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Silencing by raised extracellular Ca^{2+} of pre-Bötzinger complex neurons in newborn rat brainstem slices without change of membrane potential or input resistance

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ABSTRACT

Breathing is controlled by inspiratory pre-Bötzinger complex (preBötC) networks that remain active in transversal brainstem slices from perinatal rodents. In 600 μm thick preBötC slices, inspiratory-related bursting in physiological (3 mM) [K^+] is depressed by <1 mM elevation of superfusate [Ca^{2+}]. Here, we studied underlying cellular mechanisms in whole-cell-recorded neurons of 400 μm thin newborn rat slices with the <200 μm thin preBötC in the middle ("m-preBötC[400]" slices). Extracellular activity in the ventrolateral slice area in 3 mM K^+ and a most common physiological Ca^{2+} range (1–1.2 mM) stopped spontaneously within 2 h ("in vitro apnea"). Contrary, rhythm was stable for >3 h at 6–8 bursts/min in 7 mM K^+ and 1.2 mM Ca^{2+} solution. In non-pacemaker preBötC inspiratory cells and neighboring inspiratory or tonically active neurons, block or frequency depression by >90% of rhythm in the latter solution by 2–3 mM Ca^{2+} changed neither resting potential nor input resistance. High Ca^{2+} silenced inspiratory neurons and depressed tonic discharge of non-respiratory neurons. However, in both cell types current injection evoked normal action potentials with unchanged threshold potential. The findings show that m-preBötC[400] slices represent a good compromise between long term viability of rhythmogenic preBötC neurons and minimal modulation of these cells by adjacent tissue, but need to be studied in elevated K^+. The lack of postsynaptic K^+ channel-mediated hyperpolarization suggests that saturation of surface charges, presynaptic block of transmission and/or inhibition of postsynaptic burst-promoting conductances such as Ca^{2+} activated non-selective cation channels are involved in inspiratory depression by high Ca^{2+}.

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Breathing is controlled by inspiratory pre-Bötzinger complex (preBötC) networks that remain active in brainstem slices from perinatal rodents [7,15,21]. Raising superfusate Ca^{2+} in physiological (3 mM) K^+ from 1 to 1.5 mM, i.e., from the lower to the upper limit of the estimated physiological range in interstitial brain tissue [11,14,17,22], severely depresses inspiratory-related bursting in 600 μm thick preBötC slices, but rhythm is restored by elevated K^+ [18]. Similar slowing of rhythm in mouse slices by opioids has been attributed to K^+ channel-mediated hyperpolarization of preBötC neurons [7,8] whereas others proposed a major contribution of presynaptic inhibition to opioid-evoked inspiratory depression in vitro [1–3]. Here, we studied whether block of inspiratory rhythm by high Ca^{2+} is typically associated with membrane hyperpolarization of preBötC neurons and neighboring cells, or whether this phenomenon occurs in the absence of a postsynaptic change in neuronal membrane potential or input resistance.

The isolated preBötC should ideally be studied in thin slices to minimize modulation of its activity by adjacent (respiratory) brainstem structures [1,7,19,20]. We showed recently that the newborn rat preBötC is centered 0.5 mm caudal to facial (VII) motor nucleus and extends by <200 μm [19]. Slices of 200–250 μm thickness containing this kernel are capable of generating inspiratory activity in 3 mM K^+, but these rhythms (and more regular bursting in elevated K^+) appear to last for <2.5 h [19]. This limited viability may be related to the fact that dendrites of newborn rat preBötC cells extend by ∼400 μm [2,12] and are thus partly cut in thin slices. Here, we used 400 μm thin newborn rat slices with the preBötC in the middle ("m-preBötC[400]" slices) and "calibrated" margins [18] as a compromise between long term viability of rhythmogenic neurons and minimal preBötC modulation by adjacent tissue. We hypothesize that longevity of rhythm in 3 mM K^+ in such slices is sufficient for the planned intracellular analysis.

