Characterization of inward currents and channels underlying burst activity in motoneurons of crab cardiac ganglion

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Large cell motoneurons in the Cancer borealis cardiac ganglion generate rhythmic bursts of action potentials responsible for cardiac contractions. While it is well known that these burst potentials are dependent on coordinated interactions among depolarizing and hyperpolarizing conductances, the depolarizing currents present in these cells, and their biophysical characteristics, have not been thoroughly described. In this study we used a combined molecular biology and electrophysiology approach to look at channel identity, expression, localization, and biophysical properties for two distinct high-voltage-activated calcium currents present in these cells: a slow calcium current (\(I_{\text{CaS}}\)) and a transient calcium current (\(I_{\text{CaT}}\)). Our data indicate that ChCaV1 is a putative voltage-gated calcium channel subunit in part responsible for an L-type current, while ChCaV2 (formerly cacophony) is a subunit in part responsible for a P/Q-type current. These channels appear to be localized primarily to the somata of the motoneurons. A third calcium channel gene (ChCaV3) was identified that encodes a putative T-type calcium channel subunit and is expressed in these cells, but electrophysiological studies failed to detect this current in motoneuron somata. In addition, we identify and characterize for the first time in these cells a calcium-activated nonselective cationic current (\(I_{\text{CAN}}\)), as well as a largely nonactivating TTX-sensitive current reminiscent of a persistent sodium current. The identification and further characterization of these currents allow both biological and modeling studies to move forward with more attention to the complexity of interactions among these distinct components underlying generation of bursting output in motoneurons.

Characterization of action potentials is vital to appropriate motor output in central pattern generator networks. The organization of burst potentials often relies on calcium- and sodium-mediated depolarizing currents that have an influence on neuronal function beyond eliciting a direct change in membrane potential. For example, calcium influx via voltage-gated calcium currents is known to affect second messenger systems involved in transcriptional regulation (Clapham 2007), activate other ionic currents (Sah 1995; Wisgirda and Dryer 1994; Yamoah et al. 1994), as well as trigger neurotransmitter release at the synapse (Mulkey and Zucker 1993). Sodium has been shown to activate sodium-dependent potassium currents and influence neuronal excitability (Hage and Salkoff 2012; Rose 2002). These higher-order cation effects are also known to act as the mechanism behind different forms of plasticity in the ongoing output of a cell and/or network (Cummings et al. 1996; George et al. 2012; Turrigiano 2008). However, before we can fully understand the role of inward currents and resulting cation influx during a burst potential, we must have a thorough understanding of what depolarizing components exist in a given cell type.

The large cell (LC) motoneuron of the Cancer borealis cardiac ganglion (CG) has been a successful model for studying how underlying conductances interact to mediate burst potentials (Ball et al. 2010; Franklin et al. 2010; Tobin et al. 2009), and new evidence shows there is rapid calcium-dependent homeostatic compensation among potassium currents that impacts both LC intrinsic excitability as well as appropriate network bursting (Ransdell et al. 2012). However, there is little characterization of what depolarizing properties are active in the Cancer borealis LC burst, hindering a more complete understanding of how the LC bursts are generated and maintained. The goal of this study was to characterize the depolarizing components intrinsic to the LC motoneuron in the Cancer borealis CG.

Despite the extensive use of crustacean motor systems as models for understanding neuronal network function and modulation (Marder and Bucher 2007), the inward currents have been less thoroughly studied than their outward counterparts. In neurons of the stomatogastric ganglion (STG), total voltage-activated calcium current has been successfully measured by using sensitivity to cadmium chloride (CdCl2) as an effective blocker (Golowasch and Marder 1992). Further pharmacological dissection of STG calcium currents suggests there are two distinct high-threshold calcium currents present: an L-type (CaV1) and a P/Q-type (CaV2) current (Hurley and Graubard 1998; Johnson et al. 2003). Molecular cloning studies support the presence of these two calcium channel types in neurons of the STG (French et al. 2002). Because the calcium dependence of synaptic transmission in STG neurons has a more hyperpolarized threshold (~60 mV) than these two high-threshold currents, there is thought to be an additional low-threshold or T-type (CaV3) current involved in neurotransmitter release (Graubard et al. 1983; Harris-Warrick et al. 1992). In the CG, some analysis of calcium currents has taken place in the LCs (Tazaki and Cooke 1979, 1986), particularly in the lobster Homarus americanus (Tazaki and Cooke 1990). However, a more thorough analysis of these currents is necessary to understand their role in generating burst output in LC motoneurons.

