Ionic Currents and Endogenous Rhythm Generation in the Pre-Bötzinger Complex: Modelling and *In Vitro* Studies

Olivier Pierrefiche, Natalia A. Shevtsova, Walter M. St.-John, Julian F. R. Paton, and Ilya A. Rybak

1. Introduction

The pre-Bötzinger complex (pBC), a small area in the rostroventrolateral medulla, has been suggested to represent a "kernel" of the mammalian respiratory network[1−5]. The *in vitro* preparations from neonatal rodents containing this area can, under certain experimental conditions, generate an intrinsic rhythmic activity[4,5]. This activity does not require inhibitory neurotransmission[6] and, therefore, is likely to be generated by a population of pacemaker neurons in the pBC[1−5]. At the same time, the "decrementing" discharge pattern of rhythmic activity in the pBC recorded *in vitro* differs from the pattern of respiratory discharges observed under normal conditions *in vivo* ("eupnoea") and is similar to gasping pattern[7,8]. In order to establish possible relationships of the intrinsic rhythmic activity in the pBC to the respiratory rhythmogenesis *in vivo*, it is important to analyse the conditions in which this activity occurs *in vitro* and to compare these conditions with the rhythmogenic conditions during eupnoea and gasping *in vivo*. According to the preliminary modelling studies[9], the *in vitro* rhythmic activity in the pBC may be dependent on a relative expression of the voltage-gated potassium and persistent sodium currents in pBC neurons. Here we present the results of our combined modelling and *in vitro* studies performed to test this modelling prediction. Our studies focused on the involvement of the potassium and persistent sodium currents in the endogenous rhythmic activity in the pBC *in vitro* and on the possible relation of this activity to the genesis of the respiratory oscillations *in vivo*.

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2. Modeling Intrinsc Bursting Activity in the PBC

The model of a single pBC pacemaker neuron was developed using the Hodgkin-Huxley formalism. The model was based on the previous models[2,3] and included fast sodium (I_{Naf}), persistent sodium (I_{NaP}) delayed-rectifier potassium (I_K), leakage (I_{leak}), and synaptic (I_{syn}) currents which together defined the dynamics of the neuron membrane potential. The voltage-gated and kinetic parameters for I_{Naf} and I_{NaP} were drawn from recent _in vitro_ studies of isolated pBC neurons[10]. To investigate firing behaviour of a pacemaker neuron population we modelled a population of 50 neurons with all-to-all excitatory synaptic connections. Heterogeneity within the population was set by the random distribution of the maximal channel conductances ($\bar{g}_{NaP}$, $\bar{g}_K$, g_{leak} and g_{syn}). For more details see Rybak _et al._[11]

Figure 1 shows results of our simulations. We found that the population bursting activity could be induced in the model by an elevation of the external potassium concentration $[K^+]_o$ from the physiological level (3 mM) to higher levels (Figure 1A) or by changing the maximal conductances of potassium ($\bar{g}_K$, Figure 1B) and persistent sodium ($\bar{g}_{NaP}$, Figure 1C) channels at the normal level of $[K^+]_o$. Our modelling study has demonstrated that rhythmic bursting activity in a population of pacemaker neurons may be induced by either (1) an increase of the extracellular potassium concentration, or (2) a suppression of the voltage-gated potassium currents, or (3) an augmentation of the persistent sodium currents (see Figure 1).

Our _in vitro_ studies described below have been performed with the primarily goal to test our modelling predictions.

3. _In Vitro_ Studies

Experiments were performed using transverse slices (700 µm) obtained from neonatal rats (P0-P4), deeply anaesthetized with ether. Anatomical landmarks were used for the localisation of the pre-Bötzinger region. Rhythmic activity in the slices was triggered by the elevation of $[K^+]_o$ to 5–7 mM. The population activity of the pBC and the activity of XII nerve rootlet were recorded simultaneously with suction electrodes. Raw activities were filtered, amplified and integrated. Experiments were started with aCSF-5 (aCSF containing 5 mM of potassium). With aCSF-7, all slices generated stable rhythmic activity. Then we replaced aCSF-7 with an aCSF containing 3 mM of potassium (aCSF-3) which was considered as an aCSF corresponding to the normal _in vivo_ conditions. The effects of potassium current blockers (4-AP, 50–200 µM, or TEA, 2–4 mM) and of sodium cyanide (NaCN, 2 mM) were examined with aCSF-3 after rhythmic activities were completely abolished. The effects of the persistent sodium current blocker riluzole (25–50 µM) were tested using the rhythmically active slices superfused with aCSF-7.

