Participation of a persistent sodium current and calcium-activated nonspecific cationic current to burst generation in trigeminal principal sensory neurons

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Tsuruyama K, Hsiao CF, Chandler SH. Participation of a persistent sodium current and calcium-activated nonspecific cationic current to burst generation in trigeminal principal sensory neurons. J Neurophysiol 110: 1903–1914, 2013. First published July 24, 2013; doi:10.1152/jn.00410.2013.—The properties of neurons participating in masticatory rhythmogenesis are not clearly understood. Neurons within the dorsal trigeminal principal sensory nucleus (dPrV) are potential candidates as components of the masticatory central pattern generator (CPG). The present study examines in detail the ionic mechanisms controlling burst generation in dPrV neurons in rat (postnatal day 8–12) brain stem slices using whole cell and perforated patch-clamp methods. Nominal extracellular Ca$^{2+}$ concentration transformed tonic discharge in response to a maintained step pulse of current into rhythmic bursting in 38% of nonbursting neurons. This change in discharge mode was suppressed by riluzole, a persistent Na$^+$ current (I_{NaP}) antagonist. Veratridine, which suppresses the Na$^+$ channel inactivation mechanism, induced rhythmic bursting in nonbursting neurons in normal artificial cerebrospinal fluid, suggesting that I_{NaP} contributes to burst generation. Nominal extracellular Ca$^{2+}$ exposed a prominent afterdepolarizing potential (ADP) following a single spike induced by a 3-ms current pulse, which was suppressed, but not completely blocked, by riluzole. Application of BAPTA, a Ca$^{2+}$ chelator, intracellularly, or flufenamic acid, a Ca$^{2+}$-activated nonspecific cationic channel (I_{CAN}) antagonist, extracellularly to the bath, suppressed rhythmic bursting and the postspike ADP. Application of drugs to alter Ca$^{2+}$ release from endoplasmic reticulum also suppressed bursting. Finally, voltage-clamp methods demonstrated that nominal Ca$^{2+}$ facilitated I_{NaP} and induced I_{CAN}. These data demonstrate for the first time that the previously observed induction in dPrV neurons of rhythmic bursting in nominal Ca$^{2+}$ is mediated by enhancement of I_{NaP} and onset of I_{CAN}, which are dependent on intracellular Ca$^{2+}$. mastication; CPG; bursting; in vitro; patch clamp

THE TRIGEMINAL PRINCIPAL SENSORY NUCLEUS is typically thought of as a sensory relay nucleus to the thalamus. However, cells within the dorsal region of the principal sensory nucleus (dPrV) were recently implicated as key participants in the masticatory central pattern generator (CPG) (Kolta et al. 2007; Morquette et al. 2012; Tsuboi et al. 2003). These neurons reside within the boundaries of the minimal brain stem regions consistent with masticatory rhythmogenesis (Tanaka et al. 1999), have projections to oral motor nuclei (Yoshida et al. 1998), and are rhythmically active during mastication (Tsuboi et al. 2003). Furthermore, many of these neurons show positive labeling for c-Fos protein, an activity-dependent marker, in response to bouts of mastication (Athanassiadis et al. 2005).

For vertebrate central pattern-generating networks, such as locomotion, respiration, and mastication, the structure of the underlying networks and the mechanisms for rhythmogenesis have not been defined clearly, although recent progress using molecular biological methods has been made (Brownstone and Wilson 2008; Kiehn et al. 2010; Wilson et al. 2010). Most likely, masticatory rhythmogenesis is dependent on a combination of network-driven properties (emergent properties) and intrinsic conditional pacemaker properties (intrinsic bursters). Subpopulations of mesencephalic V neurons (Wu et al. 2001), supratrigeminal neurons (Hsiao et al. 2007), and dPrV neurons (Brocard et al. 2006; Sandler et al. 1998) all have intrinsic burst-generating properties and most likely contribute to masticatory rhythmogenesis. However, the precise ionic mechanisms responsible for this cellular behavior have not been defined precisely. Various combinations of activation of persistent Na$^+$ currents (I_{NaP}), Ca$^{2+}$ currents, and noninactivating low-threshold K$^+$ currents have roles in burst generation in various types of neurons (Li and Baccei 2011; Li and Hatton 1996; Su et al. 2001; Wu et al. 2001) and, in particular, are important for initiation of spikes to slowly rising depolarizing inputs (Kuo et al. 2006). For mastication and dPrV neurons, Kolta’s group (Brocard et al. 2006) showed that when extracellular Ca$^{2+}$ concentration is lowered, many of these neurons exhibit intrinsic bursting dependent on I_{NaP}, similar to that shown for supraoptic neurons (Li and Hatton 1996) and hippocampal neurons (Su et al. 2001). This is significant because naturally occurring reductions in extracellular Ca$^{2+}$ concentration occur (Pumain and Heinemann 1981). Interestingly, in some neuron types, bursting is dependent on intracellular Ca$^{2+}$ released from internal stores (Su et al. 2001; Cadzir et al. 2009; Pena et al. 2004b; Rubin et al. 2009) in addition to I_{NaP}. This suggests a possible role for a Ca$^{2+}$-activated nonspecific cationic current (I_{CAN}) in burst generation, as well (Dong et al. 2009; Cadzir et al. 2009). The aim of this study was to examine in more detail the mechanisms for intrinsic bursting of dPrV neurons evoked by low extracellular Ca$^{2+}$ concentration. We sought to determine if, in addition to I_{NaP}, I_{CAN} activated by intracellular Ca$^{2+}$ released from internal stores is important in mediating rhythmic bursting in dPrV neurons.

