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ABSTRACT

The aim of the present study was to determine the effect of hypercapnia on motor neuromuscular activity of the human triceps surae muscle. Nine subjects participated in trials in a normal breathing condition and a CO_2 rebreathing condition. In both conditions, in order to provoke self-sustained muscle activity, percutaneous electrical train stimulation was applied to the tibial nerve while each subject lay on a bed. Self-sustained muscle activity, which is an indirect observation of plateau potentials in spinal motoneurons, was measured for 30 sec after the train stimulation by using surface electromyography. The sustained muscle activity was increased by CO_2 rebreathing ($P < 0.05$). This finding suggests that motor neuromuscular activity may be linked to the respiratory system that is activated during hypercapnia.

Keywords

Hypercapnia, Central chemoreceptor, Self-sustained muscle activity, Plateau potentials

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1. Introduction

Carbon dioxide (CO_2) is a strong stimulus to central chemoreceptors located in the brainstem for ventilatory response, which is a homeostatic function for maintaining the internal environment of the body (Whipp and Ward 1998). Hypercapnia results in the generation of depolarizing currents known as central respiratory drive potentials in spinal motoneurons of respiratory muscles to increase ventilation through the output of the respiratory center in the medulla (Butler 2007). Additionally, it has been reported that hypercapnia facilitated plateau potentials in hindlimb motoneurons of a decerebrate anesthetized cat (Kirkwood et al. 2002, 2005). This indicates the possibility that a change in arterial blood CO_2 pressure ($PaCO_2$) is involved not only in respiratory control but also in limb motoneuronal activity in animals. Plateau potentials are sustained depolarizations of spinal motoneurons that are facilitated via activation of L-type calcium channels and persistent sodium channels of motoneurons (Bennett et al. 1998; Lee and Heckman 1999; Li et al. 2004). However, there has been no study in which the relationship between hypercapnia and plateau potentials of limb muscle motoneurons in humans was investigated.

In previous studies, it has been shown that plateau potentials can be measured

by electromyography (EMG) signals in animals (Crone et al. 1988; Gorassini et al. 1999; Hounsgaard et al. 1988) and humans (Collins et al. 2001, 2002; Gorassini et al. 1998, 2002; Nozaki et al. 2003; Trajano et al. 2014; Walton et al. 2002). Nozaki et al. (2003) demonstrated that autonomous neural activity that is related to the plateau potentials of limb muscle motoneurons could be recorded as self-sustained muscle contractions, which are muscle activities that involuntarily continue after the end of percutaneous electrical train stimulation to a peripheral nerve, by using surface EMG. This indicates that sustained muscle contractions are an indirect observation of plateau potentials in spinal motoneurons. Therefore, self-sustained muscle activity, which is an enhanced sustained EMG activity, was induced in the present study by using the methods of Nozaki et al. (2003). If plateau potentials are associated with hypercapnia as indicated by Kirkwood et al. (2002, 2005), EMG activity of the sustained muscle activity in humans would be affected by hypercapnia. The purpose of the present study was to investigate whether hypercapnia induced by CO_2 rebreathing alters the self-sustained muscle activity of the human triceps surae muscle.

2. Methods

2.1. Subjects

Nine healthy males, with a mean (± standard deviation) age of 29 ± 8.4 years, who had never had a nervous or motor disorder participated in the present study. All of the subjects provided informed consent for participation in the study. This study was approved by the Human Research Ethics Committee of the Graduate School of Education, Hokkaido University.

2.2. Experimental set-up

In the experiment, each subject lay on a bed on his left side, and the subject's right leg was placed on a cushion. The subject's right hip and knee joint were flexed approximately 60°. The subjects were instructed to remain relaxed throughout the experiment.

2.3. Recordings

Surface EMG signals were recorded from the soleus muscle (Sol) and from the

medial head (MG) and lateral head (LG) of the gastrocnemius muscle of the right leg via a bipolar electrode. The ground electrode was placed over the right caput fibulae. EMG signals were amplified (Amplifier: AB-611-J, Nihon-Kohden, Japan) with band-pass filtering between 1.5 Hz and 1 kHz and converted into digital signals at a sampling rate of 2 kHz using an analog-digital converter (LabChart 8, ADInstruments, Australia). Throughout the experiment, the subjects breathed through a face mask connected to a hot-wire flow meter and a respiratory gas analyzer (AE-280S, Minato Medical Science, Japan) to measure minute ventilation ($\dot{V}E$) and end-tidal carbon dioxide pressure ($PETCO_2$). Respiratory variables were measured with the breath-by-breath mode. A zirconium sensor and infrared absorption analyzer were used to analyze inspired and expired fractions of O_2 and CO_2, respectively. Inspired and expired flows were measured using the hot-wire flow meter. The gas analyzer was calibrated with precision reference gas (O_2, 15.17%; CO_2, 4.92%). The flow meter was calibrated with a standard 2-liter syringe.

