4 activity in response to the application of 200 µM of lidocaine and the subsequent reapplication of 400 µM of lidocaine. B, The faster sweep representations of the membrane potential trajectory in control. C, The activity after the 8 min application of 200 µM lidocaine. In this case, 200 µM lidocaine caused the cessation of inspiratory activity (which partially recovered after washout).

Figure 5. Changes in membrane excitability after lidocaine treatment. A, The firing properties of respiratory-related neurons. Data from 8 inspiratory neurons. The numbers of action potentials induced by 500 ms depolarizing current pulse stimulation (20 -160 pA) are plotted according to the current intensity. Solid circles, control. Open circles, with 400 µM lidocaine. Inset: responses to 50 pA depolarizing current pulses in control (upper) and with 400 µM lidocaine. Note that lidocaine depressed the induction of the spike train in response to membrane depolarization. B, Negative slope conductance in preinspiratory (Pre-I) neurons. Upper trace, ramp voltage command (46.7 mV/s; -70 to 0 mV/1.5 s). Lower traces, current responses. Dark line: before the application of lidocaine. Grey line: after the 15 min application of 100 µM of lidocaine. Note that lidocaine significantly suppressed the negative slope component, although it was less effective at suppressing transient, unclamped action potential-generating Na+ current which appeared as downward spikes in the current trace. The double-headed arrow denotes the approximate point of the measurement of the peak current in the negative slope. The dotted line shows the extrapolation of a passive leak current trace.

Figure 6. The effects of lidocaine on spinal reflex response and respiratory burst activity. A, The time course of C4 inspiratory rate (solid squares), amplitude change in peak values of inspiratory C4 burst activity (open circles) and C4 reflex responses (solid circles) following the application of 20 µM of lidocaine. Note the slight increase of C4 burst rate and the decrease of C4 reflex responses. B, Traces of C4 inspiratory activity (left) and reflex responses induced by C8 dorsal root stimulation (right). B-1, control. B-2, after the application of 20 µM of lidocaine. All traces are averaged from 5 cycles.
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- Conclusions
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Manuscript Title: The effects of lidocaine on central respiratory neuron activity and nociceptive-related responses in the brainstem-spinal cord preparation of the newborn rat

First Author: Tomoharu Shakuo

Disclosing Author: Hiroshi Onimaru

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**Figure 1**

A. 200 μM and 300 μM concentrations with 5 min recordings.

B-C. Control and 200 μM lidocaine (15 min).

D. 300 μM lidocaine (15 min).

E. 20 μM lidocaine with 5 min recording.

F. Graph showing C4 rate (bursts/min) over 60 min with 20 μM lidocaine.

G. Bar graph showing change in C4 burst rate with different lidocaine concentrations.
A  Control

B  200 µM lidocaine (15 min)

C  15 min after washout of 200 µM lidocaine
Figure 3

A  

B Control

C 200 µM lidocaine (15 min)
Figure 4

A

Vm

200 µM

400 µM

mV

5 min

C4

B  Control

Vm

C4

C  200 µM lidocaine (8 min)

Vm

C4
原 善

小児における術前経口補水液と
炭水化物含有飲料の比較
——無作為化比較試験——

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鈴木 尚志 世良田和幸

昭和大学新潟病院こどもセンター・小児外科
田山 愛 大橋 祐介
鈴木 孝明 渡井 有実

要約：定期手術をうける患者は、術当日を絶飲食とする術前管理が従来一般であった。これに対し、炭水化物含有飲料を術前に摂取させると、術後の回復が促進されることが、欧州を中心とした近年の研究で明らかになった。本邦でも術前に飲料を摂取させる施設が増えてつつあるが、この管理方法に関する小児での検討は少なく、本邦で入手可能な飲料も欧州と異なる。今回、小児の定期手術を対象とし、飲料の術前摂取を許可した際の、摂取状況、麻酔導入時における脱水および凍結の発生状況と誤嚥の危険性などの検討を目的に、以下の要領で無作為化比較試験を行った。腹部縫合根治術ヘルニア手術が予定された1歳から10歳までの小児120名を無作為に5群に割り付け、各群34例とした。炭水化物を25%含有する經口補水液であるオーエスワン®（OSB）、OSBと同一の熱量と電解質組成を保ちながらゼリー状であるオーエスワン®ゼリー（OSJ）、アルギニンを含有し、炭水化物も18%含有する栄養飲料であるアルジェネード®ウォーター（ArgW）、水道水（TW）のみのいずれかの飲料を許可した4群に加え、無飲食（NPO）を続ける患者群の計5群を設定した。いずれの患者群も、術当日0時からNPOとしたが、飲料を許可した4群では手術室入室予定時刻の2〜3時間前に、体重が15kg未満の場合は最大100 ml、体重が15 kg以上の場合は最大200 mlを患児の要請に応じて飲用させた。麻酔の導入、維持はセボフランで行い適宜フェンタニルも併用した。静脈路確保時に対脈を測り、気管挿管後に腹部超音波検査によって胃幽門部断面積（CSA）を計測し、胃管の挿入により胃内容を吸い、計量した。血液および尿の試料は、遊離脂肪酸（FFA）、血清総タンパク、血清アルブミン、血糖および尿比重の測定に供した。統計学的検定は、一元配置分散分析法およびScheffeの方法による多重比較検定と、Spearmanの順位相関係数を用いた。有意水準はp < 0.05とした。飲料摂取を許可した4群の実際の摂取量に差はなかった。一方、胃内容量は患者による差異が多く、その平均値はArgW群が他群に比べ、約3倍と突出していた。CSAは群間に差がなく胃内容量も同様であった。FFAはArgW群が有意に低く、尿比重はNPO群がOSJ群に対してのみ有意に高かった。使用した3種の飲料に対する患児の受容と脱水の予防効果は水道水と同様と考えられたが、FFAから評価した食餌の予防効果はArgWのみに認められた。浸透圧や熱量が高い飲料は胃内滞留時間の延長傾向が指摘されているが、小児を対象とした本研究の結果も、これを支持した。したがって、本邦でも浸透圧や熱量が高い飲料はArgWよりも低く、OSBあるいはOSJよりも高い製品の発売が望まれる。現時点で対応可能な方策としてArgWの希釈が考えられるが、至適術後栄養は今後の検討課題となる。

