NEUROSCIENCE

Effects of aconitine on the respiratory activity of brainstem-spinal cord preparations isolated from newborn rats

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Abstract

Aconitine is a sodium channel opener, but its effects on the respiratory center are not well understood. We investigated the dose-dependent effects of aconitine on central respiratory activity in brainstem-spinal cord preparations isolated from newborn rats. Bath application of 0.5–5 μM aconitine caused an increase in respiratory rhythm and decrease in the inspiratory burst amplitude of the fourth cervical ventral root (C4). Separate application of aconitine revealed that medullary neurons were responsible for the respiratory rhythm increase, and neurons in both the medulla and spinal cord were involved in the decrease of C4 amplitude by aconitine. A local anesthetic, lidocaine (100 μM), or a voltage-dependent sodium channel blocker, tetrodotoxin (0.1 μM), partially antagonized the C4 amplitude decrease by aconitine. Tetrodotoxin treatment tentatively decreased the respiratory rhythm, but lidocaine tended to further increase the rhythm. Treatment with 100 μM riluzole or 100 μM flufenamic acid, which are known to inhibit respiratory pacemaker activity, did not reduce the respiratory rhythm enhanced by aconitine + lidocaine. The application of 1 μM aconitine depolarized the preinspiratory, expiratory, and inspiratory motor neurons. The facilitated burst rhythm of inspiratory neurons after aconitine disappeared in a low Ca^{2+}/high Mg^{2+} synaptic blockade solution. We showed the dose-dependent effects of aconitine on respiratory activity. The antagonists reversed the depressive effects of aconitine in different manners, possibly due to their actions on different sites of sodium channels. The burst-generating pacemaker properties of neurons may not be involved in the generation of the facilitated rhythm after aconitine treatment.

Keywords Aconitine · Sodium channel · Respiratory rhythm · Newborn rat · Brainstem-spinal cord

Introduction

Aconitine is a lipid-soluble cardiotoxin and neurotoxin found in *Aconitum* species [7, 28]. It targets the type 2 receptor site of sodium channels and, at the cellular level, induces a depolarizing shift of the resting membrane potential and causes repetitive firing of action potentials in excitable membranes [5, 28]. In clinical settings, aconitine is known to cause systemic paralysis and nausea and vomiting, followed by dizziness, palpitation, hypotension, arrhythmia, shock, dyspnea, and coma [1, 29]. However, aconitine is exclusively used as an ingredient in traditional medicine [28]. The intravenous administration of aconitine to rats leads to irregular respiratory activity, exophthalmia, Straub reaction, convulsions, disturbed locomotion activity, mydriasis, and loss of body weight. Higher doses can result in gasping for breath, convulsions of higher intensity and frequency, lacrimation, salivation, reduced locomotion, loss of orientation, ventral or ventro-lateral recumbency, and loss of different reflexes [9]. At the tissue level, aconitine is known to trigger ventricular arrhythmias in the heart and promote spontaneous transmitter release in ventral medial hypothalamic neurons [10, 29]. However, its effects on the respiratory center in the medulla are not well understood.

The voltage-dependent fast sodium channels and persistent sodium channels are involved in respiratory burst generation [13, 15, 17, 21, 27]. Moreover, sodium leak channels can affect the excitability of neurons in the respiratory center by influencing the resting membrane potential [24, 30]. Thus, we considered that analysis of the effects of aconitine on respiratory neuronal activity would

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be of help to understand the roles of sodium channels in respiratory modulation.

Brainstem-spinal cord preparation isolated from newborn rats preserves the neuron networks essential for respiratory rhythm generation and can produce respiratory activity for several hours under in vitro conditions [3, 12, 25]. This preparation has great advantages for pharmacological study of the respiratory center because it can be maintained in anesthetic-free conditions, and drugs can be applied by superfusion at known concentrations. Therefore, in the present study, we investigated the effects of aconitine on respiratory activity in isolated brainstem-spinal cord preparations from newborn rats.

Methods

Preparations and solutions

The experimental protocols were approved by the Animal Research Committee of Showa University (approval nos. 09049, 02022, 03066) in accordance with Law No. 105 for the care and use of laboratory animals of the Japanese Government. All efforts were made to minimize the number of animals used and their suffering.

Brainstems and spinal cords were isolated from Wistar rats at postnatal days 0–4 under deep anesthesia with isoflurane (approximately 0.13%). The specimens were cut slightly rostral to the level of the anterior inferior cerebellar artery and were continuously perfused at a rate of 2.5–3 ml/min with artificial cerebrospinal fluid (ACSF) [25] composed of (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 30 glucose, equilibrated with 95% O_2 and 5% CO_2, pH 7.4, at 25–26 °C. The respiratory activity was monitored by amplifying ($\times$10,000) the C4 activity through a glass capillary suction electrode and a 0.5 Hz high-pass filter (MEG-5200, Nihon Kohden, Tokyo, Japan). The electrical signals were digitized at 4 kHz (PowerLab, ADInstruments, Sydney, Australia) and stored on a personal computer using Lab Chart 7 Pro software program (ADInstruments). In some experiments, drugs were applied separately to the medulla or spinal cord [2, 12, 25]. Two thin plastic partitions were placed at the level of the medulla-spinal cord junction and C2 of the spinal cord. The solution was introduced from both ends of the perfusion chamber (i.e., the compartments of the medulla and the spinal cord) and sucked from the central compartment between the partitions.

Drugs

Aconitine was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA) and was stocked as a 2.5 mM solution in 63% ethanol. Riluzole and lidocaine were purchased from Sigma-Aldrich (Tokyo, Japan) and were stocked as a 100 mM solution in dimethyl sulfoxide. Flufenamic acid was purchased from Sigma-Aldrich and was stocked as a 100 mM solution in 0.1 N NaOH. Tetrodotoxin (TTX) was purchased from Latoxan Laboratory (Portes lés Valence, France) and was stocked as a 1 mM solution. All stock solutions were stored at 4 °C.

Experimental protocols

1. Dose-dependent effects of aconitine on C4 activity: As there were no similar previous studies on the concentration of aconitine and its dose dependence was unknown, we first conducted experiments with four concentration levels of aconitine (0.1, 0.5, 1.0, and 5.0 μM). The perfusion time for all drugs was 15 min. Activity after the washout of drugs was followed for a minimum of 15 min. Separate perfusion experiments were performed for 1.0 μM aconitine.

2. Antagonistic effects of lidocaine or TTX on C4 activity after aconitine treatment: There are studies reporting that the effects of aconitine could be antagonized by a local anesthetic, lidocaine and a voltage-dependent sodium channel blocker, TTX [10, 20, 26]. Our previous study showed that lidocaine dose-dependently decreased the C4 burst rate, and 100 μM lidocaine induced an approximately 20% decrease in the burst rate [23]. We also examined the effects of various concentrations of TTX on C4 burst activity (Suppl. Figure 2 and Suppl. Table 1). On the basis of these results, we chose 100 μM lidocaine and 0.1 μM TTX for this protocol. First, 1 μM aconitine was applied for 15 min and then washed out. After 15 min of washout, 100 μM lidocaine or 0.1 μM TTX was applied for 15 min and then washed out.