2.4. Experimental protocol

Each subject participated in trials in two respiratory conditions, a CO_2

rebreathing condition and a normal breathing condition, which were conducted on the same day (Fig. 1). The subjects remained relaxed for 152 sec (- 90 to 62 sec) in each trial. In the CO_2 rebreathing condition, respiratory dead space (1500 ml) was added to the respiratory mask from 30 sec after the initiation of the trial to the end of the trial (- 60 to 62 sec) in order to make the subjects rebreathe expired CO_2. In the normal condition, each subject remained rested and spontaneously breathed room air through the respiratory mask. At 90 sec after the start of a trial in the both conditions, 2-sec train stimulation was delivered to the right tibial nerve. Then each subject remained relaxed for 60 sec after the 2-sec train stimulation in both conditions. This trial was repeated 6 times with intervals of 5-10 min. During each interval, maximal M-wave (M-max) and Hoffmann's reflex (H-reflex) of the soleus muscle were measured three times and five times respectively. Three of the 6 trials were performed in the CO_2 rebreathing condition. Three trials in each condition were sequentially performed and the order of the two conditions was random among subjects. It has been shown that $PaCO_2$ is increased by rebreathing expired air through the increased respiratory dead space (Koppers et al. 2006; Smolka et al. 2014).

Electrical stimulation. While each subject remained reclined on the bed, the tibial nerve was percutaneously stimulated by applying a rectangular electrical pulse of 1 msec in

duration to the popliteal fossa using a constant current stimulator (DS7AH, Digitimer Ltd, UK). Sustained muscle activity was elicited by 50-Hz electrical train stimulation for 2 sec (Nozaki et al. 2003). In this study, activity of the soleus muscle was mainly assessed sample. Thus, the stimulation intensity was 120% of the H-reflex threshold of the soleus muscle. The cathode of the stimulation electrodes was secured to the right leg popliteal fossa, and the anode was attached to the patella. The subjects were asked to ignore the electrical train stimulation as much as possible. In order to measure M-max during intervals between trials, the electrical stimulus delivered by the stimulator was increased gradually until the M-wave of the soleus muscle reached a plateau while the subject was at rest. The level of stimulation was then set 20% above this point to ensure maximal activation of these muscles. Additionally, the stimulus intensity of H-reflex was 120% of the H-reflex threshold of the soleus muscle during an interval.

Safety of subjects. In both respiratory conditions, the subjects were asked about their sensation of dyspnea after recording self-sustained muscle activity using the modified Borg scale (Borg 1982) in order to determine whether the experiment could be continued. Using a pulse oximeter (PULSOX-300i, Konica Minolta, Japan), arterial oxygen saturation of pulse oximetry (SpO_2) was measured noninvasively to check whether SpO_2

was within safety level (over 90%).

2.5. Data analysis

The root mean squares (RMSs) of EMG of the Sol, MG, and LG muscles were calculated from 10-sec and 30-sec windows immediately before and after the train stimulation. The RMSs in each respiratory condition were averaged by the 3 trials. The magnitudes of M-max and H-reflex were measured as peak-to-peak amplitude and averaged over 3 times and 5 times, respectively, for use in the following analyses. The H-reflex amplitude was evaluated as percentage of M-max amplitude.

In order to obtain continuous data for arterial carbon dioxide pressure ($PaCO_2$), $PaCO_2$ was calculated from $PETCO_2$ using the formula of Jones et al. (1979).

Predicted $PaCO_2$ ($PaCO_{2pred}$) = 5.5 + 0.90 $PETCO_2$ – 0.0021 tidal volume (VT).

To obtain continuous averaged data for changes in $\dot{V}E$ and $PaCO_{2pre}$ in all subjects, the variables were interpolated second-by-second using the 3-dimensional spline technique. The interpolated data were for the period from 30 sec before the start of CO_2 rebreathing to 60 sec after the end of the train stimulation (i.e., a 152-sec period) in each trial.