Beyond calcium currents, evidence for persistent sodium currents [tetrodotoxin (TTX) sensitive] has also been reported in both the STG (Elson and Silverston 1997; Turrigiano et al. 1995) and the CG LCs of the lobster H. americanus (Berlind 1993). The somata of these crustacean motoneurons do not
contain spike-mediating sodium currents (Golowasch and Marder 1992). However, a characterization of persistent sodium currents has yet to be performed in these crustacean systems. In addition, plateau potentials in the dorsal gastric neurons of the STG depend on activation of a calcium-dependent voltage-independent cationic current, or “CAN current” (Zhang and Harris-Warrick 1995; Zhang et al. 1995), but this current has yet to be identified or characterized in LC motoneurons.

With the *C. borealis* LC motoneuron being the focus of several recent studies exploring how intrinsic conductances and channels are organized and conserved during burst generation (Ball et al. 2010; Franklin et al. 2010; Ransdell et al. 2012; Tobin et al. 2009), a more thorough characterization of the inward currents in this species and cell type is both important and valuable to moving research in this area forward. Here we utilize multiple techniques, including two-electrode voltage clamp, immunohistochemistry, and single-cell PCR analysis of channel gene expression, to explore these depolarizing components in the LCs of *C. borealis*.

**MATERIALS AND METHODS**

*C. borealis* crabs were purchased and shipped overnight from The Fresh Lobster Company (Gloucester, MA). Crabs were kept between 24 h and 2 wk in artificial seawater at 12°C before use. Crabs were anesthetized in ice for 15 min prior to dissection. The dissection took place in chilled physiological saline comprised of (in mM) 440 NaCl, 26 MgCl₂, 13 CaCl₂, 11 KCl, and 10 mM HEPES (pH = 7.4). Details of the CG dissection can be found in Cruz-Bermudez and Marder (2007). All experiments were conducted on the three anterior LCs (see Fig. 1, A and C). To isolate LC somata from action potentials and network activity, individual strands of bulking nylon (made by Güttermann Creative) were used to ligate the nerve at the anterior branch point of the CG (Fig. 1). To impale LCs, each cell was desheathed with a desheathing pin. Anterior LCs on the same branch were isolated from the rest of the network in a pair.

**Electrophysiology.** All experiments were done in physiological saline cooled to 12°C. Two-electrode voltage clamp (TEVC) and two-electrode current clamp (TECC) protocols were carried out by impaling a LC with two glass electrodes filled with 3 M KCl (8- to 17-MΩ resistance) with an Axoclamp 2A amplifier (Axon Instruments, Union City, CA). In TEVC experiments when all potassium currents needed to be blocked, the current-injecting electrode was filled with 1 M tetraethylammonium (TEA) + 1 M cesium chloride (CsCl) (17- to 18-MΩ resistance). All recordings were made from LC somata; action potential conductances were blocked (unless otherwise noted) by tightening a thread ligature past the anterior branch point on the nerve in which the LC was located (Fig. 1A). TEVC and TECC protocols were created, driven, and recorded with Clampex 9.2 software (Molecular Devices). Current and voltage recordings were analyzed with Clampfit 9.2 software (Molecular Devices). Current and voltage traces were filtered with a digital low-pass boxcar filter using seven smoothing points at a sample frequency of 5 kHz.

**Pharmacology.** Pharmacological blockers were dissolved in physiological saline and perfused onto the CG with a Rabbit peristaltic...
pump (Raimin Instrument) at a rate of 1.5 ml/min or added from a concentrated stock solution via pipette. Prior to TEVC or TECC protocols, perfusion was stopped for at least 5 min to let the preparation temperature stabilize.

