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INHIBITION OF ENDOPLASMIC RETICULUM CA^{2+} ATPASE IN PREBÖTZINGER COMPLEX OF NEONATAL RAT DOES NOT AFFECT RESPIRATORY RHYTHM GENERATION

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Abstract—PreBötzinger complex (preBötC) neurons in the brainstem underlie respiratory rhythm generation *in vitro*. As a result of network interactions, preBötC neurons burst synchronously to produce rhythmic premotor inspiratory activity. Each inspiratory neuron has a characteristic 10–20 mV, 0.3–0.8 s synchronous depolarization known as the inspiratory drive potential or inspiratory envelope, topped by action potentials (APs). Mechanisms involving Ca^{2+} fluxes have been proposed to underlie the initiation of the inspiratory drive potential. An important source of intracellular Ca^{2+} is the endoplasmic reticulum (ER) in which active Ca^{2+} sequestration is mediated by a class of transporters termed sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs). We aim to test the hypothesis that disruption of Ca^{2+} sequestration into the ER affects respiratory rhythm generation. We examined the effect of inhibiting SERCA on respiratory rhythm generation in an *in vitro* slice preparation. Bath application of the potent SERCA inhibitors thapsigargin or cyclopiazonic acid (CPA) for up to 90 min did not significantly affect the period or amplitude of respiratory-related motor output or integral and duration of inspiratory drive in preBötC neurons. We promoted the depletion of intracellular Ca^{2+} stores by a transient bath application of 30 mM K^+ (high K^+) in the continuous presence of thapsigargin or CPA. After washing out the high K^+, respiratory rhythm period and amplitude returned to baseline values. These results show that after inhibition of SERCA and depletion of intracellular Ca^{2+} stores, respiratory rhythm remains substantially the same, suggesting that this source of Ca^{2+} does not significantly contribute to rhythm generation in the preBötC *in vitro*.

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Abbreviations: ACSF, artificial cerebrospinal fluid; APs, action potentials; CICR, Ca^{2+}-induced Ca^{2+} release; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; I_{CAN}, Ca^{2+}-activated non-specific cationic current; InsP3R, inositol 1,4,5-trisphosphate receptor; preBötC, preBötzinger complex; SERCAs, sarco/endoplasmic reticulum Ca^{2+} ATPases; VGCC, voltage-gated Ca^{2+} channels.

Key words: respiratory rhythm generation, breathing, thapsigargin, SERCA, preBötzinger complex, I_{CAN}.

INTRODUCTION

PreBötzinger complex (preBötC) neurons in the brainstem underlie respiratory rhythm generation *in vitro* (Smith et al., 1991). The inspiratory phase of the respiratory cycle results from preBötC neurons firing action potentials (APs) on top of inspiratory drive potentials. The mechanisms underlying the initiation and termination of the inspiratory drive potential are unclear. The critical role of the persistent sodium current in bursting activity in the preBötC and its contribution to shaping the inspiratory drive potential remain debated (Butera et al. 1999a,b; Del Negro et al., 2001; Pace et al., 2007a; Rybak et al., 2007; Koizumi and Smith, 2008). A mechanism dependent on a Ca^{2+}-activated non-specific cationic current (I_{CAN}) has been proposed as the main current underlying inspiratory drive potential initiation (Pace et al., 2007b). In this mechanism, I_{CAN} activation requires Ca^{2+} flux, so three sources of Ca^{2+} have been proposed: NMDA receptor-mediated Ca^{2+} influx, Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC) and inositol 1,4,5-trisphosphate receptor (InsP3R)-mediated intracellular Ca^{2+} release (Pace et al., 2007b; Pace and Del Negro, 2008).

Evidence suggests that NMDAR is not essential for respiratory rhythm generation or drive transmission (Funk et al., 1997; Morgado-Valle and Feldman, 2007). In non-neuronal systems, Ca^{2+} flux through L-type VGCCs has been suggested as a source for I_{CAN} activation (Wu et al., 1998). In neurons however, the membrane potential necessary to open VGCCs is mainly achieved during APs. In active preBötC neurons Ca^{2+} enters to the soma only during APs, several milliseconds after initiation of the inspiratory drive (Morgado-Valle et al., 2008).

The ER accumulates and releases Ca^{2+} via the activity-dependent mechanism Ca^{2+}-induced Ca^{2+} release (CICR) or after the activation of InsP3R. CICR is proposed as relevant for dendritic Ca^{2+} transients associated with inspiratory activity (Mironov, 2008); however, there is no evidence showing that CICR plays an obligatory role in respiratory rhythm generation.

To investigate how disruption of ER Ca^{2+} sequestration affects respiratory rhythm generation, we examined the effects of the potent, lipid soluble,

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cell-permeable SERCA inhibitors thapsigargin and CPA on respiratory-related rhythmic activity in a neonatal rat medullary slice preparation. These drugs deplete Ca^{2+} stores by preventing SERCA from counterbalancing the passive and activity-dependent Ca^{2+} fluxes from stores to the cytosol (Seidler et al., 1989; Plenge-Tellechea et al., 1997; Treiman et al., 1998).

We tested the hypothesis that depletion of intracellular Ca^{2+} stores by inhibiting SERCA affects respiratory rhythm generation *in vitro*. We bath applied thapsigargin or CPA while recording respiratory-related motor-output. Neither period nor amplitude of respiratory-related motor output was affected by thapsigargin or CPA. 30 mM K^+ (high K^+) applied in the presence of thapsigargin or CPA as a method to deplete intracellular Ca^{2+} stores (Friel, 2004) transiently decreased respiratory period, which returned to the baseline after removal of high K^+. These results show that inhibition of SERCA and subsequent depletion of intracellular Ca^{2+} stores do not alter respiratory rhythm, suggesting that this source of intracellular Ca^{2+} does not play a relevant role for rhythm generation.

