Inspiratory bursts in the preBötzinger complex depend on a calcium-activated non-specific cation current linked to glutamate receptors in neonatal mice

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Inspiratory neurons of the preBötzinger complex (preBöC) form local excitatory networks and display 10–30 mV transient depolarizations, dubbed inspiratory drive potentials, with superimposed spiking. AMPA receptors are critical for rhythmogenesis under normal conditions in vitro but whether other postsynaptic mechanisms contribute to drive potential generation remains unknown. We examined synaptic and intrinsic membrane properties that generate inspiratory drive potentials in preBöC neurons using neonatal mouse medullary slice preparations that generate respiratory rhythm. We found that NMDA receptors, group I metabotropic glutamate receptors (mGluRs), but not group II mGluRs, contributed to inspiratory drive potentials. Subtype 1 of the group I mGluR family (mGluR1) probably regulates a K+ channel, whereas mGluR5 operates via an inositol 1,4,5-trisphosphate (IP3) receptor-dependent mechanism to augment drive potential generation. We tested for and verified the presence of a Ca2+-activated non-specific cation current (I CAN) in preBöC neurons. We also found that high concentrations of intracellular BAPTA, a high-affinity Ca2+ chelator, and the I CAN antagonist flufenamic acid (FFA) decreased the magnitude of drive potentials. We conclude that I CAN underlies robust inspiratory drive potentials in preBöC neurons, and is only fully evoked by ionotropic and metabotropic glutamatergic synaptic inputs, i.e. by network activity.

Neurons in the preBötzinger complex (preBöC) of the ventrolateral medulla synchronously produce 300–500 ms bursts during the inspiratory phase of the respiratory cycle in vitro (for review see Feldman & Del Negro, 2006). Each inspiratory burst in a single neuron is characterized by action potentials superimposed on a 10–30 mV envelope of depolarization, i.e. inspiratory drive potential. Less than 20% of neonatal preBöC neurons express intrinsic bursting properties, i.e. pacemaker properties, in vitro (Peña et al. 2004; Del Negro et al. 2005). Therefore, most preBöC neurons generate inspiratory drive potentials by synaptic input evoking postsynaptic currents that depend on intrinsic membrane properties.

AMPA receptors (AMPARs) are critical for production of inspiratory drive potentials in vitro (Greer et al. 1991; Funk et al. 1993; Ge & Feldman, 1998; Koshiya & Smith, 1999). However, AMPARs rapidly and strongly desensitize (Trussell & Fischbach, 1989; Patneau et al. 1992; Trussell et al. 1993; Attwell & Gibb, 2005), which affects inspiratory rhythm (Funk et al. 1995) and probably limits the contribution of AMPARs in inspiratory drive potential generation. Therefore, we posit that under normal circumstances other postsynaptic mechanisms, such as those associated with NMDA receptors (NMDARs) and metabotropic glutamate receptors (mGluRs), are linked to the activation of intrinsic conductances that play an integral role in inspiratory drive potential generation, an idea introduced by Rekling and colleagues (Rekling et al. 1996; Rekling & Feldman, 1998; Feldman & Del Negro, 2006).

Both NMDARs and group I mGluRs contribute to burst-like discharges associated with Purkinje neuron slow EPSPs (Canepari et al. 2001), neocortical epileptiform discharges (Schiller, 2004), and subthalamic neuron rhythmic bursting (Zhu et al. 2004a,b). NMDARs are widely expressed in preBöC neurons (Funk et al. 1993, 1997; Paarmann et al. 2000, 2005) and mGluRs, notably

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groups I and III, are also present (Mironov & Richter, 2000; Lieske & Ramirez, 2006; Ruangkittisakul et al. 2006). Although the roles of these glutamate receptors in the generation of respiratory motor output in en bloc and slice preparations in vitro have been examined, the contributions of NMDARs and mGluRs to inspiratory drive potential generation in preBötzC neurons remains to be thoroughly investigated.

We hypothesized (Feldman & Del Negro, 2006) that intrinsic conductances such as the persistent sodium current (I_{NaP}) (Del Negro et al. 2002a,b) and the calcium-activated non-specific cation current (I_{CAN}) (Peña et al. 2004; Del Negro et al. 2005) are normally evoked by, and augment, synaptic input, which underlies the inspiratory drive potential (Rekling & Feldman, 1998; Feldman & Del Negro, 2006). Recently, we demonstrated that I_{NaP} does not contribute to inspiratory drive potential generation in the vast majority of preBötzC neurons (Pace et al. 2007). However, the role of I_{CAN} remains largely speculative. I_{CAN} activation can be triggered by either NMDARs (Zhu et al. 2004a,b) or group I mGluRs (Congar et al. 1997; Partridge & Valenzuela, 1999), which makes it a candidate for amplifying glutamatergic synaptic drive by utilizing NMDAR-mediated Ca^{2+} flux and mGluR- and IP3-mediated intracellular Ca^{2+} release to directly gate I_{CAN} and cause postsynaptic potentials.

We tested the role of non-AMPA postsynaptic glutamate receptors in drive potential generation. NMDARs and group I mGluRs, but not group II mGluRs, contribute substantially to inspiratory drive potentials. We found that the subtype 5 of the group I mGluRs (mGluR5) leads to inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) activation. Our findings suggest that postsynaptic Ca^{2+} transients induced by glutamatergic transmission normally recruit I_{CAN} to amplify synaptic currents to generate robust inspiratory drive potentials.

