Activity-dependent outward currents contribute to inspiratory burst termination in the preBötzinger Complex neurons of the neonatal mouse studied in vitro

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by

Rebecca Allison Krey

Accepted for Honors
Christopher A. Del Negro, Ph.D., Director
John D. Griffin, Ph.D.
Robin C. Looft-Wilson, Ph.D.
Margaret S. Saha, Ph.D.

Williamsburg, VA
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ABSTRACT

We examined the neural bases of inspiratory bursts in the preBötzinger Complex (preBötC) in slice preparations of neonatal mice that retain respiratory network functions in vitro. It is well established that inward currents including persistent Na$^+$ current ($I_{\text{NaP}}$) and Ca$^{2+}$-activated nonspecific cation current ($I_{\text{CAN}}$) initiate and maintain inspiratory bursts, but the ionic mechanisms of burst termination remain unknown. Since $I_{\text{NaP}}$ and $I_{\text{CAN}}$ flux Na$^+$, we hypothesized that activity-dependent processes coupled to Na$^+$ accumulation may act to hyperpolarize the membrane and hasten or cause burst termination. We tested for the contribution of the Na$^+$/K$^+$ ATPase electrogenic pump current ($I_{\text{pump}}$), Na$^+$-dependent K$^+$ current ($I_{\text{K-Na}}$), and ATP-dependent K$^+$ current ($I_{\text{K-ATP}}$). Pharmacological blockade of each of these three currents depolarized preBötC neurons and attenuated inspiratory bursts, which ultimately lead to a reversible cessation of respiratory motor output. In some cases the fictive respiratory rhythm transiently sped up before slowing down and then stopping altogether. We also estimated the post-burst hyperpolarization attributable to $I_{\text{pump}}$, $I_{\text{K-Na}}$, and $I_{\text{K-ATP}}$ by simulating inspiratory bursts with current step commands in synaptically isolated preBötC neurons. Each current produced 3-8 mV transient hyperpolarization responses lasting 50-160 ms. We conclude that pharmacologically removing any of these activity-dependent outward currents induces a state of pathological depolarization that can extinguish spiking and thus inhibit further inspiratory burst activity. We posit that a substantial fraction of the preBötC enters this depolarized state, which prevents it from participating in rhythm generation and the respiratory motor output ceases. Activity-dependent (specifically Na$^+$ dependent) outward currents appear to play a significant role in terminating inspiratory bursts, and should be integrated into cellular-level models of respiratory rhythm generation.
INTRODUCTION

Breathing in mammals depends on inspiratory rhythms that originate in the preBötzheimer Complex (preBötC) of the medulla (Feldman and Del Negro 2006; Smith et al. 1991). From a neurophysiological perspective the respiratory oscillator is an advantageous model system because spontaneous inspiratory motor activity can be maintained in thin slice preparations in vitro. Thus, rhythm-generating neurons in the preBötC can be investigated in the context of network function.

We know a great deal about the inward currents that underlie inspiratory bursts, the persistent Na\(^+\) current (\(I_{\text{NaP}}\)) (Del Negro et al. 2002; Koizumi and Smith 2008; Pace et al. 2007; Ptak et al. 2005; Rybak et al. 2003) and the Ca\(^{2+}\)-activated nonspecific cation current (\(I_{\text{CAN}}\)) (Del Negro et al. 2002; Pace and Del Negro 2008; Pena et al. 2004). However, the mechanisms that terminate inspiratory bursts remain speculative and have, until now, mostly been studied in models (Butera et al. 1999; Del Negro et al. 2009; Mironov 2009; Mironov 2008; Rubin et al. 2009). As a general problem for understanding central pattern generators (CPGs), determining the postsynaptic mechanisms that terminate the active burst phase is notoriously difficult, and the answer often involves several types of outward currents (Ballerini et al. 1997; Darbon et al. 2003; Del Negro et al. 1999).

In the respiratory system, the mechanism(s) of burst termination may involve pre- and postsynaptic factors (Rubin et al. 2009). Here we focused on potential postsynaptic mechanisms. SK-type and BK-type Ca\(^{2+}\)-dependent K\(^+\) currents (\(I_{\text{K,Ca}}\)) can regulate respiration in vivo and in vitro as well as influence the onset of gasping in hypoxia (Büsselberg et al. 2003; Zavala-Tecuapetla et al. 2008). However, \(I_{\text{K,Ca}}\) is not essential for rhythmogenesis under normal conditions in vitro (Crowder et al. 2007; Onimaru et al. 2003). Thus, we did not study Ca\(^{2+}\)-dependent currents. Alternative burst-terminating mechanisms could include Na/K ATPase electrogenic pump current (\(I_{\text{pump}}\)), which has been linked to burst termination in other CPG
systems (Ballerini et al. 1997; Darbon et al. 2003; Del Negro et al. 1999; Johnson et al. 1992). In addition to generating a net outward current, Na/K ATPase pumps consume ATP, which could conceivably evoke ATP-sensitive K\(^+\) current (\(I_{\text{K-ATP}}\)) (Mironov et al. 1998). Na\(^+\)-dependent K\(^+\) current (\(I_{\text{K-Na}}\)) could also be an important burst-terminating factor. Slack-like \(I_{\text{K-Na}}\) responds directly to cytosolic Na\(^+\) (Wallen et al. 2007; Yuan et al. 2003; Franceschetti et al. 2003). Slick-like \(I_{\text{K-Na}}\) activates in response to Na\(^+\) and is inhibited by ATP (Bhattacharjee et al. 2003; Franceschetti et al. 2003; Wallen et al. 2007). Depending on subunit composition, \(I_{\text{K-Na}}\) could act in conjunction with both \(I_{\text{pump}}\) and \(I_{\text{K-ATP}}\).

