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

Kentaro Tsuruyama, Chie-Fang Hsiao, and Scott H. Chandler

Department of Integrative Biology and Physiology, and the Brain Research Institute, University of California at Los Angeles, Los Angeles, CA, 90095, USA

Running Head: Ionic mechanisms underlying burst generation

Correspondence to: Scott H. Chandler PhD
Department of Integrative Biology and Physiology, UCLA, 2024 Terasaki Bld, Young Dr. East, Los Angeles, CA 90095
Tel: 310-206-6636
E-mail: schandler@physci.ucla.edu

247 words in abstract
Number of figures: 9
Number of tables: 1
Number of pages: 34

Key Words: mastication, CPG, bursting, in vitro, patch clamp

Contribution of Authors: Tsuruyama: performed experiments and writing; Hsiao: performed experiments; Chandler: Directed project and participated in writing.

ABSTRACT

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 (p8-12) brainstem 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 rhythmical bursting in 38% of non-bursting neurons. This change in discharge mode was suppressed by riluzole, a persistent sodium current (I\(_{NaP}\)) antagonist. Veratridine, which suppresses the Na\(^+\) channel inactivation mechanism, induced rhythmical bursting in non-bursting neurons in normal ACSF, 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 (FFA) acid, a Ca\(^{2+}\) activated non-specific cationic channel (I\(_{CAN}\)) antagonist, extracellularly to the bath, suppressed rhythmical 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 rhythmical bursting in nominal calcium is mediated by enhancement of I\(_{NaP}\) and onset of I\(_{CAN}\), which are dependent upon intracellular Ca\(^{2+}\).
Introduction

The trigeminal principal sensory nucleus is typically thought of as a sensory relay nucleus to the thalamus. However, recently, cells within the dorsal region of the principal sensory nucleus (dPrV) have been 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 brainstem 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 obtained (Brownstone and Wilson 2008; Kiehn et al. 2010; Wilson et al. ; 2010). Most likely masticatory rhythmogenesis is dependent upon 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 sodium currents ($I_{NaP}$), $Ca^{2+}$
currents, and non-inactivating low threshold potassium 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 upon I\(_{\text{NaP}}\), 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 upon intracellular Ca\(^{2+}\) released from internal stores (Dong et al. 2009; Kadiri et al. 2011; Pena et al. 2004b; Rubin et al. 2009) in addition to I\(_{\text{NaP}}\). This suggests a possible role for I\(_{\text{CAN}}\) in burst generation as well (Dong et al. 2009; Kadiri et al. 2011; Rubin 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\(_{\text{NaP}}\), I\(_{\text{CAN}}\) activated by intracellular Ca\(^{2+}\) released from internal stores is important in mediating rhythmical 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 since 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 UCLA.

**Solutions**

Solutions were bubbled with 95% O₂-5% CO₂ and maintained at a pH of 7.25–7.3 (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 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-gluconate, 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 TTX.

For voltage-clamp experiments performed to isolated I₅ᵥ, 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; the external solution contained the following (in mM): 115.25 NaCl, 3 KCl, 26 NaHCO₃, 10 glucose, 2 CaCl₂, 2 MgCl₂, and 10 TEA-Cl, 4-AP (1 mM), CdCl₂ (100 µM), TTX (0.5 µM) and antagonists of AMPA, NMDA, glycine and GABA receptors (DNQX 20 µM, APV 50 µM, strychnine 20 µM, bicuculline 20 µM) were added to the external solution to minimize network interactions. BAPTA (10 mM), and Xestospongin C (XeC) (1 µM) were applied intracellularly through the patch pipette. Veratridine (300 nM) and Thapsigargin (Thaps, 3 µM) were bath-applied. All drugs were purchased from Sigma (St. Louis, MO).