MATERIALS AND METHODS

Preparation of brain stem slices. Whole cell patch-clamp experiments were performed on dPrV neurons obtained from coronal slices of 8- to 12-day-old neonatal Sprague-Dawley rat brain stems as
described in detail previously (Enomoto et al. 2007; Hsiao et al. 2009). This age was chosen because the development of chewing from suckling occurs during this period (Westneat and Hall 1992). Briefly, rats were anesthetized by 2-bromo-2-chloro-1,1,1-trifluoroethane inhalation (Sigma, St. Louis, MO). The brain was removed and placed in oxygenated ice-cold cutting solution. Coronal sections (300 μm) containing PrV were obtained and then placed in an incubation solution at 37°C for 30 min. Animal protocols were approved by the Institutional Animal Care and Use Committee at University of California, Los Angeles.

**Solutions.** Solutions were bubbled with 95% O₂-5% CO₂ and maintained at a pH of 7.25–7.33 (22–24°C). The cutting solution was composed of (in mM) 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 1 CaCl₂, 5 MgCl₂, and 4 lactic acid. The recording solution consisted of (in mM) 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 2 CaCl₂, and 2 mM MgCl₂. When Ca²⁺ concentration was lowered, equal amounts of MgCl₂ were added to the artificial cerebrospinal fluid (ACSF) to maintain divalent cation concentration. The incubation solution was identical to the recording solution except for the addition of 4 mM lactic acid. The normal pipette solution used for current-clamp recording contained (in mM) 140 K-glucuronate, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 2 K₂-ATP, and 0.4 Na-GTP, with a pH of 7.25–7.30 and osmolarity of 280–290 mosM.

For voltage-clamp experiments that focused on isolating sodium currents (modified ACSF), K⁺ currents were blocked using an intrapipette solution containing the following (in mM): 130 CsF, 9 NaCl, 10 HEPES, 10 EGTA, 1 MgCl₂, 3 K₂-ATP, and 1 Na-GTP, with a pH of 7.25–7.30 and osmolarity of 280–290 mosM. The external solution contained the following (in mM): 131 NaCl, 10 HEPES, 3 KCl, 10 glucose, 2 CaCl₂, 2 MgCl₂, 10 tetraethylammonium (TEA)-Cl, 10 CsCl, 1 4-aminopyridine (4-AP), and 0.3 CdCl₂. Sodium currents were defined by subtraction of the currents remaining in 0.5 μM tetrodotoxin (TTX).

For voltage-clamp experiments performed to isolated I_{CAN}, the internal solution contained the following (in mM): 125 CsMeSO₃, 10 CsCl, 1 NaCl, 10 HEPES, 0.5 EGTA in 350 mM KOH, 3 Mg-ATP, and 1 Na-GTP, with a pH of 7.25–7.30 and osmolarity of 280–290 mosM. The external solution contained the following (in mM): 131 NaCl, 10 HEPES, 3 KCl, 10 glucose, 2 CaCl₂, 2 MgCl₂, 10 tetraethylammonium (TEA)-Cl, 10 CsCl, 1 4-aminopyridine (4-AP), and 0.3 CdCl₂. Sodium currents were defined by subtraction of the currents remaining in 0.5 μM tetrodotoxin (TTX).

**Histological procedures.** To visualize neurons using confocal microscopy, we used Alexa Fluor 568 hydrazide or Alexa Fluor 488 (n = 6). The patch pipette was carefully detached from the cell after recording, and slices were fixed with 4% paraformaldehyde in phosphate buffer (0.05 M, pH 7.4) for 2–3 days at 4°C. Subsequently, slices were rinsed in PBS for 10–15 min and repeated again with a fresh batch of PBS. The slice was then mounted on a coverslip for imaging. All of the procedures were performed at room temperature. A confocal microscope (Zeiss LSM 5) attached to an upright microscope (Zeiss AxiosImager) using Zen software (Carl Zeiss MicroImaging, Zeiss) was equipped with single-photon argon (488 and 514 nm), HeNe (543 nm), and red diode (633 nm) lasers. Z-plane image stacks of each cell were taken at high (×63) and low (×10) magnifications. Optimal Z-plane slice sizes were determined on a case-by-case basis with the increment for low magnification always being between 2 and 3 μm and the increment for high magnification always being between 1 and 2 μm. Two-dimensional maximum intensity projection images were then created from the Z-plane image stacks using the Zeiss LSM Image.
Browser software and analyzed to confirm cell location and examine cell morphology. In some experiments, Lucifer yellow was used to visualize the neuron during the experiment and photography but was not further processed.

Data analysis. Data were collected and analyzed with a combination of software [Clampfit (Molecular Devices), Sigma Plot (version 9.0, Systat Software), StatView (SAS Institute)]. The results are means ± SE. Comparisons of group means were performed with one-way ANOVA (ANOVA and post hoc analysis using Bonferroni test for differences between multiple group means) and Student’s t-test, with differences considered significant at \( P < 0.05 \) unless otherwise stated.

RESULTS

The data presented in this study are based on patch-clamp recordings from over 200 neurons located within the dPrV from 150 Sprague-Dawley rats. The initial criteria for inclusion in the database were resting potential more negative than \(-50\) mV, action potential amplitude at least 80 mV, and input resistance \(\geq 100\) m\(\Omega\). Figure 1 shows a schematic of the region from which recordings were obtained. In Fig. 1A the relationship between the dPrV, mesencephalic nucleus of V, and trigeminal motor nucleus is shown. Figure 1, B and C, shows typical examples of a recorded neuron during whole cell patch recording and a confocal image of a different neuron. Of the 6 neurons imaged, all were multipolar with a mean diameter of \(15.8 \pm 2.14\) \(\mu\)m for the long axis and \(8.5 \pm 0.80\) \(\mu\)m for the short axis, and \(3.8 \pm 0.31\) primary dendrites.