EXPERIMENTAL PROCEDURES

Medullary slice preparation

Experiments were performed on neonatal rat transverse brainstem slices that generate respiratory-related motor output (Smith et al., 1991). The Ethics Committee of the Universidad Veracruzana approved all protocols. Neonatal rats (0–3 days old) were anesthetized with isoflurane and decerebrated. The neuroaxis was isolated, the cerebellum was removed and the brainstem block mounted with the rostral side up. Under a microscope the brainstem was sectioned serially in the transverse plane using a VT-1000 Vibratome (Vibratome, St Louis, MO) until neuroanatomical landmarks, i.e., nucleus ambiguus and inferior olive, were visible. A transverse slice (550 μm) containing the preBötC was cut. The slice obtained is similar to the c + preBötC slice described by Ruangkittisakul et al. (2008) but with less caudal tissue. The dissection was performed in artificial cerebrospinal fluid (ACSF) containing (in mM): 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgSO_4$, 23.5 $NaHCO_3$, 0.5 NaH_2PO_4 and 30 glucose, bubbled with 95% O_2/5% CO_2 at 27 °C. The slice was transferred to a 1-ml recording chamber and anchored using a grid of nylon fibers. The chamber was mounted to a fixed-stage microscope and perfused with ACSF (6 ml/min).

Electrophysiological recording

Rhythmic respiratory-related motor output was recorded from the hypoglossal nerve (XIIn) using fire-polished glass suction electrodes and a differential amplifier. To obtain a robust and stable rhythm, ACSF K^+ concentration was elevated to 9 mM and slices were perfused for 30 min before any experimental manipulation. XIIn activity was amplified, bandpass filtered (0.3–1 kHz), rectified and integrated ($\int$XIIn). Whole-cell patch-clamp recordings were performed using an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA) in current-clamp mode. Inspiratory neurons from the preBötC were visualized using an infrared-enhanced oblique illumination videomicroscopy system. Electrodes were pulled from borosilicate glass (O.D., 1.5 mm; I.D., 0.86 mm) on a horizontal puller (Model P-97, Sutter Instruments Co., Novato, CA). Electrodes (6–8 MΩ resistance) were filled with standard intracellular solution containing (in mM): 140 K-gluconate, 5 NaCl, 10 HEPES, 0.1 $CaCl_2$, 1.1 EGTA, 2 Mg-ATP (pH = 7.3). Electrophysiological signals were acquired digitally at 4–20 kHz using pCLAMP software and a Digidata 1200 AD/DA board (Molecular Devices, Sunnyvale, CA) after low-pass filtering. Series resistance and cell capacitance were estimated under voltage-clamp conditions and compensated as previously described (Morgado-Valle and Feldman, 2007). Once whole-cell recording was established the mode was switched to current clamp. Membrane potential was continuously monitored and maintained at ≅−60 mV by adjusting bias current. Only stable active preBötC inspiratory neurons were considered for analysis.

Drugs

Thapsigargin and CPA were obtained from Sigma Chemical (St. Louis, MO) and dissolved in DMSO. Thapsigargin was applied at the following concentrations: in the bath: 2 and 20 μM; pressure ejected in the preBötC: 200 μM; intracellularly via patch solution: 20 μM. CPA (30 or 50 μM) was bath applied. To maintain a safe toxicity level, the final DMSO concentration in working solutions was always less than 0.01% (Tsvyetlynska et al., 2005).

In determining whether or not the resulting network remains functional for rhythm generation, we consider the presence of $\int$XIIn rhythmic bursts, and the duration of interburst intervals expressed as period. We also measured the amplitude of inspiratory bursts, which under normal conditions reflects the level of rhythmic inspiratory drive originating in the preBötC in the *in vitro* network. However, a significant decrease in $\int$XIIn amplitude and even the complete elimination of inspiratory bursts have been seen in conditions where the core mechanisms for rhythm generation are not altered (Del Negro et al., 2005).

Data collection and analysis

Only one experiment was performed *per* slice. In the figures and text, ''Control'' always refers to the rhythmic activity before the application of thapsigargin or CPA, unless otherwise stated. To analyze the integral of inspiratory drives, spikes were eliminated by applying a lowpass Gaussian filter (3 dB cutoff frequency = 20 Hz) offline. Clampfit 8.0 (Molecular Devices, Sunnyvale, CA), Igor Pro (Wave Metrics, Inc., OR), Chart and Microsoft Excel were used for data analyses. Results are expressed as mean ± SEM. ANOVA and Tukey HSD test were used.

RESULTS

Effects of inhibiting SERCA on respiratory-related motor output

To explore the effects of SERCA inhibition on respiratory-related motor output, we analyzed the effect of chronic exposure (90 min) to 20 μM thapsigargin or 30 μM CPA on the period and amplitude of $\int$XIIn bursts. Based on the pharmacological properties of thapsigargin and CPA described in previous reports (see Discussion) we assumed that during chronic exposure, thapsigargin and CPA concentrations would reach equilibrium in the slice. We averaged the $\int$XIIn bursts occurring in 1-min segments before and every 10 min after thapsigargin or CPA application. We did not find statistically significant changes in $\int$XIIn burst period or amplitude after 10-, 30-, 60- or 90-min bath applications of 20 μM thapsigargin with respect to control (n = 6; Fig 1A, C). In the