**Voltage-clamp protocols.** To voltage clamp inward currents, potassium currents were blocked by perfusion of 50 mM TEA and 1 mM 4-aminopyridine (4-AP) and injection of 1 M TEA + 1 M CsCl by iontophoresis (300-ms, 1.2-nA pulses at 2 Hz) for a minimum of 1 h. To isolate calcium currents, 10^-6 M TTX also was added for a minimum of 45 min. When necessary, calcium currents were blocked with 250 μM CdCl2 perfusion for a minimum of 45 min. Because of difficulty in fully blocking outward currents, we often carried out trace subtractions before and after CdCl2 (45 min) and before and after TTX (45 min) to isolate Cb-sensitive currents and TTX-sensitive currents, respectively.

To separate inactivating and noninactivating high-voltage-activated calcium currents, two TEVC protocols were carried out: one from a holding potential of −40 mV with 680-ms depolarizing steps from −50 mV to +15 mV in 5-mV increments (holding potential = −40 mV) and a second protocol identical except from a holding potential of −80 mV. Trace subtractions were used to isolate the additional inward current clamped with the −80-mV holding potential; this current is labeled I_CaS, while the inward current clamped from +40-mV holding potential is labeled I_CaT (Liu et al. 1998; Prinz et al. 2003). I_CaT inactivation was measured in TEVC with a 800-ms prestep (−80 mV to −40 mV, 5-mV increments) prior to a 500-ms, −20-mV depolarizing step. To remove I_CaT, the current generated with the −40-mV prestep was subtracted from each sweep. I_CaS inactivation was measured in TEVC with 2-s presteps (−50 mV to +5 mV, 5-mV increments) followed by a 500-ms, 0-mV depolarizing step.

Inward tail current was isolated with the same pharmacological agents as voltage-gated calcium currents, and its magnitude was measured at −80-mV holding potential after 680-ms depolarizing presteps between −50 and +15 mV. To isolate I_CaT contribution to tail current, a −40-mV holding potential was used prior to the depolarizing voltage step; in this protocol the tail current was still measured at −80 mV (see Fig. 5B). Reversal of I_CaS was measured after a 1-s, +20-mV depolarizing voltage step. Magnitude of I_CaT was always measured at peak tail current after a depolarizing voltage step.

TTX-sensitive current was isolated with the same pharmacological agents used to isolate calcium currents, and its magnitude was measured at −80-mV holding potential after 680-ms depolarizing presteps between −50 and +15 mV. To isolate I_CaS contribution to tail current, a −40-mV holding potential was used prior to the depolarizing voltage step; in this protocol the tail current was still measured at −80 mV (see Fig. 5B). Reversal of I_CaS was measured after a 1-s, +20-mV depolarizing voltage step. Magnitude of I_CaT was always measured at peak tail current after a depolarizing voltage step.

**Sequence of calcium channels.** We obtain de novo partial ORF sequence corresponding to three calcium channel subunit cDNAs to aid in characterizing the channels potentially underlying calcium currents in LC motoneurons. The methods for this degenerate PCR approach are as described in detail previously (Schulz et al. 2006, 2007; Tobin et al. 2009). Briefly, ganglia were desheathed around the area where LCs were visually identified, and to facilitate removal of the neurons the ganglia were exposed to protase (Sigma) for several minutes. The protease was replaced with a cold (−2°C) solution of 70% ethylene glycol and 30% saline, and the ganglia were put in a −20°C freezer for up to 1 h. The cells were then manually removed with fine forceps, and each cell was placed in a cryogenic tube with 350 ml of lysis buffer (buffer RLT; Qiagen) and 1% β-mercaptoethanol. The tubes were immediately placed on ice and then stored at −80°C until RNA extraction.