At 7 mM $[K^+]_o$, all slices demonstrated rhythmic activity which was synchronous in pBC and XII recordings. This activity was abolished when $[K^+]_o$ was reduced to 3 mM (Figure 2A). Application of potassium channels blockers (4-AP or TEA) at 3 mM $[K^+]_o$ triggered rhythmic activity in the pBC and on XII nerve rootlet (Figure 2B). Application of riluzole (25–50 µM), a persistent sodium channel blocker, at 7 mM $[K^+]_o$ abolished rhythmic activity in the majority of slices (Figure 2C). Finally, histotoxic hypoxia induced by application of NaCN (2 mM) also triggered synchronous rhythmic activity in pBC and XII nerve rootlet at 3 mM $[K^+]_o$ (Figure 2D).
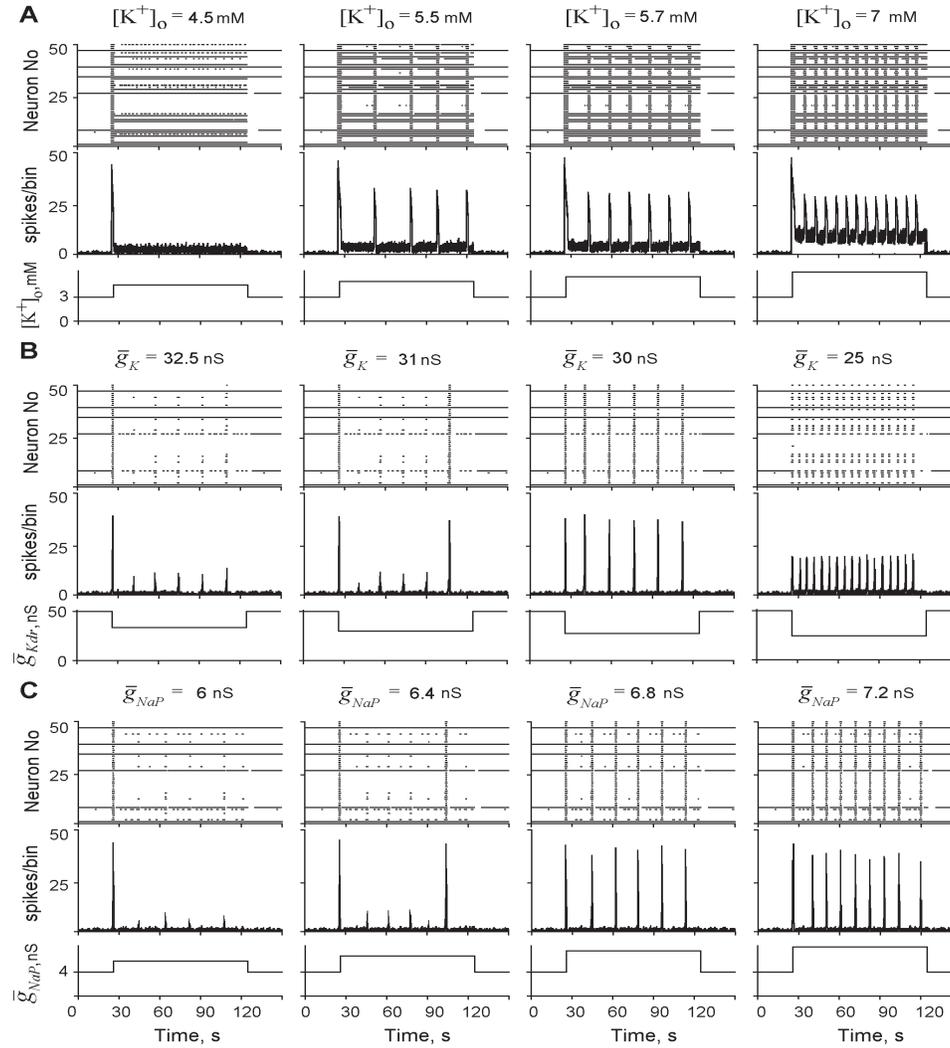
Figure 1.   Triggering bursting activity in the model of pBC population of pacemaker neurons. The result of each simulation is represented by two diagrams: the top diagram is a raster plot for spike times in all 50 cells, sorted on the ordinate axis by cell index number; the bottom diagram is a corresponding integrated histogram of population activity (bin size = 10 ms). **A.** Triggering endogenous bursting activity by elevation of $[K^+]_o$ from 3 mM to higher levels from left to right. An increase of $[K^+]_o$ increases the burst frequency and the level of background asynchronous activity and decreases the burst amplitude. **B.** Triggering bursting activity by reduction of the mean value of $\bar{g}_K$ in the population. The mean value of $\bar{g}_K$ is reduced from 50 nS to lower values from left to right. **C.** Triggering bursting activity by augmentation of the mean value of $\bar{g}_{NaP}$ in the population from 4 nS to higher values from left to right.