**Methods**

We used neonatal C57BL/6 mice aged 0–6 days (P0–6) for experiments in vitro. The Office for the Protection of Research Subjects (University of California Animal Research Committee) and the Institutional Animal Care and Use Committee (The College of William and Mary) approved all protocols.

Neonatal mice were anaesthetized using hypothermia and then rapidly decerebrated. The neuraxis was dissected in normal artificial cerebrospinal fluid (ACSF) containing (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 with pH = 7.4. Using a vibrating microslicer and landmark criteria recently characterized via an online histology atlas (Ruangkittisakul et al. 2006), we cut transverse slices (550 μm thick, Fig. 1A) that contained the preBötzC at the rostral surface and hypoglossal (XII) motoneurons. The rostral cut captured the rostral-most XII nerve roots, the dorsomedial cell column and principal lateral loop of the inferior olivary nucleus, which places the preBötzC at or near the rostral surface (Ruangkittisakul et al. 2006). The caudal cut captured the obex.

Slices were perfused with 27°C ACSF at 4 ml min⁻¹ in a 0.5 ml chamber mounted rostral side up in a fixed-stage microscope equipped with Koehler illumination, infrared-enhanced differential interference contrast videomicroscopy and epifluorescence. ACSF K⁺ concentration was raised to 9 mm and respiratory motor output was recorded from XII nerve roots using suction electrodes and a differential amplifier. The XII discharge was conditioned using a true RMS-to-DC converter (Analog Devices, One Technology Way, Norwood, MA, USA), which produces a full-wave rectified and smoothed
XII waveform (e.g. XII trace in Fig. 1A) based on the root-mean-square of voltage input to the differential amplifier (Dagan Instruments, Minneapolis, MN, USA).

All electrical recordings were performed on inspiratory preBöT neurons, visually localized ventral to the semicompact division of the nucleus ambiguous (Fig. 1A), which exhibited an inspiratory discharge pattern (Fig. 1A). We did not attempt to identify neurons with pacemaker properties and discarded recordings from expiratory neurons. Current-clamp recordings were performed using a Dagan IX2-700 amplifier. Data were digitally acquired at 4–20 kHz using a 16-bit A/D converter after low-pass filtering at 1 kHz to avoid aliasing. Intracellular pipettes (with 3–4 MΩ resistance) were fabricated from capillary glass (o.d., 1.5 mm; i.d., 0.87 mm) and filled with one of three different intracellular solutions. Standard potassium gluconate solution contained (mM): 140 potassium gluconate, 5 NaCl, 0.1 EGTA, 10 Heps, 2 Mg-ATP, and 0.3 Na-GTP. Second, a Cs⁺-based solution contained: 140 cesium gluconate (or cesium methane sulphonate), 10 NaCl, 1.5 BAPTA (tetra-acetic acid), 10 Heps, 0.7 CaCl₂, 2 Mg-ATP, and 0.3 Na-GTP. Third, 30 mM BAPTA solution contained: 30 K₄-BAPTA, 20 potassium gluconate, 10 NaCl, 10 Heps, 2 MgCl₂, 50 sucrose, and 0.3 Na-GTP. The K₄-BAPTA-based patch solution contained either CaCl₂ or Ca(CF₃SO₃)₂ to buffer the steady-state intracellular Ca²⁺ concentration (i.e. [Ca²⁺]ᵢ). To obtain nominally 0 mM [Ca²⁺]ᵢ we added nothing, for 25 mM [Ca²⁺]ᵢ we added 1.6 mM CaCl₂, and for 120 mM [Ca²⁺]ᵢ we added 6 mM Ca(CF₃SO₃)₂ according to calculations by Maxchelator/WebMaxC software for determining the free metal concentration in the presence of chelators (www.stanford.edu/~cpatton/maxc.html). The liquid junction potentials of the potassium gluconate (8 mV), Cs⁺-based patch (3 mV), and BAPTA-based (8 mV) patch solutions were corrected offline.

In current clamp, we continuously monitored membrane potential and adjusted the bias current to maintain a baseline membrane potential of −60 mV to provide a uniform standard for comparison of drive potentials among preBöT neurons. In some experiments using Cs⁺-based patch solution, endogenous EPSPs generated plateau potentials during the interburst interval when baseline membrane potential was held at −60 mV. In these experiments baseline membrane potential was held at −80 mV so that plateau-like inspiratory potentials occurred only during the inspiratory phase.

We applied drugs such as xestospongicin-C (Xes) and BAPTA (Sigma-Aldrich, St Louis, MO, USA) intracellularly through the patch pipette solution. Xes was added to the standard potassium gluconate patch solution immediately prior to use and discarded after 2 h. We used nystatin perforated patches to obtain baseline control data after obtaining a gigaohm seal and prior to intracellular drug application (Sakmann & Neher, 1995). Nystatin (250 μg ml⁻¹) was added to the patch solution immediately prior to use. To verify the integrity of the patch, we backfilled our pipettes with a patch solution containing 0.5% Lucifer yellow. After ~20 min of exposure to nystatin the amplitude and area of the underlying inspiratory drive potentials could be accurately measured, even though the high-impedance of the perforated patch partially attenuated action potentials. Fluorescence was confined to the pipette in this condition (Fig. 1B). Subsequently, the patch solution containing BAPTA or Xes was delivered intracellularly via patch rupture, which dialysed the cytosol and filled the neuron with fluorescent dye reflecting the whole-cell configuration (Fig. 1B).