Quantitative RT-PCR was performed as in Schulz et al. (2006) and modified as in Tobin et al. (2009). Primers specific for real-time PCR detection of CbCaV1, CbCaV2, and CbCaV3 with SYBR Green were developed and designed with Primer3 software as follows: CbCaV1-F 5'-GATGAGGGAGGAGCAGCAAGA-3'; CbCaV1-R 5'-GGTTGTTTGAGGCCTGTCATT-3'; CbCaV2-F 5'-GTATCCGGCGGCACTAGTAAAG-3'; CbCaV2-R 5'-AAACTTGGGAGAAATGGCG-3'; CbCaV3-F 5'-CCAGTGTGATGTTGGTACTCG-3'; CbCaV3-R 5'-TGTAGGTGTTGGTTAGG-3'. PCR amplicon sizes were 90 bp (CbCaV1), 120 bp (CbCaV2), and 93 bp (CbCaV3). Primer sets were validated for PCR efficiency, linear dynamic range, and limit of detection with both a plasmid template that contained the gene of interest and cDNA generated from total RNA extracted from crab neural tissue. All qPCR reactions were carried out with a final primer concentration of 0.25 μM for each primer, using RT2 SYBR Green qPCR Mastermix (SABiosciences). RNA from single neurons was extracted with a RNAeasy mini kit (Qiagen) and reverse transcription with SuperScript III (Invitrogen). See Schulz et al. (2006, 2007) for more details. Assay quality and variability were monitored via 18S rRNA quantitation (as in Ransdell et al. 2010; Schulz et al. 2006, 2007; Tobin et al. 2009). Data in this study are presented as raw copy number per cell (see Tobin et al. 2009).

**Cell fills and immunohistochemistry.** To visualize stereotypical localization of LC somata and projections in the CG, we iontophoresed a fluorescent tracer (Alexa Fluor 568; Invitrogen) with backfilled microelectrodes by overriding the electrode capacitance compensation for 5 min. Dye was allowed to diffuse throughout the ganglia for 24 h in physiological saline at 4°C, and then preparations were directly imaged with a Leica M205F stereomicroscope and a Leica DFC 345 FX camera.

For immunohistochemistry, CGs with desheathed LCs were pinned to Sylgard and fixed in 0.1 M phosphate-buffered saline (PBS; in mM: 440 NaCl, 11 KCl, 10 Na2HPO4, 2 K2HPO4, pH 7.4) containing 2% paraformaldehyde for 40–90 min and then washed 2 × 10 min in PBS containing 0.1% Triton X (PBT). Rabbit panpolyclonal primary antibody to CaV1 subunit (Anti-CaVpanα1; Alomone Labs) was applied overnight at a concentration of 1:100 in PBT at 4°C. Ganglia were rinsed 4 × 10 min in PBT. CaV1-like immunoreactivity was investigated with 1:250 dilution goat anti-rabbit IgG conjugated to Alexa Fluor 568 dye in PBT (Molecular Probes/Invitrogen) for 12–20 h. Ganglia were rinsed again in PBS at least 5 × 15 min and mounted in ProLong Gold that contained DAPI (Invitrogen). Images were acquired with an Olympus IX81 epifluorescent microscope and MicroSuite software.

**Note on authorship.** The first author of a study is responsible for its conception and design, acquisition of data, analysis and interpretation of data, writing and critical revision of the manuscript, and final approval of the submitted version. The last author is responsible for overall project management, providing resources, supervision, and final approval of the published version.
RESULTS

Calcium channel expression in LCs. To determine which calcium current subtypes may be present in LC motoneurons, we decided first to adopt a molecular approach and look for expression of voltage-gated calcium channels at the mRNA level. The most thorough combined molecular and electrophysiological characterization of voltage-gated calcium channels in invertebrates has been performed for the snail *L. stagnalis* (Senatore and Spafford 2010; Spafford et al. 2003, 2006). Thus we used direct comparisons of our channels and currents to these well-characterized invertebrate examples as the basis for interpretation of our molecular calcium channel data. We obtained de novo sequence, or extended partial sequences, for three channel genes that appear to be orthologous to single representatives for each of three calcium channel gene families CaV1 (L type), CaV2 (P/Q type), and CaV3 (T type) found in most invertebrates (Jeziorski et al. 2000; Spafford and Zamponi 2003). We refer to each crab gene as CbCaV1, CbCaV2, and CbCaV3, Cb to denote *C. borealis* and 1, 2, or 3 for the gene family to which the sequence is most orthologous. We assigned gene identities to our channel transcripts by comparing the sequence of the calcium channel genes allowed us to develop primers to analyze single LC mRNA expression of each channel gene (Fig. 2B). LC motoneurons expressed detectable amounts of all three putative calcium channel subunits, with CbCaV1 having the lowest mRNA copy number while higher levels of both CbCaV2 and CbCaV3 were detected (Fig. 2B).