キーワード：小児、経口補水、炭水化物負荷、ERAS
小児における術前飲料の比較

患者の術後回復強化を目的に、ESVEN（European Society for Clinical Nutrition and Metabolism）によりERAS（enhanced recovery after surgery）プログラムが開発されている。近年では本邦においても多くの施設で術前期管理にこのプログラムが組み込まれつつある。その導入項目として術前および術後の経食飲食時間の短縮や、術前の炭水化物負荷などが含まれる1)。ASA（American Society of Anesthesiologists）の術前経食飲料ガイドライン2)や、日本麻酔科学会による術前経食飲料ガイドライン3)によれば、小児においても術前2時間前までの清禁水の摂取が許可されていることから、術前の積極的な飲料摂取は小児で有益性を評価すべきと考えられる。これに対して報告は少ないが、特に小児では適切な飲料を選択し、摂取することで、術前の口渴感や空腹感によるストレスを軽減することが期待でき、麻酔導入時の不快感を抑制し、導入を円滑にできる可能性がある。現在ERASプログラムで推奨されている12.6％炭水化物含む飲料は本邦では市販されていないため、成人を対象とした場合には経口補水液の1つで、炭水化物を2.5％含有するオースワン®（大塚製薬工業：以下OS-1）や、アルギニン製錬飲料で、炭水化物を18％含有するアルジェイド®ウォーター（ネスレ日本株式会社：以下ArgW）を術前飲料として用いた運用がなされている。本研究においては全身麻酔手術症例の小児患者を対象に、OS-1、OS-1をゼリー状にした製品であるオースワン®ゼリー（大塚製薬工業：以下OS-1ゼリー）、ArgWまたは水道水のみを摂取した後の、胃内での残存の多寡、脱水の予防効果、飢餓状態の予防効果について、それぞれの飲料を比較した。

研究方法

1. 対象
本研究を開始するに際し、昭和大学横浜病院倫理委員会の承認を得た（受付番号：1100-06）。1歳から10歳までの術前の消化器系下腹部ヘルニア手術（LPEC）が予定された、American Society of Anesthesiologists physical status（ASA PS）I - IIの患者を対象とした。基礎疾患として消化器系、代謝系に何かの疾患を有する症例は除外した。対象となる患者の親または者には研究の目的と安全性について十分説明し、同意を得られた場合に被験者となった。

2. 投与方法
患者は手術前12時間前に絶食とし、手術前第1日朝から2時間前までにOS-1を摂取する群（OSB群）、OS-1ゼリーを摂取する群（OSJ群）、ArgWを摂取する群（ArgW群）、清禁水を摂取する群（TW群）、飲料を摂取しない群（NPO群）の5群に、術前日に無作為に割り付けをし、各飲料の主成分はTable 1に記載した。飲料摂取は術前1日朝に常温で保存される飲料を、体重15kg未満の場合には100mlを上限として、体重15kg以上の場合に200mlを上限として、要求に応じて摂取させた。

3. 麻酔方法
手術室入室予定時刻の1時間前にジアゼパムシロップ0.5mg/kg、経口投与した。飲料摂取の終了から2時間以内に麻酔を起動した。全身麻酔を確認後、アトロビン0.01mg/kg、フェンタニル2μg/kg、ロクロニウム0.1mg/kgも静脈内に投与した。気管挿管を行い、酸素－空気－セロフレンを用いて麻酔を維持した。手術終了後に最大1ml/kgの0.375％ロピバカインを用い、粘膜波ガイド下で間閉腹直筋隔ブロックを施行した。

4. 検体の採取と体重重量、PVI®の測定
全身麻酔導入時、気管挿管時の確保時に採血し、血糖値、血清総タンパク値（TP）、血清アルブミン値（Alb）、血清遊離脂肪酸値（FFA）を測定した。気管挿管後に、患者がどの群に割り付けられているかを告知されていない麻酔担当医が超音波断層（アロカ社製ProSound SSD-5000、5 MHzコンベックスプローブ）により仰臥位での心窩部断面を、中肝静脉と同一平面上の胃底断面断面積（cross-sectional area：CSA）を計測した（Fig. 1）。CSAは3個ずつ計測し、平均値を検討に用いた。さらに10Frのサフィード®吸引チューブ（テルモ社製）を鼻腔もちくは口腔を介して胃内に留置し、吸引により得られた量を胃内容物量とした。同時にカテーテル尿を採取し、尿比重を計測した。挿刀直前には、仰臥位、人工呼吸中でパルスオキシメータ Radical™（マシモ社製）によりPleth Variability Index（PVI®）を測定した。

5. 統計学的検討
結果は平均値±標準偏差で表し、有意水準に5％以下のもの有意差があるとした。群間比較には一元配置分散分析法
Table 1 Ingredients of beverages for preoperative ingestion

<table>
<thead>
<tr>
<th></th>
<th>OS-1&lt;sup&gt;d&lt;/sup&gt;</th>
<th>OS-1 Jelly&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Arginoid Water&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>10</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>2.5</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Arginine (g)</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>115</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>78</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>2.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>0.6</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>-</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>-</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Osmotic pressure (mOsm/l)</td>
<td>270</td>
<td>545</td>
<td></td>
</tr>
</tbody>
</table>

<sup>*" - " means that it was not mentioned in the ingredient labelings of references.</sup>

Table 2 例が嘔吐をきたした（Table 3）。全症例で明らかな誤嘔や嘔吐をきたすことなく、術後翌日に退院した。

考察

1. 胃内識残の評価方法

本研究では胃内識残の多寡の指標として超音波検査を用いた胃内識残面積（CSA）と、胃内の吸引により胃内容物量を測定する方法を採用した。前者は非侵襲的で簡便だが、客観性や再現性に乏しいという所がある。本研究においては胃内容物量とCSAに関する相関がみられなかった。小児は解剖学的に個体差が大きいので、CSAを用いた胃内識残の評価するためには、飲料摂取前にもCSAを測定し、麻酔導入時のCSAと相対的に比較するなど工夫が必要と考える。胃管を用いた胃内容物量の測定は全身麻酔下では低侵襲で、CSAの計測より客観性が高い。他にも放射線同位元素等を用いた方法<sup>7</sup>や呼気<sup>8</sup>、MRI<sup>9</sup>を用いた方法もあり、いずれも客観性が高いが、安静や検査への協力を得にくいため、小児患者には適応しきにくい。

2. 術前飲料の安全性

成人症例においてはOS-1やArgWの飲料摂取を術前管理の一環として採用している施設もあり、その良好な結果も報告されている<sup>5,10,11</sup>。いずれの報
小児における術前飲料の比較

告においても術前2時間前までの経口摂取の安全性が確認されている。2012年に日本麻酔科学学会により公表された術前飲食ガイドライン30では「複数の研究が最大で体重あたり10mlあるいは無制限を採用しており、患者が飲める範囲内で摂取が可と考えられる」と記載されている。Splinterら6は5〜10歳の小児を対象として、術前25時間前におリンゴジュースを10ml/kgを飲ませた患者群を、経口摂取を禁じた群と比較した。その結果、リンゴジュースを飲用した患者群は、胃液量および胃液pHに変化を及ぼさず、口渴感は有意に減少した。しかし本研究におけるArgW群はSplinterらの研究と同様の摂取量であるにも関わらず、胃内容が残存した。摂取飲料の浸透圧とその胃排出時間には正の相関があると報告されている13が、ArgWの浸透圧は545mosm/Lであり、リンゴジュースの浸透圧である650〜730mosm/Lと比べて低い。