We bath-applied these drugs obtained from Sigma-Aldrich: flufenamic acid (FFA), and β-2-amino-5-phosphonovaleric acid (APV). We obtained tetrodotoxin (TTX) from EMD Biosciences (San Diego, CA, USA). (RS)-1-Amino-5-phosphonoind-1-carboxylic acid (APICA), 6-methyl-2-phenylethynyl)pyridine hydrochloride (MPEP), and (S)-(+) amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) were obtained from Tocris Bioscience (Ellisville, MO, USA). We substituted choline for Na⁺ in some experiments (Fig. 8B–D) thus the ACSF contained (mM): 124 choline chloride, 9 KCl, 25 choline bicarbonate, 30 n-glucose, 1.5 CaCl₂ and 1 MgSO₄. H₂PO₄⁻ omitted to prevent precipitation.

Respiratory period in vitro was computed from the average of 10 consecutive inter-inspiratory burst intervals, where each cycle was triggered by XII motor output. We measured the amplitude and area of inspiratory drive potentials and XII motor output. The inspiratory drive potential, i.e. the envelope of depolarization that underlies spike bursts during the inspiratory phase, was obtained by digitally filtering the intracellular voltage trajectory to remove spikes but preserve the amplitude and area of the underlying voltage trajectory. The mean drive potential and XII motor output were computed by averaging 10 consecutive cycles. We compared all of these measures in control and in the presence of various drugs using paired t tests or an analysis of variance (ANOVA, Fig. 6D), with significance for the two-tailed test set at a minimum of P < 0.05.

**Results**

Rhythmically active preBöT neurons generate inspiratory bursts that collectively drive inspiratory motor output. Medullary slice preparations that retain the preBöT generate inspiratory rhythm and motor output that can be monitored via the XII nerve roots (Smith et al. 1991), while providing optimal experimental access to preBöT neurons. We tested the roles of postsynaptic conductances.
in shaping the inspiratory drive potentials that underlie inspiratory bursts in rhythmically active preBöC neurons. Left unperturbed, inspiratory drive potentials and XII motor output normally remain stable for 60–80 min (see Figure S1 in online Supplemental material).

**Role of glutamatergic synaptic input in preBöC neurons**

We tested the NMDAR contribution using the antagonist APV (30–50 μM, bath-applied for > 20 min), which significantly decreased the amplitude and area of drive potentials to 79 ± 4% and 76 ± 2% of control (both P < 0.05). APV had a negligible effect on the amplitude (93 ± 12% of control), area (91 ± 2%), and frequency (88 ± 2%) of XII discharge (all P > 0.17, n = 5, Fig. 2A).

PreBöC neurons express mGluRs (Mironov & Richter, 2000; Lieske & Ramirez, 2006; Ruangkittisakul et al. 2006). Group III mGluRs are typically presynaptic (Stuart et al. 1999; Ferraguti & Shigemoto, 2006; Lieske & Ramirez, 2006) so we focused on the postsynaptic roles of groups I and II.

**Figure 2. Effects of bath applications of non-AMPA glutamate receptor antagonists on drive potential generation and XII motor output**

*Figure 2.* Effects of bath applications of non-AMPA glutamate receptor antagonists on drive potential generation and XII motor output

A, APV (50 μM) attenuates inspiratory bursts, but has no effect on XII motor output. B, the mGluR1 antagonist LY367385 (LY, 15 μM) attenuates inspiratory bursts, but has no effect on XII motor output. C, the mGluR5 antagonist MPEP (10 μM) attenuates inspiratory bursts, but has no effect on XII motor output. D, the general group II mGluR antagonist APICA (300 μM) significantly reduces the frequency of XII motor output but has no effect on inspiratory bursts. In A–D, arrows indicate inspiratory bursts shown at high time resolution in the insets at right. Voltage calibration bars in the insets apply to all traces in A–D. Individual time calibration bars are illustrated for traces and insets. Baseline membrane potential was −60 mV.
Group I includes subtypes 1 and 5 (mGluR1 and mGluR5). Bath-application of the specific mGluR1 antagonist LY367385 (LY; 15 μM, > 15 min) significantly reduced drive potential amplitude and area to 84 ± 4% and 73 ± 8% of control (both P < 0.05, n = 5). LY had no effect on the amplitude (99 ± 8% of control), area (104 ± 8%), or frequency (97 ± 4%) of XII motor output (all P > 0.6, n = 9, Fig. 2B). Similarly, bath-application of MPEP (10 μM, > 15 min), a selective mGluR5 antagonist, significantly reduced drive potential amplitude and area to 76 ± 4% and 61 ± 4% of control (both P < 0.01). MPEP had no effect on the amplitude (95 ± 5% of control), area (79 ± 5%), or frequency (103 ± 13%) of XII motor output (all P > 0.14, n = 7, Fig. 2C). These results suggest that both mGluR1 and mGluR5 contribute to inspiratory drive potential generation that is without effect on frequency or amplitude of motor output.

We also tested the role of group II mGluRs using bath application of the general group II antagonist APICA (300 μM, > 20 min). APICA had no significant effect on the amplitude (90 ± 7% of control) or area (90 ± 9%) of the drive potential (both P > 0.3), nor had any effect on the amplitude (102 ± 4%) or area (107 ± 10%) of the XII motor output (both P > 0.5). However, APICA significantly reduced the frequency of XII motor output to 88 ± 4% of control (P < 0.05, n = 4, Fig. 2D). These findings suggest that group II mGluRs can affect respiratory frequency without an obligatory change in inspiratory drive potential generation in preBötC neurons.