Across the respective stretches of these channel sequences between *C. borealis* and *L. stagnalis*, CbCaV1 sequence shares 581 of 801 identical amino acids with *L. stagnalis* (LCaV1; 73%), CbCaV2 (initially referred to as cacophony; see Tobin et al. 2009) shares 970 of 1,483 identical amino acids (65%) to LCaV2, and CbCaV3 shares 325 of 428 identical amino acids (76%) to LCaV3. The locations of these regions of overlap between *C. borealis* and *L. stagnalis* are shown in Fig. 2C with respect to putative transmembrane domains and IQ-like binding motifs of these channels (Van Petegem et al. 2005). These binding domains and their amino acid similarity to mammalian orthologs are shown in Fig. 2D. In addition, to confirm their identity as calcium channels we identified and aligned the EEEE and EEDD loci of the pore-forming loops with consensuss mammalian sequences. These EEEE/EEDD loci are known to confer divalent cation selectivity to these channels (Cens et al. 2007; Tang et al. 1993; Yang et al. 1993) and are a hallmark for the identification of voltage-gated calcium channels.

Calcium currents. To isolate voltage-gated calcium currents, we minimized competing sodium and potassium currents with pharmacological blockers (see MATERIALS AND METHODS). Total inward current under these conditions was measured (Fig. 3A). This revealed what appeared to be a faster transient inward current (ICaT; Fig. 3A), a slower, more persistent inward current (ICaS; Fig. 3A), a late outward current, and an inward tail current (Fig. 3A). Addition of CdCl2 (see Fig. 4F) completely eliminates the inward currents and reduces or eliminates the tail current, leaving behind only a small residual outward current that is presumably due to an incomplete block of ICaT and/or ICaA. Taken together, these data plus the gene expression results suggest the presence of multiple distinct calcium and calcium-dependent inward currents in the LC motoneurons.

We then attempted to characterize these distinct currents by a combination of pharmacology and voltage-clamp protocol manipulation. Low-threshold, T-type calcium currents in *L. stagnalis* (LCaV3) activate at hyperpolarized voltages around −60 mV, are transient, and are sensitive to block with nickel (Senatore and Spafford 2010). We were unable to elicit inward current in LC motoneuron somata for any voltages lower than −30 mV (see Fig. 3D), and application of NiCl2 (75–250 μM, n = 4) resulted in no change in inward current magnitude or kinetics (data not shown). Therefore, although we detected relatively abundant transcripts for CbCaV3 in LC motoneurons (Fig. 2B), we were unable to detect any active T-type current from somatic voltage-clamp experiments in these cells.

P/Q-type calcium currents (e.g., LCaV2) are more rapidly transient than L-type calcium currents (e.g., LCaV1) and activate at more hyperpolarized holding potentials (Hille 2001; Tsien et al. 1988). Therefore, we set out to separate these potentially distinct native high-threshold calcium currents by subtracting inward current clamped with a −40-mV holding potential from inward current with a −80-mV-holding potential in LC motoneurons. The more persistent calcium current (ICaS; Fig. 3B) activates at −30 mV and reaches its peak near 0 mV (Fig. 3D). The transient current (ICaT; Fig. 3C) activates at a voltage similar to ICaS (i.e., −30 mV) but reaches its peak at more hyperpolarized voltages near −20 mV (Fig. 3D).

Native current levels for ICaS and ICaT in individual LC motoneurons were variable from cell to cell and ranged from −3 to 6 nA for both currents (Fig. 3E). However, the most noticeable distinction between ICaS and ICaT in these cells is the voltage dependence of inactivation. ICaT shows an −30 mV more hyperpolarized half-inactivation voltage than ICaS (Fig. 3F). In addition, ICaS and ICaT are distinguished by differences in time constant of inactivation (Fig. 3G), and time to peak (Fig. 3H), as a function of voltage. There is a consistent relationship between time to peak current for ICaS and ICaT that would suggest these currents are able to maintain distinct impacts on cell voltage across a range of activation voltages (Fig. 3I). These characteristics are consistent with differences reported between high-voltage activated calcium currents LCaV1 and LCaV2 in *L. stagnalis* (Spafford et al. 2006) and similar to differences among L- and P/Q-type calcium currents in mammals (Hille 2001; Tsien et al. 1988). On the basis of these results, we propose that ICaS current in crab LC motoneurons is homologous to CaV1 current as described in *L. stagnalis* (Spafford et al. 2006) and reminiscent of mammalian L-type calcium currents (Hille 2001; Tsien et al. 1988), while ICaT is homologous to CaV2 as described in *L. stagnalis* (Spafford et al. 2003, 2006) and reminiscent of mammalian P/Q-type currents (Hille 2001; Jeziorski et al. 2000; Tsien et al. 1988).

