Selective revealing of gap junction currents in single inspiratory tracheal motor neurons

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Abstract: Little is known about how gap junctions are involved in the respiratory-related or other types of physiological neuronal activity since physiologically active gap junction currents (GJCs) have never been characterized from single respiratory-related neurons or from single neurons of any other types. In the present study we hypothesized that GJCs could be selectively revealed from single neurons by elimination of transmembrane electrochemical gradients in voltage patch-clamp recording, and this hypothesis was tested in single inspiratory tracheal preganglionic vagal motor neurons (I-TPVMs). The results showed that GJCs were selectively revealed in all I-TPVMs when the transmembrane electrochemical gradients were eliminated in voltage patch-clamp recording, and were rhythmically activated by central inspiratory activity. Therefore, this method may be used as a fast way to detect GJCs within spontaneously active neuronal networks.

Key words: gap junction; respiration; motor neurons; patch-clamp techniques

电突触电流在单个吸气性气管运动神经元中的选择性显示

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摘 要：在呼吸相关神经元或其它任何类型的神经元中，与生理性自发活动相对应的电突触电流(gap junction currents, GJCs)尚未在单个神经元中被记录到，因此电突触如何参与呼吸相关的或其它类型的生理性活动，目前所知甚少。在本研究中，我们假设GJCs可在电压钳记录条件下通过消除跨膜电化学梯度在单个神经元实现选择性记录，并在单个吸气性气管迷走神经节前神经元(inspiratory tracheal preganglionic vagal motor neurons, I-TPVMs)进行验证。结果显示，用这种方法在所有I-TPVMs中均记录到GJCs，且这些神经元的GJCs可被节律性中枢吸气活动所激活。此法可用于快速探测具有自发活动的神经元网络中的GJCs。

关键词：电突触；呼吸；运动神经元；膜片钳技术

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Gap junctions are being found in more and more areas or neuronal networks in the central nervous system[1]. In brainstem gap junctions have been found in respiratory rhythmogenesis neurons in the pre-Botzinger complex[2,3], the inspiratory motor neurons of the hypoglossal nucleus and the nucleus ambiguus (NA)[4,5], and many other areas related to the regulation of central respiratory activity. Investigation of gap junction activity in respiratory-related neurons is critical in recognizing the functions of gap junctions in respiratory control. Previous studies have attempted to obtain gap junction currents (GJCs) by paired patch-clamp recording of two respiratory-related neurons (dual patch)[3,5]. Using this method GJCs were obtained from one of the paired neurons by stimulation of the other. Also, this method has been proved useful in appraising the coupling ratio of connected neurons. However as to the detection of GJCs, much patching effort is needed for dual patch to find a connected neuronal pair, and the GJCs recorded
did not reflect the ongoing activity of gap junctions in this neuronal network.

We speculate whether it is feasible to detect GJCs from single neurons in a spontaneously active neuronal network. The major difficulty resides on how to selectively isolate GJCs without using blockers of chemical synapses, which might interfere with or even prevent the occurrence of GJCs in single neurons. The electrochemical gradients between the inside and outside of neurons are the basis for chemical synapses to generate ligand-gated currents. In voltage patch-clamp recording these gradients can be eliminated artificially. However, it is not known whether GJCs can be selectively revealed under such condition.

Tracheal preganglionic vagal motor neurons (TPVMs) in the ventrolateral medulla have been indicated to play an important role in respiratory control. Ablation of these neurons significantly suppressed the ventilatory responses to hypercapnia and hypoxia in conscious rats⁹. In a recent study of our laboratory the respiratory-related synaptic control of these neurons has been characterized electrophysiologically⁷. However, whether these neurons have gap junctions is not known. The present study is based on the hypothesis that inspiratory TPVMs (I-TPVMs) have gap junctions, and the GJCs in single I-TPVMs can be selectively revealed by elimination of the electrochemical gradients in voltage patch-clamp recording.