3. Effects of riluzole or flufenamic acid on C4 activity after aconitine + lidocaine treatment: To clarify the involvement of pacemaker properties in rhythm generation after aconitine + lidocaine treatment, the effects of riluzole or flufenamic acid which are known to inhibit respiratory pacemaker activity were examined. We previously reported that riluzole (10–100 μM) alone induced a dose-dependent decrease in the C4 burst rate without a noticeable change in burst amplitude [15]. The application of 100 μM riluzole induced an approximately 35% decrease in the burst rate. We also tested the effects of flufenamic acid because this drug inhibited Ca^{2+}-dependent pacemaker activity [17, 21, 22], although this drug (100 μM) induced no significant effects on the C4 burst activity of brainstem-spinal cord preparations in the standard solution (unpublished observation). On the basis of these results, we chose

100 µM riluzole and 100 µM flufenamic acid for this protocol. First, 1 µM aconitine + 100 µM lidocaine was applied for 15 min. After 15-min washout, 100 µM riluzole or 100 µM flufenamic acid was applied for 15 min and then washed out.

4. The involvement of pacemaker properties in rhythm generation after aconitine was also examined by perfusion with a low Ca^{2+}/high Mg^{2+} (synaptic blockade) solution during monitoring firings of inspiratory neurons in the medulla because burst generation of neurons that have pacemaker properties could persist, even in the synaptic blockade solution [15, 18]. First, 1 µM aconitine was applied for 15 min. After 15 min of washout, the perfusate was changed to low Ca^{2+}/high Mg^{2+} solution for 30–40 min. Then, the perfusate was changed to normal ACSF.

Membrane potential recordings

We recorded the membrane potential of respiratory-related neurons, i.e., pre-inspiratory (Pre-I), expiratory (Exp), and inspiratory (Insp) neurons, at the level 0–400 µm caudal to the caudal end of the facial nucleus [4] in the ventrolateral region of the medulla where respiratory-related neurons were frequently found (i.e., respiratory regions) by a ventral approach. Pre-I neurons were characterized by firings that started preceding the Insp phase and continued during the post-Insp phase. Exp neurons showed tonic firings that were inhibited during the Insp phase (or Insp and pre- and post-I phases). Insp neurons showed firings that were limited during the Insp phase. Intracellular recordings were conducted using a blind whole-cell patch-clamp method [12, 19] with a high input impedance DC amplifier (CEZ-3100; Nihon Kohden). The electrical signals were amplified (× 10) and digitized at 4 kHz (PowerLab, ADInstruments), and then stored on a personal computer using the Lab Chart 7 Pro software program (ADInstruments). The electrodes were created by pulling thin-wall borosilicate glass (TW100F-4; World Precision Instruments, Inc., Sarasota, FL, USA) while heating. The inner tip diameter was 1.2–2.0 µm, and the resistance was 4–8 MΩ. The electrodes were filled with a pipette solution containing 130 mM K-gluconate, 10 mM EGTA, 10 mM HEPES, 2 mM Na_2-ATP, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and KOH at pH 7.2–7.3. The electrode tip was filled with a 0.5% solution of Lucifer Yellow (lithium salt, Sigma-Aldrich) dissolved in the same solution for the histological analysis of the recorded cells. In some experiments, we also recorded the membrane potential of Insp motor neurons at the C4 level. Insp motor neurons were identified by antidromic activation by C4 ventral root stimulation and the location of Lucifer yellow-stained cells in the ventral horn.

After the experiment, the preparations were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution at 4 °C and stored for histological analysis. Transverse 50-µm slices were cut using a vibrating-blade tissue slicer (PR07, Dosaka EM CO. Ltd., Osaka, Japan). The locations of the cell bodies of the recorded neurons were confirmed after staining with NeuroTrace™ Nissl staining (435/455 blue or 530/615 red fluorescence, Invitrogen). Labeled neurons were photographed using a fluorescence microscope (BX60; Olympus Optical, Tokyo, Japan). The cell body locations of the recorded neurons were identified and plotted in a figure.

Data analysis

The initial data analysis was performed using the Lab Chart 7 Pro software program (ADInstruments). The burst rate was calculated as an average of 3–5 min. After passing the normality test by the method of Kolmogorov and Smirnov, the significance of the values was analyzed by a paired t-test or one-way repeated-measures ANOVA, followed by a Tukey–Kramer multiple comparisons test at a confidence level of $P < 0.05$ using the GraphPad Prism 6 software program (GraphPad Software Inc., La Jolla, CA, USA).

Results

Dose-dependent effects of aconitine on C4 activity

To examine the effects of aconitine, we perfused the preparation with 0.1, 0.5, 1.0, and 5 µM aconitine for 15 min, followed by a washout of at least 15 min (n = 5 in each experimental condition). The effects of aconitine were observed at concentrations of > 0.5 µM. Bath application of 0.5 or 1.0 µM aconitine caused an increase in respiratory rhythm and a decrease in amplitude (Fig. 1 A, B). The effects were sustained even after washout for > 15 min. The inspiratory burst duration was not significantly changed at these concentrations of aconitine (Fig. 1F). Bath application of 5 µM aconitine caused the disappearance of C4 activity without recovery after washout (Fig. 1C). A summary is shown in Fig. 1D-F, and detailed values are shown in Table 1.

Fig. 1 Examples of C4 activity changes following the application of 0.5 μM aconitine (**A**), 1 μM aconitine (**B**), and 5 μM aconitine (**C**). a, Activity in control. b, Activity after 15-min application of aconitine. c, Activity after 15 min of washout. D-F, Summary of changes in the amplitude (**D**), burst rate (**E**), and burst duration (**F**) of C4 inspiratory activity, which are presented as relative values to the control (before the application of aconitine). Five samples were analyzed in each condition. Abscissa axis: a, activity in control; b, activity after 15-min application of aconitine; c, activity after 15-min washout. Dark blue, 0 μM; brown, 0.5 μM; light blue, 1 μM; yellow, 5 μM aconitine application. Note that the amplitude of the C4 inspiratory burst decreased extremely in association with the application of 0.5 or 1 μM aconitine and was not detectable after the application of 5 μM aconitine. Statistical significance was determined by a one-way repeated-measures ANOVA followed by Tukey–Kramer multiple comparisons test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) in comparison to the control values
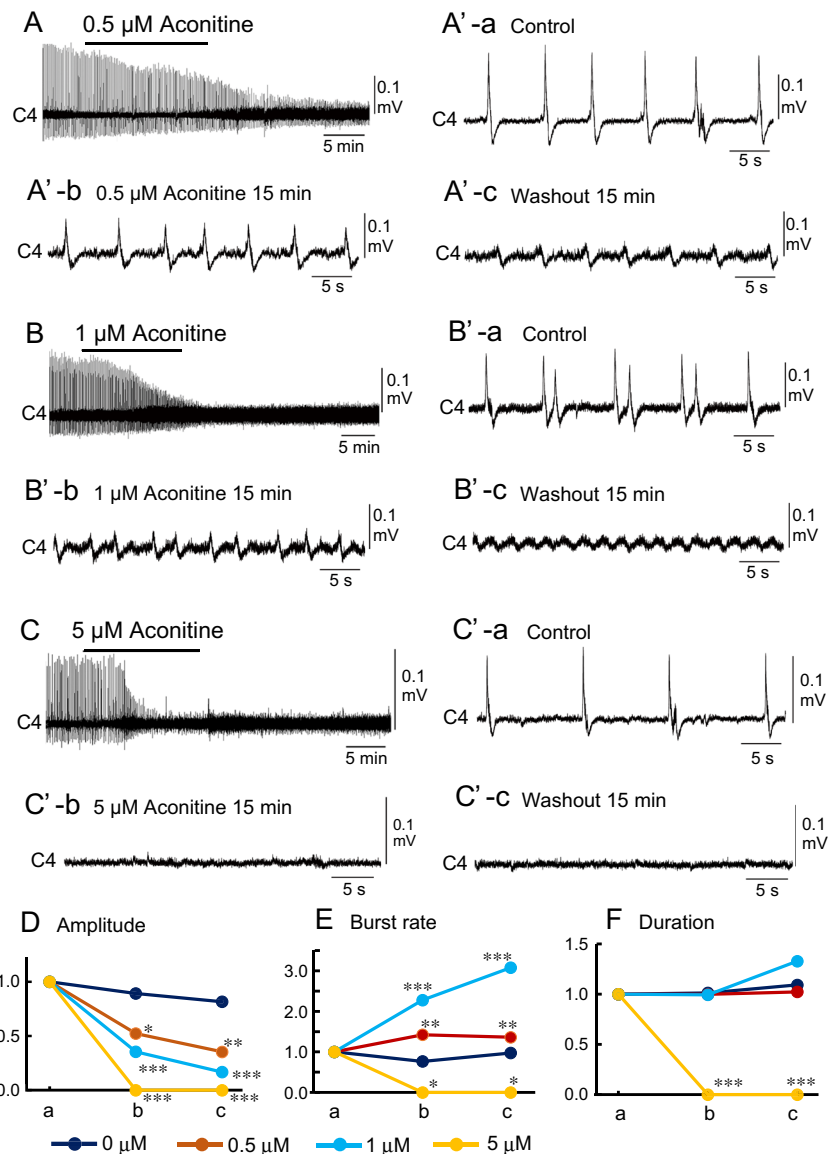
Effects of separate perfusion of the medulla or spinal cord with aconitine on C4 activity

