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ROLE OF NEUROKININ RECEPTORS AND IONIC MECHANISMS WITHIN THE RESPIRATORY NETWORK OF THE LAMPREY

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Abstract—We have suggested that in the lamprey, a medullary region called the paratrigeminal respiratory group (pTRG), is essential for respiratory rhythm generation and could correspond to the pre-Bötzinger complex (pre-BötC), the hypothesized kernel of the inspiratory rhythm-generating network in mammals. The present study was performed on *in vitro* brainstem preparations of adult lampreys to investigate whether some functional characteristics of the respiratory network are retained throughout evolution and to get further insights into the recent debated hypotheses on respiratory rhythmogenesis in mammals, such as for instance the "group-pacemaker" hypothesis. Thus, we tried to ascertain the presence and role of neurokinins (NKs) and burst-generating ion currents, such as the persistent Na^+ current (I_{NaP}) and the Ca^{2+}-activated non-specific cation current (I_{CAN}), described in the pre-Bötzinger complex. Respiratory activity was monitored as vagal motor output. Substance P (SP) as well as NK1, NK2 and NK3 receptor agonists (400–800 nM) applied to the bath induced marked increases in respiratory frequency. Microinjections (0.5–1 nl) of SP as well as the other NK receptor agonists (1 μM) into the pTRG increased the frequency and amplitude of vagal bursts. Riluzole (RIL) and flufenamic acid (FFA) were used to block I_{NaP} and I_{CAN}, respectively. Bath application of either RIL or FFA (20–50 μM) depressed, but did not suppress respiratory activity. Coapplication of RIL and FFA at 50 μM abolished the respiratory rhythm that, however, was restarted by SP microinjected into the pTRG. The results show that NKs may have a modulatory role in the lamprey respiratory network through an action on the pTRG and that I_{NaP} and I_{CAN} may contribute to vagal burst generation. We suggest that the "group-pacemaker" hypothesis is tenable for the lamprey respiratory rhythm generation since respiratory activity is abolished by blocking both I_{NaP} and I_{CAN}, but is restored by enhancing network excitability.

Key words: flufenamic acid, paratrigeminal respiratory group, respiration-related neurons, respiratory rhythm generation, riluzole, substance P.

In mammals, it has been proposed that the pre-Bötzinger complex (pre-BötC) contains the kernel of the inspiratory rhythm-generating network (reviewed in Feldman and Del Negro, 2006; Smith et al., 2009). Neurons specifically expressing μ-opioid receptors and neurokinin1 (NK1) receptors localized within the pre-BötC of neonatal rodents have been reported to modulate respiratory frequency and to be essential for respiratory rhythm generation (Gray et al., 1999, 2001). It has also been shown that the μ-opioid receptor agonist [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) microinjected into the pre-BötC causes depressant effects on respiratory activity (Gray et al., 1999). Substance P (SP) is the endogenous agonist of neurokinin (NK) receptors that preferentially acts via NK1 receptors, but may also activate the other tachykinin receptors, that is NK2 and NK3 receptors (Pennefather et al., 2004 also for further Refs). Interestingly, SP microinjections into the pre-BötC region exert excitatory effects on respiratory activity in *in vitro* (Gray et al., 1999, 2001; Hayes and Del Negro, 2007) and *in vivo* (Bongianni et al., 2008) preparations. Little knowledge is available on the respiratory role played by NKA and NKB, the endogenous tachykinins which interact with NK2 and NK3 receptors, respectively (Haxhiu et al., 1990; Monteau et al., 1996; Bongianni et al., 2008). An important and controversial issue is the role of pre-BötC neurons that show bursting-pacemaker activity in the absence of synaptic transmission in respiratory rhythm generation. In particular, bursting-pacemaker activity can have two different ionic mechanisms (Thoby-Brisson and Ramirez, 2001; Del Negro et al., 2002a, 2005; Peña et al., 2004; reviewed in Feldman and Del Negro, 2006). One ionic mechanism for pacemaker activity is proposed to generate bursts via a Ca^{2+}-activated non-specific cation current (I_{CAN}) and its bursting properties are selectively blocked by flufenamic acid (FFA). The other ionic mechanism generates bursting-pacemaker activity through the persistent Na^+ current (I_{NaP}) and its bursting properties are blocked by riluzole (RIL). Co-application of RIL and FFA eliminates the respiratory rhythm in rodent medullary slices *in vitro*, while respiratory activity persists upon blockade of either I_{NaP} or I_{CAN} alone (Del Negro et al., 2005). The two drugs probably induce a lowering of the excitability of all neurons which express I_{NaP} or I_{CAN}, regardless of their effects on pacemaker neurons (Del Negro et al., 2002b, 2005; Pace et al., 2007b; reviewed in Feldman and Del Negro, 2006). Accordingly, the rhythmogenic role of pacemaker neurons does not seem to be obligatory since rhythmic activity could be restored by increasing network excitability with SP application in the presence of RIL and FFA (Del Negro et al., 2005). Therefore, rhythm generation appears to be an emergent property of non-pacemaker neurons that are interconnected by excitatory synaptic interactions. All these neuronal features have been incor-