4. Discussion

This study reveals that rhythmic activity in the pBC is triggered by a reduction of I_K. The suppression of I_K can be obtained by either an increase in $[K^+]_o$ (via the reduction of the
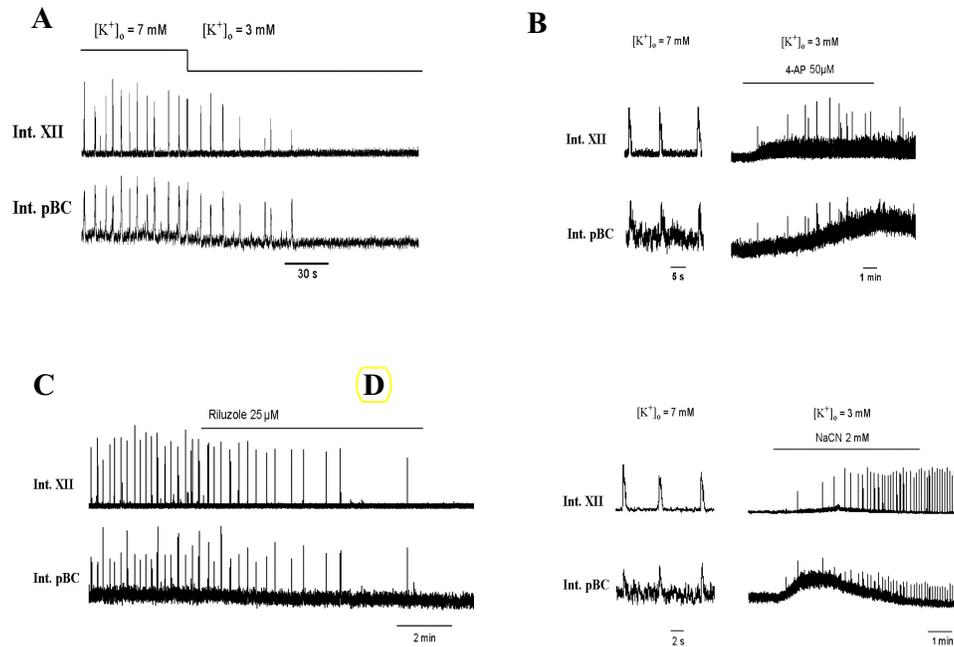
Figure 2. Results of *in vitro* studies. **A.** Reducing $[K^+]_o$ from 7 to 3 mM stopped rhythmic activity in the slice. **B.** Triggering endogenous bursting activity in the slice by application of 4-AP (3 mM) at $[K^+]_o = 3$ mM. Left column shows the intrinsic activity in the same slice at $[K^+]_o = 7$ mM before the application of the blocker. **C.** Riluzole (25 µM) abolished rhythmic activity in the slice at $[K^+]_o = 7$ mM. **D.** Triggering endogenous bursting activity in the slice with NaCN (2 mM). Left column shows the activity in the same slice at $[K^+]_o = 7$ mM, before the application of NaCN.

potassium reversal potential) or a direct suppression of the voltage-gated potassium channels. Our simulations suggest that at the physiological level of $[K^+]_o$, the I_{NaP}-dependent intrinsic oscillations in the pBC are restrained by the normally expressed voltage-gated potassium currents. Consequently, a suppression of I_K or an activation of I_{NaP} can trigger endogenous bursting oscillations in the pBC.