2.6. Statistical analysis

Measured data are presented as means ± standard error (SE). A paired *t*-test was used to compare $\dot{V}E$, $PaCO_{2pred}$, sensation of dyspnea and SpO_2 between the two respiratory conditions and RMSs between pre- and post-train stimulation. Two-way repeated measures analysis of variance (ANOVA) was performed to examine the effects of the two respiratory conditions and three muscles on RMSs of the muscles during and before the self-sustained muscle activity period. To confirm that the activities of peripheral nerves were not different among all of the trials before the CO_2 rebreathing period, the effects of trials and respiratory conditions on the amplitudes of M-max and H-reflex were examined by using two-way repeated measures ANOVA. After ANOVA, the Bonferroni post hoc test was performed for multiple comparisons. If a significant interactive effect was indicated, a simple main effect test was performed. SPSS (Version 20, IBM, USA) was used for statistical analysis. Statistical significance was set at $P < 0.05$.

3. Results

Fig. 2A shows changes in $\dot{V}E$ and $PaCO_{2pred}$. Before the start of CO_2 rebreathing (30-sec period), $\dot{V}E$ and $PaCO_{2pred}$ were not different between the two conditions (Fig. 2B, a^1, b^1). During self-sustained muscle activity (Fig. 2B, a^2, b^2), the variables in the CO_2 rebreathing condition ($\dot{V}E$: 17.3 ± 1.2 l/min, $PaCO_{2pred}$: 53.6 ± 0.7 mmHg) were significantly ($P < 0.05$) larger than those in the normal breathing condition ($\dot{V}E$: 8.9 ± 0.3 l/min, $PaCO_{2pred}$: 39.0 ± 0.7 mmHg). In the CO_2 rebreathing condition, $\dot{V}E$ and $PaCO_{2pred}$ were 1.9 ± 0.1-times and 1.4 ± 0.02-times larger, respectively, than those in the normal breathing condition. $PaCO_{2pred}$ continuously increased immediately after the start of CO_2 rebreathing, while $\dot{V}E$ increased gradually at a later timepoint than $PaCO_{2pred}$. $\dot{V}E$ and $PaCO_{2pred}$ did not reach a plateau during the CO_2 rebreathing period as shown in Fig. 2A. The sensation of dyspnea was 4.3 ± 0.4 (moderate – very severe) in the CO_2 rebreathing condition. SpO_2 decreased during CO_2 rebreathing (93.6 ± 0.7%) compared to that during normal breathing (97.8 ± 0.3%), but it did not reach a dangerous level.

M-max amplitudes, which were evoked before the start of each trial in both conditions, were 9.6 ± 1.3 mV, 9.9 ± 1.3 mV and 10.1 ± 1.2 mV in the normal breathing condition and 10.1 ± 1.2 mV, 9.9 ± 1.3 mV and 9.5 ± 1.3 mV in the CO_2 breathing condition. H-reflex amplitudes (%M-max) were 58.1 ± 7.1%, 57.8 ± 8.2% and 54.9 ± 7.5% in the normal breathing condition and 53.5 ± 6.7 mV, 53.9 ± 6.3 mV and 54.0 ± 6.9

mV in the CO_2 breathing condition. Two-way repeated measures ANOVA did not indicate a significant interaction between conditions and trials for M-max and H-reflex (Fig. 3). There were no differences in M-max and H-reflex in the soleus muscle measured before electrical train stimulation among all trials and between conditions.

During the electrical train stimulation (1 msec pulse, 50 Hz, 2 sec) period, EMG reflex responses were elicited in the Sol, MG and LG (Fig. 4). The first stimulus induced a large reflex response, and then several subsequent responses were depressed (lower left panel). However, the reflex response recovered with repetitive stimuli (lower right panel).