Next, we conducted an experiment to determine whether changes of respiratory activity induced by aconitine were attributable to effects in the medulla or spinal cord (n = 5 in each experimental condition). We perfused the preparation with 1 μM aconitine for 15–20 min on either the medullary or spinal side and then washed it out. Application to the medulla caused a decrease in C4 amplitude and an increase in respiratory rhythm. These effects persisted even after washout for > 15 min (Fig. 2A). In contrast, application to the spinal cord

caused no significant change in inspiratory burst rate, but this resulted in a decrease in C4 amplitude (Fig. 2B). A summary is shown in Fig. 2C-E, and the detailed values are shown in Table 2.

Antagonistic effects of lidocaine or TTX on C4 activity after aconitine treatment

We examined the antagonistic effects of lidocaine or TTX on the effects of aconitine (n = 5 in each experimental condition). The preparation was perfused with 1 μM aconitine for 15–20 min and washed out. After 15-min washout of aconitine, 100 μM lidocaine or 100 nM TTX

Table 1 Dose-dependent effects of aconitine on C4 activity (n = 5 in each experiment)

| A. Amplitude of C4 activity (µV)					
Concentration	0 µM	0.1 µM	0.5 µM	1 µM	5 µM
Control	156.7 ± 61.8	138.1 ± 109.9	141.9 ± 107.7	186.1 ± 57.7	143.4 ± 80.1
15 min	144.0 ± 68.2	129.8 ± 106.9	60.5 ± 32.2*	78.2 ± 60.3***	0.0***
Wash 15 min	130.8 ± 60.5	112.5 ± 85.0	38.0 ± 16.4**	45.5 ± 34.0***	0.0***
B. C4 burst rate (bursts/min)					
Concentration	0 µM	0.1 µM	0.5 µM	1 µM	
Control	9.5 ± 2.3	6.5 ± 2.0	8.2 ± 1.2	7.4 ± 1.9	
15 min	7.2 ± 1.8	7.9 ± 1.5	11.7 ± 3.4**	16.0 ± 3.4***	0.0*
Wash 15 min	8.9 ± 2.0	8.3 ± 2.5	11.9 ± 2.6**	21.8 ± 5.7***	0.0*
C. Inspiratory burst duration (s)					
Concentration	0 µM	0.1 µM	0.5 µM	1 µM	5 µM
Control	0.96 ± 0.05	0.91 ± 0.15	0.91 ± 0.08	0.91 ± 0.12	1.08 ± 0.21
15 min	0.98 ± 0.14	0.91 ± 0.05	0.90 ± 0.07	0.90 ± 0.20	0.000***
Wash 15 min	1.05 ± 0.18	0.97 ± 0.09	0.93 ± 0.07	1.21 ± 0.20	0.000***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, in comparison to Control

(by a one-way repeated measures ANOVA followed by Tukey–Kramer multiple comparisons test)

"Control", values before the application of aconitine; "15 min", values after 15-min application of aconitine;