1 MATERIALS AND METHODS

1.1 Retrograde fluorescent labelling of tracheal motor neurons

Tracheal motor neurons were retrogradely labeled by injection of fluorescent dye into the tracheal muscles, as we have described before⁷. Briefly, halothane (0.5 mL) was dripped into a glass box (5 cm × 5 cm × 5 cm) with a lid and cotton pad on the bottom and 3- to 4-day old Sprague-Dawley rats (Shanghai Institute for Family Planning) were put into the box for 30 s with the lid on. This procedure anaesthetized the rats but kept their breathing in a relatively normal state. When the rats lost responsiveness to a needle prick stimulus to the limbs, the body was surrounded by ice-filled bags to lower the body temperature and slow-down the heart beat. Once spontaneous breathing stopped (usually within 2 min), the animal was put on an ice-filled bag (tip diameter 30 µm), which was attached to a syringe through polyethylene tubing. The incision was sutured, and the animals were heated with a thermostatically controlled (30 °C) pad to help recovery. During the whole surgery period (about 5 min), the body temperature of the animal was below 10 °C and the animal exhibited no spontaneous breathing or struggling. After the surgery, the animals usually started spontaneous breathing within 3 min and started moving freely within another 5 min. The animals were allowed 48-52 h to recover.

1.2 Slice preparation

The animal was anaesthetized deeply with halothane applied as before and decapitated at the supracollicular level. The brain was submerged in cold (4 °C) artificial cerebral spinal fluid (ACSF) with the following composition (in mmol/L): NaCl 124, KCl 3.0, KH₂PO₄ 1.2, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 26, D-glucose 10; constantly bubbled with 95% O₂-5% CO₂, pH 7.4. The cerebellum was removed and the hindbrain was isolated using a dissection microscope. The brainstem was then secured in the slicing chamber of a vibratome (Leica VT 1000S) filled with the same ACSF. The rostral end of the brainstem was set upwards and the dorsal surface was glued to an agar block facing the razor. The brainstem was sectioned serially at variable thickness in the transverse plane. Once the NA was visible under the microscope, a single medulla slice of 500-800 µm thick, of which one to two hypoglossal rootlets in each side were retained, was taken for experiment. The thick medullary slice preparation contained the pre-Botzinger complex, local circuits for motor output generation, and respiratory hypoglossal motor neurons, which generate inspiratory-phase motor discharge in hypoglossal cranial nerves⁸. The slice was transferred into the recording chamber and submerged in flowing ACSF (flow rate, 8-11 mL/min). The rostral cutting plane of the slice was set upwards to allow fluorescent identification and patch-clamp recording of tracheal motor neurons in the ventrolateral medulla. The temperature was maintained at (23±0.5) °C, and the concentration of KCl in the ACSF was increased to 10 mmol/L to allow steady recording of the respiratory rhythm.

1.3 Electrophysiological recording

Individual tracheal motor neurons were identified in the area ventral/ventrolateral to the compact portion of the NA by the fluorescent tracer using an Olympus upright microscope with a 40× water immersion objective. The patch pipettes (2.0-5.0 MΩ) were normally filled with a solution consisting of (in mmol/L): NaCl 124, KCl 3.0, KH₂PO₄,
1.2. CaCl₂ 1.0, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 10, Mg-ATP 2, NaHCO₃ 26, and D-glucose 10 (pH 7.4). QX314 (lidocaine N-ethyl bromide, 2.0 mmol/L) was included in the pipette solution to prevent action potential generation. The cells were voltage clamped at 0 mV under whole-cell configuration. With this pipette solution and a holding voltage of 0 mV the transmembrane currents mediated by chemical synapses were assumed to be undetectable since there is little ion gradient and no voltage gradient between the intra- and extracellular spaces. Voltage cell-attached recording (holding voltage, 0 mV) was made to observe the respiratory-related discharge behaviours of tracheal motor neurons before gaining into intracellular access, and to identify I-TPVMs. The membrane resistance and capacitance were not compensated either before or after gaining into intracellular access. In some experiments the patch pipettes were filled with a solution consisting of (in mmol/L): K⁺ gluconate 130, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, EGTA 10, CaCl₂ 1.0, and MgCl₂ 1.0 (pH 7.3). Once a tracheal motor neuron was identified as I-TPVM under cell-attached configuration (holding voltage, 0 mV), the cell was voltage clamped at -80 mV after gaining into intracellular access. Action potential currents were evoked in I-TPVMs by gradedly depolarizing the cells from -80 to 0 mV (10 mV step).