### Mechanisms of group I mGluRs

By what mechanisms do group I mGluRs contribute to inspiratory drive potentials? Group I mGluRs are linked to L-type Ca2+ channel regulation in preBötC neurons (Mironov & Richter, 2000) so we tested the contribution of these channels. Bath-applied nifedipine (NIF, 10 μM, > 15 min) had no significant effect on drive potentials: amplitude (97 ± 8% of control) and area (93 ± 8%; both P > 0.3). Additionally, NIF had no significant effect on the amplitude (129 ± 14% of control), area (124 ± 22%) or frequency (132 ± 30%) of the XII motor output (all P > 0.15, n = 4, Fig. 5A).

Group I mGluRs contribute to rhythm-related burst generation by blocking K+ currents that are active at subthreshold membrane potentials, causing transient depolarization in lamprey spinal potentials (Kettunen et al. 2003) and in neonatal mice XII motoneurons (Sharifullina et al. 2004). We tested whether group I mGluRs were coupled to K+ channels in preBötC neurons. When we blocked K+ channels with a Cs+-based patch solution, LY (15 μM) no longer caused significant reductions in inspiratory drive potentials; area and amplitude were 91 ± 5% and 80 ± 12% of control (both P > 0.12, n = 5, Figs 3A and 5B); in contrast, MPEP (10 μM) reduced the amplitude and area of the inspiratory drive potentials to 72 ± 5% and 45 ± 6% of control (both P < 0.01, n = 3, Figs 3B and 5B).

Group I mGluRs catalyse IP3 production, which triggers intracellular Ca2+ release. We tested whether IP3R-mediated intracellular Ca2+ release plays a role in
If group I mGlur act exclusively through IP$_3$-dependent Ca$^{2+}$ release, then blocking either receptor (mGlur1 or mGlur5) after dialysis with Xes should not have any additional effect on the inspiratory drive potential. In the presence of intracellular Xes, LY (15 μM) significantly attenuated the amplitude and area of the drive potential to 71 ± 6% and 68 ± 5% of steady-state Xes conditions ($P < 0.05$, $n = 4$, Figs 4A and 5B), which suggests that mGlur1 acts independently of IP$_3$R. In contrast, MPEP (10 μM) had no effect on drive potentials in the presence of intracellular Xes; the amplitude and area of drive potentials remained at 100 ± 12% and 99 ± 19% of steady-state Xes conditions ($P > 0.5$, $n = 3$, Figs 4B and 5B), which suggests that mGlur5 acts exclusively via IP$_3$Rs.

Intracellular Ca$^{2+}$ signalling and inspiratory drive potential generation

NMDARs and IP$_3$Rs contribute to inspiratory drive potentials (Figs 2A, 4 and 5A) and utilize intracellular Ca$^{2+}$ as a second messenger. Therefore, we tested the role of Ca$^{2+}$ signalling using a high concentration of intracellular BAPTA (30 mM). Nystatin perforated patches were used to measure inspiratory drive in control (e.g. Fig. 1B). After rupturing the membrane and establishing whole-cell dialysis for ~20 min, BAPTA significantly decreased the amplitude and area of inspiratory drive potentials to 36 ± 5% and 42 ± 7% of control (both $P < 10^{-8}$, $n = 5$, Fig. 6A and B). In 24 additional experiments without nystatin we used the first min of whole-cell recording as control and intracellular BAPTA yielded identical results; the amplitude and area of inspiratory drive potentials decreased to 32 ± 3% and 33 ± 3% of control (both $P < 10^{-9}$, $n = 24$, Fig. 6B).
To examine whether resting [Ca\(^{2+}\)], influenced inspiratory drive potentials, we formulated 30 mM BAPTA patch solutions with [Ca\(^{2+}\)], buffered to nominally 0, 25 or 120 nM (see Methods). Baseline [Ca\(^{2+}\)], had no significant effect on the BAPTA-mediated attenuation of drive potentials (P > 0.25 with regard to both amplitude and area, Fig. 6C). These results suggest that intracellular Ca\(^{2+}\) transients, but not the resting [Ca\(^{2+}\)], affect drive potential generation. These data are consistent with intracellular Ca\(^{2+}\) transients activating an inward current that enhances inspiratory drive potentials. \(I_{\text{CAN}}\) is an obvious candidate.

**\(I_{\text{CAN}}\) in preB\(\text{"otC}\) neurons**

\(I_{\text{CAN}}\) activation requires intracellular Ca\(^{2+}\) transients, which should be blocked by 30 mM intracellular BAPTA. To test whether BAPTA was in fact blocking \(I_{\text{CAN}}\), we bath-applied the \(I_{\text{CAN}}\) antagonist FFA (100 \(\mu\)M, > 15 min) after 30 mM BAPTA reached steady-state conditions (≥ 30 min), which avoids falsely attributing the effects of 30 mM BAPTA to bath-applied 100 \(\mu\)M FFA. Previously, we showed that 100 \(\mu\)M FFA attenuates drive potential generation in preB\(\text{"otC}\) neurons (Del Negro et al. 2005). Here in the presence of intracellular BAPTA, we found that 100 \(\mu\)M FFA had no effect on the amplitude (131 ± 22% of control) or area (129 ± 17% of control) of inspiratory drive potentials (both P > 0.3, n = 5, Fig. 6A and B). This suggests that both BAPTA and FFA (100 \(\mu\)M) act on the same mechanism, consistent with \(I_{\text{CAN}}\).