**Whole Cell Recording**

Slices were perfused with an oxygenated recording solution (2 mL/min) while secured in a recording well mounted on a Zeiss fixed-stage Axioskop microscope equipped with bright-field and fluorescence optics. Neurons were visualized with enhanced resolution using infrared differential video microscopy. Patch recordings were obtained with an Axopatch 1D (Axon Instruments, Foster City, CA) in concert with pCLAMP acquisition software (version 9.2, Medical Devices, Sunnyvale, CA) operating on a desk top personal computer. Signals were digitized online, filtered at 2 kHz (voltage clamp) or 5-10 kHz (current clamp), and sampled at 10–50 kHz depending on the experiment. Patch pipettes were fabricated from conventional thin-walled glass (1.5 mm OD, 0.86 mm ID; Warner Instrument, Hamden, CT) and pulled on a Brown-Flaming P-97
micropipette puller (Sutter Instruments, Novato, CA). Their bath resistance was 3-5 MΩ. Signals were grounded by a 3M KCl agar bridge electrode (Ag/AgCl wire) mounted in the recording well. Liquid junction potentials were measured between the pipette and bath solutions and varied between 5 mV (normal pipette solution, \(I_{\text{CAN}}\) solution), and 7 mV (sodium current isolation recording solution) and were corrected offline. Whole-cell capacitance \(C_{\text{inp}}\) was determined from the integral of capacity current in response to 15-msec hyperpolarizing voltage commands or directly obtained by the pCLAMP software during the experiment. Uncompensated series resistance \((R_s)\) was calculated from the decay time constant \((\tau)\) of the transient and was usually less than 10 MΩ. Sixty to eighty percent compensation was routinely employed. Data were not collected in the first 10 min after gaining whole-cell access. Spike threshold and depolarization amplitude to burst onset were measured. Threshold for action potential initiation in response to a brief 3 ms current pulse was defined as the membrane potential on the rising phase of the spike where the \(dV/dT\) inflection was most prominent (typically greater than 50 mV/sec). Spike amplitude was measured from baseline to peak. Input resistance \((R_{\text{inp}})\) was determined from the slope of the steady-state voltage-current relationship in response to a series of small hyperpolarizing current pulses from the resting potential (within 10mV). The area of the ADP was measured by using Clampfit v9.2 (Molecular Devices). The peak of the AHP immediately following the spike was measured from the most negative trough to the initial baseline, while the post spike ADP amplitude was measured from the most negative trough of the preceding AHP to the peak of the ADP.
Nystatin patch experiments. We used the nystatin perforated patch method to obtain baseline control data after obtaining a gigaohm seal and prior to intracellular drug application (Sakmann and Neher 1995). Nystatin (250 µg/mL) 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. Fluorescence was confined to the pipette in the perforated patch condition. Subsequently, the patch solution containing BAPTA, or XeC was delivered to the intracellular environment via patch rupture, which allowed diffusion of drug from pipette to cytosol and filled the neuron with fluorescent dye reflecting the whole-cell configuration.

Identification of trigeminal principal sensory neurons

Trigeminal principal sensory nuclei in a coronal slice were identified bilaterally under low magnification (50×) as an opaque oval region lateral to the trigeminal motor nucleus (Fig. 1A). Following identification of the TMN, the visual field was moved to an area within 200 µm lateral and 300 µm dorsal to the border of the center TMN. This placed the recording pipette in approximately the dorsal region of PrV (gray lines). These neurons were visualized at higher magnification (400×) and clearly identified as neurons with small somata (around 8–20 µm) with various morphologies. Initial experiments indicated that fusiform and pyramidal shaped neurons, in contrast to multipolar neurons, seldom showed bursting in low Ca^{2+} solution. Therefore, this study focused on multipolar neurons only.

Histological procedures

To visualize neurons using confocal microscopy Alexa Fluor 568 Hydrazide or Alexa Fluor 488 (n=6) was used. 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 are rinsed in PBS for 10-15 mins 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, AxioImager) using Zen Software (Carl Zeiss MicrolImaging Inc., Thornwood, NY) was used. The confocal microscope was equipped with single-photon (Argon (488, 514 nm), HeNe (543 nm) and Red Diode (633 nm)) lasers. Z-plane image stacks of each cell were taken at high (63X) and low (10X) magnifications. Optimal Z-plane step 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 to examine cell morphology. In some experiments, Lucifer yellow was used to visualize the neuron during the experiment and photograph, but not further processed.