Spike discharge characteristics and rhythmical bursting. In normal ACSF all neurons showed repetitive discharge in response to a long step pulse of current (>5 s) or maintained DC adjustment of the membrane potential \((n = 267; \text{Fig. 2A})\). However, when \(Ca^{2+}\) was removed from the ACSF (nominal \(Ca^{2+}\)), in 38% of the neurons \((n = 102)\), the pattern changed from repetitive discharge to rhythical burst discharge. A characteristic of burst discharge was repetitive spike discharge occurring on top of a plateau-like wave of depolarization (Fig. 2B and inset). Bursting characteristics were not affected by bath application of antagonists to either excitatory or inhibitory amino acid receptors (glutamate, glycine, or GABA; not shown). A frequency histogram of sampled membrane potential (Fig. 2C) more clearly shows the lack of, and presence of, slow membrane oscillation before and after removal of \(Ca^{2+}\). Before \(Ca^{2+}\) removal, the histogram shows a uniform distribution with peak around \(-47\) mV (onset of spike discharge), whereas in the presence of nominal \(Ca^{2+}\), two peaks are evident, with the more depolarized peak indicating the induced region of plateau potential and the more negative peak voltage indicating the trough of the interburst interval.

Table 1 shows some electrical properties for the burst and nonburst neurons. Interestingly, although the resting membrane potential and action potential amplitudes were not significantly different between the two groups, for bursting neurons, \(R_{\text{onp}}\) was significantly lower and \(C_{\text{onp}}\) was significantly higher compared with the nonbursting neurons. These data suggest a qualitative difference in discharge characteristic between the two groups based on cell size.

Additional differences between burst and nonburst neurons in normal \(Ca^{2+}\) medium were elucidated by examining changes in spike and AHP characteristics induced by a short current pulse (3 ms) sufficient to elicit a single spike off the membrane capacitance immediately following the pulse. The passive capacitive response just subthreshold has been subtracted to give a more accurate representation of the spike

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**Fig. 1.** Location and morphological identification of neurons within the dorsal trigeminal principal sensory nucleus (dPrV neurons). A: schematic of region within PrV where neurons were recorded (horizontal lines). B: example of Lucifer yellow-filled neuron during experiment. Patch pipette and dye-filled multipolar neuron are visible. C: confocal image of Alexa Fluor-filled multipolar neuron (different neuron from that shown in B).
current-clamp methods that \( I_{\text{NaP}} \) contributes to bursting in low-Ca\(^{2+}\) medium (Brocard et al. 2006). To confirm these observations and examine in more detail the participation of these currents to bursting and ADP production, we performed additional current- and voltage-clamp experiments. In current-clamp mode in nominal Ca\(^{2+}\), riluzole (5–10 \( \mu \)M) was applied to the bath, and the effects on both rhythmic bursting in response to maintained membrane depolarization and on spike characteristics induced by a short stimulus pulse were examined. Typically, riluzole transformed rhythmic bursting into tonic discharge (\( n = 5/5 \) neurons; Fig. 3, A and B) and shifted the sampled membrane potential histogram from two peaks to one peak during drug application (Fig. 3D). Simultaneously, the amplitude and duration of the underlying depolarization (Fig. 3B, inset) were reduced. This is more clearly seen when a single spike was induced by a short stimulus pulse. During nominal Ca\(^{2+}\) conditions, the brief stimulus evoked a long-duration plateau potential with a series of repetitive spikes (Fig. 3E, control trace). As shown, riluzole reduced the amplitude of the poststimulus plateau potential (~17%; control: 21.5 ± 1.08 mV vs. riluzole: 17.8 ± 0.93 mV, \( P \leq 0.001, n = 5 \); Fig. 3E, dashed trace) and the area of the plateau potential (~27%; control: 3,415.4 ± 1,048.5 mV·s vs. riluzole: 940.2 ± 177.28 mV·s, \( P \leq 0.05, n = 5 \)), as well as the number of evoked spikes.

Additional evidence for a role for \( I_{\text{NaP}} \) in bursting comes from experiments using veratridine, a toxin that allows Na\(^{+}\) channels to pass current longer by blocking the Na\(^{+}\) channel inactivation mechanism (Hille 2001). Bath application of 300 nM veratridine produced rhythmic bursting in normal ACSF in 6/8 neurons tested, as shown in Fig. 4. In control conditions, regardless of stimulus intensity, bursting was not induced, but repetitive discharge was present without an underlying envelope of membrane depolarization. As shown, in the presence of veratridine, rhythmic bursting was induced with spikes occurring on top of a prominent membrane depolarization. Figure 4B, inset, shows a single burst and the underlying depolarization. Figure 4C shows the sample membrane potential before and after veratridine application. Note the two prominent peaks in membrane potential after drug application. The effects of veratridine on the short pulse-induced spike are shown in Fig. 4D. After drug application, the ADP was enhanced and a continuous depolarization and spike train were induced.

### Table 1. Membrane properties of PrV neurons

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Values are means ± SE for burst and nonburst neurons of the dorsal trigeminal principal sensory nucleus (PrV). \( * P < 0.05 \). RMP, resting membrane potential; \( R_{\text{input}} \), input resistance; AP, action potential amplitude; \( V_{\text{threshold}} \), AP threshold potential; AHP, afterhyperpolarizing potential; ADP, afterdepolarizing potential. All data were obtained in normal artificial cerebrospinal fluid from a 3-ms pulse.