Fig. 5 shows typical data for self-sustained muscle activities in both respiratory conditions that were induced following electrical train stimulation in the same subject. We attempted to measure self-sustained muscle activities for 1 min. However, in three subjects, the activities in several trials were suppressed within 1 min. Thus, RMSs of EMG signals during the sustained muscle activity were evaluated for 30 sec after the end of the train stimulation. RMSs increased from pre- to post-train stimulation in both the normal breathing condition (Sol, from 1.6 ± 0.3 μV to 11.1 ± 3.1 μV; MG, from 1.6 ± 0.3 μV to 5.4 ± 1.4 μV; LG, from 2.2 ± 0.4 μV to 8.7 ± 2.8 μV; $P < 0.05$) and the CO_2 rebreathing condition (Sol, from 1.7 ± 0.3 μV to 18.4 ± 4.9 μV; MG, from 1.4 ± 0.2 μV to 9.2 ± 1.8 μV; LG, from 1.7 ± 0.1 μV to 10.7 ± 2.7 μV; $P < 0.05$). In pre-train stimulation,

there were no main effects or interactions between conditions and muscles for RMSs. In post-train stimulation (i.e., during the sustained muscle activity), there was no significant interaction between conditions and muscles for RMSs. RMSs of the CO_2 rebreathing condition were significantly larger than those of the normal breathing condition for all muscles ($P < 0.05$), though there were no differences among muscles.

4. Discussion

The purpose of this study was to investigate the effect of hypercapnia on self-sustained muscle activity in humans. $PaCO_{2pred}$ showed an exponential increase immediately after the start of CO_2 rebreathing, and $\dot{V}E$ showed a constant increase about 30 sec after the rise in $PaCO_{2pred}$ (Fig. 2A). There were no differences between the two conditions in $\dot{V}E$ and $PaCO_{2pred}$ before the start of the CO_2 rebreathing period (Fig. 2B, a^1, b^1), indicating that the internal environment of the body was likely to be the same in the two conditions until the start of CO_2 rebreathing. An increase in $PaCO_2$ is a stimulus to activate central chemoreceptors located in the medulla (Whipp and Ward 1998). Therefore, an increase of $\dot{V}E$ during CO_2 rebreathing is thought to be due to activation of the chemoreceptors (Fig. 2B, a^2, b^2).

In order to induce self-sustained muscle activity, 2-sec train stimulation was delivered to the right tibial nerve 3 times with intervals of 5-10 min in each condition. To confirm that the electrical train stimulation electrode was in the same position in both conditions, we measured M-max of the soleus muscle three times in each interval between trials. In this study, the magnitude of M-max was not different among the trials (Fig. 3). Thus, the stimulation electrode position was not likely to be different among the trials. Also, the magnitude of H-reflex of the soleus muscle, which was measured five times at the same time as measurement of M-max, was not different among the trials. This suggests that activities of peripheral nerves were not different among the trials before the CO_2 rebreathing period.

During the electrical train stimulation period, reflex responses were elicited in the triceps surae muscles (Fig. 4). Nozaki et al. (2003) found that although the first electrical stimulus induced the largest reflex response in the soleus muscle and several subsequent responses were depressed as shown in the lower left panel of Fig. 4, responses recovered with repetitive stimuli as shown in the lower right panel of Fig. 4. They explained this phenomenon as depolarization of membrane voltage of motoneurons due to the emergence of plateau potentials because this phenomenon resembled firing frequency acceleration of motoneurons during elicitation of plateau potentials

(Hounsgaard et al. 1984, 1988). In the present study, we induced self-sustained muscle activity by using the methods of Nozaki et al. (2003). Reflex responses, which are similar to those in the previous study, were elicited during train stimulation. Therefore, we probably induced neuromuscular activity as self-sustained muscle activity that is likely to be associated with plateau potentials.

We attempted to measure self-sustained muscle activity of the triceps surae muscle for 1 min in all subjects, but the sustained muscle activity was depressed within 1 min in three subjects. Thus, RMS of EMG of the Sol, MG, and LG muscles was calculated from a 30-sec window immediately after the train stimulation. Although we could not determine the cause of these results, we thought that sustained muscle activities of the three subjects in several trials were suppressed by slight limb movement. Indeed, the sustained muscle activity was suppressed after the measuring period by voluntary dorsiflexion in our study. In a previous study, sustained muscle contraction that was induced by the same methods as those used in this study was shown to be depressed by electrical stimulation to an antagonist muscle nerve (Nozaki et al. 2003). Therefore, it is possibly because of slight contraction of an antagonist muscle such as the tibialis anterior muscle that the sustained muscle activity was depressed within 1 min in the three subjects.

Before the electrical train stimulation, RMSs of all muscles were not different

between the two conditions, while after stimulation, self-sustained muscle activities were significantly larger for the CO_2 rebreathing condition (Fig. 6). This suggests that an increase in sustained muscle activity was not due to differences in EMG activity before the train stimulation between conditions.