Data analysis

Data were collected and analyzed with a combination of software [Clampfit, Molecular Devices), Sigma Plot v9.0 (Systat Software), StatView (SAS Institute)]. The results are reported as mean ± SEM. Comparisons of group means were performed with one-way ANOVA (ANOVA and post hoc analysis using Bonferonni test for differences between multiple group means) and Student $t$-test, with differences considered significant at $P < 0.05$ unless otherwise stated.
RESULTS

The data presented in this study are based upon patch clamp recordings from over 200 neurons located within the dorsal region of the trigeminal principal sensory nucleus 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 greater than or equal to 100 mΩ. Figure 1 shows a schematic of the region from which recordings were obtained. In figure 1A the relationship between the dPrV, Mesencephalic nucleus of V (Mes V) and trigeminal motor nucleus (MoV) is shown. Figure 1B and C show 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 the long axis of 15.8 µm ± 2.14, and 8.5 ± 0.80 for the short axis, and 3.8 ± 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 sec), or maintained DC adjustment of the membrane potential (n=267) (Figure 2A). However, when calcium was removed from the ACSF (nominal Ca²⁺), in 38% of the neurons (n=102), the pattern changed from repetitive discharge to rhythmical burst discharge. A characteristic of burst discharge was repetitive spike discharge occurring on top of a plateau-like wave of depolarization (Figure 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 Ca²⁺.
Prior to nominal Ca\textsuperscript{2+}, the histogram shows a uniform distribution with peak around -47 mV (onset of spike discharge), while in the presence of nominal Ca\textsuperscript{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 non-burst neurons. Interestingly, although the resting membrane potential and action potential amplitudes were not significantly different between the two groups, for bursting neurons, the input resistance (R\text{inp}) was significantly lower and the input capacitance (C\text{inp}) was significantly higher compared to the non-bursting neurons. These data suggest a qualitative difference in discharge characteristic between the two groups based upon cell size.

Additional differences between burst and non-burst neurons in normal Ca\textsuperscript{2+} media 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 characteristics. In particular, compared to non-burst neurons, burst neurons had a significantly more negative spike threshold, a smaller post spike 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 Ca\textsuperscript{2+} action potential waveform evoked by a short current pulse prior to, and in nominal, Ca\textsuperscript{2+} solution. Most notable is the prolonged ADP and multiple spiking after Ca\textsuperscript{2+} removal.
Persistent sodium currents contribute to burst depolarization

Previously, it was shown in dPrV neurons using current clamp methods that $I_{\text{Na}^P}$ contributes to bursting in low Ca$^{2+}$ media (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 μM) was applied to the bath and the effects on both rhythmical bursting in response to maintained membrane depolarization and on spike characteristics induced by a short stimulus pulse were examined. Typically, riluzole transformed rhythmical bursting into tonic discharge ($n=5/5$ neurons) (Fig. 3A,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 plateau potential following the stimulus (Fig. 3E, long dashed trace) (~17%, control 21.5 mV ± 1.08 vs riluzole 17.8 mV ± 0.93, $p \leq 0.001$, $n=5$), and the area of the plateau potential (~27%, control 3415.4 mV · sec ± 1048.5 vs riluzole 940.2 ± 177.28, $p \leq 0.05$, $n=5$), as well as the number of evoked spikes.

Additional evidence for a role for $I_{\text{Na}^P}$ in bursting comes from experiments using veratridine, a toxin that allows sodium channels to pass current longer by blocking the sodium channel inactivation mechanism (Hille 2001). Bath application of 300 nM veratridine produced rhythmical bursting in normal ACSF in 6/8 neurons tested, as
shown in figure 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 rhythmical bursting was induced with spikes occurring on top of a prominent membrane depolarization (Fig. 4B, inset arrow). The inset shows a single burst and the underlying depolarization. Figure 4C shows the sample membrane potential prior to, and after, veratridine. Note the two prominent peaks in membrane potential after drug application. The effects of veratridine on the short pulse induced spike are shown in figure 4D. After drug application, the ADP was enhanced and a continuous depolarization and spike train were induced.