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Fig. 2. Nominal extracellular Ca\(^{2+}\) transforms tonic spike discharge into rhythmic burst discharge in dPrV neurons. A: example of tonic discharge in dPrV neuron in normal artificial cerebrospinal fluid (ACSF). B: rhythmic burst discharge in nominal extracellular Ca\(^{2+}\) solution (0 Ca\(^{2+}\)). \( \text{Inset} \) shows 1 burst. C: frequency distribution of sampled membrane potential (1-mV bins) before (control) and after removal of Ca\(^{2+}\). Note the 2 peaks in the histogram, indicative of holding potential and plateau potential in nominal Ca\(^{2+}\). D: short pulse stimulation (3-ms duration) in normal (control) and nominal Ca\(^{2+}\) solutions. Note the enhanced afterdepolarizing potential (ADP) and spiking in nominal Ca\(^{2+}\). Voltage calibration in B was applied to D.

Characteristics. In particular, compared with nonburst neurons, burst neurons had a significantly more negative spike threshold, a smaller postspike early AHP, and larger subsequent ADP (Table 1), suggesting that burst neurons in general are more excitable. Figure 2D shows the effects on the single action potential waveform evoked by a short current pulse before Ca\(^{2+}\) removal and in nominal Ca\(^{2+}\) solution. Most notable is the prolonged ADP and multiple spiking after Ca\(^{2+}\) removal. Persistent Na\(^{+}\) currents contribute to burst depolarization. Previously, it was shown in dPrV neurons with the use of NaP contributes to bursting in low-Ca\(^{2+}\) medium (Brocard et al. 2006). To confirm these observations and examine in more detail the participation of these currents to bursting and ADP production, we performed additional current- and voltage-clamp experiments. In current-clamp mode in nominal Ca\(^{2+}\), riluzole (5–10 \( \mu \)M) was applied to the bath, and the effects on both rhythmic bursting in response to maintained membrane depolarization and on spike characteristics induced by a short stimulus pulse were examined. Typically, riluzole transformed rhythmic bursting into tonic discharge (\( n = 5/5 \) neurons; Fig. 3, A and B) and shifted the sampled membrane potential histogram from two peaks to one peak during drug application (Fig. 3D). Simultaneously, the amplitude and duration of the underlying depolarization (Fig. 3B, inset) were reduced. This is more clearly seen when a single spike was induced by a short stimulus pulse. During nominal Ca\(^{2+}\) conditions, the brief stimulus evoked a long-duration plateau potential with a series of repetitive spikes (Fig. 3E, control trace). As shown, riluzole reduced the amplitude of the poststimulus plateau potential (~17%; control: 21.5 ± 1.08 mV vs. riluzole: 17.8 ± 0.93 mV, \( P \leq 0.001, n = 5 \); Fig. 3E, dashed trace) and the area of the plateau potential (~27%; control: 3,415.4 ± 1,048.5 mV·s vs. riluzole: 940.2 ± 177.28 mV·s, \( P \leq 0.05, n = 5 \)), as well as the number of evoked spikes.

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Fig. 3. Persistent Na⁺ current ($I_{NaP}$) contributes to burst generation in dPrV neurons. A–C: rhythmic bursting in control bursting (nominal Ca²⁺; A), after riluzole (5 μM) application (B), and after washout (C). Inset shows example of a single burst before and after drug. Note the reduction of the ADP.

D: frequency histogram of sampled membrane potential in nominal Ca²⁺ and during riluzole application. E: short pulse stimulation before (control; solid line), during application (dashed line), and after (dotted line) washout of riluzole. Note that riluzole substantially attenuates, but does not abolish, the ADP.

Fig. 4. Veratridine induces rhythmic burst discharge in dPrV neurons. A and B: tonic discharge in normal ACSF (A) is transformed into rhythmic burst discharge (B) in the presence of veratridine (300 nM). Inset shows a single burst discharge. C: frequency histogram of sampled membrane potential before (control) and during veratridine application. D: short pulse stimulation before and after veratridine application. Note the enhanced ADP and continuous discharge in the presence of veratridine.
Voltage-clamp experiments were performed to determine the direct effects of nominal Ca$^{2+}$ medium on membrane currents and provide evidence for the presence of $I_{\text{Nap}}$. In modified ACSF (see MATERIALS AND METHODS), a slow voltage ramp command (Fig. 5A, inset) sufficient to inactivate the fast Na$^+$ current (18 mV/s, 5 s) produced a small negative slope conductance region and prominent outward rectification at more depolarized potentials (solid circles, leak subtracted). Subsequent recording in nominal Ca$^{2+}$ (open circles) significantly ($P < 0.01$) enhanced the peak inward current by $\sim 46\%$ [control: 30.9 ± 11.7 pA ($n = 5$) vs. 0 Ca$^{2+}$: 45.2 ± 9.7 pA] and shifted the voltage of the peak by $\sim 11\%$ (control: $-37.2 ± 2.1$ mV vs. 0 Ca$^{2+}$: $-41.2 ± 1.4$ mV) but was without effect on the onset of activation of the inward current (control: $-61.1 ± 1.7$ mV vs. 0 Ca$^{2+}$: $-62.8 ± 1.5$, V). This change for the inward current after nominal Ca$^{2+}$ effectively enhanced the negative slope conductance region of the current-voltage (I-V) current, thus facilitating burst generation in current-clamp mode. This current is reminiscent of $I_{\text{Nap}}$ recorded in trigeminal mesencephalic V neurons (Wu et al. 2001, 2005). Application of TTX to the nominal Ca$^{2+}$ condition abolished the region of negative slope conductance, indicative of $I_{\text{Nap}}$ (shaded circles).