To further confirm the characteristics of these calcium currents, as well as investigate any calcium-dependent aspects of their kinetics, we performed our voltage-clamp experiments with BaCl2 substituted for CaCl2 in the saline as well as with addition of CdCl2 to the saline. Both ICaS and ICaT are carried by Ba2+ and show activation characteristics similar to the
native calcium currents (Fig. 4, A and C). Furthermore, both $I_{\text{CaS}}$ and $I_{\text{CaT}}$ are eliminated by CdCl2 administration (Fig. 4F).

This further supports the hypothesis that these inward currents are indeed calcium mediated. We also noted that the slow inactivation of $I_{\text{CaS}}$ is eliminated or significantly reduced ($P < 0.01; n = 5$) in the corresponding $I_{\text{Ba}}$ (Fig. 4, B and E), suggesting that the inactivation of this current is not purely voltage dependent, but rather a calcium-dependent inactivation (CDI). This is also a characteristic consistent with mammalian and other invertebrate L-type calcium currents (Hille 2001).

We were able to identify in our CbCaV1 sequence an IQ-binding motif that has been shown to strongly influence CDI in mammalian-L-type channels (Zühlke et al. 2000). Site-directed mutagenesis shows that the IQ amino acid residues of this motif, as well as an FRK motif within the domain, are significant mediators of CDI, with the mutagenesis of K to A having the least impact in the motif (Zühlke et al. 2000). Our putative IQ domain shows this same pattern of amino acid residues (Fig. 2D), differing only in the K residue, which is consistent with the observation of CDI in our $I_{\text{CaS}}$ characterization.

Additionally, we sometimes detected a decrease in the magnitude of $I_{\text{CaT}}$ following barium substitution (Fig. 4D, E and F), which could be indicative of a calcium-dependent facilitation of the native transient calcium current in these cells (Tazaki and Cooke 1990). While these collective data are consistent with CDI of $I_{\text{CaS}}$ as suggested by barium substitution, it is possible
Fig. 3. Properties of voltage-gated calcium currents in LC motoneurons. A: raw trace of total current elicited at 0 mV from a holding potential of −80 mV in saline containing tetraethylammonium (TEA), 4-aminopyridine (4-AP), and tetrodotoxin (TTX). Four distinct aspects of the total current are labeled, including “transient” inward (I\text{CaT}), “slow” inactivating inward (I\text{CaS}), late outward (late current), and inward tail (tail current) current. The voltage trace used to elicit the current trace is shown above the current recording. B: typical recording of I\text{CaS} at 0 mV from a holding potential of −40 mV in saline containing TEA, 4-AP, TTX, and Cs+ loaded to reduce K+ currents to the minimum possible. The voltage trace used to elicit the current is shown above the current recording. C: representative recording of I\text{CaT} at −20 mV from a holding potential of −80 mV in saline containing TEA, 4-AP, TTX, and Cs+ loaded to reduce K+ currents to the minimum possible. The voltage trace used to elicit the current is shown above the current recording. D: mean (±SD) current-voltage (I-V) relationship for I\text{CaS} and I\text{CaT} indicating the voltage dependence of activation. E: mean (±SD) current levels for I\text{CaS} and I\text{CaT} across LC motoneurons. Individual points represent 1 cell. These data are from the same cells used to generate the I-V curves in D (n as shown). I\text{CaS} measurements were taken at 0 mV and I\text{CaT} at −20 mV. F: mean (±SD) I-V relationships for voltage dependence of inactivation of I\text{CaS} and I\text{CaT} as shown by normalized currents (I/I\text{max}) measured at 0 mV and −20 mV, respectively, from a varying holding potential plotted on the x-axis. G: mean (±SD) time constant of inactivation (\tau\text{inact}) of I\text{CaS} and I\text{CaT} as a function of voltage. H: mean (±SD) time to peak current of I\text{CaS} and I\text{CaT} as a function of voltage. I: correlation of time to peak current of I\text{CaS} and I\text{CaT} across voltages from −20 to +10 mV (data are from cells used in Fig. 2H). r Value is the result of a Pearson correlation test.
that barium influences other currents that may be incompletely blocked by our experimental protocol, altering the apparent inactivation kinetics of $I_{CaS}$.