The patch-clamp signal was amplified with an Axopatch 200B amplifier (10 kHz sampling frequency; 1 kHz filter frequency), digitized with Digidata 1322A, and collected with Clampex 9.0 software (Axon Instruments, USA). The activity of the hypoglossal rootlets was recorded using a suction electrode. Respiratory-related hypoglossal signal was amplified with a BMA-931 bioamplifier (5 kHz sampling frequency; 10 000 Hz band-pass; 20 000 times), and electronically integrated (time constant τ = 200 ms) with MA-1000 Moving Averager (CWE Inc., Ardmore, PA, USA). Both the raw and the integrated hypoglossal signals were digitized with Digidata 1322A, and were fed into the computer simultaneously with the patch-clamp signal. All animal procedures were performed in compliance with the Institutional Guidelines at Fudan University, and were in accordance with the internationally accepted principles in the Care and Use of Experimental Animals.

1.4 Drug application
Gap junction blocker carbeneoxolone (CBX, 100 µmol/L) was included in the pipette solution to block gap junctions from the inside of putative TPVMs. CBX was purchased from Sigma-Aldrich (St Louis, MO, USA).

1.5 Data analysis
The inspiratory-related slow inward currents were analyzed with Clampfit 9.0 (Axon Instrument, USA). The inspiratory-related spikelets were analyzed with MiniAnalysis (Synaptosoft, version 4.3.1) with minimal acceptable amplitude at 10 pA. Data were presented as means±SEM and statistically compared with independent Student’s t test. Significant difference was set at P<0.05.

2 RESULTS

2.1 Electrophysiological identification of I-TPVMs
Fluorescently labeled TPVMs were characteristically located in the area ventral/ventrolateral to the compact portion of the NA. These neurons were larger in size than the neurons within the compact portion of the NA, and were typically multipolar or spindle in shape (Fig.1A). Those TPVMs that exhibited trains of inspiratory discharges under cell-attached patch-clamp configuration were defined as I-TPVMs (Fig.1B).

2.2 CBX-sensitive inspiratory-related currents in I-TPVMs
Upon gaining into intracellular access I-TPVMs generated an abrupt outward current at a holding voltage of 0 mV, and the activity of chemical synapses, which was featured by the greatly varied amplitude, duration and shapes, could still be observed initially. The abrupt outward current, which in the beginning could be over 1 000 pA in some individual neurons, decayed rapidly within the first 30 s to 1 min, and the activity of chemical synapses quickly disappeared in this short period (Fig.2A).

After the rapid decay of the abrupt outward current the baseline current became relatively stable but was still outward [namely the baseline outward current; (141±21) pA, averaged at 5 min after rapture of cell membrane]. In this state, while no activity of chemical synapses was shown in I-TPVMs, inspiratory-related slow inward current was exclusively (examined in 27 neurons) generated, on which trains of inspiratory-related spikelets were superimposed (Fig.2B). The duration of the inspiratory-related spikelets [2.98±0.01 ms, n=27] was quite consistent either in individual I-TPVMs or among different I-TPVMs, suggesting that they might come from the propagation of the action potential current of its neighbouring I-TPVMs through gap junctions. To strengthen this assumption action potential current was evoked in an I-TPVM with the patch pipette filled with K⁺ gluconate-dominated pipette solution, and the duration of the action potential
current was compared with that of the inspiratory-related spikelet. The result showed that the action potential current in an I-TPVM had a duration of \((3.03\pm0.01)\) ms \((n=5)\), not significantly different from that of the inspiratory-related spikelet. A sample inspiratory-related spikelet was compared with the action potential current in an I-TPVM in Fig.2C.