Next, we quantified the effects of bath application of FFA (10, 100, 300 and 350 \(\mu\)M) on drive potential generation and XII motor output. FFA at 10 \(\mu\)M (15 min) had no significant effect on the amplitude (86 ± 8% of control) or area (88 ± 13%) of inspiratory drive potentials nor on the amplitude (106 ± 11%) or area (112 ± 18%) or frequency of XII motor output (98 ± 3%) (all P > 0.25, n = 4, not shown). At 100 \(\mu\)M, FFA reduced drive potential amplitude and area significantly to 70 ± 5% (P < 0.01) and 65 ± 10% (P < 0.05) of control. FFA at 100 \(\mu\)M had no effect on the amplitude (79 ± 11%), area (89 ± 11%) or frequency (89 ± 13%) of XII motor output (all P > 0.18, n = 6, Fig. 7A). Note that the actions of 100 \(\mu\)M FFA reached steady state in ~12 min and its effects on inspiratory drive potentials were not fully reversible.

In contrast, 300 \(\mu\)M FFA caused rhythm cessation after 10–15 min in 7 of 10 slices tested (Fig. 7B). In the remaining 3 slices, raising the concentration of FFA to 350 \(\mu\)M silenced the rhythm (Fig. 7C). In all cases, XII motor output was never revived by adding 1 \(\mu\)M of the excitatory neuropeptide substance P (SP, bath-applied > 10 min) but recovered in washout (n = 10, Fig. 7C).

The FFA-sensitive current in preB\(\text{"otC}\) neurons has been attributed to \(I_{\text{CAN}}\) (Peña et al. 2004), but this issue is not yet fully resolved. FFA also affects gap junctions and Ca\(^{2+}\)-dependent K\(^{+}\) channels (Ottolia & Toro, 1994; Greenwood & Large, 1995; Kochetkov et al. 2000; Harks et al. 2001). To verify the presence of \(I_{\text{CAN}}\) in preB\(\text{"otC}\) neurons, we bath-applied 350 \(\mu\)M FFA (10, 100, 300, or 350 \(\mu\)M) on drive potential generation and XII motor output. FFA at 100 \(\mu\)M (15 min) had no significant effect on the amplitude (131 ± 22% of control) or area (129 ± 17% of control) of inspiratory drive potentials (both P > 0.3, n = 5, Fig. 6A and B). This suggests that both BAPTA and FFA (100 \(\mu\)M) act on the same mechanism, consistent with \(I_{\text{CAN}}\).

**Figure 6. Intracellular Ca\(^{2+}\) transients are important for inspiratory bursts**

A, perforated-patch recordings and intracellular dialysis using 30 mM BAPTA patch solution. Control conditions in the perforated-patch configuration are shown at 35 min. BAPTA once introduced into the cytosol via patch rupture causes a progressive attenuation of the inspiratory burst. Inset shows that action potentials could be evoked with 5 ms-long current pulses at rheobase in steady-state intracellular BAPTA conditions (> 20 min). Subsequent bath-application of 100 \(\mu\)M flufenamic acid (FFA) has no additional attenuating effects even after 15 min of exposure to the drug. Baseline membrane potential was ~60 mV throughout the experiment. B, time course of the effects of 30 mM BAPTA on the amplitude of inspiratory bursts (\(V_{\text{M}}\)). The first minute of whole-cell configuration is indicated by the grey bar at zero time. Subsequent bath-application of 100 \(\mu\)M FFA causes no additional attenuation. C, bar chart showing that BAPTA reduces both the amplitude (amp.) and area of inspiratory drive potentials to the same extent whether WC (n = 24) or PP (n = 5) recording configurations are used for control. D, patch solutions that buffered baseline [Ca\(^{2+}\)], to nominally 0 (n = 17), 25 (n = 4), or 120 nM (n = 8) have no significant effect on the BAPTA-mediated attenuation of inspiratory drive potential amplitude or area. C and D show mean ± S.E.M., and number of experiments in parentheses.

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neurons we tested for its hallmark properties: activation by intracellular Ca\(^{2+}\), with Na\(^{+}\) as the primary inward charge carrier (Teulon, 2000). To isolate the effects of Na\(^{+}\) substitution on putative \(I_{\text{CAN}}\), we bath-applied 1 \(\mu\)M TTX, which also blocks rhythmogenesis in the slice. Therefore, we used a Cs\(^{+}\)-based patch-pipette solution and depolarizing current steps to reliably evoke \(I_{\text{CAN}}\)-mediated plateau-like potentials.

Intracellular Cs\(^{+}\), which attenuates K\(^{+}\) currents and reduces electrotonic length, transformed the inspiratory drive potential present in control into a long-lasting plateau potential (Fig. 8A, traces 1 and 2) that could also be evoked exogenously with a 50–150 ms current pulse (Fig. 8A, trace 3). The evoked plateaus were attenuated by 100 \(\mu\)M FFA (\(n=5\), Fig. 8A, traces 2–5) in a manner consistent with the effects of 100 \(\mu\)M FFA on endogenous drive potentials, e.g. Fig. 7A.