Voltage clamp experiments were performed to determine the direct effects of nominal Ca\(^{2+}\) media on membrane currents and provide evidence for the presence of I\(_{\text{NaP}}\). In modified ACSF (see methods), a slow voltage ramp command (Fig. 5 inset) sufficient to inactivate the fast Na\(^{+}\) current (18 mV/sec, 5 sec) produced a small negative slope conductance region and prominent outward rectification at more depolarized potentials (black circles, leak subtracted). Subsequent recording in nominal Ca\(^{2+}\) (open circles) significantly (p<0.01) enhanced the peak inward current by ~46% (control 30.9 pA ± 11.7 SE (n=5) vs 0 Ca\(^{2+}\) 45.2 ± 9.7), shifted the voltage of the peak by ~ 11% (control -37.2 mV ± 2.1 vs 0 Ca\(^{2+}\) -41.2 ± 1.4), but was without effect on the onset of activation of the inward current (control -61.1 mV ± 1.7 vs 0 Ca\(^{2+}\) -62.8 ± 1.5). This change for the inward current after nominal Ca\(^{2+}\) effectively enhanced the negative slope conductance region of the I-V current, thus facilitating burst generation in current clamp mode. This current is reminiscent of I\(_{\text{NaP}}\) recorded in trigeminal Mes V neurons.
Application of TTX to the nominal Ca\textsuperscript{2+} condition abolished the region of negative slope conductance, indicative of I_{NaP} (gray circles).

An example from one neuron of the isolated I_{NaP} obtained by subtraction is shown in Fig. 5B. Subtraction of the current in nominal Ca\textsuperscript{2+} and TTX from that recorded in nominal Ca\textsuperscript{2+} exposed the TTX-sensitive component (I_{NaP}) of the total current. Figure 5C shows a summary plot for I_{NaP} of normalized conductance (normalized to g_{max}) versus command potential for 4 neurons. Conductance (G) was calculated as $G = I/(V - E_{rev})$, the calculated reversal potential based upon pipette and extracellular solutions ($E_{rev}$) was +64 mV. The data were fit with a single Boltzmann function (solid line), $1/(1 + \exp[-(V - V_{1/2max})/k]}$. The $V_{1/2max}$ was -53.0 mV ± 0.6, and the slope factor, K, in millivolts, was 5.8 ± 0.5. The mean onset voltage was -62.6mV ± 1.2.

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

Evidence for the presence of I_{CAN} after nominal Ca\textsuperscript{2+} application comes from voltage clamp experiments in the presence of modified ACSF to block I_{NaP} (see methods). Bath application of nominal Ca\textsuperscript{2+} elicited a steady inward current when the neuron was clamped to -70 mV (Fig. 6A, n=5). Membrane currents produced by slow ramp voltage 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\textsuperscript{2+} and blocked by addition of 50 µM FFA, a \textit{l}_{\text{CAN}} channel blocker (Gogelein et al. 1990; Harks et al. 2001). The \textit{I}/\textit{V} relation of \textit{l}_{\text{CAN}} was obtained by subtracting the current recorded in the presence of nominal Ca\textsuperscript{2+} from that recorded in control. Figure 6C shows an example of an \textit{I}-\textit{V} plot for \textit{l}_{\text{CAN}} (black 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 (\textit{n} = 12). Bath application of FFA (50 µM), in the presence of the nominal Ca\textsuperscript{2+} solution, substantially reduced \textit{l}_{\text{CAN}} in this neuron (open circles). A summary dot plot for the effects of FFA on \textit{l}_{\text{CAN}} obtained at a holding potential of -83 mV for all neurons is shown in Fig. 6D.