An example from one neuron of the isolated $I_{\text{Nap}}$ obtained by subtraction is shown in Fig. 5B. Subtraction of the current in nominal Ca$^{2+}$ and TTX from that recorded in nominal Ca$^{2+}$ exposed the TTX-sensitive component ($I_{\text{Nap}}$) of the total current. Figure 5C shows a summary plot for $I_{\text{Nap}}$ of normalized conductance (normalized to $g_{\text{max}}$ vs. command potential for four neurons. Conductance ($G$) was calculated as $G = I/(V - E_{\text{rev}})$. The calculated reversal potential based on pipette and extracellular solutions ($E_{\text{rev}}$) was $+64$ mV. The data were fit with a single Boltzmann function (solid line): $I/[1 + \exp(-(V - V_{1/2\text{max}})/k)]$. The value for $V_{1/2\text{max}}$ was $-53.0 ± 0.6$ mV, and the slope factor $k$ was $5.8 ± 0.5$ mV. The mean onset voltage was $-62.6 ± 1.2$ mV.

Two observations suggest that in addition to $I_{\text{Nap}}$, $I_{\text{CAN}}$ (Formenti et al. 2001; Smith et al. 2004) is activated as well in nominal Ca$^{2+}$ conditions and contributes to the production of the ADP and bursting. First, in the presence of TTX, nominal Ca$^{2+}$ induced a steady inward current at command potentials more negative than at the onset of $I_{\text{Nap}}$ (Fig. 6A). Second, the observation that riluzole reduced, but did not abolish (Fig. 3E), the ADP suggests a role for intracellular Ca$^{2+}$ and activation of an $I_{\text{CAN}}$ channel, as well.

Evidence for the presence of $I_{\text{CAN}}$ after nominal Ca$^{2+}$ application comes from voltage-clamp experiments in the presence of modified ACSF to block $I_{\text{Nap}}$ (see MATERIALS AND METHODS). Bath application of nominal Ca$^{2+}$ elicited a steady inward current when the neuron was clamped to $-70$ mV ($n = 5$; Fig. 6A). Membrane currents produced by slow voltage ramp commands from $-83$ to $+7$ mV (18 mV/s) were used to generate I-V curves in various ACSF/drug conditions. Figure 6B shows that a linear voltage-dependent current was produced by application of nominal Ca$^{2+}$ and blocked by addition of 50 μM flufenamic acid (FFA), a $I_{\text{CAN}}$ channel blocker (Gogelein et al. 1990; Harks et al. 2001). The I-V relation of $I_{\text{CAN}}$ was obtained by subtracting the current recorded in the presence of nominal Ca$^{2+}$ from that recorded in control. Figure 6C shows an example of an I-V plot for $I_{\text{CAN}}$ (filled circles) obtained for one neuron. This current was linear across the range of membrane potentials examined and reversed polarity near 0 mV ($-1.6 ± 1.2$ mV, $n = 12$). Bath application of FFA (50 μM), in the presence of the nominal Ca$^{2+}$ solution, substantially reduced $I_{\text{CAN}}$ in this neuron (open circles). A summary dot plot for the effects of FFA on $I_{\text{CAN}}$ obtained at a holding potential of $-83$ mV for all neurons is shown in Fig. 6D.

To determine whether $I_{\text{CAN}}$ induced by nominal Ca$^{2+}$ solution depends on intracellular Ca$^{2+}$, we compared currents
recorded using standard internal pipette solution with those obtained in a separate series of experiments with BAPTA in the internal solution. The composite $I-V$ relationships for $I_{\text{CAN}}$ in standard internal solution (Fig. 6E, filled circles, $n = 12$) and in BAPTA internal solutions (Fig. 6E, open circles, $n = 10$) are shown. In all cells tested, BAPTA (10 mM) substantially reduced $I_{\text{CAN}}$. Figure 6F shows a summary dot plot of the effects of BAPTA on $I_{\text{CAN}}$ (recorded at $-83$ mV). The above experiments demonstrate that nominal Ca$^{2+}$ solutions induce, in addition to $I_{\text{Na}^+}$, an $I_{\text{CAN}}$ that, as expected, requires intracellular Ca$^{2+}$. The following set of experiments examines the role of intracellular Ca$^{2+}$ and its source in more detail.

**Bursting is dependent on intracellular Ca$^{2+}$.** To determine if rhythmical bursting induced by replacement of the ACSF with nominal Ca$^{2+}$ is dependent on intracellular Ca$^{2+}$, we introduced 10 mM BAPTA, a Ca$^{2+}$ chelator, into the neuron via the pipette. The nystatin perforated-patch recording method (Sakmann and Neher 1995) was used to obtain control bursting in nominal Ca$^{2+}$ solution before diffusion of BAPTA into the cell. The integrity of the perforated patch was verified by the lack of diffusion of Lucifer yellow into the soma and the attenuated spike amplitudes accompanied with high input resistance, typically observed with perforated recording (Fig. 7, A and B). After rupture of the membrane to obtain whole cell configuration, Lucifer yellow diffused rapidly into the neuron, as indicated by the fluorescence observed in the soma (Fig. 7B). As BAPTA diffused into the neuron, the burst cycle duration became prolonged, until tonic discharge ensued without an underlying large depolarizing potential and rhythmical bursting (22/25 neurons). This typically occurred within the first 20 min of rupture. Once tonic spike discharge was observed, the prolonged plateau potential (ADP) with overriding spikes induced by a short 3-ms stimulus pulse was reduced, as well (Fig. 7B). BAPTA significantly reduced the peak amplitude of the ADP induced by a short pulse stimulus by ~48% (control: $17.5 \pm 1.2$ mV vs. BAPTA: $15.0 \pm 1.3$ mV, $n = 10$, $P < 0.001$) and area by 90% (control: $2,410 \pm 751.3$ mV·s vs. BAPTA: $2,490 \pm 751.3$ mV·s, $n = 8$, $P < 0.01$). Typically, one or two spikes following the stimulus were observed after BAPTA application. These data demonstrate that intracellular Ca$^{2+}$ is necessary for rhythmical bursting in low-Ca$^{2+}$ medium and contributes to the postspike ADP.