Common among the last three mechanisms is activity dependence, i.e., outward currents developing in response to bursts of spiking activity. Since \(I_{\text{NaP}}\) and \(I_{\text{CAN}}\) are inward currents that flux mainly Na\(^+\) to initiate and maintain inspiratory bursts, we posited that burst termination might depend on activity-dependent outward currents linked to Na\(^+\) accumulation, as well as the related depletion of ATP due to Na\(^+\) pumping. From this hypothesis comes the straightforward prediction that blocking any of these putative burst-terminating outward currents will cause larger or more long-lasting inspiratory bursts, and concomitant depolarization of baseline membrane potential.

We measured the burst-terminating contributions of \(I_{\text{pump}}\), \(I_{\text{K-Na}}\), and \(I_{\text{K-ATP}}\) by blocking each current pharmacologically and measuring the changes in activity patterns at the cellular level in the preBötC and via respiratory-related network motor output from the hypoglossal (XII) cranial motor nerve root. In addition, we synapticly isolated preBötC neurons and then measured the outward currents attributable to \(I_{\text{pump}}\), \(I_{\text{K-Na}}\), and \(I_{\text{K-ATP}}\) in simulated bursts in current clamp. Here we show that \(I_{\text{pump}}\), \(I_{\text{K-Na}}\), and \(I_{\text{K-ATP}}\) are significant postsynaptic factors that contribute to, and/or hasten inspiratory burst termination. These postsynaptic mechanisms may not be the only factors contributing to burst termination, but \(I_{\text{pump}}\), \(I_{\text{K-Na}}\), and \(I_{\text{K-ATP}}\) each play a measurable role in inspiratory burst termination, which should be considered when evaluating contemporary models of respiratory rhythm generation in the preBötC.
METHODS

We used neonatal C57BL/6 mice aged 0-6 days for experiments *in vitro*. The Institutional Animal Care and Use Committee at The College of William & Mary approved all protocols.

Neonatal mice were anesthetized by hypothermia and de-cerebrated. The brainstem was isolated in normal artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgSO₄, 25 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose, equilibrated with 95% O₂ and 5% CO₂ (pH of 7.4). We used a vibrating microslicer in conjunction with a calibrated atlas for rat and mouse slice anatomy (Ruangkittisakul et al. 2006) to cut 550-µm-thick transverse slices with the preBötC on the rostral surface, and which retained hypoglossal (XII) nerve roots. Slices were secured rostral side up in a 0.5 ml recording chamber on a fixed-stage microscope equipped with Koehler illumination. ACSF K⁺ concentration was raised to 9 mM and perfused at 4 ml/min at 27°C. Respiratory motor output was recorded from the XII nerve roots using suction electrodes and a differential amplifier.

We performed whole-cell recordings of preBötC neurons that demonstrated an inspiratory pattern of activity in current clamp. Candidate preBötC neurons were selected visually in the region ventromedial to the semicompact division of the nucleus ambiguous, which is a reliable and easy to visualize landmark sharing a contiguous dorsal border with the preBötC (Rekling and Feldman 1997; Ruangkittisakul et al. 2006). Patch pipettes (3-4 MΩ) made from capillary glass (o.d, 1.5 mm; i.d., 0.87 mm) were filled with solution containing (in mM): 140 K-Gluconate, 5 NaCl, 1 EGTA, 10 Hepes, 2 Mg-ATP, and 0.3 Na-GTP (pH = 7.3 by KOH). Pipettes were guided to individual preBötC neurons using a hydraulic micromanipulator and infrared-enhanced differential interference contrast (IR-DIC) videomicroscopy. We used a Dagan IX2-700 amplifier (Minneapolis, MN) for current clamp. Bridge balance was adjusted to offset access resistance. Data were digitally recorded at 4-10 kHz using a 16-bit A/D converter after low-pass filtering at 1 kHz to avoid aliasing. Test pulses (20-ms steps at 0.1 Hz) were applied periodically to monitor
the input resistance of the cell and access resistance of the pipette on a TTL-synced oscilloscope with 100 ms sweep speed.

To study $I_{\text{pump}}$, $I_{\text{K-}Na}$, and $I_{\text{K-ATP}}$, we bath-applied appropriate antagonists obtained from Sigma-Aldrich (St. Louis, MO, USA). We used strophanthidin (10 µM, dissolved in ethyl alcohol) to block $I_{\text{pump}}$, quinidine (100 µM, dissolved in DMSO) to block $I_{\text{K-}Na}$, and glybenclamide (500 µM, dissolved in DMSO) to block $I_{\text{K-ATP}}$. Quinidine and glybenclamide tend to precipitate so the reservoir for ACSF was continually mixed with an electric stirrer prior to and during each experiment. We obtained dose-response curves for quinidine and glybenclamide, bath applying quinidine at (in µM): 1, 10, 100, 550 and 1000 and glybenclamide at (in µM): 100, 500, 1000 and 2000 (n=3 at each level). We measured XII amplitude and area, normalized with respect to control, as well as half-width and frequency reported in ms and Hz, respectively.