Our *in vitro* data confirmed our modelling predictions. We conclude that the reduction of I_K via the elevation of $[K^+]_o$ is critical for triggering and maintenance of stable rhythmic activity within the pBC. In addition, blocking the intrinsic activity in the pBC by riluzole applications provides strong support for the previous suggestions[1-3] that rhythmic activity recorded from the pBC *in vitro* is persistent-sodium dependent.

We have also demonstrated that histotoxic hypoxia induced by application of NaCN (2 mM) can also trigger rhythmic activity in the pBC. The decrementing shape of the bursts triggered by NaCN were not different from the shape of bursts triggered by the elevation of $[K^+]_o$ or application of potassium current blockers.

The role of pacemaker-driven oscillations in the pBC in the generation of the eupnoeic respiratory rhythm and its possible involvement in the generation of other patterns of breathing, such as gasping, is the subject of debate. As mentioned above, the neuronal bursts recorded *in vitro* (as seen in both the hypoglossal nerve activity and the population activity from the pBC area) have a "decrementing" pattern differs from the "augmenting" pattern of
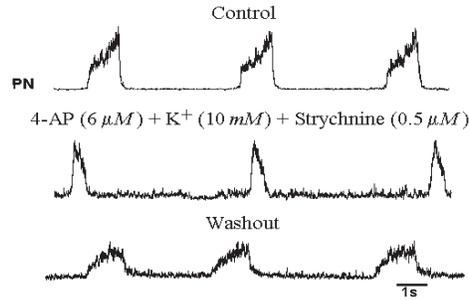
Figure 3.   The results of our studies using the perfused *in situ* preparation. Application of 4-AP and strychnine (to block glycinargic inhibition) in combination with an increase of $[K^+]_o$ converted the eupnoeic pattern of phrenic nerve discharge to a decrementing discharge similar to that recorded *in vitro* and during ischemia-induced gasping *in situ*. This effect was fully reversible following washout of the drugs and returning the $[K^+]_o$ to the control levels (see "Washout").

the phrenic nerve discharges recorded *in vivo* during eupnoea but is similar to gasping[7,8]. Similar to the *in vitro* rhythm, gasping is resistant to blockade of inhibitory transmissions, and hence it has been suggested that gasping is also generated by a pacemaker-driven mechanism[12,13]. We suggest that the pacemaker-driven activity in the pBC is not expressed during eupnoea but plays a fundamental role in the generation of gasping. According to the *switching concept* [9,13] the pBC is considered a region explicitly responsible for switching from eupnoea, generated by a network mechanism distributed in the pontomedullary region, to gasping driven by pacemaker-based oscillations in the pBC.

The decrementing high-amplitude, short-duration discharges recorded from the phrenic nerve during gasping have a similar shape to that of endogenous discharges recorded from *in vitro* preparations. Therefore, hypoxia may indeed produce a switch in the respiratory rhythm generation from a network mechanism for eupnoea to a pacemaker-driven mechanism for gasping. In support of this concept, it should be noted that hypoxia causes (1) a suppression of voltage-gated potassium currents, I_K[14−16], (2) an augmentation of the persistent sodium current, I_{NaP}[17,18], and (3) an increase of external potassium concentration $[K^+]_o$[19], which produces an additional suppression of I_K.

In the present study, by combining *in vitro* experiments and computational modelling, we have shown that each of the above factors accompanying hypoxia may trigger endogenous bursting activity in the pBC. These results support the concept that gasping *in vivo* is driven by a pacemaker mechanism originated in the pBC.

Our recent study of the neurogenesis of gasping *in situ* provided additional experimental evidence for this concept. Specifically, application of 4-AP and strychnine (to block glycine receptor mediated inhibition) in combination with an increase of $[K^+]_o$ converted the eupnoeic pattern of phrenic nerve discharge to a decrementing discharge similar to that recorded *in vitro* and during ischemia-induced gasping *in situ*[13] (Figure 3).

5. Acknowledgements

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