Barium did not cause a change in inactivation kinetics of $I_{CaT}$ (Fig. 4, D and E), suggesting that this current does not show CDI. There is less known about the molecular basis of CDI and the IQ domain in P/Q-type currents. However, CDI in these channels has been linked to an interaction with an IQ-like binding domain (including IM amino acid residues; see Fig. 2D) with a calmodulin binding domain within CaV2 channels.
in mammals (Lee et al. 2003). While this interaction is not yet well characterized, it is noteworthy that our CbCaV2 IQ-like domain lacks the IM motif implicated in CDI, consistent with our lack of evidence for CDI for $I_{CaT}$.

Calcium-activated nonselective cationic current. We observed a native inward tail current (Fig. 3A) that is eliminated after CdCl$_2$ administration (Fig. 4F) and significantly decreased after BaCl$_2$ substitution (pre-Ba$^{2+}$ mean = $-5.7 \pm 0.5$ nA; post-Ba$^{2+}$ mean = $-2.7 \pm 1.0$ nA; $n = 3$; $P = 0.03$) (Fig. 4, B and C), suggesting the presence of a calcium-activated depolarizing conductance in these cells. The observed characteristics of this current are very similar to those of a calcium-activated nonselective cationic current ($I_{CAN}$) found in STG neurons of lobster and described in detail by Zhang et al. (1995). We performed subsequent voltage-clamp and pharmacological manipulations to determine whether the current we observe in LC motoneurons shares the same kinetics and properties of $I_{CAN}$ as described in lobster (Zhang et al. 1995).

To analyze $I_{CAN}$ in LC motoneurons, we bath applied TTX, TEA, and 4-AP and iontophoresed Cs$^+$ intracellularly as described in MATERIALS AND METHODS. We then elicited inward currents with increasing depolarizing voltage steps, followed by clamping tail currents at $-80$ mV. As seen in Fig. 3A as well as Fig. 5A (inset), these protocols resulted in initial inward current activation, usually a late outward current, and then an inward tail current measured at $-80$ mV (see Fig. 5A, inset).

The tail current activated at voltages above $-30$ mV (concomitant with $I_{CaT}$; see Fig. 3D), reaching a peak around $+15$ mV (Fig. 5A).

We next investigated whether the prepulse voltage and thus activation of distinct voltage-dependent calcium currents ($I_{CaS}$ and $I_{CaT}$) is correlated to tail current activation by clamping the LC to depolarized potentials that activate both currents (holding potential = $-80$ mV). We found that while using a holding potential of $-80$ mV (eliciting both $I_{CaS}$ and $I_{CaT}$) results in a larger overall tail current (Fig. 5B), holding at $-40$ mV (activating only $I_{CaS}$) still is sufficient to activate the tail current (Fig. 5B), but reduced in magnitude $\sim 35\%$. These results suggest that the tail current is activated by calcium influx via both $I_{CaS}$ and $I_{CaT}$.

To measure the reversal potential of the tail current, inward current was activated with a depolarizing step from a holding potential of $-40$ mV up to $+20$ mV and then the tail current clamped across a range of voltages from $-85$ mV to $-20$ mV (Fig. 5C). Through these experiments we obtained a current-voltage ($I$-$V$) curve based on measurements of the peak tail current, from which we extrapolated the reversal potential of the tail current to be approximately $-30$ mV (Fig. 5D).

Finally, we investigated whether the tail current is sensitive to increases in intracellular calcium release. Figure 5E demonstrates that caffeine exposure (bath applied for 10 min at 10 mM), known to stimulate intracellular calcium release (Friel......