Endoplasmic reticulum is a source of intracellular calcium required for bursting. The condition of nominal extracellular Ca$^{2+}$ and requirement of intracellular Ca$^{2+}$ for bursting suggest that a source for Ca$^{2+}$ could be from intracellular stores such as endoplasmic reticulum (ER). If this hypothesis is correct, then one would expect depletion of stores to block
Fig. 7. Bursting in nominal Ca\(^{2+}\) solution is dependent on intracellular Ca\(^{2+}\). A: time course of effects of BAPTA diffusion from pipette into neuron after rupture of perforated patch. Recording at 0 min shows bursting during nystatin patch configuration and immediately after rupture to form whole cell configuration (dotted vertical line). B: short pulse stimulus evokes plateau potential and discharge in dPrV neuron before and after rupture of perforated patch. Note the reduction in plateau potential over time. Insets shows absence of dye into neuron during perforated-patch configuration and after rupture of membrane to obtain whole cell configuration. Note the Lucifer yellow-filled neuron indicating whole cell configuration was obtained.

bursting over time. To test this hypothesis, we applied thapsigargin (Thaps), a blocker of the ER Ca\(^{2+}\) pump, to the bath during bursting induced by nominal extracellular Ca\(^{2+}\). A typical example is shown in Fig. 8. After Thaps application, bursting was transformed into spontaneous low-frequency discharge with a reduced underlying wave of depolarization (9/14 neurons; Fig. 8B). Figure 8C shows that after Thaps application, the sampled membrane potential showed one peak, indicating elimination of bursting. Furthermore, in response to a single short pulse stimulus, the postspike ADP amplitude and area were reduced, significantly, by 28.8% (control: 19.5 ± 1.2 mV vs. Thaps: 14.3 ± 0.67 mV, \(n = 7\), \(P < 0.001\)) and 83.0% (control: 2.353 ± 1.9333 mV mV·s vs. Thaps: 401.4 ± 174.1 mV·s, \(n = 7\), \(P < 0.02\)), respectively (Fig. 8D).

Finally, we examined whether inositol 1,4,5-trisphosphate (IP\(_3\)) receptor activation, which triggers Ca\(^{2+}\) release from ER, contributes to burst generation. Intracellular application of XeC (1 \(\mu\)M in pipette), an IP\(_3\) receptor antagonist (Gafni et al. 1997), using the nystatin perforated-patch method allowed us to identify a neuron as burst generating in nominal Ca\(^{2+}\) before diffusion into the cell (Fig. 9A). Once the patch was ruptured and whole cell configuration obtained, XeC diffusion transformed bursting into tonic discharge (Fig. 9B). Concomitantly, the sampled membrane potential showed one peak in the histogram, indicating that bursting was abolished and the underlying rhythmical plateau potential suppressed (Fig. 9C). This is more clearly demonstrated by the significant reduction in postspike ADP amplitude induced by a short pulse (22.7%; control: 17.0 ± 1.60 mV vs. XeC: 13.9 ± 1.43 mV, \(n = 3\), \(P < 0.001\); Fig. 9D). Taken together, the above data suggest a link between reduction of extracellular Ca\(^{2+}\) concentration, IP\(_3\) receptor activation, and burst generation in dPrV neurons.

**DISCUSSION**

An interesting observation is that dPrV neurons in response to reduced extracellular Ca\(^{2+}\) show intrinsic bursting (Sandler et al. 1998), which is blocked by low doses of riluzole, an \(I_{\text{NaP}}\) antagonist (Urbani and Belluzzi 2000), and suppressed after reduction of extracellular Na\(^{+}\) (Brocard et al. 2006; Tazerart et al. 2008). However, the precise ionic mechanisms responsible for this observation are not completely understood. As argued in previous reports (Kolta et al. 2007; Tsuboi et al. 2003), these neurons could well be part of a masticatory CPG, since they exhibit intrinsic burst-generating properties under specific conditions, receive input from oral cavity sensory structures (Shigenaga et al. 1986), project to oral motor nuclei (Kolta et al. 2000; Turman and Chandler 1994a, 1994b), and are rhythmically active during oral motor activity (Kolta et al. 2007; Tsuboi et al. 2003). Furthermore, they are located within the minimal brain stem region shown to support rhythmical jaw movements in reduced preparations (Kogo et al. 1998). Thus understanding the electrical properties of these neurons and the factors that govern their discharge characteristics is important for understanding the mechanisms responsible for masticatory pattern generation.

This study is the first to demonstrate in dPrV neurons that reduction in extracellular Ca\(^{2+}\) facilitates \(I_{\text{NaP}}\) and is dependent on intracellular Ca\(^{2+}\) release from internal stores for the postspike ADP and rhythmical burst production. Furthermore, we show that the source of internal Ca\(^{2+}\) is partly from ER, since the IP\(_3\) antagonist XeC and the ER calcium pump blocker, Thaps substantially reduced the ADP amplitude and suppressed maintained bursting. The data also suggest that in addition to \(I_{\text{NaP}}\), an \(I_{\text{CAN}}\) participates in ADP and burst production, as well.