We recorded preBötC neurons via whole cell recordings as the antagonists were bath applied and measured the inspiratory burst amplitude, area, half width and frequency. Since each experiment varied slightly (see Figs. 2 and 7), inspiratory burst area and amplitude are reported as a ratio of control to steady state antagonist so that experiments could be compared.

We applied 500-ms current pulses to simulate inspiratory-like bursts in synaptically isolated preBötC neurons. A cocktail of ionotropic synaptic receptor antagonists was used to stop network activity including: 50 µM DL-2-amino-5-phosphonovaleric acid (APV), 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 5 µM picrotoxin (PTX), and 5 µM strychnine (STR). The amount of current injected was adjusted, depending on input resistance, to a level that evoked at least nine spikes per pulse (~20 Hz).

We used two-tailed Student’s t-tests to evaluate changes in: 1) the XII discharge properties, 2) the magnitude of inspiratory bursts in preBötC neurons during network rhythms, and 3) the transient hyperpolarization responses evoked in synaptically isolated neurons. Significance level was 0.05 or less. Significance of inspiratory burst changes in amplitude and area were tested for
each experiment due to an inconsistent baseline. In all other cases significance was tested for using the averages of the experiments.

**RESULTS**

**Contribution of \( I_{pump} \)**

We tested whether \( I_{pump} \) contributes to burst termination by first exposing rhythmically active slice preparations to strophanthidin (10 \( \mu \)M) while recording XII output and inspiratory preBötC neurons (Fig. 1). Bias current was adjusted to maintain a -65 mV baseline in control, and was not adjusted after strophanthidin application. Within 5.3 ± 1.0 min, we consistently observed tonic discharge from the XII nerve (n= 11 of 12 slices tested) (Fig. 1Ab). Strophanthidin caused some preBötC neurons to tonically spike between inspiratory bursts within 2.8 ± 1.3 min (n=4, not shown). Within 6 minutes, all preBötC neurons depolarized by 8.8 ± 1.5 mV (n=7) (Fig. 1Ab, Bb). Respiratory frequency sped up significantly from 0.23 ± 0.03 Hz to 0.41 ± 0.07 Hz (n=7; p<0.05). In addition to nearly doubling frequency, blocking \( I_{pump} \) decreased inspiratory burst amplitude by 52 ± 12% (p<0.005) and decreased area by 68 ± 7% (p<0.05) (n=7, Fig. 2). In two cases, the amplitude of the drive potentials was initially very low (7 mV) and the changes induced by strophanthidin were not statistically significant (Fig. 2, see NS). However, in all cells tested inspiratory burst duration decreased 50%, from 245 ± 46 ms to 122 ± 29 ms (p<0.05) (n=7).

The strophanthidin-induced seizure-like XII discharge phase eventually subsided, lasting 30 seconds or less. Within 7.7 ± 4.8 min, more than half of the preBötC neurons tested became quiescent at a depolarized baseline membrane potential, typically –55 mV (n=4 of 7 tested) (Fig. 1Ac, Bc). After 8.3 ± 6.0 min of continuous exposure to strophanthidin, the XII output stopped in four preparations, three of which demonstrated this quiescence at a depolarized baseline membrane potential in the preBötC. Another subset of slices maintained rhythmogenesis throughout the experiment, including one experiment in which the preBötC neuron depolarized
to the quiescent state described above. In these experiments, the XII output persisted at a higher frequency and lower amplitude compared to control, and the recorded preBötc neuron concurrently showed greatly diminished drive potentials (n=3) (Fig. 1 Ac, Bc).

All the effects of strophanthidin were reversible. After 20-40 min of washout, preBötc neurons returned to their former baseline membrane potential and inspiratory bursts resumed. XII activity also returned to control frequency and XII burst magnitude was reestablished at control levels (Fig. 1Ad, Bd).

The systems-level pharmacology experiments shown in Figs. 1 and 2 provide limited insights into the burst-terminating role of the pump because all constituent neurons in the preBötc experienced a profound shift in baseline membrane potential, and their inspiratory synaptic drive consequently appeared to change in terms of frequency and magnitude in response to strophanthidin. Thus we sought to study $I_{\text{pump}}$ without the confounding network effects that sped up the rhythm yet diminished synaptic drive. We recorded synaptically isolated preBötc neurons and applied current pulses to simulate burst-like activity. After applying the cocktail of ionotropic receptor antagonists (APV, CNQX, PTX, STR, see Methods) to block network activity and isolate $I_{\text{pump}}$, baseline membrane potential was biased to -70 mV, which is very close to $E_K$ in 9 mM $K^+$ ACSF and thus minimizes $K^+$ currents. We then injected 500-ms current steps to evoke burst-like responses in control and in the presence of 10-µM strophanthidin to obtain the $I_{\text{pump}}$ contribution by subtraction (Fig. 3A). $I_{\text{pump}}$ was responsible for a -6.4 ± 1.7 mV post-burst hyperpolarization that decayed back to baseline in 1.6 ± 0.6 s (n=9 neurons tested, 32 subtractions). This experiment in current clamp was commensurate with prior experiments that examined $I_{\text{pump}}$ using a hybrid voltage clamp (Del Negro et al. 2009).