一方、胃から十二指腸へ排出される、ブドウ糖あるいはエネルギーの移動速度が一定になるよう胃と十二指腸の間で何らかのフィードバックにより調節されていることも示されている15。ArgWの熱量は0.80kcal/mlであり、一般的なリンゴジュースのエネルギーの約0.44kcal/mlより高いことが、胃排泄が遅延する原因となっている可能性がある。

また、飲料へグルタミンを添加すると胃排泄が遅延すると報告されている7。アルギニンにおいても同様の現象が生じる可能性がある。

日本麻酔科学会のガイドラインでは「浸透圧や熱量が高い飲料、アミノ酸含有飲料は胃排泄時間が遅くなる可能性がある」10と記載されており、ArgW

Fig. 1 Measurements of cross sectional area of the pyloric region of the stomach
Representative ultrasonic observation for tracing of cross-sectional area of the pyloric region of the stomach was shown.
A: pyloric region of the stomach
B: middle hepatic vein
C: inferior vena cava

Table 2 Patient characteristics

<table>
<thead>
<tr>
<th>Item</th>
<th>OSB (n = 24)</th>
<th>OSJ (n = 24)</th>
<th>ArgW (n = 24)</th>
<th>TW (n = 24)</th>
<th>NPO (n = 24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>4.6 ± 2.3</td>
<td>4.6 ± 2.2</td>
<td>5.0 ± 2.2</td>
<td>4.6 ± 2.2</td>
<td>4.5 ± 1.9</td>
<td>0.738</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>9 / 15</td>
<td>3 / 21</td>
<td>7 / 17</td>
<td>17 / 7</td>
<td>9 / 15</td>
<td>0.278</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>16 ± 5</td>
<td>17 ± 5</td>
<td>18 ± 7</td>
<td>17 ± 5</td>
<td>16 ± 3</td>
<td>0.706</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>103 ± 16</td>
<td>107 ± 15</td>
<td>105 ± 17</td>
<td>104 ± 16</td>
<td>101 ± 13</td>
<td>0.756</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation or number.
There are no significant difference among the groups.
OSB: OS-1
OSJ: OS-1 jelly
ArgW: Argnaid Water
TW: tap water
NPO: non per os

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Table 3 Examination findings

<table>
<thead>
<tr>
<th>beverage</th>
<th>OSB (n = 24)</th>
<th>OSJ (n = 24)</th>
<th>ArgW (n = 24)</th>
<th>TW (n = 24)</th>
<th>NPO (n = 24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingested volume* (ml)</td>
<td>6.7 ± 3.3</td>
<td>8.7 ± 3.3</td>
<td>8.5 ± 3.6</td>
<td>6.9 ± 2.9</td>
<td>-</td>
<td>0.0553</td>
</tr>
<tr>
<td>VGC (ml)</td>
<td>7.0 ± 6.9</td>
<td>9.0 ± 8.4</td>
<td>25.7 ± 26.5**</td>
<td>8.2 ± 8.7</td>
<td>8.0 ± 8.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>VGC (≥ 25ml/ &lt; 25ml)</td>
<td>1 / 23</td>
<td>2 / 22</td>
<td>10 / 14</td>
<td>1 / 23</td>
<td>1 / 23</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>VGC / Body weight (ml / kg)</td>
<td>0.40 ± 0.37</td>
<td>0.54 ± 0.57</td>
<td>1.39 ± 1.37**</td>
<td>0.45 ± 0.43</td>
<td>0.54 ± 0.61</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CSA (cm²)</td>
<td>2.07 ± 1.15</td>
<td>2.02 ± 0.85</td>
<td>1.88 ± 0.77</td>
<td>2.08 ± 1.30</td>
<td>1.82 ± 0.89</td>
<td>0.8689</td>
</tr>
<tr>
<td>CSA (cm²) / BSA (m²)</td>
<td>3.09 ± 1.58</td>
<td>2.97 ± 1.50</td>
<td>2.8 ± 1.45</td>
<td>3.03 ± 1.85</td>
<td>2.77 ± 1.38</td>
<td>0.9398</td>
</tr>
<tr>
<td>Blood glucose (mg / dl)</td>
<td>86.5 ± 10.6</td>
<td>80.8 ± 9.2</td>
<td>84.5 ± 10.7</td>
<td>83.8 ± 8.9</td>
<td>79.2 ± 15.2</td>
<td>0.1807</td>
</tr>
<tr>
<td>Serum total protein (g / dl)</td>
<td>6.46 ± 0.30</td>
<td>6.50 ± 0.39</td>
<td>6.44 ± 0.12</td>
<td>6.50 ± 0.11</td>
<td>6.51 ± 0.11</td>
<td>0.9457</td>
</tr>
<tr>
<td>Serum albumin (g / dl)</td>
<td>4.17 ± 0.26</td>
<td>4.16 ± 0.20</td>
<td>4.14 ± 0.18</td>
<td>4.21 ± 0.17</td>
<td>4.18 ± 0.20</td>
<td>0.7953</td>
</tr>
<tr>
<td>Serum free fatty acid (μEq / l)</td>
<td>1.10 ± 0.42</td>
<td>1.18 ± 0.42</td>
<td>0.58 ± 0.43**</td>
<td>1.13 ± 0.34</td>
<td>1.16 ± 0.54</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Urine specific gravity (g / ml)</td>
<td>1.021 ± 0.007</td>
<td>1.018 ± 0.006***</td>
<td>1.022 ± 0.007</td>
<td>1.019 ± 0.008</td>
<td>1.025 ± 0.005***</td>
<td>0.0011</td>
</tr>
<tr>
<td>PVIR (%)</td>
<td>143 ± 62</td>
<td>118 ± 39</td>
<td>145 ± 38</td>
<td>127 ± 68</td>
<td>136 ± 66</td>
<td>0.5965</td>
</tr>
<tr>
<td>Postoperative vomiting****</td>
<td>1 / 23</td>
<td>0 / 24</td>
<td>0 / 24</td>
<td>1 / 23</td>
<td>2 / 22</td>
<td>0.798</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation or number.

*: estimated ingested volume of beverages

**: statically different among the groups by Sheffe's post hoc test

***: statically different between the both groups

****: vomiting at the time of first oral intake in recovery period

VGC: volume of gastric contents

CSA: cross-sectional area of the pyloric region of the stomach

BSA: body surface area

OS-1, ORS: oral rehydration solution

PVIR: Pletth Variability Index

OSB: OS-1

OSJ: OS-1 jelly

ArgW: Argna1d Water

TW: tap water

NPO: non per os
Table 4 Scheffe's test of Gastric contents volume

<table>
<thead>
<tr>
<th></th>
<th>OSJ</th>
<th>ArgW</th>
<th>TW</th>
<th>NPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSB</td>
<td>0.9938</td>
<td>0.0070*</td>
<td>0.9992</td>
<td>0.9996</td>
</tr>
<tr>
<td>OSJ</td>
<td>-</td>
<td>0.9936*</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>ArgW</td>
<td>-</td>
<td>-</td>
<td>0.0019*</td>
<td>0.0017*</td>
</tr>
<tr>
<td>TW</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are P value. *: significant
OSB: OS-1
OSJ: OS-1 jelly
ArgW: Argnaid Water
TW: tap water
NPO: NPO

Fig. 2 Scatter plot of volume of gastric contents versus CSA
CSA: cross-sectional area of the pyloric region of the stomach
Spearman's rho = -0.070
Correlation is not significant.