Experiments were done on slices from forty 0–5 days old Sprague–Dawley and Wistar rats in compliance with the guidelines of the Canadian Council for Animal Care and with approval of the University of Alberta Health Animal Care and Use Committee for Health Sciences. Rats were anesthetized with isoflurane until

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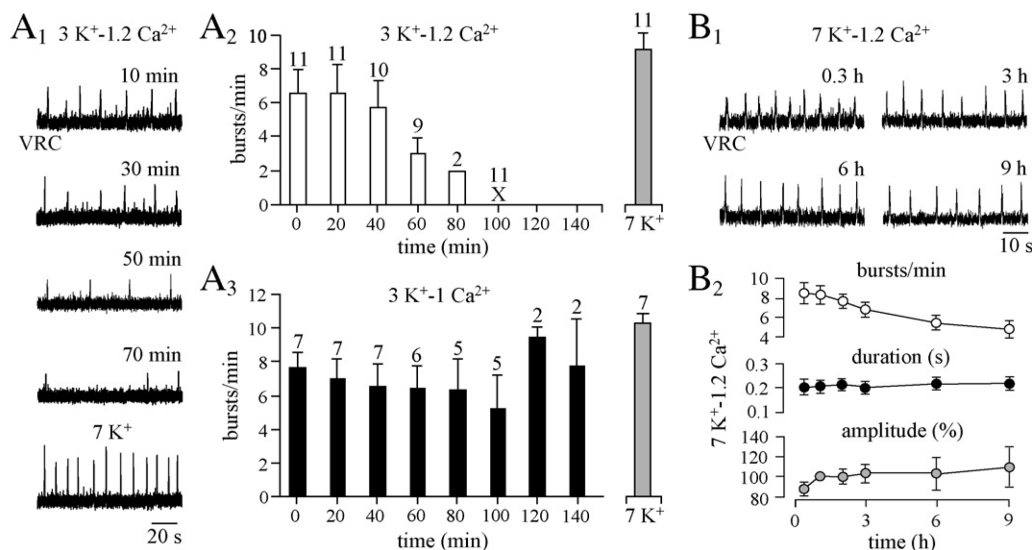

Fig. 1. Rhythmic activity of inspiratory pre-Bötzinger complex (preBötC) networks in 400 μm thin newborn rat brainstem slices. (A_1) Suction electrode recording from the ventrolateral slice area of a 400 μm thin slice containing the ventral respiratory column (VRC) with the preBötC in the middle ("m-preBötC[400]" slice) that was generated with "online histology" [18]. The differentially amplified, bandpass-filtered and integrated VRC recordings show that inspiratory-related rhythm in physiological K^+ (3 mM) and most common physiological Ca^{2+} (1.2 mM) ("$3K^+$–$1.2Ca^{2+}$") [11,14,17,22] arrested spontaneously after slightly more than 70 min ("in vitro apnea" [18,19]), but was restored by raising superfusate K^+ to 7 mM. (A_2) The plot of the time dependence of burst rates shows that only 2 of 11 (slice numbers above bars) m-preBötC[400] slices were active for >1 h in $3K^+$–$1.2Ca^{2+}$. '*x*' indicates the time for arrest of rhythm in all slices. (A_3) Rhythm was more robust in seven slices, that were both generated and studied in 3 mM K^+ and 1 mM Ca^{2+} solution ("$3K^+$–$1Ca^{2+}$") corresponding to the low end of the estimated physiological Ca^{2+} range [11,14,17,22]. Bars represent means (±SEM) of burst rates in slices that were still active at indicated times. (B_1) The original recording indicates that rhythm was stable for 9 h in slices generated in $3K^+$–$1.2Ca^{2+}$ and studied in $7K^+$–$1.2Ca^{2+}$. This was confirmed in the plots in B_2 of burst rate, duration and amplitude in seven slices that did not significantly differ from control (20 min) at any time until 9 h of recording.