**Contribution of $I_{\text{K-Na}}$**

We assessed the pharmacology of $I_{\text{K-Na}}$ by measuring quinidine dose-response curves for the amplitude, area, half-width, and frequency of XII motor discharge (Fig. 4, Table 1). At 1 µM we observed no change in the XII area or frequency. Amplitude significantly increased by 23%
(n=3, p<0.05). In addition, XII half width decreased from 588 ± 60 ms to 437 ± 186 ms significantly (n=3, p<0.001). Quinidine at 10 µM (and all higher concentrations) significantly attenuated every measure of XII output. We chose 100 µM for subsequent experiments that involved preBötC neuron recordings because 100 µM exceeds the IC50 ~20 µM, could be reliably maintained in aqueous solution, and was fully reversible. At 100 µM, quinidine stopped the rhythm in 46.3 ± 1.7 min and was recovered in 24.4 ± 5.7 min (Table 1).

To begin examining the role of I\textsubscript{K-\textsubscript{Na}} at the cellular level we bath applied 100 µM quinidine while monitoring XII output and recording inspiratory preBötC neurons (Fig. 5). Membrane potential was biased to -65 mV initially and not adjusted further during drug application and washout. Because we needed to monitor inspiratory neurons for >40 min (and often >1 hr) test pulses were used to periodically monitor seal resistance and bridge balance, and thus assess the viability of the whole-cell recording conditions (see Methods). At steady state, quinidine depolarized preBötC neurons by 14.1 ± 5.4 mV (n=4) (Fig. 7).

However, before reaching steady state, two preBötC neurons transiently hyperpolarized. In one case the amplitude and area of the inspiratory bursts increased (Figs. 5 and 6). This transient phase lasted around 4 min (the cells ultimately depolarized) thus we could not compute reliable burst statistics. Nevertheless, Fig. 6 serves as a useful illustration of the effect of removing I\textsubscript{K-\textsubscript{Na}} in a recorded cell, before the drug has affected the entire rhythmogenic network. In this example, the decremental burst pattern in control (Fig. 6B, grey trace) transformed to an incrementing burst pattern in quinidine with greater amplitude and area, which terminated with a Ca\textsuperscript{2+} spike-like event. These data suggest that the mechanism of burst termination had been inhibited by the quinidine. The neuron shown in Figs. 5 and 6 illustrates the steady-state effect of quinidine, which was consistent in all experiments (Figs. 5 and 7), but also provided an early, brief window into the role of I\textsubscript{K-\textsubscript{Na}} in which its pharmacological blockade transiently increased inspiratory burst amplitude and area (Fig. 6A [15 min], B).
These early data suggest that removing $I_{KNa}$ hinders the neuron’s ability to self-terminate inspiratory bursts. However, prolonged exposure to quinidine impedes rhythmogenesis ($n=4$). Within $14.8 \pm 4.0$ minutes, inspiratory burst amplitude decreased by $65 \pm 18\%$ ($p<0.001$) and burst area decreased by $74 \pm 8\%$ ($p<0.001$) ($n=4$). The effects of quinidine on half width were inconsistent, increasing significantly in two cases from an average of $75.1$ ms to $95.6$ ms ($n=2$, $p<0.05$) and decreasing in two others, but here only one case showed a statistically significant change (from $391.0$ ms to $99.1$ ms, $p<0.05$) (Fig. 7). Burst frequency also slowed from $0.20 \pm 0.03$ Hz to $0.07 \pm 0.02$ Hz ($n=4$, $p<0.024$). Within $23.8 \pm 5.3$ minutes, any evidence of inspiratory burst activity stopped altogether and in $36.0 \pm 5.4$ minutes the XII rhythm was abolished.

We studied $I_{KNa}$ in isolated preBötC neurons to avoid the confounding network-level effects of quinidine, which change inspiratory burst dynamics. We used the current-pulse protocol shown in Fig. 3A for $I_{pump}$ to test the contribution of $I_{KNa}$. Since $I_{KNa}$ is a $K^+$ current we used a $-50$ mV baseline, which is physiologically realistic for preBötC neurons in the context of network function with $9$ mM $K^+$ ACSF, and exceeds $E_K$ by $20$ mV so it could be observed. $I_{KNa}$ was obtained by subtraction ($100 \mu M$ quinidine – control) (Fig. 3B).

Applying the same magnitude of step current in the presence of quinidine evoked greater depolarization than in control, most likely due to effects on input impedance, which often caused depolarization block of spiking (Fig. 3B left). Therefore, we repeated the $500$-ms current-pulses but lowered the magnitude of the pulse to evoke bursts similar to those in control (Fig. 3B right). In response to the lesser current-pulse command, the quinidine-sensitive response that we attribute to $I_{KNa}$ caused a $7.7 \pm 1.1$ mV hyperpolarization lasting $0.54 \pm 0.14$ s ($n=3$ slices tested, 11 subtractions). In response to the larger amplitude step (equivalent to the pulse magnitude in control) $I_{KNa}$ caused a $7.6 \pm 1.2$ mV hyperpolarization lasting $0.74 \pm 0.13$ s ($n=5$ neurons, 53 subtractions). Despite the disparity in the magnitudes of the injected currents and the amount of depolarization they evoked, neither the amplitude nor the duration of these $I_{KNa}$-mediated responses were significantly different ($p>0.05$ for amplitude and duration).
**Contribution of $I_{\text{K-ATP}}$**