Table 5 Scheffe's test of FFA

<table>
<thead>
<tr>
<th></th>
<th>OSJ</th>
<th>ArgW</th>
<th>TW</th>
<th>NPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSB</td>
<td>0.9826</td>
<td>0.0023*</td>
<td>0.9998</td>
<td>0.9963</td>
</tr>
<tr>
<td>OSJ</td>
<td>-</td>
<td>0.0002*</td>
<td>0.9960</td>
<td>0.9999</td>
</tr>
<tr>
<td>ArgW</td>
<td>-</td>
<td>-</td>
<td>0.0013*</td>
<td>0.0005*</td>
</tr>
<tr>
<td>TW</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.9995</td>
</tr>
</tbody>
</table>

Values are P value. *: significant
OSB: OS-1
OSJ: OS-1 jelly
ArgW: Argnaid Water
TW: tap water
NPO: NPO

Table 6 Scheffe's test of Urine specific gravity

<table>
<thead>
<tr>
<th></th>
<th>OSJ</th>
<th>ArgW</th>
<th>TW</th>
<th>NPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSB</td>
<td>0.7805</td>
<td>0.5860</td>
<td>0.3746</td>
<td>0.3851</td>
</tr>
<tr>
<td>OSJ</td>
<td>-</td>
<td>0.4435</td>
<td>0.5835</td>
<td>0.0028*</td>
</tr>
<tr>
<td>ArgW</td>
<td>-</td>
<td>-</td>
<td>0.7931</td>
<td>0.7602</td>
</tr>
<tr>
<td>TW</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1163</td>
</tr>
</tbody>
</table>

Values are P value. *: significant
OSB: OS-1
OSJ: OS-1 jelly
ArgW: Argnaid Water
TW: tap water
NPO: NPO

指標となることが人工呼吸中の小児においても示されている(18). 本研究においては TP, Alb は各群間に差はなかったが、尿比重においてのみ有意差が認められた。PTV(19)においても統計学的有意差を得るにはわずかに至らなかったが、OSJ 群が低い傾向が認められる。これは、OSB 群よりも OSJ 群の方が飲料を飲みやすく、実際に飲料を摂取した量が多い傾向があったことが理由の1つと推測している。したがって、味や飲みやすさを改善し、十分な経口補水をすることができれば、麻酔導入時飲水を予防できる可能性はある。

ArgW は創傷治癒にかかわるとされるアルギニンが強化された。食事あるいは経腸栄養の補助となる食品である(19)。18%の炭水化物を含み、清澄であるため、ERAS プログラムで推奨されている12.6%炭水化物合有飲料に比較的近い。

FFA は飢餓などの生理的ストレス下で著明に上昇する(19). 本研究では手術室入室時に、ArgW 群の FFA が他の群に対して有意に低値であったことから、手術室入室予定時点の 3 時間前から 2 時間前までの、患者の要求に応じた ArgW の摂取は、飢餓による代謝変動を抑制することが示された。

また、成人の乳癌手術後の患者における ArgW の飲用の効果が検討され、その結果 Postoperative Nausea and Vomiting (PONV) の減少が報告されている(20). 小児の乳癌ヘルニア手術において、LPEC は従来の Potts 法よりも PONV の発生頻度が高い(21) ことが知られている。本研究においても ArgW 群では PONV をきたした症例はなかったことから、炭水化物負荷による PONV の抑制効果の有無は今後も
検討すべきだと考えられる。
本研究の結果に基づくと、胃腸の予防と胃内遺残の回遊の両立を目的とした飲料が小児の術前飲料として適切であると考えられる。したがって、本邦においても炭水化物濃度がOSBあるいはOSJよりも高く、ArgWより低い、すなわち25%と18%の中間的な製品の発売が利用可能となることが望まれ、今後では対応可能な方策としてArgWを希釈した利用法が考えられる。さらには細菌に伴う問題点や、高還白アミノ酸含有による胃内遺残の可能性について検討し、それらの飲料を用いて小児患者の口渴感や空腹感を減少させることができることが知られ、評価する必要がある。

利益相反
本研究に関して開示すべき利益相反はない。

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COMPARISON OF PRODUCTS FOR PREOPERATIVE ORAL REHYDRATION THERAPY AND CARBOHYDRATE LOADING IN CHILDREN

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Ai TAYAMA, Yusuke OHASHI, Komei SUZUKI and Yu WATARAI
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Abstract — We compared the effects of different preoperative beverages on children undergoing elective laparoscopic percutaneous extraperitoneal closure for inguinal hernia repair. In this study, 120 low-risk patients (aged 1–10 years) scheduled for surgery were randomly assigned to an oral rehydration liquid (OSB, n = 24) group, oral rehydration jelly (OSJ, n = 24) group, carbohydrate-rich drink (ArgW, n = 24) group, tap water (TW, n = 24) group, or a fasting (NPO, n = 24) group. The patients in the NPO group fasted from midnight the night before surgery. Approximately 2 to 3 hours before entering the operating room, patients in the OSB, OSJ, ArgW, and TW groups consumed ≤ 100 ml (< 15 kg in weight) or ≤ 200 ml (≥ 15 kg in weight) of OS-1, OS-1 Jelly, Arginaid® Water, and tap water, respectively. Patients in the NPO group continued fasting until surgery. Anesthesia was induced by inhalation of sevoflurane and oxygen. When venous access was obtained, we collected blood samples to measure free fatty acid (FFA) levels. After intubation, we measured the volume of gastric contents (VGC) using a gastric tube, and the cross-sectional area of the pyloric region of the stomach (CSA) using ultrasonography. The groups were compared using analysis of variance and Scheffe’s post hoc test. A p value of < 0.05 was considered statistically significant. The volume of the stomach content was notably large for the ArgW group. In the ArgW group, 10 patients had VGC of ≥ 25 ml. CSA did not significantly differ between the groups. FFA during induction of anesthesia was significantly lower in the ArgW group. In children, carbohydrate loading with ArgW may prevent preoperative starvation. However, it may remain in the stomach for more than two hours.