Self-sustained muscle activity is an indirect measure of plateau potentials (Nozaki et al. 2003). It is known that plateau potentials are facilitated via activation of L-type calcium channels and persistent sodium channels of spinal motoneurons (Bennett et al. 1998; Lee and Heckman 1999; Li et al. 2004). The channels are strongly activated by serotonin (5-HT) released from serotonergic neurons in the medullary raphe (Harvey et al. 2006a, 2006b; Perrier & Hounsgaard, 2003). Walton et al. (2003) demonstrated that self-sustained firing of the limb muscle motor unit, which is associated with plateau potentials, was facilitated by an increase in 5-HT via a dose of caffeine. Previous studies have shown that the 5-HT system of the brain is involved in spinal motor output (Heckman et al. 2005; Jacobs and Fornal 1993; Jacobs et al. 2002). In addition, plateau potentials are likely to be a fundamental component in the maintenance of posture (Hounsgaard et al. 1988; Lee and Heckman 1998). Furthermore, serotonergic neurons, which are a part of central chemoreceptors, are activated by an increase in $PaCO_2$ (Corcoran et al. 2013; Mitchell et al. 2008; Veasey et al. 1995, 1997; Wang et al. 2002).

Speculatively, if serotonergic projection from the medulla to spinal motoneurons is increased during a high $PaCO_2$ level of the body and if 5-HT can facilitate plateau potentials of spinal motoneurons, an increase in self-sustained muscle activity might be associated with activation of central chemoreceptors. However, there are several other possibilities for the increase in sustained muscle activity.

First, there is a matter of an increase in ventilatory activity per se during CO_2 rebreathing that might have influenced the sustained muscle activity. Balzamo et al. (1997) found that respiratory resistive breathing enhanced tonic vibratory response in an arm and a leg. They suggested that activation of respiratory muscle afferents facilitated limb motoneurons reflex. Second, the effect of a decrease in SpO_2 induced by CO_2 rebreathing on sustained muscle activity is not clear. It was shown that H-reflex amplitude in an arm muscle was increased during hypoxia induced by inhalation of hypoxic gas (Delliaux and Jammes 2006). This indicates that there is a direct or indirect effect of hypoxia on excitability of motoneurons. Therefore, to establish that CO_2 is indeed necessary to facilitate self-sustained muscle activity, the effect of hyperventilation at a rate under normoxic/isocapnic conditions should be investigated. Third, there was an increase in dyspnea sensation of the subjects during CO_2 rebreathing in the present study. In previous studies, a slight increase of H-reflex amplitude in the soleus muscle occurred

with respiratory discomfort caused by doses of lobeline and/or inspiratory threshold loading (Gandevia et al. 1998; Morélot-Panzini et al. 2007). It is possible that emotion alters the function of afferent fibers. With regard to serotonergic neurons, a link between the 5HT neuron system and the "emotional" system has been shown (Bowker et al., 1982; Holstege and Kuypers 1987) Therefore, further research in sleeping subjects to remove the emotional effect on sustained muscle activity may be required (Collins et al. 2001).

We measured self-sustained muscle activity for 30 sec in the two respiratory conditions and evaluated the effect of hypercapnia on self-sustained muscle activity. Self-sustained muscle activities were increased during CO_2 rebreathing. Kirkwood et al. (2005) reported that plateau potentials triggered by central respiratory drive potentials were observed in hindlimb motoneurons of a decerebrate anesthetized cat during hypercapnia. They indicated that activation of the plateau potentials of spinal motoneurons was dependent on the physiological state of the cat. Consequently, our study is the first study showing that hypercapnia is likely to influence self-sustained muscle activity originating from plateau potentials of triceps surae muscle motoneurons in humans. We conclude that self-sustained muscle activity in humans may be linked to the respiratory system, which is activated during hypercapnia.

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Figure 1. Schematic representation of the experimental protocol of the normal breathing condition and/or CO_2 rebreathing condition. The sustained muscle activity trial was repeated 6 times with intervals of 5-10 min. M-max and H-reflex of the soleus muscle were measured three times and five times, respectively, before the trial (*vertical arrows*). CO_2 rebreathing was performed from 30 sec after the initiation of the trial to the end of the trial (*horizontal arrow*). The vertical gray bar indicates the electrical train stimulation period.