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

\textbf{Bursting is dependent upon intracellular Calcium}

To determine if rhythmical bursting induced by replacement of the ACSF with nominal Ca\textsuperscript{2+} is dependent upon intracellular Ca\textsuperscript{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 prior to diffusion of BAPTA into the cell. The integrity of the perforated patch was verified by the lack of diffusion of Lucifer Yellow (LY) into the soma and the attenuated spike amplitudes accompanied with high input resistance, typically observed with perforated recording (Fig. 7A and B). After rupture of the membrane to obtain whole cell configuration, LY 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 minutes of rupture. Once tonic spike discharge was observed, the prolonged plateau potential (ADP) with over-riding 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 \(\sim 48\%\) (control: 17.5 mV ± 1.8 vs BAPTA 10.0 mV ± 1.3, \(n=8\), \(p \leq 0.001\)) and area by 90% (control: 2410.9 mV · sec ± 751.3 vs BAPTA 249.0 mV · sec ± 751.3, \(n=8\), \(p \leq 0.01\), \(n=8\), \(p \leq 0.01\)). 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\(^{2+}\) media and contributes to the post spike 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 bursting over time. To test this hypothesis, we applied thapsigargin (Thaps), a blocker of the ER Ca\textsuperscript{2+} pump, to the bath during bursting induced by nominal extracellular Ca\textsuperscript{2+}. A typical example is shown in figure 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 now showed one peak, indicating elimination of bursting. Furthermore, in response to a single short pulse stimulus, the post spike ADP amplitude and area were reduced, significantly, by 28.8% (control: 19.5 mV ± 1.2 vs Thaps 14.3 mV ± 0.67, n=7, p ≤ 0.001, and 83.0% (control: 2353.8 mV · sec ± 1933.3 vs Thaps 401.4 mV · sec ± 174.1, n=7, p ≤ 0.02) respectively (Fig. 8D).

Finally, we examined whether IP\textsubscript{3}R activation, which triggers Ca\textsuperscript{2+} release from ER, contributes to burst generation. Intracellular application of xestospongin-C, an IP\textsubscript{3} receptor antagonist (Gafni et al. 1997) (XeC 1 μM in pipette), using nystatin-perforated patch method, allowed us to identify a neuron as burst generating in nominal Ca\textsuperscript{2+} prior to 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 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 mV ± 1.60 vs XeC 13.9 mV ± 1.43, n=3, p ≤ 0.001) (Fig. 9D). Taken together, the above data suggest
a link between reduction of extracellular Ca\(^{2+}\) concentration, IP\(_3\)R 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 sodium (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 papers (Kolta et al. 2007; Tsuboi et al. 2003), these neurons could well be part of a masticatory central pattern generator 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; Turman and Chandler 1994b), and are rhythmically active during oral-motor activity (Kolta et al. 2007; Tsuboi et al. 2003). Furthermore, they are located within the minimal brainstem 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 which govern their discharge characteristics is important for understanding the mechanisms responsible for masticatory pattern generation.

This paper is the first to demonstrate in dPrV neurons that reduction in extracellular Ca\(^{2+}\) facilitates I\(_{\text{NaP}}\) and is dependent upon intracellular Ca\(^{2+}\) release from internal stores for the post spike ADP and rhythmical burst production. Furthermore, we show that the source of internal Ca\(^{2+}\) is partly from endoplasmic reticulum (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 $I_{NaP}$, an $I_{CaN}$ 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 phenomena 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 post spike 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 upon activation of $I_{NaP}$ (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 after veratridine application, and the voltage clamp data showing enhancement of $I_{NaP}$ 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 that in addition to $I_{NaP}$, 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 $I_{NaP}$ compared to 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 sodium to more hyperpolarized directions (Hille 2001), 2) reduction in gK\(^+\)/Ca\(^{2+}\), and/or 3) activation of non-specific 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 gK\(^+\)/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 gK\(^+\)/Ca\(^{2+}\), never induced bursting in our study (unpublished observations). However, more recently, extracellular Ca\(^{2+}\) was shown to regulate a sodium 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 knock-out 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 post spike 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 µM flufenamic acid, 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 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 flufenamic acid, it must be emphasized that results from studies using this drug must be tempered by the fact that flufenamic acid is non-specific at doses greater than ~100 µM 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 post spike ADP, bursting and the underlying envelope of depolarization during rhythmical bursting are dependent upon 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 supraoptic 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 post spike ADP. Involvement of release of Ca\(^{2+}\) from internal stores and I\(_{\text{CAN}}\) currents were reported for brainstem 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 (Lu et al. 2010).