"Wash", values after 15-min washout of aconitine

Fig. 2 Examples of C4 activity changes following the separate application of 1 µM aconitine to the medulla (**A**) and spinal cord (**B**). a, Activity in control. b, Activity after 15-min application of aconitine. c, Activity after 15-min washout. C-E, Summary of changes in the amplitude (**C**), burst rate (**D**), and burst duration (**E**) of C4 inspiratory activity, which are presented as relative values to the control (before the application of aconitine). Five samples were analyzed in each condition. Abscissa axis: a, activity in control; b, activity after 15-min application of aconitine; c, activity after 15-min washout. Orange, Application to the medulla; green, application to the spinal cord. Note that the amplitude of the C4 inspiratory burst decreased extremely in association with the application of aconitine. Statistical significance was determined by a one-way repeated-measures ANOVA followed by Tukey–Kramer multiple comparisons test (**$P < 0.01$, ***$P < 0.001$) in comparison to the control values
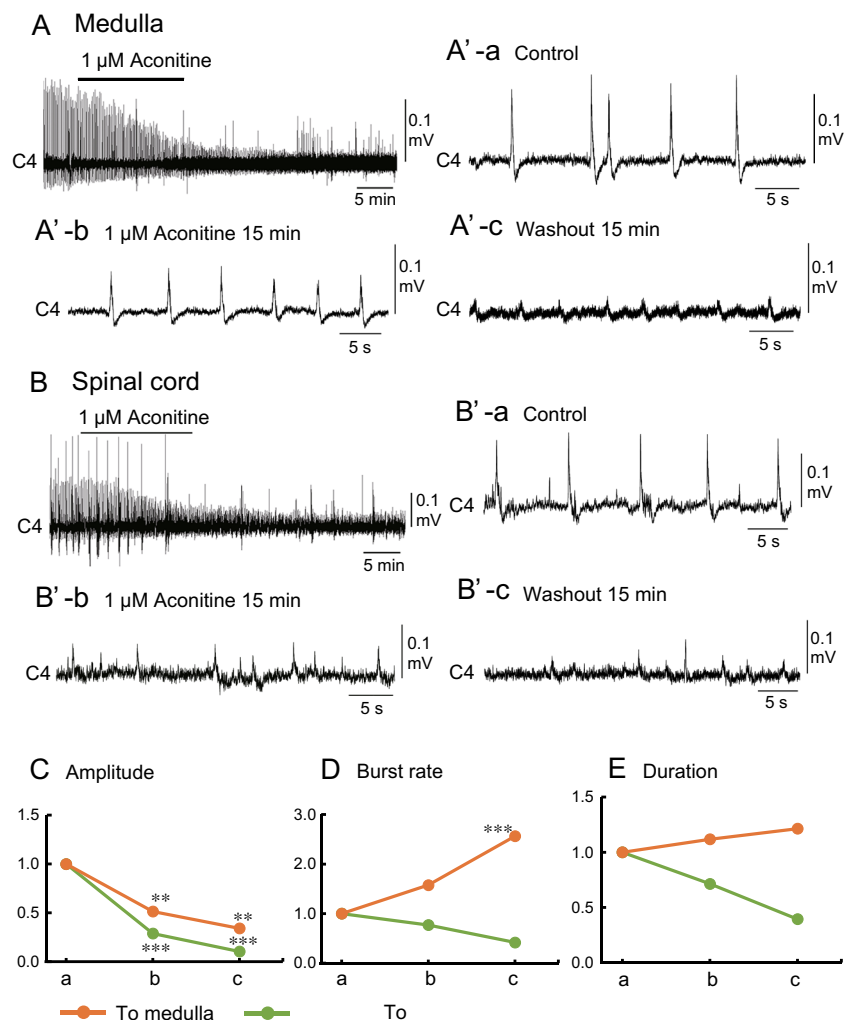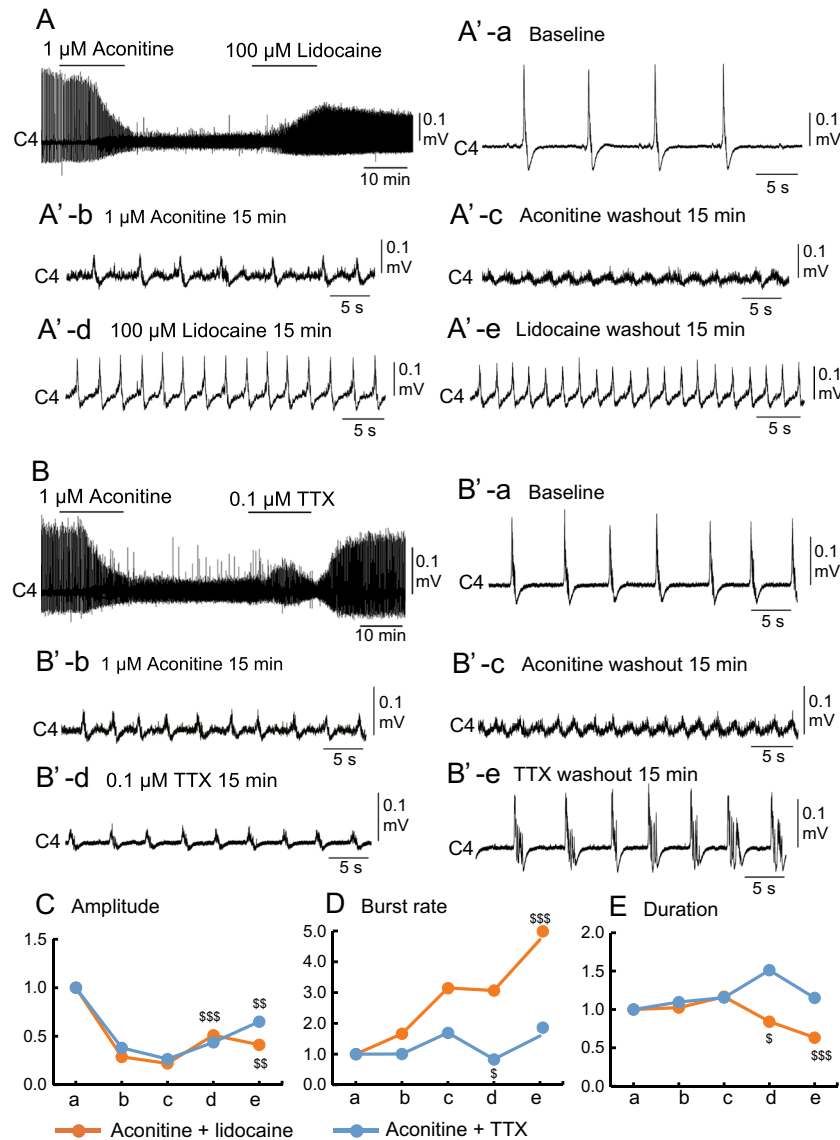
Fig. 3 Examples of C4 activity changes following the application of 100 μM lidocaine (**A**) and 0.1 μM tetrodotoxin (TTX) (**B**) after treatment with 1 μM aconitine. a, Activity in control. b, Activity after 15-min application of aconitine. c, Activity after 15-min washout of aconitine. d, Activity after 15-min application of 100 μM lidocaine (**A'**) or 0.1 μM TTX (**B'**). e, Activity after 15-min washout of lidocaine or TTX. C-E Summary of changes in the amplitude (**C**), burst rate (**D**), and burst duration (**E**) of C4 inspiratory activity, which are presented as relative values to the control (before the application of aconitine). Five samples were analyzed in each condition. Abscissa axis: a, activity in control; b, activity after 15-min application of aco- nitine; c, activity after 15-min washout of aconitine; d, activity after 15-min application of 100 μM lidocaine or 0.1 μM TTX; e, activity after 15-min washout of lidocaine or TTX. Orange, application of aconitine + lidocaine; blue, application of aconitine + TTX. Note that the amplitude of the C4 inspiratory burst decreased extremely in association with the application of aconitine and partially recovered after the application of lidocaine or TTX. Statistical significance was determined by a one-way repeated-measures ANOVA followed by Tukey–Kramer multiple comparisons test ($^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) in comparison to values immediately before the applica- tion of lidocaine or TTX. See Table 3 for other detailed statistics

was perfused for 15–20 min. The application of lidocaine led to a partial recovery of C4 amplitude, a further increase in respiratory rhythm, and a decrease in inspiratory burst duration. These effects persisted after washout of lidocaine

for > 15 min (Fig. 3A). Similarly, the application of TTX after aconitine treatment caused a partial recovery of C4 amplitude and a decrease in respiratory rhythm. The recovery of C4 amplitude was sustained even after washout

Table 2 Effects of separate perfusion of medulla or spinal cord with aconitine (1 µM) on C4 activity (n = 5 in each experiment)

A. Amplitude of C4 activity (µV)				
	Medulla		Spinal cord	
Control	143.8±75.7		187.3±62.3	
15 min	70.3±35.1	**	57.1±45.1	***
Wash 15 min	48.4±28.6	***	16.9±23.3	***
B. C4 burst rate (bursts/min)				
	Medulla		Spinal cord	
Control	7.0±0.9		7.2±1.1	
15 min	10.7±2.7		5.7±4.3	
Wash 15 min	18.1±4.0	***	3.0±4.4	
C. Inspiratory burst duration (s)				
	Medulla		Spinal cord	
Control	0.95±0.16		1.04±0.20	
15 min	1.10±0.24		0.77±0.44	
Wash 15 min	1.15±1.16		0.46±0.63	

** $P<0.01$, *** $P<0.001$, in comparison to Control

(by a one-way repeated measures ANOVA followed by Tukey–Kramer multiple comparisons test)

"Control", values before the application of aconitine; "15 min", values after 15-min application of aconitine;

"Wash", values after 15-min washout of aconitine

of TTX for > 15 min (Fig. 3B). Bath application of TTX did not result in a significant difference in inspiratory burst duration. A summary is shown in Fig. 3C-E, and the detailed values are shown in Table 3.