Figure 2. A: Changes in minute ventilation ($\dot{V}_E$) and predicted arterial carbon dioxide pressure ($PaCO_{2pred}$) in the CO_2 rebreathing condition (*closed circle*) and normal breathing condition (*open circle*). A horizontal arrow indicates the CO_2 rebreathing period. Four squares drawn by dotted lines indicate the periods before CO_2 rebreathing (a^1, b^1,) and during the self-sustained muscle activity period (a^2, b^2). Vertical gray bars indicate the electrical train stimulation period. B: Comparisons of $\dot{V}_E$ and $PaCO_{2pred}$ between the CO_2 rebreathing condition (*black bar*) and normal breathing condition (*white bar*). a^1 and b^1 were before the CO_2 rebreathing period (data for 30 sec), and a^2 and b^2 were during the self-sustained muscle activity period (data for 1 min). Data are means ± SE for $n = 9$. * = significant difference between the two respiratory conditions ($P < 0.05$).

Figure 3. Comparison among all trials of the amplitudes of M-max and H-reflex in the soleus muscle measured before electrical train stimulation. H-reflex is expressed as a percentage of M-max. Data are means ± SE for $n = 9$.

Figure 4. EMG signals of the soleus muscle (Sol) and the medial head (MG) and lateral head (LG) of the gastrocnemius muscle during the electrical train stimulation period (*horizontal arrow*) in one subject. The first electrical stimulus induced the largest reflex response in the soleus muscle, but several subsequent responses were depressed (*lower left panel*) Then the reflex responses recovered with repetitive stimuli (*lower right panel*).

Figure 5. EMG signals in the soleus muscle (Sol) and the medial head (MG) and lateral head (LG) of the gastrocnemius muscle in one subject. In the CO_2 rebreathing condition (*left panel*) and the normal breathing condition (*right panel*), self-sustained muscle activities were induced for 1 min after electrical train stimulation (*vertical gray bars*) to the tibial nerve.

Figure 6. Comparisons of root mean squares (RMSs) of the soleus muscle (Sol) and the medial head (MG) and lateral head (LG) of the gastrocnemius muscle for 10 sec before the train stimulation (upper panel) and for the 30 sec after the train stimulation (lower panel) between the CO_2 rebreathing condition (*black bar*) and the normal breathing condition (*white bar*). Data are means ± SE for $n = 9$. * = significant difference between the two respiratory conditions ($P < 0.05$).

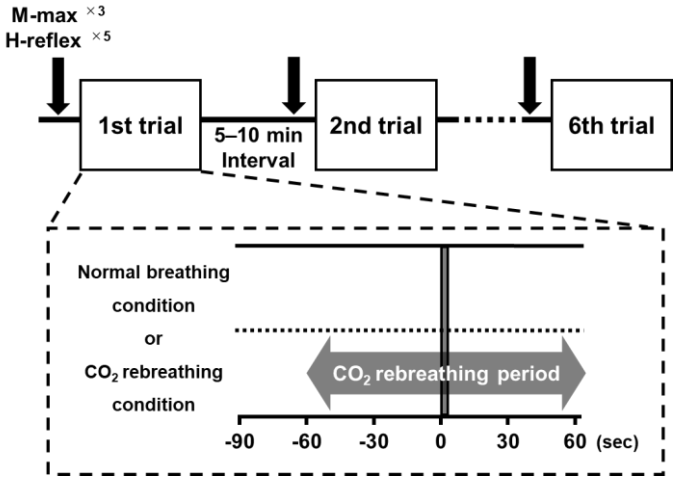

M-max ×3
H-reflex ×5
1st trial
5–10 min
Interval
2nd trial
6th trial
Normal breathing
condition
or
CO2 rebreathing
condition
CO2 rebreathing period
-90
-60
-30
0
30
60
(sec)