We studied the pharmacology of $I_{\text{K-ATP}}$ by measuring glybenclamide dose-response curves for the amplitude, area, half-width, and frequency of XII output during respiratory network activity (Fig. 8, Table 2). At 100 µM XII frequency and half significantly decreased (p<0.05) and peak amplitude significantly increased (p<0.01). However, peak area was not significantly altered (p>0.05). At concentrations ≥500 µM glybenclamide significantly perturbed all measures of respiratory network output. Glybenclamide precipitates if not subject to constant stirring. In our experience this frequently blocked the ACSF perfusion lines and perturbed laminar perfusion of the recording chamber. This limitation does not disrupt XII recording but generally dislodges a whole-cell recording pipette. To obtain long-lasting whole-cell recordings in the context of network activity (e.g., Figs. 1, 5, and 6) we employed 500 µM glybenclamide since this concentration (on the lower side of the dose-response curve) could be maintained in solution, and perturbs rhythmogenesis without completely stopping it (Fig. 8, Table 2). At 500 µM, glybenclamide significantly decreased XII burst amplitude by 54 ± 23% and attenuated burst area by 47 ± 23% (n=3, both p<0.001). XII half width also decreased from 305 ± 59 ms to 163 ± 44 ms, but this was only statistically significant in two of the three experiments (p<0.05). Respiratory frequency slowed significantly from 0.26 ± 0.03 Hz to 0.06 ± 0.03 Hz (n=3, p<0.01).

Due to the limited solubility of glybenclamide, obtaining cellular and systems-level data for $I_{\text{K-ATP}}$ was inherently difficult. Many preBötC neurons recordings in rhythmically active slices were lost in the initial stages of glybenclamide application. Figure 9A shows a complete experiment in which we measured the reversible effects of glybenclamide on both the XII and a representative preBötC neuron. The neuron depolarized nearly 30 mV, from -60 to -30 mV. The inspiratory burst amplitude decreased by 49% and burst area decreased by 55% at steady state. Half-width decreased from 185 ms to 85 ms and burst frequency slowed from 0.21 Hz to 0.09 Hz. Ultimately the preBötC neuron locked into a non-terminating plateau-like state in the continued presence of glybenclamide. After 44 minutes of washout the preBötC neuron returned.
to -65 mV when we applied -20 pA to trigger the termination of the plateau-like state (Fig. 9B). At -65 mV baseline, attenuated inspiratory bursts after >45 min of washout indicated that the cell and the network rhythm had substantially recovered. All measures of XII output returned fully to control levels in washout.

We also employed the current-pulse protocol to study the contribution of $I_{K}$-ATP, but again many whole-cell recordings were lost during the initial stages of 500-μM glybenclamide application. In three successful experiments we obtained reliable measurements in control, glybenclamide, and washout conditions. Current-pulses were again applied from a baseline membrane potential of -50 mV to measure this $K^+$ current. In two neurons we measured a glybenclamide sensitive afterhyperpolarization (Fig. 3C). The subtracted response that we attribute to $I_{K}$-ATP showed a $-3.24 \pm 0.63$ mV afterhyperpolarization returning to base line in 41.7 ± 7.5 ms (n=3, 57 subtractions). In one cell, however, we observed a very brief hyperpolarization followed by a transient depolarization of ~2 mV. We speculate that $I_{K}$-ATP current may not be expressed in all inspiratory neurons and the drug may have modified membrane properties in some other way. For example, glybenclamide also dramatically inhibited excitability, as evidenced by its tendency to abolish repetitive spiking behaviors in most neurons tested within 6.35 ± 0.47 minutes (n=2 of 3 neurons tests) (Fig. 3C).

**DISCUSSION**

We examined three post-synaptic mechanisms that may contribute to burst termination: $I_{pump}$, $I_{K}$-Na, and $I_{K}$-ATP. Each of these outward currents can be described as activity-dependent, particularly sensitive to the accumulation of Na$^+$ or the depletion of ATP due to Na$^+$ pumping. We found that pharmacological blockade of each of these currents caused reversible depolarization of baseline membrane potential in preBötC neurons, and attenuated inspiratory bursts. In many instances, their blockade stopped XII output gradually decreasing respiratory frequency, which suggests a direct impact on the mechanism of rhythmogenesis and not merely
an effect on the amplitude of motor output.

Although there are significant caveats associated with interpreting the pharmacology data at the cellular level, as well as disentangling the cellular versus network-level effects of the drugs, we were still able to glean important insights into the potential burst-terminating contributions of $I_{\text{pump}}$, $I_{\text{K-Na}}$, and $I_{\text{K-ATP}}$.

**Caveats and limitations**

One of the most difficult experiments to interpret is bath application of pharmacology. Since the entire network is affected, it is difficult to understand the behavior of individual neurons. Systems level experiments (Figs. 1, 5, and 9) on their own provide nearly no insights. However, if you look at these pharmacological experiments coupled with experiments done in isolated cells as well as a theoretical framework from a model that can predict will happen when activity-dependent outward currents are removed, then we can begin to make sense of the data. In a recent paper, Rubin et al (2009) proposed a model of outward activity dependent burst terminating currents. This model predicts that eliminating these types of currents results in depolarization block and longer, bigger bursts that continue spiking in a ringing-like fashion. What is clear from models and experimental data is that $I_{\text{CAN}}$ is necessary for burst generation and thus the outward currents depend on Na$^+$, which is consistent with our hypothesis and results. The model also speculates that at least one activity dependent outward current is necessary for proper burst termination and rhythmic activity, although which current does not appear to be important. From this model we can begin to predict what we will see experimentally: depolarization and larger, longer lasting inspiratory bursts.