In 1 \(\mu\)M TTX, addition of Cd\(^{2+}\) (200 \(\mu\)M) to block Ca\(^{2+}\) influx attenuated the evoked plateau to 29 ± 9% of control (\(P<0.05\), \(n=3\)); the subsequent equimolar replacement of Na\(^{+}\) with choline caused no significant change in the evoked response (Fig. 8B, \(n=3\)). Reversing the order of these tests, choline substitution attenuated the evoked plateau to 13 ± 2% of control (\(P<0.05\), \(n=3\)); further addition of Cd\(^{2+}\) (200 \(\mu\)M) caused no further reduction (Fig. 8C, \(n=6\) total). Also in the presence of 1 \(\mu\)M TTX, 100 \(\mu\)M FFA attenuated the evoked plateau to 39 ± 8% of control (\(P<0.05\), \(n=6\)). Choline substitution further reduced the response by an additional 31 ± 11% (\(P<0.05\), \(n=4\), Fig. 8D). These data indicate that \(I_{\text{CAN}}\) is expressed in preBöC neurons and is substantially, but incompletely, reduced by 100 \(\mu\)M FFA.

**Discussion**

Inspiratory drive potentials are a signature of preBöC neurons during the inspiratory phase of the respiratory cycle. We show that inspiratory drive potentials depend on both ionotropic and metabotropic glutamate receptors to evoke postsynaptic membrane properties. AMPARs are essential for respiratory rhythmogenesis (Greer *et al.* 1991; Funk *et al.* 1993; Ge & Feldman, 1998; Koshiya & Smith, 1999) and thus are pivotal for inspiratory drive potential generation *in vitro*. However, the roles of

**Figure 7. The \(I_{\text{CAN}}\) antagonist, flufenamic acid (FFA), attenuates inspiratory drive potentials**

A, 100 \(\mu\)M FFA reduces drive potentials but not the magnitude or frequency of XII motor discharge. The letters ‘a’ and ‘b’ indicate inspiratory bursts that are shown at high resolution in the inset (at right) for control (a) and 100 \(\mu\)M FFA (b). B, 300 \(\mu\)M FFA blocks inspiratory activity in 7 of 10 slices after 12 or more minutes of drug exposure. C, in the 3 remaining slices, 350 \(\mu\)M FFA blocks the respiratory rhythm, which does not restart in the presence of 1 \(\mu\)M substance P (SP), but does recover after > 1 h of washout. Baseline membrane potential was −60 mV for \(V_{\text{M}}\) traces in A (inset) and B. A (inset), B and C all have separate time calibrations.
NMDARs and mGluRs in drive potential generation have largely been overlooked. We show that both NMDARs and group I mGluRs contribute significantly to inspiratory drive potential generation. Inspiratory drive potentials required intracellular Ca\(^{2+}\) transients and decreased after exposure to 100 \(\mu\)M FFA. This suggests that \(I_{\text{CAN}}\) is involved in inspiratory drive potential generation.

Additionally, evoked plateau potentials under intracellular Cs\(^+\) conditions were attenuated by FFA and also sensitive to external Na\(^+\) substitution and Cd\(^{2+}\), which is indicative of \(I_{\text{CAN}}\). These properties are also hallmarks of unconventional members of the transient receptor potential, i.e. TRP, family of ion channels, namely TRPM4 and/or TRPM5, which are FFA-sensitive, monovalent cation channels gated by intracellular Ca\(^{2+}\). Therefore TRPM4 and/or TRPM5 may underlie \(I_{\text{CAN}}\) in preB\(\ddot{\text{o}}\)'tC neurons (Launay et al. 2002; Hofmann et al. 2003; Montell, 2005; Ullrich et al. 2005).

**Figure 8. The ionic basis of \(I_{\text{CAN}}\)**

A, preB\(\ddot{\text{o}}\)'tC neuron recorded with Cs\(^+\)-patch solution shown immediately after onset of whole-cell recording (4, trace 1). After 2 min, the drive potential is transformed into an inspiratory plateau response (2), which can also be evoked using 150 ms somatic current pulses (3). At 100 \(\mu\)M FFA reduces the endogenous (4) and evoked (5) responses. B, inspiratory plateaus evoked with 50 ms current steps were subjected to cumulative applications of 1 \(\mu\)M TTX, 200 \(\mu\)M Cd\(^{2+}\) and choline substitution for Na\(^+\) in the ACSF. Inset shows superimposition of the active responses immediately following current-pulse stimuli. C, inspiratory plateaus subjected to the same agents as in B (above), but with Cd\(^{2+}\) and choline substitution in reverse order. Inset is similarly constructed. D, inspiratory plateaus subjected to 1 \(\mu\)M TTX, and then cumulatively followed by 100 \(\mu\)M FFA and choline substitution. Inset is similarly constructed to B and C. Calibration bars in A apply to A–D and baseline membrane potential was \(-60\) mV.