As reported initially, a subpopulation of dPrV neurons have intrinsic burst-generating properties (Sandler et al. 1998).
The proportion of cells goes up significantly when the extracellular Ca\(^{2+}\) concentration is reduced to below 1.2 mM (Brocard et al. 2006), a phenomenon similar to that observed in hippocampal pyramidal neurons, supraoptic neurons, and putative spinal cord CPG interneurons after a similar reduction of external Ca\(^{2+}\) (Li and Hatton 1996; Su et al. 2001; Tazerart et al. 2008). The induction of bursting in these neurons is most likely related to the reduction of the medium-duration Ca\(^{2+}\)-dependent postspike AHP and emergence of a prominent ADP and plateau potential (Brocard et al. 2006). As shown here and previously, the ADP and bursting are partially dependent on activation of \(I_{\text{NaP}}\) (Brocard et al. 2006; Su et al. 2001; Tazerart et al. 2008), and this is supported by our data showing induction of bursting in normal ACSF following veratridine application and the voltage-clamp data showing enhancement of \(I_{\text{NaP}}\) after reduction of extracellular Ca\(^{2+}\). However, the data also show that although TTX application abolished the inward current associated with the negative slope conductance region of the \(I-V\) curve, a steady-state voltage-dependent inward current was still present in nominal Ca\(^{2+}\) conditions, suggesting a role for other voltage-dependent currents.

**Fig. 8.** Depletion of endoplasmic reticulum (ER) Ca\(^{2+}\) stores suppresses rhythmical burst activity. A and B: bath application of 3 μM thapsigargin (Thaps) transformed burst activity into tonic firing (9/14 neurons). C: frequency histogram of sampled membrane potential in nominal Ca\(^{2+}\) and Thaps. D: application of 3 μM Thaps reduced both the amplitude and duration of the ADP induced by short pulse stimulation.

**Fig. 9.** Block of inositol 1,4,5-trisphosphate (IP\(_3\)) receptor activation suppresses bursting in nominal Ca\(^{2+}\). A and B: internal application of 1 μM xestospongin C (XeC) through a patch pipette attenuated the rhythmical burst activity (5/5). C: application of 1 μM XeC reduced both the amplitude and duration of the ADP in response to short pulse stimulation. D: frequency histogram of sampled membrane potential in response to short pulse stimulation. D: frequency histogram of sampled membrane potential in response to short pulse stimulation.
ing that in addition to \( I_{\text{NaP}} \), other steady-state inward currents are activated by the reduction in Ca\(^{2+} \) concentration. Our current-clamp data support this hypothesis, since riluzole, which is more selective for \( I_{\text{NaP}} \) compared with transient fast Na\(^+ \) currents (Urbani and Belluzzi 2000; Wu et al. 2005), substantially reduced, but never abolished, the ADP following activation of a single spike. It is unlikely that the residual ADP results from current through Ca\(^{2+} \) channels, since the driving force on Ca\(^{2+} \) is substantially reduced in nominal extracellular Ca\(^{2+} \).

Changes in extracellular cation concentration are well known to alter membrane excitability (Frankenhaeuser and Hodgkin 1957; Hille 2001). Typically, a decrease in extracellular Na\(^+ \) or K\(^+ \) suppresses excitability, whereas such a change in extracellular Ca\(^{2+} \) has the opposite effect. The mechanism(s) for this is varied and could result from 1) membrane charge screening, which shifts activation and inactivation curves of Na\(^+ \) to more hyperpolarized directions (Hille 2001); 2) reduction in \( g_{\text{K}}/\text{Ca}^{2+} \); and/or 3) activation of nonspecific cationic currents (Formenti et al. 2001; Hablitz et al. 1986). Charge screening is unlikely in our case, since divalent cation concentration was maintained in all cases during ionic substitutions. Although we did not test for changes in \( g_{\text{K}}/\text{Ca}^{2+} \), others have shown that specific block of those channels did not induce bursting in PrV neurons, spinal motoneurons, or hippocampal neurons (Brocard et al. 2006; Su et al. 2001; Tazerart et al. 2008). Furthermore, in normal ACSF, direct application of Cd\(^{2+} \), which blocks Ca\(^{2+} \) channels and indirectly \( g_{\text{K}}/\text{Ca}^{2+} \), never induced bursting in our study (unpublished observations). However, more recently, extracellular Ca\(^{2+} \) was shown to regulate a Na\(^+ \) leak channel (NALCN) via a G protein-coupled extracellular Ca\(^{2+} \)-sensing receptor and G protein-mediated intracellular pathway (Lu et al. 2010). In that study, reduction in extracellular Ca\(^{2+} \) enhanced membrane excitability, whereas selective knockout of that channel protein eliminated the changes in excitability in response to Ca\(^{2+} \) reduction. We cannot eliminate the possibility that similar mechanisms are responsible for the effects of nominal Ca\(^{2+} \) on dPrV neurons and are responsible for the residual inward current observed in nominal Ca\(^{2+} \) and TTX. However, we also cannot rule out the possibility that a \( I_{\text{CAN}} \) current participates, since intracellular BAPTA in nominal extracellular Ca\(^{2+} \) reduced the postspike ADP and transformed bursting into tonic discharge. This possibility is supported by our observation that in voltage clamp, in nominal Ca\(^{2+} \) and after TTX application to block \( I_{\text{NaP}} \), an inward current with similar characteristics to \( I_{\text{CAN}} \) was still present. Finally, in current clamp after 50 \( \mu \)M FFA, bursting was abolished and tonic firing ensued. Participation of \( I_{\text{CAN}} \) in Aplysia burst-firing neurons has been demonstrated (Kramer and Zucker 1985) and was more recently shown to be important for dopamine-induced bursting from lobster pyloric neurons (Kadiri et al. 2011). Additionally, \( I_{\text{CAN}} \) was implicated in bursting for respiratory neurons in mice (Del Negro et al. 2005) and spinal cord dorsal lamina bursting neurons related to pain pathways (Li and Baccei 2011). Although we used low doses of FFA, it must be emphasized that results from studies using this drug must be tempered by the fact that FFA is nonspecific at doses greater than \( \sim 100 \mu \)M and can affect other cellular processes (Gogelein et al. 1990; Kochetkov et al. 2000; Ottolia and Toro 1994). Clearly, more specific antagonists of \( I_{\text{CAN}} \) must be developed.