Key words: children, oral rehydration therapy, carbohydrate loading, ERAS

[受理 : 11月5日, 受理 : 11月27日, 2013]
EFFECTS OF OUABAIN ON RESPIRATORY RHYTHM GENERATION IN BRAINSTEM-SPIAL CORD PREPARATION FROM NEWBORN RATS AND IN DECREBERATE AND ARTERIALLY PERFUSED IN SITU PREPARATION FROM JUVENILE RATS

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Abstract—The significance of Na/K-ATPase on respiratory rhythm generation is not well understood. We investigated the effects of the Na/K-ATPase blocker, ouabain, on respiratory rhythm. Experiments were performed with brainstem-spial cord preparation from 0 to 3-day-old Wistar rats and with decerebrate and arterially perfused in situ preparation from juvenile rats (postnatal day 11–13). Newborn rat preparations were superfused at a rate of 3.0 ml/min with artificial cerebrospinal fluid, equilibrated with 5% CO2 and 95% O2, pH 7.4, at 26–27 °C. Inspiratory activity was monitored from the fourth cervical ventral root (C4). Application of ouabain (15–20 min) resulted in a dose-dependent increase in the burst rate of C4 inspiratory activity. After washout, the burst rate further increased to reach quasi-maximum values under each condition (e.g., 163% of control in 1 μM, 253% in 10 μM, and 303% in 20 μM at 30 min washout). Inspiratory or pre-inspiratory neurons in the rostral ventrolateral medulla were depolarized. We obtained similar results (i.e., increased phrenic burst rate) in an in situ perfused preparation of juvenile rats. Genes encoding the Na/K-ATPase α subunit were expressed in the region of the parafacial respiratory group (pFRG) in neonatal rats, suggesting that cells (neurons and/or glia) in the pFRG were one of the targets of ouabain. We concluded that Na/K-ATPase activity could be an important factor in respiratory rhythm modulation. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: ouabain, Na/K-ATPase, respiratory rhythm.

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Abbreviations: ACSF, artificial cerebrospinal fluid; C4, fourth cervical ventral root; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP3, inositol trisphosphate; PBS, phosphate-buffered solution; pFRG, parafacial respiratory group; preBötC, pre-Bötzinger complex; Pre-I, pre-inspiratory.

http://dx.doi.org/10.1016/j.neuroscience.2014.12.006
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INTRODUCTION

Na/K-ATPase (sodium pump) is a plasma membrane protein essential for maintaining Na+ and K+ gradients across the animal cell membrane. Glucose and amino acids, calcium ions, and various neurotransmitters are transported using the Na+ gradients generated by this pump. The ion gradients are also critical for maintaining osmotic balance and cytosolic pH and supporting and modulating the electrical activity of excitable cell membranes. Na/K-ATPase also functions as a signal transducer. Several of the signal transduction pathways through which it works have been reviewed (Zhang et al., 2008). Previous studies in α2 subunit knockout mice demonstrated that normal expression of this protein is essential for the development of the respiratory neuron network in the fetal period (Ikeda et al., 2004; Onimaru et al., 2007). Recently, experiments using Na/K-ATPase blocker have shown the significance of Na/K-ATPase in respiratory rhythm generation in mouse slices preparations, including the pre-Bötzinger complex (preBötC) in the medulla (Del Negro et al., 2009; Krey et al., 2010). The same authors proposed that the Na/K-ATPase pump has dynamic and tonic components, which are important in the regulation of respiratory rhythm. In contrast, there is, to the authors' knowledge, only one report on experiments regarding the effects of Na/K-ATPase blocker in en block preparation (Brockhaus et al., 1993), which includes wider respiratory neuron networks such as the preBötC and the parafacial respiratory group (pFRG).

One of the cardiac glycosides, ouabain, is a specific inhibitor of the α subunits of Na/K-ATPase. In the present study, we examined the effects of ouabain on the respiratory rhythm of brainstem-spial cord preparation from newborn rats. We also examined the effects of ouabain in decerebrate and arterially perfused in situ rat preparation from juvenile rats (postnatal day 11–13). The distribution of α subunits of Na/K-ATPase in the "restricted region" of the rodent brain has been reported using the immunostaining method ( McGrail et al., 1991; Pietrini et al., 1992) and in situ hybridization (Schneider et al., 1998; Hieber et al., 1991). A full, comprehensive study of the distribution of the α3 subunit protein in the adult mouse brain has also been reported (Böttger et al., 2011). Thus far, however, there have been no reports on the detailed expression of the three subunits in the rostral medulla, including the pFRG of neonatal
rats. We therefore examined the distribution of Na/K-ATPase α subunits (α1, α2, and α3) in the rostral medulla of neonatal rats. The goal of the present study is to clarify the effects of Na/K-ATPase activity and α subunit distribution on respiratory rhythm and pattern generation and to verify the involvement of the previously proposed dynamic and ionic components of the Na/K-ATPase pump.

**EXPERIMENTAL PROCEDURES**

**Ethical approval**

Experimental protocols were approved by the Animal Research Committee of the Showa University, which operates in accordance with Law No. 105 of the Japanese Government for the care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

**In vitro preparation**

Experiments were performed with brainstem-spinal cord preparations from 0 to 3-day-old Wistar rats. Newborn rats were deeply anesthetized with isoflurane. The brainstem and spinal cord were isolated and superfused at a rate of 3.0 ml/min with the following artificial cerebrospinal fluid (ACSF) (Suzue, 1984) (in mM): 124 NaCl, 5.0 KCl, 1.24 KH2PO4, 2.4 CaCl2, 1.3 MgCl2, 26 NaHCO3 and 30 glucose, equilibrated with 95% O2 and 5% CO2, pH 7.4, at 26–27°C. The preparations were cut transversely at a level just rostral to the anterior inferior cerebellar artery. Ouabain (octahydrate) was purchased from Sigma–Aldrich (Tokyo, Japan) and was stocked as 10 mM solution in ultrapure water. Drugs were dissolved with the above-described ACSF and bath-applied.

Inspiratory activity corresponding to phrenic nerve activity was monitored from the fourth cervical ventral root (C4). Membrane potentials of pre-inspiratory (Pre-I) or inspiratory (Inap) neurons in the rostral ventrolateral medulla, corresponding to the caudal part of the pFRG in which respiratory neurons have been recorded in many previous studies (Onimaru et al., 2003; Onimaru et al., 2008; Ballanyi et al., 2009), were recorded by a blind whole-cell patch-clamp method (Onimaru and Homma, 1992). The electrodes, which had an inner tip diameter of 1.2–2.2 μm and a resistance of 4–8 MΩ were filled with the following pipette solution (in mM): 130 K-gluconate, 10 EGTA, 10 HEPES, 2 Na2-ATP, 1 CaCl2, and 1 MgCl2, with pH 7.2–7.3 adjusted with KOH. For histological analysis of the recorded cells, the electrode tips were filled with 0.5% Lucifer Yellow (lithium salt). After the experiments, preparations were fixed for 2–3 h at 4°C and 4% paraformaldehyde in 0.1 M phosphate-buffered solution (PBS), transferred into 18% sucrose/PBS and cut into 50 μm-thick transverse sections. Lucifer-Yellow-filled neurons were visualized using a fluorescence microscope (BX60, Olympus Optical, Tokyo, Japan), and photographed. We confirmed that most of the intracellularly recorded neurons were located in the caudal part of the pFRG that corresponded to the level within ±100 μM rostro-caudal to the caudal end of the facial nucleus (Ballanyi et al., 2009).