Effects of riluzole or flufenamic acid on C4 activity after aconitine + lidocaine treatment

Aconitine + lidocaine treatment induced a remarkable increase in the C4 burst rate. To investigate which mechanisms are involved in this increased respiratory rhythm, we examined the effects of riluzole or flufenamic acid, which are known to inhibit inspiratory pacemaker cells [22] (n = 5 in each experimental condition). The preparation was perfused with 1 µM aconitine plus 100 µM lidocaine for 15–20 min. After washout for > 15 min, 100 µM riluzole or 100 µM flufenamic acid was applied for 15–20 min. Application of riluzole after aconitine + lidocaine treatment induced an increase in C4 amplitude without change in the burst rate and duration (in comparison to values immediately before the application of riluzole) (Fig. 4 Ac-e). In contrast, the application of flufenamic acid after aconitine + lidocaine treatment induced a significant decrease in C4 amplitude without change in the burst rate and duration (compared to values immediately before flufenamic acid application) (Fig. 4 Bc-e). A summary is shown in Fig. 4C-E, and the detailed values are shown in Table 4.

Effects of aconitine on respiratory-related neurons in the medulla and spinal cord

We examined the effects of 1 µM aconitine on 7 Insp neurons, 6 Pre-I neurons, and 5 Exp neurons in the rostral ventrolateral medulla, as well as 5 Insp motor neurons in the spinal cord at the C4 level. The application of aconitine depolarized Pre-I neurons, Exp neurons, and Insp motor neurons, whereas no significant change was observed in the membrane potential of Insp neurons in the medulla (Figs. 5 and 6, Table 5). After aconitine treatment, the firing pattern of Pre-I neurons changed from a burst pattern to a tonic firing pattern, indicating the disappearance of burst activity (Fig. 5Bbc). Inspiratory motor neurons showed tonic firings during the interburst interval (i.e., Exp phase) (Fig. 6Abc). The above results of experiments with riluzole and flufenamic acid implied that the facilitated rhythm after treatment with aconitine (+ lidocaine) might be produced by independent mechanisms of burst-generating

Table 3 Antagonistic effects of lidocaine or TTX on C4 activity after aconitine treatment (n = 5 in each experiment)

A. Amplitude of C4 activity (µV)		
	ACO+LIDO	ACO+TTX
Baseline	216.5±59.7	138.6±43.5
ACO (15 min)	62.6±23.7***	51.1±13.2***
Control (wash 15 min)	47.8±18.6***	35.0±7.8***
LIDO or TTX (15–20 min)	111.7±48.4***$$$	56.5±15.9***
Wash 15 min	90.7±37.4***$$	85.6±26.9*$$
B. C4 burst rate (bursts/min)		
	ACO+LIDO	ACO+TTX
Baseline	8.1±2.1	10.6±1.8
ACO (15 min)	12.3±1.9	10.4±2.2
Control(wash 15 min)	22.7±6.7***	18.8±8.4*
LIDO or TTX (15–20 min)	22.8±4.4***	8.6±3.7$
Wash 15 min	35.2±2.9***$$$	16.1±3.5
C. Inspiratory burst duration (s)		
	ACO+LIDO	ACO+TTX
Baseline	0.93±0.14	0.89±0.15
ACO (15 min)	0.94±0.17	0.97±0.15
Control (wash 15 min)	1.06±0.19	1.20±0.22
LIDO or TTX (15 min)	0.77±0.13$	1.33±0.24**
Wash 15 min	0.59±0.10*$$$	1.00±0.22

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, in comparison to Baseline. $ $P<0.05$, $$ $P<0.01$, $$$ $P<0.001$, in comparison to Control (immediately before the application of lidocaine or TTX

"Baseline", values before the application of aconitine (ACO); "ACO (15 min)", values after the 15-min application of 1 µM aconitine;

"Control (wash 15 min)", values after 15-min washout of aconitine;

"LIDO or TTX (15 min)", values after 15-min application of lidocaine (100 µM) or TTX (0.1 µM);
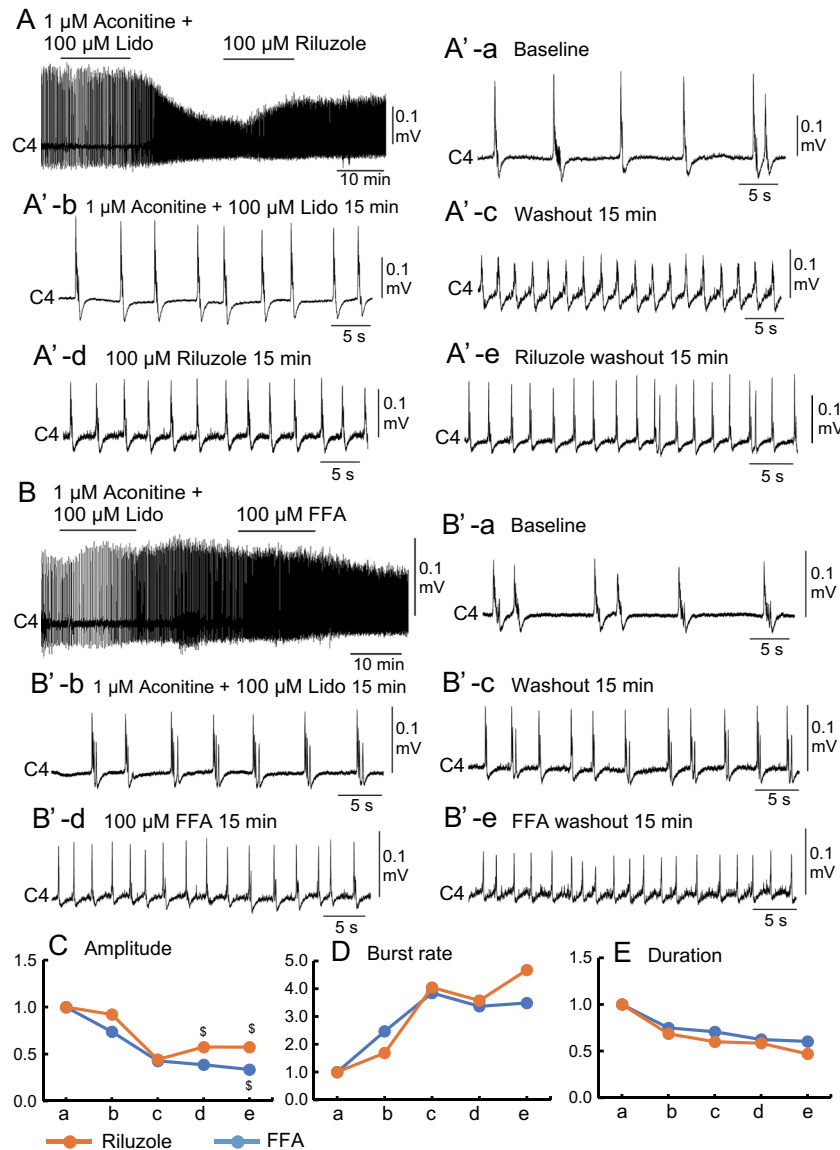
"Wash 15 min", values after 15-min washout