Interestingly, even in a relatively stable state the baseline outward currents in I-TPVMs still declined progressively and slowly, and the inspiratory-related slow inward currents and spikelets also declined in amplitude correspondingly. Usually the inspiratory-related currents became undetectable about 1 h after gaining into intracellular access (observed in 5 I-TPVMs). However, on a time scale of several minutes both kinds of inspiratory-related currents were quite consistent either in the amplitude or in the current area. Figure 2D showed the slow baseline current declination with time in a representative I-TPVM. The declination with time in the amplitude and the current area of the inspiratory-related spikelet in this I-TPVM were shown in Fig.2E and 2F, respectively.

Elimination of the transmembrane electrochemical gradients might have caused progressive loss of the functions of the patched I-TPVMs, which might contribute to the baseline declination and prevention of the neurons from responding to any inputs. To rule out this possibility 5 I-TPVMs were repolarized to -80 mV after the inspiratory-related currents had decayed and became undetectable. As shown in Fig.2G, all these neurons still showed active synaptic activity with inspiratory enhancement, suggesting that their membrane functions were still normal to a large extent.

To test whether gap junctions were involved in the generation of the inspiratory-related currents in I-TPVMs, we initially globally applied 100 µmol/L CBX in the bath. Unfortunately, this protocol eliminated the inspiratory hypoglossal bursts in all the 3 slices tested, as reported previously\[3\]. We next applied 100 µmol/L CBX in the pipette solution. In 3 of the 7 I-TPVMs examined no inspiratory-related currents were detected from the time of gaining into intracellular access. In 4 of these 7 neurons the inspiratory-related slow inward currents and spikelets initially appeared in much smaller amplitude than that in the control, and became neglectable within 1-3 min. In the presence of intracellular CBX there was also an abrupt outward current upon gaining into intracellular access. After the rapid decay process of the abrupt outward current the baseline current was close to zero \((7.2\pm3.1)\) pA, averaged at 5 min after rapture of cell membrane; \(P<0.05\) compared with control] and remained stable. At 5 min after rapture of cell membrane, the measured inspiratory-related slow inward current was \((4.6\pm0.3)\) pA \((n=7)\) in the intracellular presence of CBX, and was \((36.3\pm3.7)\) pA \((n=27)\) in the control \((P<0.05)\). An example of such experiment in Fig.2H showed the absence of inspiratory-related currents in these neurons in the presence of intracellular CBX. These
Fig. 2. I-TPVMs generated CBX-sensitive currents upon elimination of the electrochemical gradients. A: The abrupt outward current upon gaining into intracellular access in I-TPVMs. Note that the activity mediated by chemical synapses disappeared quickly along with the rapid decay of the abrupt outward current. B: The inspiratory-related slow inward current and spikelet in a representative I-TPVM. Note the absence of the activity mediated by chemical synapses in this I-TPVM during inspiratory intervals. C: A sample inspiratory-related spikelet was compared with the action potential current in a sample I-TPVM. Note their similarity in the duration. D: The slow declination of the baseline outward current with time in a sample I-TPVM. E: The declination of the amplitude of the inspiratory-related spikelet with time in the same I-TPVM as in D. F: The declination of the current area of the inspiratory-related spikelet with time in the same I-TPVM as in D. G: Active synaptic activity upon repolarization was still shown in I-TPVM even when the inspiratory-related spikelets had become undetectable. H: In the presence of intracellular CBX the inspiratory-related slow inward current and spikelet were neglectable.
results suggested that I-TPVMs exclusively had gap junctions. To test whether the inspiratory-related slow inward currents and spikelets were specific to neurons with gap junctions, 8 neurons within the compact portion of the NA were randomly selected and examined in the same way as those I-TPVMs examined. Under cell-attached configuration, most of these neurons (5 out of 8) were silent and 3 of them were spontaneously active with no respiratory-related rhythm. In none of these neurons were inspiratory-related slow inward currents or spikelets detected. There was also an abrupt outward current upon gaining into intracellular access in these neurons. However, this abrupt outward current disappeared or became neglectable after a similar decay process as that in I-TPVMs, and the baseline current remained stable. A sample experiment was shown in Fig.3A. In contrast, hypoglossal motor neurons, which had been reported to have gap junctions[4,5], exclusively (examined in 6 neurons) showed similar inspiratory-related slow inward currents and spikelets (Fig.3B).