**Decerebrate and arterially perfused in situ rat preparation**

Experiments in a decerebrate and arterially perfused in situ rat preparation (Pickering and Peton, 2006; Yazawa, 2014) were performed on 12 juvenile Wistar rats (postnatal day 11–13; body weight 24.8–29.3 g). Isoflurane concentration was maintained at 2.0–2.5% and depth of anesthesia was assessed through lack of limb withdrawal to tail pinch. Laparotomy was performed for the removal of the stomach, small and large intestines, spleen, and pancreas. After administration of heparin (10 U/I) via intracardiac injection, the animal was immediately submerged in Ringer’s solution that had been maintained at a temperature of 5–10°C. The Ringer’s solution was composed of (mM): 125 NaCl, 3 KCl, 24 NaHCO3, 1.25 KH2PO4, 1.25 MgSO4, 2.5 CaCl2, and 10 glucose, equilibrated with 96% O2 and 4% CO2, pH 7.4, at 25–27°C. After cardiac arrest, cranialotomy was performed, with decerebration performed with suction at the precollicular level to render insensible level. The lungs were removed and an incision made through the apex of the left ventricle.

While being held in a supine position in the recording chamber, a double-lumen catheter (DL-AS-040; Braintree Scientific, Braintree, MA, USA) was inserted into the heart through an incision in the left ventricle. Arterial perfusion was immediately performed with carbogen-gassed, heparinized (10–20 U/I) Ringer’s solution containing Ficoll-70 (1.28%), an oncotic agent, at 26°C. Subsequently, an incision was made in the right atrium. After resumption of spontaneous breathing, at ≤5 min from the initiation of perfusion at 26°C, the muscle relaxant d-tubocurarine (2 μM) was added to the perfusate to induce immobilization. Using a peristaltic pump (model 323U pump, model 318MC pump head; Watson-Marlow, Wilmington, MA, USA) to generate the perfusion flow, the perfusate was pumped from a reservoir flask through a bubble trap and a nylon net filter. The flow rate was set above 5x the total blood volume (calculated using 1/13 of the body weight as measured in grams) per minute at 26°C. The left phrenic nerve was identified and detached from both blood vessels and connective tissues, and severed at the distal end. Although bradycardia was pronounced at the initiation of perfusion, ventricular fibrillation never developed. Suction electrodes constructed of polyethylene tubing (PE 50; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) were used to record neuronal discharge from the left phrenic nerve. Ouabain was dissolved with the above-described Ringer’s solution for perfused preparations and applied by perfusion.

All data analyses were performed by LabChart 7 Pro (ADInstruments, Castle Hill, Australia). To assess the effects of ouabain on C4 (in the brainstem-spinal cord preparation) or phrenic nerve activity (in the arterially perfused in situ preparation), the burst rate (bursts/min) was calculated from the mean rate for 3–5 min. Burst amplitude and duration of C4 activity or phrenic nerve activity were averaged from 5–10 consecutive...
respiratory cycles. Analysis of the consecutive time course of change in respiratory rate was performed by the peak analysis program of LabChart 7 Pro. We also analyzed membrane potential, input resistance and burst duration (mean for 8–10 cycles) of Pre-I and InsP neurons in the brainstem-spinal cord preparation. Since the burst activity of Pre-I neurons was typically divided into pre-and post-inspiratory phases with inspiratory inhibition, burst duration was calculated as the sum of both phases. Data are presented as mean and standard deviation (SD) for all preparations. The significance of values was analyzed by a one-way ANOVA, followed by a Bonferroni Multiple Comparison Test (GraphPad InStat; GraphPad Software Inc., La Jolla, CA, USA) at a confidence level of P < 0.05.

RNA probes and in situ hybridization and immunofluorescence

in situ hybridization and immunofluorescence experiments were performed on brainstems isolated from three independent Wistar rat neonates. The brainstem was isolated and further fixed at 4°C in fixation solution for 1–2 h. Samples were immersed in 16% sucrose/PBS, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA), then frozen on dry ice, and cut into 12-μm-thick cryosections. We prepared 5 sets of alternate sections. In situ hybridization was performed, as described previously, using three sets of sections (Ikeda et al., 2013), with isoform-specific digoxigenin-UTP (Roche Diagnostics, Basel, Switzerland)-labeled riboprobes for rat Atp1a1, rat Atp1a2, and rat Atp1a3 at 46–48°C (Ikeda et al., 2013). Partial cDNAs of rat Atp1a1 (nucleotide number 1–260 and 3281–3540 of NM_012504), rat Atp1a2 (nucleotide number 1–300 and 3191–3410 of NM_012505), and rat Atp1a3 (nucleotide number 1–214 and 3191–3410 of NM_012506), were obtained by RT-PCR using rat brain total RNA, subcloned into pGEM-T easy Vector (Promega, Madison, WI, USA), and confirmed by sequencing. Proteinase K (1 μg/ml) was applied for 3–4 min at 26°C. Signals were detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche) and NBT/BCIP (Roche) for chromogen. We were not able to detect immunofluorescent signals from Phox2b proteins after in situ hybridization because the administration of proteinase K broke down most of the proteins on the sections. Therefore, one set of alternate sections was provided (out of five sets) for immunofluorescence detection using anti-Phox2b antibody (Onimaru et al., 2008). Alexa fluor 488 anti-guinea pig antibody (Molecular Probes, Invitrogen, Eugene, Oregon, USA) was used as the secondary antibody.

RESULTS

Electrophysiological analyses of in vitro brainstem-spinal cord preparation from newborn rats

The effects of ouabain on C4 burst rate were examined in the concentration range of 0.1–20 μM in 49 preparations. The average C4 burst rate in the control solution was 6.0 ± 1.3 (n = 49). Application of ouabain (15–20 min) resulted in a dose-dependent increase in the burst rate of C4 Inspiratory activity, which was accompanied by a temporal induction of background ionic activity in the C4 nerve. At concentrations of 1–20 μM, initial transient changes of the burst rate (increase and subsequent decrease at 2–6 min after ouabain application) were observed in half of the preparations, before reaching the steady state (e.g. Fig. 2). A typical example of 2 μM ouabain application is shown in Fig. 1. At these higher concentrations, the burst rate tended to increase further after washout by 15–26%, to reach quasi-maximum values under each condition at a 30-min washout. The facilitated frequency was maintained for more than 1 h after washout (Fig. 1Bd). When 20 μM ouabain was applied, the C4 burst disappeared in 4/5 preparations at a 10–40-min washout (Fig. 2). The dose-dependent effect on C4 burst rate is summarized in Fig. 3. There was a notable decrease in burst duration at higher concentrations of ouabain; 65 ± 8.1% (P < 0.001) of control at a 30-min washout, following the application of 10 μM ouabain (n = 6). Since the C4 burst gradually disappeared after washout of 20 μM ouabain, the burst duration was measured just before the disappearance at a 10–30-min washout; 55 ± 16% (P < 0.05, n = 4) of the control. The amplitude (peak value) also tended to decrease after the application of 20 μM ouabain (Fig. 2), although the mean value (41 ± 43% of control; n = 4) was not significant when the value was measured at the
Fig. 2. A typical example of the effects of 20 μM ouabain on C4 activity. Upper traces, raw data; lower traces, integrated C4 activity. Note that a 15-min application of 20 μM ouabain induced strong tonic discharges as well as facilitation of respiratory rhythm. The amplitude gradually decreased and C4 bursts disappeared at 10 min after washout.