Fig. 4 Examples of C4 activity changes following the application of 100 µM riluzole (**A**) and 100 µM flufenamic acid (FFA) (**B**) after treatment with 1 µM aconitine + 100 µM lidocaine (Lido). a, Activity in control. b, Activity after 15-min application of aconitine + lidocaine. c, Activity after 15-min washout of aconitine + lidocaine. d, Activity after 15-min application of 100 µM riluzole (A') or 100 µM FFA (**B'**). e, Activity after 15-min washout of riluzole or FFA. C-E Summary of changes in the amplitude (**C**), burst rate (**D**), and burst duration (**E**) of C4 inspiratory activity, which are presented as relative values to the control (before the application of aconitine). Five samples were analyzed in each condition. Abscissa axis: a, activity in control; b, activity after 15-min application of aconitine + lido-

caine; c, activity after 15-min washout of aconitine + lidocaine; d, activity after 15-min application of 100 µM riluzole or 100 µM FFA; e, activity after 15-min washout of riluzole or FFA. Orange, application of riluzole; blue, application of FFA. Note that the amplitude of the C4 inspiratory burst partially recovered after the application of riluzole, and the burst rate tended to increase after the application of riluzole or FFA. Statistical significance was determined by a one-way repeated-measures ANOVA followed by Tukey–Kramer multiple comparisons test ($^{\$}P < 0.05$) compared with values immediately before the application of riluzole or FFA. See Table 4 for other detailed statistics

pacemaker properties of respiratory neurons [13, 15]. Intrinsic burst generation should be preserved in low Ca^{2+}/high Mg^{2+} synaptic blockade solution [15, 18]. Therefore, we examined the effects of perfusion with a low Ca^{2+}/high Mg^{2+} solution on the burst generation of medullary

inspiratory neurons (n = 4) after treatment with 1 µM aconitine. After 30–40 min in the low Ca^{2+}/high Mg^{2+} solution, C4 inspiratory activity and firings of inspiratory neurons disappeared, whereas excitatory postsynaptic potentials remained. This suppression was reversed when

Table 4 Effects of riluzole or flufenamic acid on C4 activity after aconitine+lidocaine treatment (n=5 in each experiment)

A. Amplitude of C4 activity (µV)		
	Riluzole	Flufenamic acid
Baseline	115.6 ± 50.7	178.2 ± 100.3
ACO+LIDO (15 min)	107.9 ± 52.7	116.9 ± 49.8
Control(wash 15 min)	57.2 ± 38.3***	64.2 ± 30.0**
Riluzole or Flufenamic acid (15 min)	72.4 ± 49.3***[$]	55.3 ± 26.6**
Wash 15 min	72.8 ± 51.1***[$]	48.1 ± 20.5**[$]
B. C4 burst rate (bursts/min)		
	Riluzole	Flufenamic acid
Baseline	7.5 ± 0.8	7.7 ± 1.7
ACO+LIDO (15 min)	12.5 ± 2.8	18.1 ± 6.4
Control(wash 15 min)	29.5 ± 8.5***	27.6 ± 5.8***
Riluzole or Flufenamic acid (15 min)	26.5 ± 10.4**	24.5 ± 9.4**
C. Inspiratory burst duration (s)		
	Riluzole	Flufenamic acid
baseline	0.97 ± 0.10	0.97 ± 0.14
ACO+LIDO (15 min)	0.66 ± 0.08**	0.71 ± 0.20
Control(wash 15 min)	0.56 ± 0.08***	0.68 ± 0.15
Riluzole or Flufenamic acid (15 min)	0.56 ± 0.08***	0.58 ± 0.18*
Wash 15 min	0.45 ± 0.19***	0.57 ± 0.26*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, in comparison to Baseline. [$] $P < 0.05$, in comparison to Control (immediately before riluzole or flufenamic acid application)

(by a one-way repeated measures ANOVA followed by Tukey–Kramer multiple comparisons test)

"Baseline", values before the application of aconitine+lidocaine (ACO+LIDO);

"ACO+LIDO (15 min)", values after the 15-min application of 1 µM aconitine+100 µM lidocaine;

"Control (wash 15 min)", values after 15-min washout of aconitine+lidocaine;

"Riluzole or Flufenamic acid (15 min)", values after 15-min application of riluzole (100 µM) or flufenamic acid (100 µM);

"Wash 15 min", values after 15-min washout

the perfusate was returned to the normal solution (Fig. 7). Figure 8 shows the location of the Lucifer yellow-stained medullary cells that were successively identified in the histological examination after the experiments. Recorded cells were located in the rostral ventrolateral medulla of the rostral part of the preBötzinger complex from the caudal part of the parafacial respiratory group [4].

Discussion

The application of aconitine to brainstem-spinal cord preparations of neonatal rats resulted in a dose-dependent depression of the Insp burst amplitude and an increase in the burst rate (rhythm-facilitating effect). Separate perfusion experiments showed that application of aconitine to the medulla led to a rhythm-facilitating effect and an amplitude-suppressing effect. Conversely, the application of aconitine to the spinal cord only produced an amplitude-suppressing effect. Thus, the amplitude decrease in Insp burst was attributable to the effects of aconitine on motor neurons in the spinal cord as well as on medullary neurons, including interneurons and premotor neurons, whereas the rhythm-facilitating effect was due to aconitine effects on medullary neurons. The effects of aconitine persisted after washout. This could be explained by the characteristics of aconitine, which is a lipophilic substance that binds to site 2 of sodium channels with strong affinity [28].

It was reported that the LD50 value of aconitine was approximately 0.1 mg/kg in mice [9]. On the basis of this value, we calculated the concentration in the plasma as approximately 2 µM, if this substance moved into the plasma. This concentration was comparable to the concentrations of the present study, which produced obvious effects on respiratory activity.