Figure 1

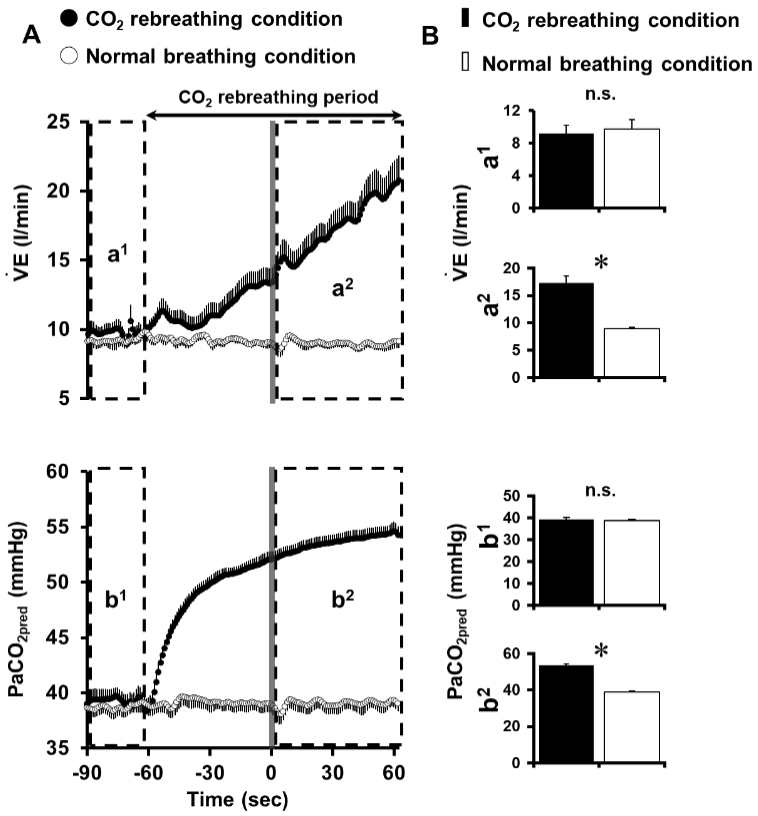

A
CO2 rebreathing condition
Normal breathing condition
CO2 rebreathing period
V̇E (l/min)
a1
a2
PaCO2pred (mmHg)
b1
b2
Time (sec)
B
CO2 rebreathing condition
Normal breathing condition
n.s.
*
n.s.
*

Figure 2

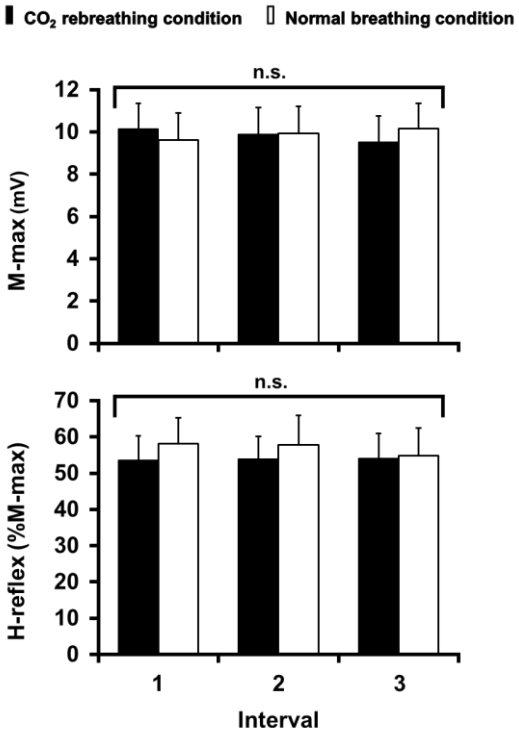

CO_2 rebreathing condition
Normal breathing condition
n.s.
M-max (mV)
12
10
8
6
4
2
0
n.s.
H-reflex (%M-max)
70
60
50
40
30
20
10
0
1
2
3
Interval