**Role of \(I_{\text{CAN}}\) in inspiratory drive potentials and respiratory rhythmogenesis**

Testing the role of \(I_{\text{CAN}}\) in inspiratory drive potential generation using FFA proved difficult. At 100 \(\mu\)M FFA incompletely blocked \(I_{\text{CAN}}\) (Fig. 8D and Teulon, 2000) whereas higher concentrations stopped respiratory rhythmogenesis altogether. Although it is tempting to conclude that FFA at doses of 300–350 \(\mu\)M, e.g. Fig. 7B, stopped the rhythm by fully blocking \(I_{\text{CAN}}\), FFA at concentrations exceeding 100 \(\mu\)M significantly affects gap junctions, Ca\(^{2+}\)-dependent K\(^+\) channels and Cl\(^{-}\) channels (Ottolia & Toro, 1994; Greenwood & Large, 1995; Kochetkov et al. 2000; Harks et al. 2001), which are present in preB\(\ddot{\text{o}}\)'tC neurons (Rekling & Feldman, 1997a; Brockhaus & Ballanyi, 1998; Onimaru et al. 2003). Therefore, here and elsewhere, e.g. (Peña et al. 2004; Tryba et al. 2006), whether FFA is applied alone or in
combination with other drugs, one cannot assert that rhythm cessation brought on by FFA at doses exceeding 100 μM is attributable solely to the ability of FFA to block of I_{CAN}.

How are we to reconcile our finding that 300–350 μM FFA blocks rhythm generation with the prior report by Peña et al. (2004) in which 500 μM FFA was bath-applied and failed to stop respiratory rhythm in slices? This apparent discrepancy can be explained as follows: the duration of FFA application is an important parameter because we found dramatic effects of FFA within a 2 min window, e.g. Fig. 7B, during which the rhythm went from normal frequency to complete stoppage. The time course of FFA application was not specified in Peña et al. (pertaining to their Fig. 7B) thus one cannot evaluate whether FFA reached steady state in their protocol. Moreover, the slices employed by Peña et al. were 630–690 μm thick with their rostral surface at the caudal pole of the facial nucleus (see Methods in Peña et al. 2004), which suggests that critical preBötC rhythmogenic neurons were located ~200 μm deeper within their tissue compared to our slices and thus would probably require longer exposure times for FFA to achieve its steady-state effects. Lastly, one cannot rule out that rostral rhythmogenic circuits located at the caudal pole of the facial nucleus such as the retrotrapezoid-parafacial respiratory group (RTN-pFRG) (Onimaru & Homma, 2003; Janczewski & Feldman, 2006) may be present in the slices in Peña et al., as suggested by the likely cytoarchitectonic boundaries (Ruangkittisakul et al. 2006; Barnes et al. 2007). In contrast, the RTN-pFRG is not present in our slices, which isolates the preBötC at the rostral surface (see Methods and Ruangkittisakul et al. 2006).

To address the pharmacological caveats associated with use of FFA, we straightforwardly analysed the role of I_{CAN} in inspiratory drive potential generation using high levels of intracellular BAPTA, which prevented I_{CAN} activation by suppressing Ca^{2+} transients. BAPTA at 30 mM appears to substantially, if not quite fully, block I_{CAN} yet has few (if any) other effects on burst generation or membrane properties. For example: inspiratory drive potentials were not further attenuated following intracellular BAPTA dialysis by the subsequent application of 100 μM FFA. Also, 30 mM BAPTA had no effect on input resistance and did not affect action potentials evoked with current pulses (Fig. 6A inset). To the extent that BAPTA dialysis affects Ca^{2+}-dependent K^{+} channels that normally attenuate the magnitude of inspiratory drive potentials (Onimaru et al. 2003), we expect that the 60–70% BAPTA-mediated attenuation may actually underestimate the true contribution of I_{CAN} during inspiratory drive potentials. We conclude that I_{CAN} predominantly contributes to inspiratory drive on a cycle-to-cycle basis by significantly boosting the transformation of glutamatergic synaptic inputs to membrane depolarization.

I_{CAN} activation mechanisms in preBötC neurons

We hypothesize that three pathways normally activate I_{CAN} during inspiratory drive potential generation. First, synaptically activated mGluR5s trigger IP_{3}-mediated intracellular Ca^{2+} release, which activates I_{CAN}. A similar mechanism may promote network-driven oscillations in CA1 pyramidal neurons, where group I mGluRs activate I_{CAN} via a mechanism that probably involves IP_{3}-mediated Ca^{2+} release (Congar et al. 1997).

Second, inspiratory drive potentials depended on NMDAR-mediated Ca^{2+} influx, which may play a small but statistically significant role in activating I_{CAN}. While the burst-generating role of NMDARs is small in preBötC neurons, Ca^{2+} influx through NMDARs exclusively activates I_{CAN} to generate bursting oscillations in subthalamic neurons (Zhu et al. 2004a, b). AMPARs are primarily Ca^{2+} impermeable in preBötC neurons (Paarmann et al. 2000) and thus probably cannot activate I_{CAN} directly.

Third, ionotropic receptor-mediated depolarization due to AMPARs, and possibly NMDARs, opens voltage-gated Ca^{2+} channels (Fremann et al. 1999; Pierrefiche et al. 1999; Onimaru & Homma, 2003) and directly activates I_{CAN} (Fig. 8B and Peña et al. 2004). Voltage-gated Ca^{2+} channels recruited by synaptic depolarization activate I_{CAN} in motoneurons of the nucleus ambiguous (Rekling & Feldman, 1997b), layer II neurons in the entorhinal cortex (Egorov et al. 2002; Fransen et al. 2006), and Blanes cells of the olfactory bulb (Pressler & Strowbridge, 2006). Importantly, in neocortical slices NMDARs, voltage-gated Ca^{2+} channels and intracellular Ca^{2+} release all converge to activate I_{CAN} during epileptiform discharges (Schiller, 2004), which resemble a prolonged version of the inspiratory drive potentials in preBötC neurons.