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Abbreviations: CPG, central pattern generator; DAMGO, [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin; DMSO, dimethyl sulfoxide; FFA, flufenamic acid; I_{CAN}, Ca^{2+}-activated non-specific cation current; I_{NaP}, persistent Na^+ current; NK, neurokinin; pre-BötC, pre-Bötzinger complex; pTRG, paratrigeminal respiratory group; RIL, riluzole; SP, substance P.

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porated in the so-called "group-pacemaker" hypothesis (see Feldman and Del Negro, 2006).

For many years, we have investigated the role of neurotransmitters and neuromodulators within the respiratory network of the lamprey, a lower vertebrate which diverged from the main vertebrate line around 450 million years ago (Hotton, 1976) and has proved to be highly useful to identify the cellular mechanisms underlying neural control of locomotion in vertebrates (Grillner, 2006). The isolated brainstem of the adult lamprey spontaneously generates respiratory neuronal activity *in vitro*. The vast majority of respiratory motoneurons are located in the facial, glossopharyngeal and, especially, in the vagal nuclei, while the neural aggregate responsible for respiratory rhythm generation has not yet been completely defined but appears to be located in a region rostrolateral to the trigeminal motor nucleus (Rovainen, 1983, 1985; Russell, 1986; Thompson, 1985; Bongianni et al., 1999, 2002, 2006; Gravel et al., 2007; Martel et al., 2007; Mutolo et al., 2007). We have provided evidence that glutamatergic neurotransmission is crucial for the respiratory rhythmogenesis in the lamprey (Bongianni et al., 1999), while GABAergic and glycinergic inhibitory mechanisms only contribute to maintain a stable and regular breathing pattern (Bongianni et al., 2006). Recently, it has been shown that the depressant influences of opioids on respiratory activity are present not only in mammals (e.g. Bianchi et al., 1995; Gray et al., 1999; Feldman and Del Negro, 2006) but also in the lamprey and that microinjections of the μ-opioid receptor agonist DAMGO into a rostrolateral trigeminal region, where respiration-related neurons have been encountered, abolish the respiratory rhythm (Mutolo et al., 2007). This region, termed the paratrigeminal respiratory group (pTRG), likely plays a pivotal role in the lamprey respiratory rhythmogenesis. Extracellular recordings have been performed to characterize the different discharge patterns of pTRG neurons. Intracellular recordings, that are the only suitable to ascertain the presence of respiratory neurons in this region and to discriminate cells from by-passing fibers, are still lacking probably due to the small size of pTRG neurons, as revealed by histological observations (Mutolo et al., 2007). Most of the above reported findings suggest that several properties of the respiratory network are highly retained throughout evolution (Kinkead, 2009). Thus, a comparative approach could be important to derive broader biological principles and a more comprehensive view of the basic neural mechanisms responsible for respiratory rhythm generation. In fact, they are still not fully elucidated and remain highly debated (see, e.g. Feldman and Del Negro, 2006; Smith et al., 2009). In this scenario, we reasoned that the demonstration that some functional characteristics of the respiratory central pattern generator (CPG) are retained throughout evolution could provide support to some of the recent disputed hypotheses on respiratory rhythm generation in mammals and, in particular, to the "group-pacemaker" hypothesis. No information is available on the presence and role of NKs, I_{NaP} and I_{CAN} within the lamprey respiratory network. To address these issues we undertook the present study on the *in vitro* brainstem preparation of adult lampreys with a threefold purpose. First, to make an attempt to record respiratory neurons within the pTRG and further characterize their discharge patterns. Second, to ascertain whether NKs affect respiratory activity by using not only bath application of SP or NK1, NK2 and NK3 receptor agonists and antagonists, but also microinjections of the same drugs at the level of the pTRG. Third, to ascertain the presence of the inspiratory burst-promoting currents described in mammals, that is the I_{NaP} and the I_{CAN}, in the lamprey respiratory network and their contribution in the respiratory rhythm generation by bath application of RIL and FFA, either alone or combined. If blockades of these currents abolish respiratory activity, the "group-pacemaker" hypothesis was tested in the lamprey by increasing the excitability of pTRG neurons with SP.