Fig. 3. Dose-dependency of ouabain effects on C4 burst rate. Solid bars, at a 15-min application; gray bars, at a 30-min washout. Averages from five to nine experiments for each concentration. Bars show mean values and standard deviations. Significance values compared to control are *P < 0.05, **P < 0.01, and ***P < 0.001.

same timing as the above measurement of burst duration (i.e. before the disappearance of C4 activity at a 10–30-min washout.

We measured membrane potential and input resistance in 6 Pre-I and 5 Insp neurons in the caudal part of the pFRG. Pre-I neurons (Fig. 4) were significantly depolarized in response to application of 1 μM ouabain (Table 1). Insp neurons (Fig. 5) also showed a tendency for depolarization, but the change was not significant. The input resistance did not change significantly. The burst durations of Pre-I and Insp neurons were significantly decreased in response to the application of 1 μM ouabain (Table 1).

Effects of ouabain in decerebrate and arterially perfused in situ rat preparation from juvenile rats

We also examined the effects of ouabain on respiratory activity in arterially perfused in situ preparation of juvenile rats (postnatal day 11–13; n = 8). The burst rates of phrenic nerve activity increased in response to the application of 10 or 40 μM ouabain, reaching 121 ± 17% or 186 ± 58% of control, respectively, after 15 min. The long-lasting effects on the respiratory rate were examined in the case of 40 μM application. After washout, the rate further increased and reached quasi-maximum values (more than 200% of control) at a 30-min washout (Fig. 6, Table 2). The burst duration gradually increased after washout, with statistical
Table 1. Effects of ouabain on membrane potential (Vm) and input resistance (Rm) of preinspiratory (Pre-I) and inspiratory (Insp) neurons in brainstem-spinal cord preparation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vm (mV)</th>
<th>Rm (MΩ)</th>
<th>Burst duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-I (n = 6)</td>
<td>-47.5 ± 3.9</td>
<td>516 ± 306</td>
<td>2.78 ± 0.65</td>
</tr>
<tr>
<td>Insp (n = 5)</td>
<td>-48.8 ± 4.6</td>
<td>340 ± 88.6</td>
<td>0.09 ± 0.22</td>
</tr>
</tbody>
</table>

* P < 0.05.

Expression of genes encoding the Na/K-ATPase α subunit in the region of pFRG in neonatal rats

Electrophysiological data showed that the application of ouabain facilitates the respiratory activities for several hours. Here, we examined the expression of Atp1a1, Atp1a2, and Atp1a3 encoding the Na/K-ATPase α1, α2, and α3 subunits, respectively, in the pFRG region in rat neonates (postnatal day 1) by in situ hybridization. In situ hybridization studies are extremely useful for detecting the distribution of mRNA at the single-cell level. Subgroups of Pre-I neurons in the pFRG expressed a transcription factor, Phox2b (Onimaru et al., 2008). We previously reported that Phox2b expression parsafacial neurons, i.e., Pre-I neurons, play key roles in respiratory rhythm generation as well as in central chemoreception (Onimaru et al., 2008, 2012). Immunolocalization of Phox2b was therefore performed in parallel with in situ hybridization, using the alternate section to label the rostral and caudal pFRG.

Atp1a1 signals were strongly detected in the neurons of the facial nucleus (VII) (Figs. 7B, F, I, N) and weakly detected in the region of the pFRG (Fig. 7N). Atp1a2 signals were detected in some of the small cells of the VII and the region of the pFRG. The small dotted signals probably reflect its expression in glial cells (Fig. 7G, K, O). Intense Atp1a3 signals were observed in the neurons of the VII and clearly in the region of the pFRG (Fig. 7D, H, L, P). As previously reported (Onimaru et al., 2008), Phox2b product was observed in the rostral (Figs. 7A, E, M, arrows) and caudal pFRG (Fig. 7I, arrows). In the more caudal medulla, including the preBotC, we confirmed the expression of α subunits with α3 subunits being more predominantly expressed (data not shown).
Table 2. Effects of 40 μM ouabain on phrenic nerve activity in arterially perfused in situ preparations (n = 8)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ouabain (15 min)</th>
<th>Ouabain (60 min)</th>
<th>Washout (15 min)</th>
<th>Washout (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burst rate (bursts/min)</td>
<td>11.5 ± 3.2</td>
<td>18.6 ± 3.9***</td>
<td>28.1 ± 3.0***</td>
<td>60 ± 146</td>
<td>60 ± 93</td>
</tr>
<tr>
<td>Burst duration (ms)</td>
<td>661 ± 149</td>
<td>666 ± 93</td>
<td>804 ± 176*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak value (%)</td>
<td>100</td>
<td>101 ± 14.3</td>
<td>87.4 ± 28.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05.
*** P < 0.001 (paired t-test); compared with the control values.

DISCUSSION

The application of ouabain (15–20 min) resulted in a dose-dependent increase in the burst rate of C4 inspiratory activity in the brainstem-spinal cord preparation. After washout, the burst rate further increased to reach quasi-maximum values under each condition and the facilitated effect continued for more than 1 h. Respiratory neurons tended to depolarize and the burst duration was shortened. Similar effects on nerve activity were obtained in a decerebrate and arterially perfused in situ preparation of juvenile rats (postnatal day 11–13). We confirmed the mRNA expression of Atp1a1, Atp1a2, and Atp1a3 encoding Na/K-ATPase α1, α2, and α3 subunits, respectively, in the region of the pFRG by in situ hybridization. Distribution of the α subunits within the facial motor nucleus and the pFRG was heterogeneous. Expression of α3 subunits was more dominant than other two types in the pFRG. A previous study by Moseley et al. (2003) demonstrated that the α2 subunit was abundantly expressed in neurons in the lower brainstem, including the preBötC. We further confirmed the expression of α3 subunits in other medullary regions, including the preBötC. These results indicate that all cells (neurons and/or glia) in the lower brainstem, including the pFRG and preBötC, could be the target of ouabain, when it diffused to their particular level. The effects of ouabain can be explained by the depolarization of rhythm-generating neurons, linked with the inhibition of Na/K-ATPase by ouabain.