Fig. 5 Examples of membrane potential (Vm) and C4 activity changes following the application of 1 μM aconitine; inspiratory (Insp) neurons (**A**), pre-inspiratory (Pre-I) neurons (**B**), and expiratory (Exp) neurons (**C**). a, Activity in control. b, Activity after 15-min application of aconitine. c, Activity after 15-min washout. Note that after aconitine treatment, the firing pattern of Pre-I neurons changed from a burst pattern to a tonic firing pattern in association with membrane depolarization, indicating the disappearance of burst activity. d, A low magnification view of slices where recorded neurons were identified. e, A higher magnification view of the highlighted square in d. Arrows indicate the location of the recorded Lucifer yellow-labeled neuron. IO, inferior olivary nucleus
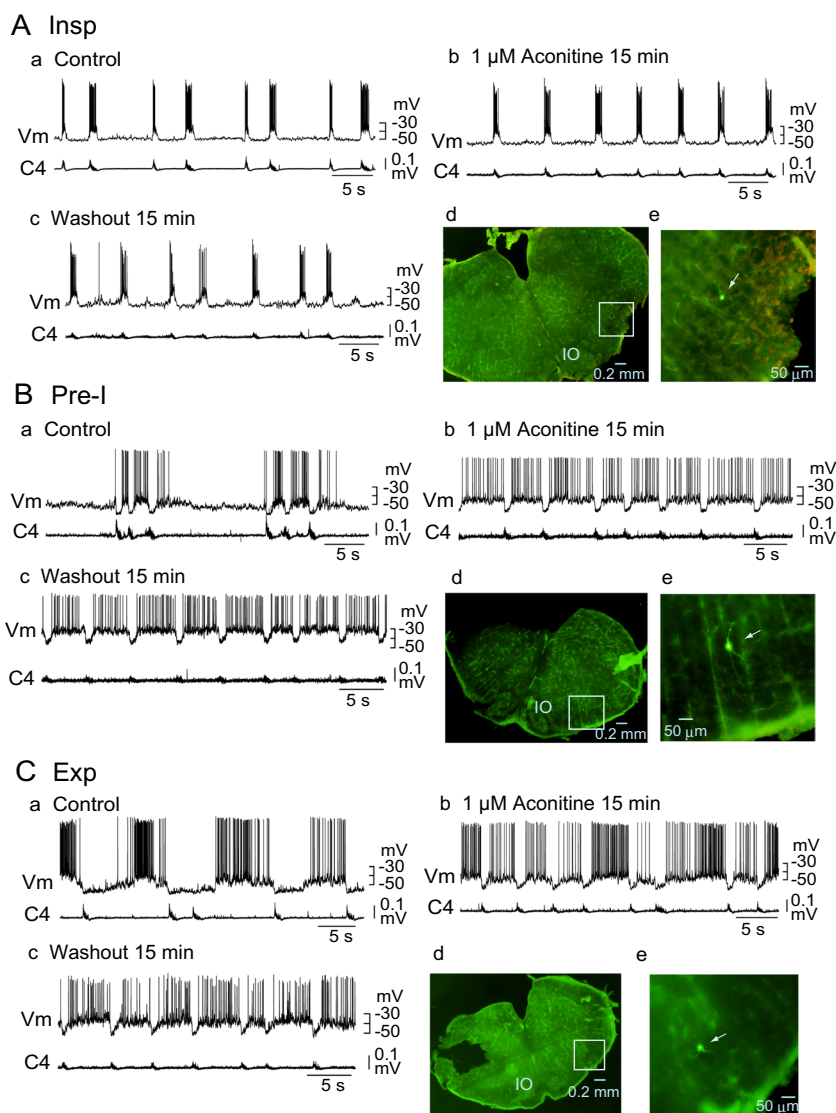
Both lidocaine and TTX showed a recovery of C4 amplitude that was decreased by aconitine treatment, but they exhibited different antagonistic effects on respiratory rhythm. Lidocaine further facilitated respiratory rhythm, whereas TTX temporarily reversed it. Lidocaine binds to the local anesthetic binding site of sodium channels, whereas TTX binds to site 1 of sodium channels [28]. These differences in binding sites might have contributed to the changes observed in respiratory rhythm.

Treatment with aconitine and subsequent lidocaine induced a rhythm-facilitating effect that persisted after washout with a burst rate that was approximately four times that of the control group. We investigated whether pacemaker properties are involved in the generation of this facilitated rhythm. This rhythm generation was not suppressed by the administration of riluzole or flufenamic acid, which are known to inhibit respiratory pacemaker activity [17, 21, 22]. Thus, these results suggest that pacemaker properties were not involved in the generation of facilitated rhythm after treatment with aconitine followed by lidocaine.

Membrane potential changes in respiratory-related neurons following 1 μM aconitine treatment differed in neuronal subtypes: depolarization in Pre-I, Exp, and spinal motor neurons but no significant change in the Insp neurons in the medulla. In particular, burst generation in Pre-I neurons was depressed, and tonic firings appeared after aconitine treatment. It is well known that the voltage-dependent sodium channels are the target site of aconitine. However, this mechanism seemed to be not

Fig. 6 An example of membrane potential (Vm) changes in C4 inspiratory motor neurons and C4 activity changes following the application of 1 μM aconitine. a, Activity in control. b, Activity after 15-min application of aconitine. c, Activity after 15-min washout. Note the membrane depolarization and appearance of tonic firings during interburst phases. B, Antidromic action potential induced by C4 ventral root stimulation. C, Location of the Lucifer yellow-labeled neuron recorded in the ventral horn
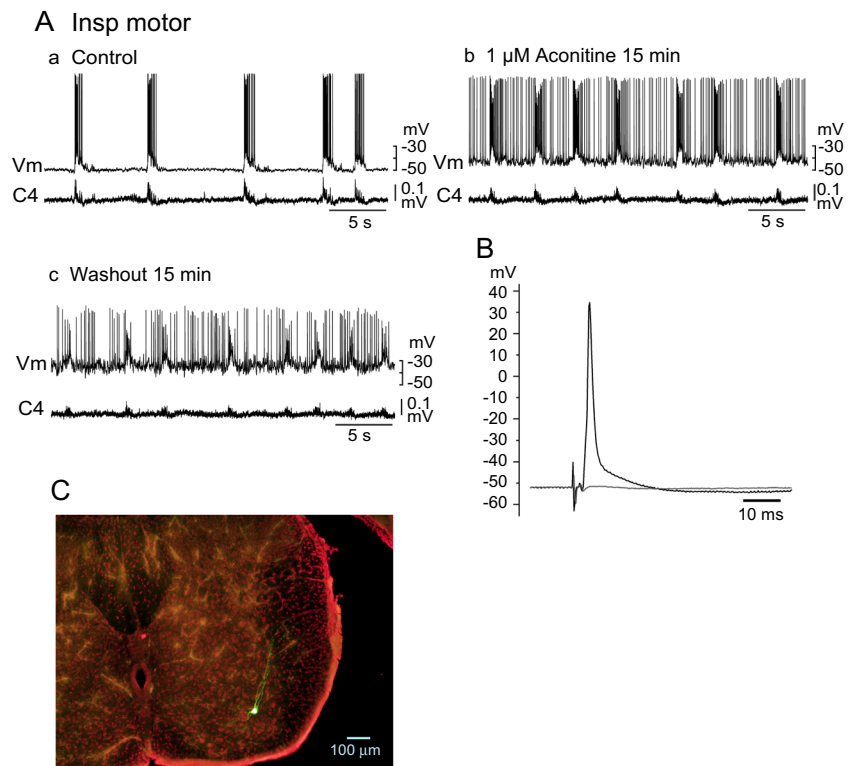

Table 5 Effects of 1 μM aconitine on respiratory related neurons in the medulla and spinal cord (n = 5 in each experiment)

A. Resting membrane potential (mV)

	Insp neuron	Pre-I neuron	Exp neuron	Motor neuron
Control	-47.4 ± 3.6	-50.1 ± 4.8	-43.3 ± 2.9	-48.5 ± 1.7
15 min	-46.8 ± 3.1	-39.4 ± 7.9*	-39.7 ± 3.6	-39.3 ± 4.5*
Wash 15 min	-45.9 ± 5.0	-34.1 ± 6.5***	-33.6 ± 1.3**	-36.1 ± 4.3**

B. Input resistance (MΩ)

	Insp neuron	Pre-I neuron	Exp neuron	Motor neuron
Control	346.2 ± 109.8	543.2 ± 206.2	770.4 ± 355.4	130.3 ± 32.6
15 min	338.4 ± 114.3	474.6 ± 171.5*	571.6 ± 473.4	118.0 ± 33.9

* $P < 0.05$, in comparison to control. ** $P < 0.01$, in comparison to control. *** $P < 0.01$, in comparison to control

(by a one-way repeated measures ANOVA followed by Tukey–Kramer multiple comparisons test)

"Control", values before aconitine application; "15 min", values after 15-min application of aconitine;