Preliminary accounts of this work have been published in abstract form (Mutolo et al., 2008, 2009).

EXPERIMENTAL PROCEDURES

Lamprey brainstem preparation

Experiments were carried out on 53 young adult lampreys (*Petromyzon marinus*). All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Communities Council on the use of laboratory animals (directive 86/609/EEC). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize the number of animals used. Animal preparation and experimental procedures were similar to those described in previous reports (Bongianni et al., 1999, 2002, 2006; Mutolo et al., 2007). The animals were anesthetized with tricaine methanesulfonate (100 mg/l; MS 222, Sigma-Aldrich, St. Louis, MO, USA) and transected below the gills. Muscles and connective tissues were removed and the isolated brain-spinal cord was mounted dorsal side up in a Sylgard-lined (Dow Corning, Midland, MI, USA) recording chamber continuously perfused with a cold solution using a peristaltic pump. The chamber volume was 3.0 ml and the perfusion rate was set at 2.5 ml/min. Bath temperature was maintained at 9–10 °C. The solution (control solution) flowed from a reservoir and had the following composition (in mM): 91 NaCl, 2.1 KCl, 2.6, $CaCl_2$, 1.8 $MgCl_2$, 4 glucose, 23 $NaHCO_3$. The solution was continuously bubbled with 95% O_2-5% CO_2 to oxygenate and maintain the pH in the bath at 7.4. The brain was exposed dorsally and the choroid plexus removed; the brain tissue rostral to the optic tectum was cut and removed. Caudally, a transection was made below the obex, maintaining a minimum length of spinal cord to hold the preparation. Recordings and microinjections within the pTRG as well as neighboring sites were performed under microscope control (Stemi 2000, Zeiss, Göttingen, Germany). These maneuvers were facilitated by cutting the roof of the isthmic region along the midline, spreading the alar plates laterally and pinning them down. In agreement with previous reports (Thompson, 1985; Russell, 1986; Mutolo et al., 2007), these procedures had no significant effect on respiratory activity.

Recording procedures

Efferent respiratory activity was recorded bilaterally from the vagal nerves by means of suction electrodes. The signals were then amplified, full-wave rectified and integrated (low-pass filter, time constant 10 ms). In control trials, brainstem preparations spontaneously produced a stable and regular fictive respiratory rhythm for at least 12 h (Rovainen, 1985; Thompson, 1985; Russell, 1986; Bongianni et al., 1999, 2002, 2006; Mutolo et al., 2007). Extracel-