Intracellular Ca\(^{2+} \) release from internal stores is necessary for bursting and enhances ADP amplitude and duration. Our data demonstrate that in nominal extracellular Ca\(^{2+} \), the postspike ADP, bursting, and the underlying envelope of depolarization during rhythmical bursting are dependent on the presence of intracellular Ca\(^{2+} \), since BAPTA application directly into the neuron suppressed these events. This indicates a role for Ca\(^{2+} \) release from internal stores in control of membrane excitability and bursting. This has not been examined in dPrV neurons previously but is consistent with that observed in supraoptoic and olfactory bulb neurons (Dong et al. 2009; Li and Hatton 1996) as well as trigeminal motoneurons that exhibit bursting in response to NMDA application (Hsiao et al. 2002). However, this is in contrast to that reported by others in hippocampal neurons and spinal CPG interneurons in low Ca\(^{2+} \) (Brocard et al. 2006; Su et al. 2001). The basis for these differences in response to BAPTA is not clear presently.

Most likely, the source of the Ca\(^{2+} \) in nominal extracellular Ca\(^{2+} \) must be release from intracellular stores such as ER and mitochondria. Although we did not study Ca\(^{2+} \) release from mitochondria, application of antagonists of IP\(_3\) receptors and disruption of the ER Ca\(^{2+} \) pump both suppressed bursting and reduced the amplitude of the postspike ADP. Involvement of release of Ca\(^{2+} \) from internal stores and \( I_{\text{CAN}} \) currents were reported for brain stem bursting neurons within the respiratory system (Del Negro et al. 2005; Pace et al. 2007; Pena et al. 2004a), as well as olfactory bulb neurons in response to metabotropic glutamate receptor stimulation (Dong et al. 2009) and more recently in lobster pyloric CPG neurons (Kadiri et al. 2011). What could trigger activation of IP\(_3\) receptors on ER? As mentioned, reduction in extracellular Ca\(^{2+} \) can activate a G protein-mediated Ca\(^{2+} \)-sensing receptor complex on the extracellular side of the membrane. Through subsequent activation of intracellular G protein-dependent pathways, this activates IP\(_3\) receptors and stimulates release of Ca\(^{2+} \) into the cytosol from ER stores (Su et al. 2010).

Functional implications. Extracellular Ca\(^{2+} \) concentration is typically \( \sim 1.3 \) mM and can fluctuate during behavior to as low as 0.1 mM during behavior (Heinemann et al. 1977). For instance, during the slow-wave sleep state, Ca\(^{2+} \) levels in cortex can drop to as low as 0.85 mM (Amzica et al. 2002), and further reduction to as low as 0.2 mM occurs during pathological states such as epilepsy (Hablitz et al. 1986; Puma and Heinemann 1981). Considering that such changes in extracellular Ca\(^{2+} \) concentration do occur and can modulate neuronal firing patterns, these alterations could control masticatory neuron patterns during chewing or transition from maintained jaw position to chewing behaviors. Koltz’s group (Brocard et al. 2006) proposed that before the onset of rhythmical mastication when cortical neurons increase their discharge and recruit pattern-generating interneurons, the level of extracellular Ca\(^{2+} \) around those neurons could be reduced. This would act as an additional stimulus in conjunction with the masticatory pattern-generating network to strengthen maintained rhythmical oscillations in interneurons involved in masticatory CPG function. The presence of rhythmical neurons within a network could also serve to reinforce the timing of information transferred within the network (Feldman and Del Negro 2006; Kiehn et al. 1996). As a consequence, such activity would impart greater robustness and stability to the output of the network (Purvis et al. 2007).
Although the structure of the masticatory CPG network has not been defined conclusively (Tanaka et al. 1999), dPrV neurons are strong candidates as intrinsic burst-generating neurons within such a network (Tsuboi et al. 2003). Participation of \( I_{NaP} \) and \( I_{CaN} \) currents in burst generation can serve as targets for modulation by various neuromessengers for the basic rhythmic pattern. The relative contributions of \( I_{NaP} \) and \( I_{CaN} \) are most likely regulated to adapt the masticatory CPG to the ongoing needs of the organism. It is clear that intrinsic ion channel properties participate in sculpting CPG output for various behaviors such as locomotion (Brownstone and Wilson 1998; Harris-Warrick 2010; Tazerart et al. 2008), respiration (Del Negro et al. 2002a, 2002b), and mastication (Del Negro and Chandler 1998; Del Negro et al. 1998, 1999; Hsiao et al. 2002; Koltz et al. 2007). Further studies on the role of intrinsic ion channels, their modulation, and their participation in masticatory central pattern-generating circuits are necessary.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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