Figure 3

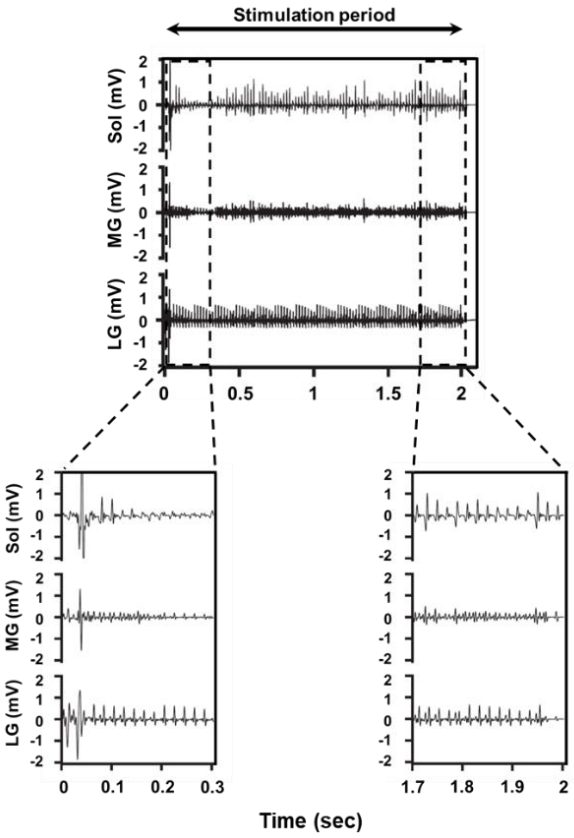

Stimulation period
Sol (mV)
MG (mV)
LG (mV)
0
0.5
1
1.5
2
0.1
0.2
0.3
1.7
1.8
1.9
Time (sec)

Figure 4

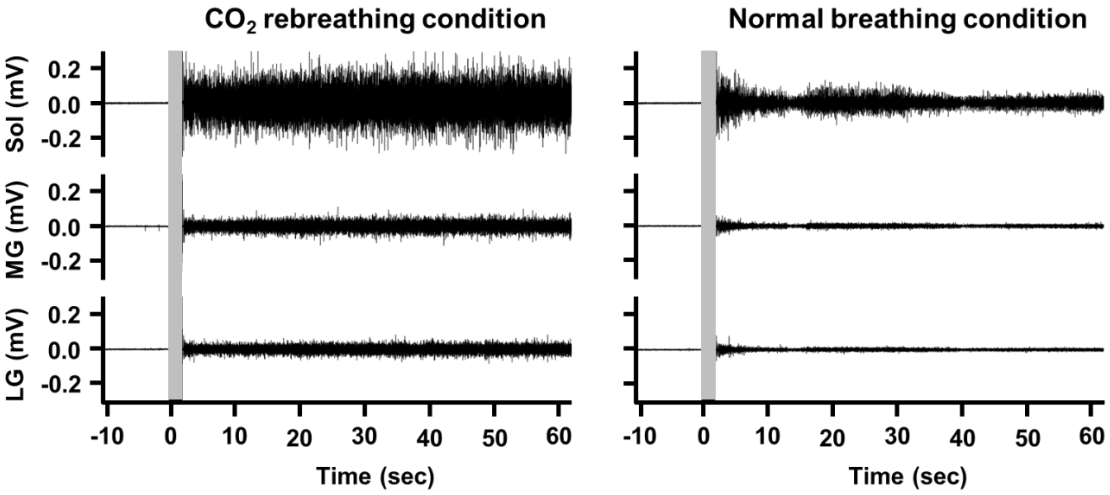

CO_2 rebreathing condition
Normal breathing condition
Sol (mV)
MG (mV)
LG (mV)
0.2
0.0
-0.2
-10
0
10
20
30
40
50
60
Time (sec)

Figure 5

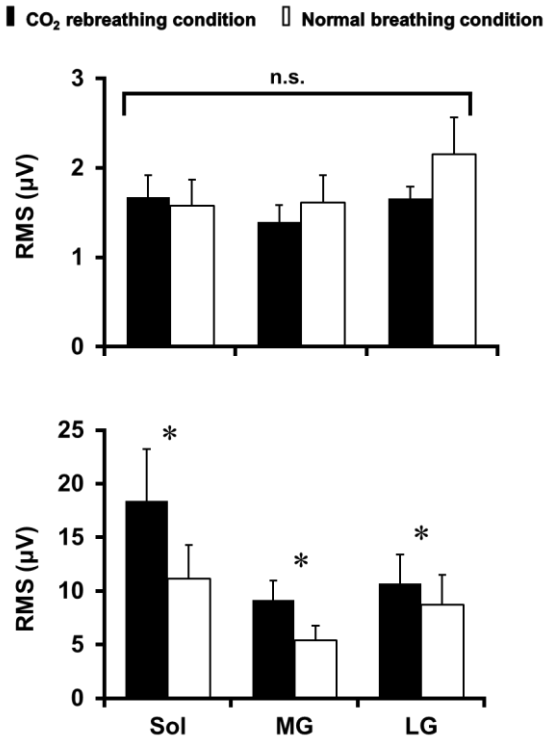

CO2 rebreathing condition
Normal breathing condition
n.s.
RMS (μV)
3
2
1
0
25
20
15
10
5
0
*
*
*
Sol
MG
LG

Figure 6