3 DISCUSSION

There are two major findings in the present study. First, I-TPVMs had gap junctions, and the currents mediated by which could be selectively revealed in single I-TPVMs by elimination of transmembrane electrochemical gradients in voltage patch-clamp recording. Second, the GJs in I-TPVMs were rhythmically activated by central inspiratory activity.

Upon gaining into intracellular access the membrane potential of I-TPVMs was suddenly changed from its original negative value to 0 mV. This sudden change would be expected to generate a temporary outward current in I-TPVMs if the intracellular fluid was not quickly replaced by the pipette solution. In the present study, the abrupt outward current occurred in I-TPVMs upon gaining into intracellular access was in accordance with this expectation. The time period of the rapid decay of this current might represent the time during which the intracellular fluid in I-TPVMs was balanced by the pipette solution. When CBX was included in the pipette solution, this time period might also represent the time for CBX to spread in the patched neuron and start to take effect.

By clamping the patched I-TPVMs at 0 mV, it is assumed that a voltage difference had occurred between the patched neuron and its neighbouring neurons to which the patched neuron was connected by gap junctions, and this voltage difference would cause ion exchange between the patched neuron and its neighbouring neurons. Thus, the baseline outward current in I-TPVMs in the present study might be caused by this assumed ion exchange. In support of this postulation, the neurons within the compact portion of the NA had little or no baseline outward currents, and I-TPVMs either had little baseline outward currents once CBX was included in the pipette solution. Theoretically if this assumed ion exchange could be compensated by the membrane mechanisms of those neighbouring neurons, the membrane potentials of those neighbouring neurons would be normal, and the ion exchange would maintain at a relatively fixed rate. As a result, the baseline outward currents recorded in the patched I-TPVMs would maintain at a fixed level. In the present study the baseline outward currents in I-TPVMs actually declined slowly and

Fig. 3. Gap junction-mediated inspiratory-related currents were specific to neurons with gap junctions. A: Neurons within the compact portion of the nucleus ambiguus (NA) showed no inspiratory-related currents in voltage patch-clamp recording when the transmembrane electrochemical gradients were eliminated. B: Similar inspiratory-related slow inward currents and spikelets were shown in hypoglossal motor neurons as that in I-TPVMs when recorded with the same method.
progressively in relatively steady state, suggesting that these neighbouring neurons could not compensate this ion exchange and they were also influenced by the clamping voltage in the patched I-TPVMs.

The inspiratory-related spikelets in I-TPVMs in the present study might be caused by the propagation of the action potential currents of its neighbouring neurons through gap junctions. First, the duration of the inspiratory-related spikelets in I-TPVMs was almost identical in individual neurons and among different neurons, and was comparable with the duration of the action potential currents in this type of neurons. Second, they were not likely caused by activation of the voltage-gated sodium channels since these channels should have been deactivated by elimination of transmembrane electrochemical gradients and by inclusion of QX314 in the pipette solution. Third, they were sensitive to intracellular CBX.

It must be noted that by elimination of the transmembrane electrochemical gradients the patched neurons in the present study had been made in non-physiological state. The slow declination of the baseline currents as well as the decay of GJCs with time in I-TPVMs might be in part due to the gradual breakdown of the patched neurons. However, since repolarization of the patched I-TPVMs showed normal membrane responses to synaptic inputs, elimination of their transmembrane electrochemical gradients might not have caused severe damage. As to the decay of the inspiratory-related spikelets, there is another possibility that QX314, by diffusing to the neighbouring neurons of the patched I-TPVMs, might decrease the amplitude of the action potentials of these neighbouring neurons and result in decreased propagation of them through gap junctions.

In the present study, GJCs, although with spontaneous decay, were detected in single neurons. Compared with dual patch, the method used in the present study might be superior in fast detection of GJCs, especially within a spontaneously active neuronal network.

In conclusion, the present study has shown that I-TPVMs have gap junctions, and the currents mediated by which could be selectively revealed in single I-TPVMs by elimination of transmembrane electrochemical gradients. The GJCs in I-TPVMs were activated by central inspiratory activity. The method used in the present study to detect GJCs may be used as a fast way to detect gap junctions within spontaneously active neuronal networks.

REFERENCES