However, difficulties with the pharmacology and confounding effects of the antagonists made it challenging to perform such experiments *in vitro*. All of the antagonists caused a profound depolarization on the network in every experiment (9-30 mV). This depolarization completely alters the dynamics of how the network functions, making it difficult to separate the depolarizing effect of all of the neurons in the preBötC and the removal of burst terminating
outward currents. In fact, the depolarization of the preBötC may actually be stimulating the network. These massive depolarizations in some cases (>30 mV) eventually become pathological, pushing the neurons far beyond their functional limit. Thus, we lose our ability to make conclusions about the removal of burst terminating currents.

Likewise, these experiments were difficult to perform due to limitations in the nature of the pharmacology of the antagonists. Quinidine eliminates $I_{KNa}$ but it is a non-selective $K^+$ channel blocker (Neff et al. 1972). So it may also block other $K^+$ currents, which may explain its profound attributable afterhyperpolarization (-8 mV). Thus, we cannot attribute our results concretely to $I_{KNa}$ alone. Quinidine also appears to alter membrane properties. Current-pulses given at the same magnitude resulted in a greatly elevated response during quinidine application (Fig. 3B). However, when current pulses were given at a lower magnitude to mimic control responses, the results were not significantly different from the larger response. This suggests that we can attribute this contribution to the loss of the burst terminating current alone.

Glybenclamide was the most challenging drug to employ. It required constant mixing to prevent precipitation, which immediately blocked the ACSF perfusion lines and disrupted cellular recordings. Thus, we applied it at a lower concentration (500 μM) to ensure it remained in solution. Observable effects at this concentrations required experiments lasting 40 min or longer, a very difficult undertaking. Long lasting recordings are difficult to maintain and often the patched seal weakens over time. Numerous experiments were lost in the early stages of application of glybenclamide. Likewise, it also altered membrane properties, abolishing spiking when current-pulses were given in the presence of glybenclamide (Fig. 3C). However, comparing early current-pulse responses that retained proper spiking with control still demonstrated a significant subtraction (Fig. 3C1). Therefore, we can still credit the subtraction to the loss of $I_{K-ATP}$. 
Elimination of activity-dependent outward currents

Elimination of $I_{\text{pump}}$, $I_{\text{K-Na}}$, and $I_{\text{K-ATP}}$ did result in pathological depolarization and eventually depolarization block, suggesting that in each case, the activity dependent outward current plays an important role in maintaining proper inspiratory burst dynamics, and that these dynamics are essential to maintain rhythmogenic function in preBötC. However, we observed burst attenuation in each case, not burst augmentation as predicted by our model. Only transiently with quinidine did we see an increase in burst size (Fig. 6). The straightforward model prediction does not take into account the reaction of the network to pharmacological blockade of outward currents. In the real system, we propose that eventually depolarization and depolarization block becomes persistent and pathological and ultimately individual preBötC neurons cannot sustain phasic activity. Initially the frequency increased as the neurons depolarized closer to burst threshold and fired at a faster rate. But as the effects propagate through the network, more neurons are locked into the depolarized state and fewer neurons are able to participate in rhythmogenesis, ultimately abolishing XII motor output. At this depolarized state, the net synaptic drive is less resulting in smaller bursts. When rhythm was maintained, we suspect that there were fewer preBötC neurons that were able to participate and thus network activity was slowed. In some preparations the entire network continued to function. From this we conclude that while these currents are playing a role in burst termination, none of these currents are the sole contributors to burst termination.

Role of $I_{\text{pump}}$

$I_{\text{pump}}$ has two components: a transient activity dependent factor and a tonic factor that is active at rest (Del Negro et al. 1999). Blocking the tonic element may result in pathological depolarization, whereas the loss of a burst terminating current occurs when the transient factor is removed. Strophanthidin blocks both of these components, causing baseline depolarization that also promotes depolarization block. The massive tonic discharge seen after 5 min of exposure to strophanthidin may be the result of a large fraction the neurons in the network
reaching this depolarized state collectively. It may also simply be the response of the XII nerve in addition to the effects on preBötc neurons. We also observed tonic Na⁺ spikes intermittently between inspiratory bursts, due mostly likely to the depolarized membrane potential. We conclude that pathological depolarization (loss of tonic component) combined with loss of transient burst termination results in highly depolarized preBötc neurons that, for the most part, no longer participate in rhythmogenesis. When rhythm continued, we speculate that few neurons are involved, in the diminished network output. The rhythm, however, was maintained at a higher frequency. We speculate that this may be attributable to the stimulating effect of the depolarization and relative high Na⁺ concentration inside the neuron as result of the inactivity of the pump.

**Roles of $I_{K_{Na}}$ and $I_{K_{ATP}}$**

We saw similar results for both of the K⁺ based currents, $I_{K_{Na}}$, and $I_{K_{ATP}}$. Removing the outward current resulted in pathological depolarization and eventually depolarization block. When a sufficient number of these neurons were incapable of maintaining rhythmogenesis, XII motor output ceased. However, all the data show that ultimately quinidine and glybenclamide are deleterious to inspiratory bursting and rhythmogenesis in the system. Thus the full effects of the drugs cause a breakdown in network activity that eliminates inspiratory bursts and XII rhythm.