Rather than acting on I_{CAN}, mGluR1 appears to promote inspiratory drive potentials by transiently closing K^{+} channels. A similar role for mGluR1 was identified in lamprey spinal neurons and neonatal mice XII motoneurons, in which mGluR1-modulated K^{+} leak channels boost membrane depolarization and increase membrane excitability in vitro (Kettunen et al. 2003; Sharifullina et al. 2004).

The specific subtype of Ca^{2+} channel involved in I_{CAN} activation in preBötC neurons remains unknown. While L-type Ca^{2+} channels are present in preBötC neurons (Onimaru et al. 1996, 2003; Mironov & Richter, 1998; Elsen & Ramirez, 2005) and are regulated by group I mGluR activation (Mironov & Richter, 2000), they do not contribute to drive potential generation or XII motor
output under standard conditions in vitro (Fig. 5A and Onimaru et al. 2003). However, the role of L-type Ca\(^{2+}\) channels may change during hypoxia (Mironov & Richter, 2000).

Onimaru et al. (1996, 2003) studied N-type and P/Q-type Ca\(^{2+}\) channels in respiratory neurons throughout the ventral medullary column, including the RTN-pFRG. N-type Ca\(^{2+}\) channel blockade actually increases the magnitude of inspiratory drive potentials because these channels are functionally linked to SK-type Ca\(^{2+}\)-dependent K\(^{+}\) channels, and the net effect of blocking them is to remove an activity-dependent outward current (Onimaru et al. 2003). In contrast, \(\omega\)-agatoxin-IVA bath application significantly reduces inspiratory drive potential amplitude (Onimaru et al. 2003), which suggests that P/Q-type Ca\(^{2+}\) channels may participate in \(I_{\text{CAN}}\) activation, although this remains to be demonstrated in preBöTC neurons specifically.

That AMPARs are critical for rhythm generation may simply reflect their role as the primary source of excitatory postsynaptic currents during the inspiratory phase. However, NMDAR, mGluR1 and mGluR5 antagonists significantly attenuated the magnitude of inspiratory drive potentials (Fig. 2), which suggests that the bulk of the inward current during inspiratory drive is not conveyed by AMPARs. To explain their critical role in vitro, we posit that AMPARs recruit and initiate the mechanisms that activate \(I_{\text{CAN}}\). These mechanisms include the AMPAR-mediated depolarization required to activate Ca\(^{2+}\) channels and partially relieve the voltage-dependent Mg\(^{2+}\) block of NMDARs.

**Physiological significance**

One might expect that reducing the magnitude of drive potentials in preBöTC neurons would necessarily result in decreased XII motor output, whereas in most of our experiments XII motor output was maintained even though drive potentials in preBöTC neurons decreased significantly. At present, how changes in the inspiratory drive potentials from preBöTC neurons affect presynaptic drive to XII motoneurons is unknown. However, inspiratory synaptic drive to XII motoneurons is primarily AMPAR mediated with little to no postsynaptic contribution from NMDARs (Funk et al. 1993) or mGluRs (Bocchiaro & Feldman, 2004; Sharifullina et al. 2004; Nistri et al. 2006). Therefore, the pharmacological approaches used in this study to attenuate inspiratory drive in the preBöTC should not have affected XII motoneurons directly. In contrast, XII motoneurons express persistent Na\(^{+}\) current (\(I_{\text{NaP}}\)) (Bellingham, 2006), which is consistent with bath-applied riluzole, the \(I_{\text{NaP}}\) antagonist, causing dose-dependent decreases in XII motor nerve output (Del Negro et al. 2002b, 2005) that is not seen when riluzole was injected directly into the preBöTC (Pace et al. 2007).

We conclude that \(I_{\text{CAN}}\) predominantly contributes to inspiratory drive on a cycle-to-cycle basis by augmenting the transformation of synaptic input to membrane depolarization. \(I_{\text{CAN}}\) only becomes fully activated by glutamate-mediated activation of AMPA, NMDA and metabotropic glutamate receptors during endogenous respiratory behaviour, and thus is properly considered a network-induced property (and would not be activated by current injection via an intracellular or patch electrode). A rhythmogenic mechanism that depends on glutamatergic recurrent excitation coupled to intrinsic burst-generating currents via intracellular signalling mechanisms was predicted by Rekling and colleagues and dubbed the group pacemaker hypothesis (Rekling et al. 1996; Rekling & Feldman, 1998). In the group pacemaker hypothesis, some or all preBöTC neurons express postsynaptic currents that are normally latent and unavailable except when recruited by synthetically activated signalling cascades. This is a major paradigm shift because the rhythmogenic population need not express intrinsic voltage-dependent pacemaker properties to play a key role in inspiratory burst generation. In light of the diminishing evidence in support of the obligatory role of pacemaker properties in respiratory rhythm generation (Del Negro et al. 2005; Pace et al. 2007), our findings demonstrate that a framework in which recurrent synaptic excitation evokes cellular burst-generating membrane properties available to all preBöTC neurons, such as the group pacemaker hypothesis, is a viable mechanism that can explain key aspects of respiratory rhythmogenesis.

**References**


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Supplemental material

Online supplemental material for this paper can be accessed at:
http://jphysiol.physoc.org/cgi/content/full/jphysiol.2007.133660/DC1