**Functional implications**

Extracellular Ca\(^{2+}\) concentration is typically \(~1.3\) 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\textsuperscript{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 occur during pathological states such as epilepsy (Hablitz et al. 1986; Pumain and Heinemann 1981). Considering that such changes in extracellular Ca\textsuperscript{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.

Kolta’s group (Brocard et al. 2006) proposed that prior to the onset of rhythmical mastication when cortical neurons increase their discharge and recruit pattern generating interneurons, the level of extracellular Ca\textsuperscript{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 2008; Harris-Warrick 2010; Tazerart et al. 2008), respiration (Del Negro et al. 2002a; Del Negro et al. 2002b) and mastication (Del Negro and Chandler 1998; Del Negro et al. 1999; Del Negro et al. 1998; Hsiao et al. 2002; Kolta et al. 2007). Further studies on the role of intrinsic ion channels, their modulation, and participation in masticatory central pattern generating circuits are necessary.

**Acknowledgements**

This work was supported by National Institute of Dental and Craniofacial Research DE 06193 to SHC. We would like to thank Takuma Sonoda for technical assistance and Victoria Nguyen for participation in data analysis. Address all correspondence to: S.H. Chandler, Dept. of Integrative Biology and Physiology, 2024 Terasaki Bld, 610 Young Dr, Los Angeles, CA 90095-7239 (E-mail: schandler@physci.ucla.edu).
References


**Figure Legends**

Fig. 1. Location and morphological identification of dPrV neurons A: schematic of region within PrV where neurons where 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 B).

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

Fig. 3. Persistent sodium current contributes to burst generation in dPrV neurons. A-C:
rhythmic bursting in (A) control bursting (nominal Ca\(^{2+}\)), after riluzole (5 µM) application
(B), and washout (C). Inset shows example of single burst before and after drug. Note
the reduction of the ADP. D: frequency histogram of sampled membrane potential in
nominal Ca\(^{2+}\) and during riluzole application. E: Short pulse stimulation before (black),
during (long dashes) and after (short dashes) washout of riluzole. Note that riluzole
substantially attenuates, but does not abolish the ADP.

Fig. 4. Veratridine induces rhythmical burst discharge in dPrV neurons. A and B: tonic
discharge in (A) normal ACSF is transformed into (B) rhythmical burst discharge in the
presence of veratridine (300 nM). Inset shows single burst discharge. C: frequency
histogram of sampled membrane potential prior to (control) and during Veratridine
application. D: short pulse stimulation prior to, and after, Veratridine application. Note
the enhanced ADP and continuous discharge in the presence of Veratridine.

Fig. 5. Nominal Ca\(^{2+}\) solution enhances I\(_{NaP}\). A: I-V relationship in response to slow
current ramp command in control ACSF (black circles), nominal Ca\(^{2+}\) (open circles), and
in the presence of TTX + 0 Ca\(^{2+}\) (0.5 µM) (gray circles). All traces were leak subtracted.
B: I\(_{NaP}\) obtained from subtraction of current in nominal Ca\(^{2+}\) from that after TTX. C:
composite normalized conductance-voltage relationship for 0 Ca\(^{2+}\) sensitive I\(_{NaP}\) current
(n= 4). Single Boltzmann function is fit to normalized conductance.