In one experiment blocking $I_{K_{Na}}$ (Figs. 5 and 6), however, we saw a brief period in which bursts did get bigger briefly. This particularly informative case showed a transient phase of burst enhancement consistent with the model’s prediction. Without the terminating outward current, we saw an incrementing pattern at the end of the burst, opposed to the decremental pattern when burst-terminating current were present. We conclude that at this early stage of the experiment, the pharmacology did not exert its full effect on the network (which is contained in 500-μm-thick slice) but did have full effect on the cell we recorded in the top ~50 μm of the slice.
And this brief window of time verified the straightforward prediction that attenuating a burst-terminating current should amplify burst amplitude and lengthen its duration.

**Physiological significance**

We concluded that $I_{\text{pump}}$, $I_{\text{K,Na}}$, and $I_{\text{K,ATP}}$ play significant roles in terminating inspiratory bursts since rhythmogenesis ultimately stopped after pharmacological antagonists were present at steady state. Of course there are many caveats associated with the pharmacology and applying ‘dirty’ drugs to whole slice preparations. Nevertheless, simulated bursts allowed us to measure the transient post-burst hyperpolarizations, which demonstrate that these afterhyperpolarizations are activity and/or $\text{Na}^+$ dependent with $I_{\text{pump}}$, $I_{\text{K,Na}}$, and $I_{\text{K,ATP}}$ each contributing a substantial fraction.

Our findings are consistent with observations in other systems. In rat spinal neurons, ouabain, an analogue of strophanthidin that also serves to block $I_{\text{pump}}$, decreased the amount of hyperpolarization following evoked bursts (Darbon et al. 2003; Ballerini et al. 1997; Johnson et al. 1992; Matsumoto et al. 2008). Application of quinidine eliminates afterhyperpolarizations in lamprey spinal neurons (Wallen et al. 2007) and rat midbrain preparations (Franceschetti et al. 2003; Bhattacharjee and Kaczmarek 2005). Likewise, glybenclamide has also been found to abolish the hyperpolarization in rat midbrain slices (Stanford and Lacey 1995; Fujimura et al. 1997).

Based on our assessment it appears that $I_{\text{K,Na}}$ is playing the largest role in burst termination (-7.7 mV lasting 540 ms). $I_{\text{pump}}$ also appears to be playing a significant role (-6.4 mV lasting 160 ms). Since we hypothesize that burst termination is dependent on $\text{Na}^+$, it is logical that both of these $\text{Na}^+$-activated currents play a larger role in termination. Glybenclamide sensitive mechanisms are clearly least significant (~3mV lasting 42 ms) and in some cases may not be involved at all. But since $I_{\text{K,ATP}}$ is mainly a chain reaction in response to the activity of the pump, we expect its contribution to be less compared to $\text{Na}^+$ mechanisms.
We applied current pulses at the soma but burst generation is most likely a dendritic process (Pace et al. 2008; Pace et al. 2007; Mironov 2008; Morgado-Valle 2008). Given the difficulty of recording at this level, the soma serves as a limited model of what is actually happening at the dendrite. Nevertheless, if anything our experiments underestimate the contribution of each current in the dendrite that has a higher resistance and smaller volume.

We also found that burst termination was a very rapid process that was accomplished within 50-160 ms, consistent with the estimated time frames for \( I_{\text{pump}} \), \( I_{\text{K-Na}} \), and \( I_{\text{K-ATP}} \). While the role of burst termination in cycle period control is not well established, we speculate that it occurs via a fast, active mechanism, which we posit involves pre- and postsynaptic factors.

As evidenced by the pharmacological blockade of these currents, \( I_{\text{pump}} \), \( I_{\text{K-Na}} \), and \( I_{\text{K-ATP}} \) are playing some role in burst termination. Without these currents preBöTc neurons depolarize and lock into an attenuated burst state, unable to maintain activity. As predicted by our model it is necessary to have a burst terminating current for proper rhythmogenesis but it does not appear to matter which burst terminating current is present. This is consistent with our findings that in some preparations rhythmogenesis continued despite eliminating an outward burst terminating current. While these currents may not be the only ones contributing burst terminating, we conclude that they are important factors that should be considered when studying and modeling preBöTc neuron activity.
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**Figure Legends**

**Figure 1.** Effects of 10 μM strophanthidin on inspiratory burst and XII motor output characteristics. XII output is plotted synchronously below the voltage response. **A.** Overview of the entire experiment with control (a), early strophanthidin (b, 3 min), late strophanthidin (c, 7 min), and washout (d, 35 min). The bar above represents the duration of strophanthidin application. A 38 min gap is not shown between late strophanthidin and washout. Time and voltage calibrations are shown. **B.** Individual traces (15 s) of the sections shows in A. $V_m$ and XII baseline were not adjusted. Time and voltage calibrations are shown.

**Figure 2.** Individual data plots of inspiratory burst characteristic changes in response to 10 μM strophanthidin. The change in baseline membrane potential ($V_m$) peak amplitude, peak area, peak half width and inspiratory burst frequency is plotted in control (c) and strophanthidin (s) for each experiment. Average measurements were made in steady state control and steady state strophanthidin before the neuron reached a quiescent state. The two-paired t-tests for peak amplitude that were not significant (NS) are noted.