It has previously been reported that the application of 20 μM ouabain induced massive tonic discharges of cervical motoneurons followed by an irreversible blockage of respiratory activity in brainstem-spinal cord preparation from 0 to 4-day-old rats (Brockhaus et al., 1993). Brockhaus et al. (1993) also reported that extracellular potassium concentration increased with ouabain application. Another study showed that bath-applied Na/K-ATPase blocker another cardiac glycoside, strophanthidin (10 μM), depolarized baseline membrane potential in preBötC neurons and increased respiratory frequency in slice preparations from neonatal mice (postnatal day 0–5) (Del Negro et al., 2009). The amplitude of inspiratory bursts decreased during drug application, which was followed within several minutes by the cessation of rhythm. The results of the present study were consistent with those of previous studies at relatively high concentrations of ouabain. Although it was not clearly described in previous studies, our present findings indicated that higher concentrations of ouabain induced a long-lasting facilitatory effect on respiratory rhythm that continued after washout. The long-term modulation of cellular components including Na/K-ATPase itself may be involved in this phenomenon. Although the time course of dissociation of ouabain after

Fig. 7. Expression of genes encoding Na,K-ATPase α subunits and Phox2b protein in the region of pFRG of rat neonates (postnatal day 1). A, E, I, M, Immunofluorescence of Phox2b (green) in cryosections. Arrows indicate Phox2b-positive cells corresponding to pre-I neurons in the region of the pFRG. Rostral (A, E, M) and Caudal (I) pFRG are indicated. B, F, J, N, in situ hybridization using riboprobes for rat Atp1a1, C, G, K, O, in situ hybridization using riboprobes for rat Atp1a2. A white asterisk indicates blot artifact signals. D, H, L, P, in situ hybridization using riboprobes for rat Atp1a3. VII, facial nucleus; Rt, reticular nucleus. Fig. 7A–I were results from a brainstem isolated from one neonate, and Fig. 7M–P were from another neonate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
binding during 15–20-min incubation is unknown, it has been reported that high-affinity sites (see below) of Na/K-ATPase in ouabain binding showed a slow time course in the dissociation process (Berresbi-Bertrand et al., 1990). Thus, the slow time course of dissociation from the enzyme-ouabain complex may explain the long-lasting effects of ouabain. We observed similar long-lasting excitatory effects of ouabain (40 μM) on respiratory rhythm in the decerebrate and arterially perfused in situ preparations of juvenile rat (postnatal day 11–13). The high-dose requirement in the latter preparation may be explained by the presence of the blood–brain barrier in the preparations. In en bloc preparations, ouabain causes the effects by diffusion from surface regions of preparation, whereas the drug reaches target tissues, including deeper regions, via blood vessels in in situ perfused preparation. Similar effects on respiratory rhythm in both types of preparations are consistent with the assumption that the effects of ouabain might be caused through common targets like the ventral respiratory groups.

We also showed that ouabain exerted the facilitation of respiratory rhythm at rather low concentrations of ouabain (0.2–2 μM). The different isoforms of Na/K-ATPase exhibit differing sensitivity to ouabain; low affinity is observed in α1, while high affinity is observed in α2 and α3 (Marks and Seeds, 1978; Blanco and Mercer, 1998). Therefore, the different isoforms may be involved in the wide range of ouabain effects. Since nanomolar concentration of ouabain stimulates cardiac Na/K Pumps through the high glycoside affinity to α2 and α3 isoforms (Gao et al., 2002), isoform-specific effects in the respiratory system of low concentrations of ouabain remain to be studied.

Del Negro et al. (Del Negro et al., 1999; Darbon et al., 2003; Del Negro et al., 2005) suggested that the Na/K-ATPase pump has dynamic and tonic components. The dynamic component of the Na/K-ATPase pump develops during the inspiratory phase and is relevant during burst termination, but its contribution is brief, decaying significantly within 25–100 ms. Recently, Gulleide et al. (2013) demonstrated that the Na/K-ATPase pump generates prolonged after hyperpolarizations after periods of action potential generation in neocortical and hippocampal pyramidal neurons. The tonic component of the Na/K-ATPase pump may influence network excitability through its tonic contribution to baseline membrane potential (see also Darbon et al., 2003). Therefore, our present results indicating membrane depolarization and the facilitation of respiratory rhythm may be attributable to the depression of the tonic component of the Na/K-ATPase pump. It is known that some neuromodulators affect Na/K-ATPase activity (Volkov et al., 2007; Zhang et al., 2012). It was suggested that the electrogenic Na/K-ATPase pump was responsible for changes in excitability in disinhibition-induced bursting in cultured spinal networks (Darbon et al., 2003). Thus, Na/K-ATPase activity could be an important target in respiratory rhythm modulation by some types of neuromodulators.

The burst duration of inspiratory activity decreased in the in vitro brainstem-spinal cord preparation, but tended to increase in the decerebrate and arterially perfused in situ preparations of juvenile rats. In the former en bloc preparation, the decrease of burst duration is presumed to be due to an activity-dependent mechanism of inspiratory burst generation; the burst duration tend to decrease in association with increase of burst frequency as discussed in (Onimaru et al., 1997). The increase of burst duration in the latter preparation may reflect the modulation of the dynamic component of Na/K-ATPase pump (see above) by ouabain (Del Negro et al., 2005). It is not clear whether such a difference is due to the differing developmental stages of the preparations or other factors, such as differences in extracellular microenvironment (Brockhaus et al., 1993) between the two preparations, in which superfusion was used in the former and blood vessel perfusion was used in the latter.

In addition to its role as an ion pump, Na/K-ATPase is involved in signal transduction from the cell membrane to the intracellular target structures in caveolae isolated from cardiac myocytes, cardiac ventricles, kidney cell lines, and elsewhere (Pierre and Xie, 2000; Zhang et al., 2008). For instance, low concentrations (submicromolar range) of ouabain binding to Na/K-ATPase have been shown to activate multiple signal transduction pathways, including the activation of Src/epidermal growth factor receptor (Haas et al., 2000, 2002), phospholipase C (PLC) (Yuan et al., 2005), and phosphatidylinositol 3-kinase in a Src-dependent manner. The activation of PLC induces the production of diacylglycerol and inositol trisphosphate (IP3) (Liu et al., 2003; Yuan et al., 2005), and activates the protein kinase C and IP3 receptors, resulting in an increase in intracellular Ca2+ concentration. In the central nervous system, ouabain has been reported to induce a significant increase in the phosphorylation levels of molecules responsible for the initiation of protein translation in the rat frontal cortex (Kim et al., 2013). The significance of these signal pathways to the modulation of respiratory rhythm is unknown. Since the concentration of ouabain that affects the signal transduction is comparable in the lower doses used in the present study, these issues are worthy of future investigation.

Conclusion

Ouabain induced the facilitation of respiratory rhythm, which lasted long after washout when administered in higher concentrations. We suggest that effects of ouabain involve the modulation of tonic and partially dynamic components in cellular function of Na/K-ATPase pump, possibly through the inhibition of α-subunits.

Acknowledgments—This work was supported by Grants-In Aid for Scientific Research (KAKENHI: 22500206, 25430012).

REFERENCES