the paw withdrawal reflex disappeared. They were then decerebrated and the neuraxis isolated at 18–21 °C in solution with (in mM) 120 NaCl; 3 KCl; 1.2 $CaCl_2$; 1 $MgSO_4$; 26 $NaHCO_3$; 1.25 NaH_2PO_4 and 20 D-glucose; pH 7.4 upon gassing with 95% O_2, 5% CO_2. The ion content of this solution resembled most common values in extracellular brain tissue in vivo [11,14,17,22]. After removal of the cerebellum and transection slightly rostral to the caudal cerebellar artery and just rostral to the C_1 spinal segment, the brainstem was glued rostral side down to a metal plate and m-preBötC[400] slices were generated using online histology [18]. Slices were fixed caudal side up with insect pins in a acrylic chamber (volume 1.5 ml) and superfusate was applied at 25–27 °C (flow rate 5 ml/min) via a peristaltic pump. After the experiments, slices were chemically fixed, stained and photographed [18]. Blockers of neuronal activity, Cd^{2+} and tetrodotoxin, were kept frozen in H_2O as 250 mM and 1 mM stock solutions, respectively. Agents were obtained from Sigma–Aldrich (Canada), except for salts for the superfusate (Fisher Scientific, Ottawa, Ontario, Canada). Suction electrodes were positioned at the surface of the ventrolateral slice area for recording neuronal population activity from the ventral respiratory column (VRC) which includes the preBötC [7,15,19,21]. Such differential "VRC" signals were amplified (×10k; DAM 50, WPI, Sarasota, FL, USA), bandpass-filtered (0.3–3 kHz), integrated (time constant 12 ms) and sampled at 1 kHz into a computer via a digital recorder [18–20]. "Blind" patch-clamp was used for membrane potential recording [4,5]. Patch pipettes (outer tip Ø 2–3 μm), pulled from borosilicate glass capillaries, were filled with (in mM): 140 K-gluconate, 1 NaCl, 0.5 $CaCl_2$, 1 $MgCl_2$, 1 K_4-BAPTA, 1 Na_2-ATP and 10 Hepes; pH was adjusted to 7.4 with KOH; dc resistance in superfusate was 5–8 MΩ. During perpendicular advancement of patch electrodes into the contralateral preBötC region not used for VRC recording, inspiratory neurons were identified via their rhythmic action potential discharge. Augmentation by up to 300% of a voltage signal in response to injection of negative dc current pulses (1 nA, 20 ms) indicated that the electrode was close to a cell membrane. Positive pressure (20 mmHg) was then released and negative pressure applied for "giga seal" (>1 GΩ) formation. Whole-cell-recording was established by abrupt suction (∼100 mmHg). Only cells were analyzed, in which resting potential was more negative than −40 mV and was stable during 10 min control periods. Input resistance was measured at resting potential via rectangular hyperpolarizing current pulses (40–250 pA, 0.5–0.7 s). Cell locations were estimated by comparing slice margins, referred to the distance from the caudal end of VII nucleus (VII_c) [18], with the recording depth indicated by the digital readout of the microdrive (7600 series, Siskiyou Corporation, Grants Pass, OR, USA). preBötC activity was quantified by measuring time-dependent changes in burst rate, plus single burst duration and amplitude in some cases. The half-width of burst duration was determined using ClampFit software (Molecular Devices Corporation, Chicago, IL, USA) as the time interval from when the signal increased above and decreased below a threshold set at 50% of the peak amplitude value for that burst. Burst amplitude for long term stability of rhythms in 7 mM K^+ was normalized referred to values after 1 h of recording. Longevity of rhythm in 3 mM K^+ solution (see below) was defined as the time from start of continuous recording until the time when the time period between consecutive bursts exceeded 5 min. Effects of test solutions were analyzed for a 2 min time period. Values are means ± SEM. Significance ($^*P < 0.05$, $^{**}P < 0.01$) was determined with one-way ANOVA with Tukey post test or Student's *t*-test (Graphpad Software Inc., La Jolla, CA, USA).

First, we tested whether longevity of inspiratory-related rhythm in solution with most common brain interstitial K^+ (3 mM) and Ca^{2+} (1.2 mM), i.e., "$3K^+$–$1.2Ca^{2+}$ solution", is sufficient for cellular analyses that require already extended time periods for cell tracking. Bursting in the VRC area at an initial rate of 6.6 ± 1.4 bursts/min was stable in 10 slices for 40 min, whereas in one slice rhythm arrested after 37 min (Fig. 1). Such "in vitro apnea" [18–20] occurred in the other slices during the next hour, resulting in a mean longevity of rhythm in $3K^+$–$1.2Ca^{2+}$ of 67 ± 4 min. Next, we tested whether rhythm persists for longer time periods in solution with 1 mM Ca^{2+} as seen in m-preBötC[600] slices [20]. Indeed, in seven slices longevity of rhythm in $3K^+$–$1Ca^{2+}$ was more pronounced

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