**Figure 3.** Cellular responses from injected current pulses. The above traces show the voltage response to the bottom traces which shows the current pulses given to evoke the cellular response. All traces have the same time calibration (3 s). The dashed boxes highlight the subtractions made and shown in the insets in each subsection (A, B, C₁ and C₂). The voltage scale bars correspond with the response (lower) and the inset (higher) **A.** Cellular responses to current step pulses given in at -70 mV are shown in control (grey) and strophanthidin (black) are superimposed. **B.** Cellular responses to current step pulses given at -50 mV in control (grey) and quinidine (black) are superimposed. **Left:** Show the response when the same magnitude of current was injected. **Right:** Show the response when the current magnitude was lowered to
mimic bursts in control. \( C_1 \). Cellular responses to current step pulses given at -50 mV in control (grey) and glybenclamide (gly) (black) are superimposed. \( C_2 \). Right: Show the normal spiking response in control. \( \text{Left: Show the abolished spiking seen in response to glybenclamide (10 min). The voltage scale bar corresponds with both left and right traces.} \)

**Figure 4.** Dose response curves for quinidine. XII peak amplitude, area, half width and frequency are plotted and fitted in response to varying dosages of quinidine. Traces (all 30s) in control, early quinidine and late quinidine are presented for each concentration at varying times. XII response is scaled the same for each concentration.

**Figure 5.** Effects of 100 \( \mu \)M quinidine on inspiratory burst and XII motor output characteristics. The cellular and XII response is shown in control, quinidine (15 min, 30 min and 35 min), and washout. All traces (30 s) are scaled to the same voltage and XII scale. The voltage scale bars on the right indicate the baseline membrane potential of the cellular response. The asterisks (\(^*\)) demote the bursts illustrated in Fig. 6.

**Figure 6.** Transient phase of cellular response to 100 \( \mu \)M quinidine. \( \text{A. Cellular and XII response are shown in control and quinidine (15 min and 30 min). Baseline membrane potential was not adjusted. Cellular responses correspond with the voltage scale bar. The XII response is also calibrated to its own scale.} \) \( \text{B. Cellular response in control (grey) and 15 min quinidine (black) are superimposed. Baseline membrane potential is normalized.} \)
Figure 7. Individual data plots of inspiratory burst characteristic changes in response to 100 µM quinidine. The change in baseline membrane potential ($V_m$) peak amplitude, peak area, peak half with and inspiratory burst frequency is plotted in control (c) and quinidine (q) for each experiment. All changes were statistically significant except one change in a half width that was not significant (NS).

Figure 8. Dose response curves for glybenclamide. XII peak amplitude, area, half width and frequency are plotted and fitted in response to varying dosages of quinidine. Traces (30s) in control, early glybenclamide and late glybenclamide are presented for each concentration at varying times. XII response is scaled the same for each concentration.

Figure 9. Effects of 500 µM glybenclamide on inspiratory burst and XII motor output characteristics. All traces are scaled to the same time scale. A. Cellular and XII response are shown in control and glybenclamide (2 min, 10 min and 15 min). All traces are scaled to the same voltage and XII response calibration. The voltage scale bars on the right indicate the baseline membrane potential of the cellular response. B. Cellular and XII response in washout are shown along with the applied current ($I_{app}$) used to bias the neuron back down to -50 mV. There is a 25 s block omitted to show that the cell eventually regains control-like inspiratory bursts.

Table 1. Systems level response of motor output (XII) to quinidine. The time for XII rhythm to stop and return in washout is reported at different concentrations. Averaged quinidine dose response measurements for XII peak amplitude, area, half width and frequency are shown at varying concentrations. Amplitude and area were calculated as the ratio of the average steady state quinidine response to the average steady state control response. Half width and frequency are reported in ms and Hz, respectively.
Table 2. Systems level response of motor output (XII) to glybenclamide. The time for XII rhythm to stop and return in washout is reported at different concentrations. Averaged quinidine dose response measurements for XII peak amplitude, area, half width and frequency are shown at varying concentrations. Amplitude and area were calculated as the ratio of the average steady state quinidine response to the average steady state control response. Half width and frequency are reported in ms and Hz, respectively.
Figure 1

A

10 μM strophanthidin

30 s

B

a control b c d washout

40 mV

4 s
Figure 2
Figure 3
Figure 4

[Graph showing concentration-response relationship with various treatments indicated.]
Figure 5

control

quinidine [100 µM]

15 min

30 min

35 min

washout

V_m

XII

5 s
Figure 6

A  control  quinidine [100 μM]
15 min  30 min

B  quinidine [15 min]

control
Figure 7
Figure 8

![Graph showing concentration vs. response with symbols and bars indicating different conditions and time points.]

- peak amplitude
- peak area
- peak half width
- frequency

Control vs. GLY at different concentrations and time points.
Figure 9

A control

\[ V_M \]

\[ V_M \]

2 min

\[ V_M \]

10 min

\[ V_M \]

15 min

\[ V_M \]

glybenclamide [500 µM]

B washout

44 min

\[ V_M \]

\[ I_{app} \]

\[ V_M \]

\[ 0 \ mV \]

\[ 0.04 \ nA \]

[25 s]

5 s
Table 1: Systems level response of motor output (XII) to quinidine

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<th>Concentration (µM)</th>
<th>Time to Stop (min)</th>
<th>Time to Washout (min)</th>
<th>Amplitude (norm)</th>
<th>Area (norm)</th>
<th>Half Width (ms)</th>
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Table 2: Systems level response of motor output (XII) to glybenclamide

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