"Wash", values after 15-min wash out of aconitine

Insp neuron, inspiratory neuron; Pre-I neuron, pre-inspiratory neuron; Exp neuron, expiratory neuron;

Motor neuron, inspiratory motor neuron at C4 level

straightforward in understanding why changes of resting membrane potentials after aconitine treatment differed in neuronal subtypes. Recently, it was reported that a channel that generates "leak" sodium currents could regulate the excitability of neurons [16, 24] and influence the resting membrane potential of respiratory neurons [30]. Although there have been no reports indicating that aconitine affects the "leak" sodium channel, this may be considered as a
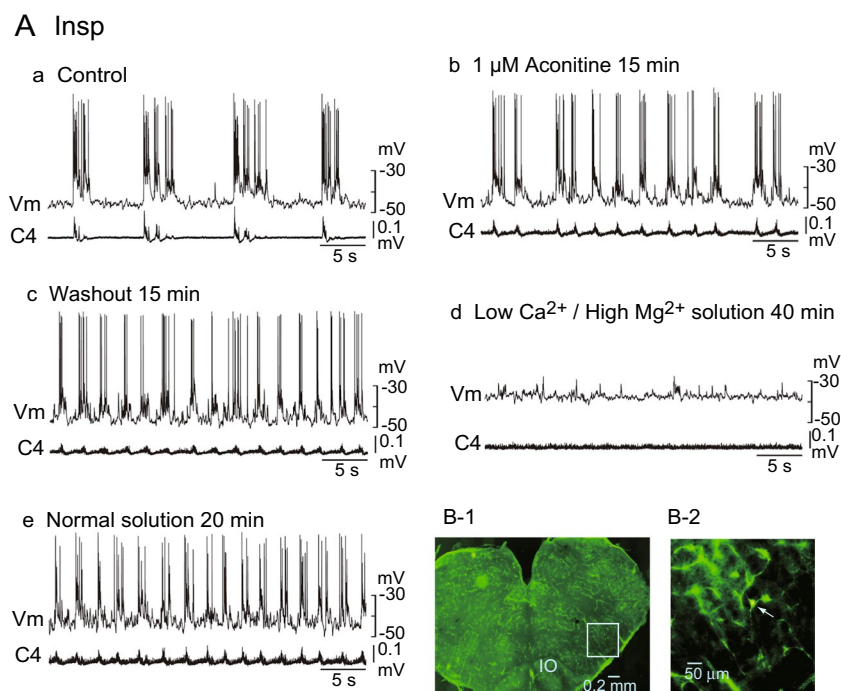
Fig. 7 Effects of low Ca^{2+}/high Mg^{2+} synaptic blockade solution on the burst activity of inspiratory neurons after aconitine treatment. a, Activity in control. b, Activity after 15-min application of 1 μM aconitine. c, Activity after 15-min washout. d, Activity after 40 min low Ca^{2+}/high Mg^{2+} solution. e, Activity after returning to normal ACSF. Note that after 40 min in low Ca^{2+}/high Mg^{2+} solution, C4 inspiratory activity and firings of inspiratory neurons disappeared, whereas EPSPs remained. B-1, A low-magnification view of slices where the recorded neuron was identified; B-2, A higher-magnification view of the highlighted square in B-1. Arrows indicate the location of the Lucifer yellow-labeled neuron recorded. ACSF, artificial cerebrospinal fluid; EPSPs, excitatory postsynaptic potentials; IO, inferior olivary nucleus

possible additional mechanism. Our findings suggest that sodium channels could work as one of factors modulating respiratory activity.

Previous studies have suggested that some Pre-I neurons express persistent sodium currents (i.e., riluzole-sensitive negative slope conductance), which are essential to produce intrinsic burst generation [14, 15, 23]. This burst-generating pacemaker property of Pre-I neurons was not involved in generation of the facilitated rhythm, which was resistant to riluzole after aconitine. Alternatively, sustained inactivation of the persistent sodium current might occur after aconitine because the burst generation of Pre-I neurons disappeared. It was also reported that some Insp neurons have intrinsic burst-generating properties [11]. Intrinsic burst generation should be retained in low Ca^{2+}/high Mg^{2+} synaptic blockade solution [15, 18]. However, the burst activity in Insp neurons disappeared after perfusion with the synaptic blockade solution. This result was also consistent with the above suggestion that the pacemaker property is not involved in the facilitated rhythm after aconitine and further suggested that mutual excitatory synaptic connections are important in fast rhythm generation. This mechanism coincides with a model of rhythm generation by the group pacemaker [8, 21]. Under standard experimental conditions, the synaptic activities of Insp neurons were almost completely silenced in low Ca^{2+}/high Mg^{2+} solution [18]. However, after aconitine treatment, some synaptic activities in Insp neurons remained in the low Ca^{2+}/high Mg^{2+} solution. We speculated that Ca^{2+}-independent transmitter release [6] might be involved in this synaptic activity.

Conclusion

We found that aconitine has the dose-dependent effects of depressing C4 amplitude and increasing respiratory rhythm. The amplitude decrease could be caused by effects on spinal motor neurons and medullary (premotor and inter) neurons, whereas effects on medullary neurons facilitated respiratory rhythm. Lidocaine and TTX reversed
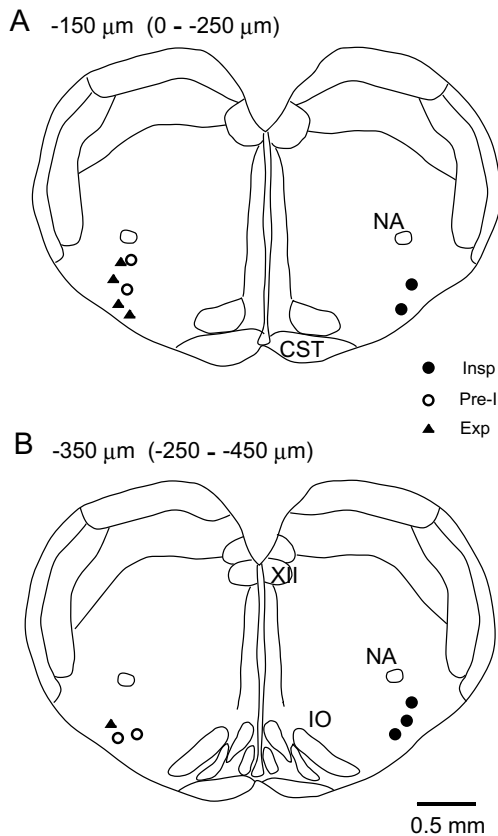
Fig. 8 Location of recorded neurons in the ventrolateral medulla. Neurons labeled with Lucifer yellow were distributed in the range of 0–450 μm caudal to the caudal end of the facial nucleus and were plotted in two sections (A and B). Closed circles, inspiratory neurons; open circles, pre-inspiratory neurons; and closed triangles, expiratory neurons. NA, nucleus ambiguus; CST, corticospinal tract; IO, inferior olivary nucleus; XII, hypoglossal nucleus

the effects of aconitine in different ways, likely because they target different sites on sodium channels. Pacemaker properties might not be involved in the facilitation of rhythm generation after aconitine treatment. The results of synaptic blockade experiments are consistent with the facilitation of rhythm generation by the group pacemaker mechanism. Overall, our findings shed light on the effects of aconitine on respiratory activity and provide new insights into a role of sodium channels involved in respiratory rhythm modulation.

Declarations

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