Fig. 6. Nominal calcium solutions induce a calcium activated non-selective cationic
current in dPrV neurons. A: nominal Ca\(^{2+}\) solution induces an inward current. Horizontal
line indicates duration of nominal Ca\(^{2+}\) application. Holding potential was -70 mV.
Modified ACSF was used (see methods). B: membrane currents recorded in response
to slow ramp voltage commands in the presence of modified ACSF (black trace),
nominal Ca\(^{2+}\), and FFA (50 µM, light gray) plus nominal Ca\(^{2+}\). Ramp voltage command
protocol is shown at the bottom. A hyperpolarizing pulse was applied immediately
following the ramp to monitor changes in input conductance. C: example of I-V
relationship for one neuron in nominal Ca\(^{2+}\) (black circles) and after addition of FFA
(open circles). D: summary dot plot showing the effects of FFA on I\(_{CAN}\). E: composite I-V
relationship for I\(_{CAN}\) induced by nominal Ca\(^{2+}\) in a control population, and in a separate
population of neurons after BAPTA diffusion into the neuron. F: summary dot plot for the
effects of BAPTA on I\(_{CAN}\). Data used for D and F were obtained at a holding potential of
-83 mV. Long horizontal lines in D and F indicate mean values, while short lines indicate
SE. Asterisks indicated significance at p < 0.05.
Fig. 7. Bursting in nominal Ca\(^{2+}\) solution is dependent upon intracellular Ca\(^{2+}\). A: time course of effects of BAPTA diffusion from pipette into neuron after rupture of perforated patch. Zero minutes 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. Inset 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.

Fig. 8. Depletion of ER calcium 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 Thapsigargin. D: application of 3 µM Thaps reduced both the amplitude and the duration of the ADP induced by short pulse stimulation.

Fig. 9. Block of IP\(_3\) receptor activation suppresses bursting in nominal Ca\(^{2+}\). A and B: internal application of 1 µM XeC through patch pipette attenuated the rhythmical burst activity (5/5). C: application of 1 µM XeC reduced both the amplitude and the duration of the ADP in response to short pulse stimulation. D: frequency histogram of sampled membrane potential in nominal Ca\(^{2+}\) and XeC.
A. Control

B. Thapsigargin

C. a. 0 Ca^{2+}  
   b. Thaps

D. Control  
   Thaps
A. Control

B. XeC

C. a) 0 Ca\(^{2+}\)  b) XeC

D. Control
Table 1. The membrane properties of PrV neurons

<table>
<thead>
<tr>
<th></th>
<th>Burst cells</th>
<th>Non-burst cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vr (mV)</td>
<td>-54.9 ± 0.7 (n = 102)</td>
<td>-55.5 ± 0.8 (n = 170)</td>
</tr>
<tr>
<td>Rinp (MΩ)</td>
<td>283.6 ± 19.9 (n = 102)</td>
<td>532.2 ± 36.3 * (n = 170)</td>
</tr>
<tr>
<td>Rinp (pF)</td>
<td>82.3 ± 6.5 (n = 102)</td>
<td>55.4 ± 2.7 * (n = 170)</td>
</tr>
<tr>
<td>AP (mV)</td>
<td>101.84 ± 12.77 (n = 43)</td>
<td>103.03 ± 9.58 (n = 59)</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>-43.39 ± 6.09 (n = 43)</td>
<td>-39.64 ± 6.29 * (n = 59)</td>
</tr>
<tr>
<td>AHP (mV)</td>
<td>-3.03 ± 3.21 (n = 43)</td>
<td>-6.10 ± 4.00 * (n = 59)</td>
</tr>
<tr>
<td>ADP (mV)</td>
<td>13.01 ± 6.24 (n = 43)</td>
<td>5.96 ± 5.69 * (n = 59)</td>
</tr>
</tbody>
</table>

*, significant at p-value < 0.05. RMP, resting membrane potential; Rinp, input resistance; AP, action potential amplitude; Vthreshold, AP threshold potential; AHP, afterhyperpolarizing potential; ADP, afterdepolarizing potential. All data obtained in normal ACSF from 3 ms pulse.