Fig. 5. Properties of calcium-activated nonselective cationic current ($I_{CAN}$) in LC motoneurons. Recordings were made in saline containing TEA, 4-AP, and TTX and cells loaded with Cs$^+$. A: mean (±SD) $I$-$V$ relationships for activation of $I_{CAN}$ measured at $-80$ mV from a varying depolarizing pulse potential ($x$-axis) to elicit calcium currents (holding potential of $-80$ mV, $n = 5$). Inset: typical recordings of the total current, including tail current (black arrow) elicited from these voltage protocols. B: mean (±SD) $I$-$V$ relationships for activation of $I_{CAN}$ from holding potentials of $-40$ mV, activating predominantly $I_{CaS}$, or $-80$ mV, which activates both $I_{CaS}$ and $I_{CaT}$ ($n = 3$). Inset: representative tail currents in the same cell elicited from a holding potential of $-40$ mV (black trace) or $-80$ mV (gray trace). C: typical recordings used to measure the reversal potential of tail current. Current traces were obtained by depolarizing the LC neuron from a holding potential of $-50$ mV to $+20$ mV and then measuring tail currents at varying tail holding potentials from $-80$ mV to $-20$ mV (as shown in voltage traces). D: mean (±SD) $I$-$V$ relationships used to interpolate the reversal potential of these tail currents ($n = 8$). E: caffeine-evoked increase in tail current amplitude in LCs. Recording of tail currents from a holding potential of $-80$ mV before (black trace/arrow) and after (gray trace/arrow) bath application of 10 mM caffeine (this experiment was performed $n = 4$ times).
and Tsien 1992a, 1992b; Yoshimura 2005; Zhang and Harris-Warrick 1995) as well as an increased level of calcium-induced calcium release (Levi et al. 2003; Mironov and Usachev 1991), causes an increase in the inward tail current ($n = 4, P = 0.047$). These results suggest that the tail current is indeed calcium activated and not solely dependent on extracellular calcium influx via voltage-gated calcium currents. Taken together, these results are very similar to the characteristics of the $I_{\text{CAN}}$ in lobster, including sensitivity to caffeine exposure (Zhang and Harris-Warrick 1995), indicating that the same current is indeed present in LC motoneurons of the crab.

**TTX application reveals a largely noninactivating TTX-sensitive current in LC motoneurons.** During our experiments, we observed that in the presence of TEA and 4-AP CdCl$_2$ alone does not consistently prevent all voltage-activated inward current (Fig. 6A), but rather CdCl$_2$ and TTX combined are required to eliminate inward current (Fig. 6A). These observations suggest that there is a TTX-sensitive current, distinct from spike-mediated sodium currents, involved in bursting in these cells. We attempted to voltage clamp this current by subtracting the TTX-sensitive inward current in both step and ramp voltage-clamp protocols, with inconsistent results. Because of the small magnitude of the TTX-sensitive current (on the order of 2 nA or smaller, clamped from the soma), voltage-clamp protocols using step depolarizations often were not adequate to clearly characterize this current above background noise, though they did suggest that the current is largely noninactivating (Fig. 6B). Voltage ramp protocols used before and after TTX ($10^{-6} \text{M}, 30 \text{ min}$) to isolate the TTX-sensitive current during a $-80$ to $+20 \text{ mV}$ ramp demonstrated that TTX-sensitive current activation began at $-37.5 \text{ mV} \pm 4.7 \text{ mV}$ and peak activation occurred at $6 \text{ mV} \pm 3.2 \text{ mV}$ (Fig. 6B; $n = 3$).

Because we had mixed success voltage-clamping the TTX-sensitive current, and because of its often smaller magnitude, we set out to determine whether this current plays an active role in the burst dynamics of LC motoneurons. To investigate this, we induced exaggerated burst potentials in isolated LCs by adding 50 mM TEA to the saline and generating large burst potentials with depolarizing current pulses (Fig. 6C). These potentials are intrinsically driven because the depolarizing potential lasts longer than the brief current injection. After burst potentials were successfully generated, $10^{-6} \text{ M} \text{ TTX}$ was added, thus eliminating the TTX-sensitive inward current. In six of six experiments, TTX administration completely eliminated these burst potentials (Fig. 6C), supporting the presence of this current and suggesting an active role for this current in burst output. However, the exact impact of this TTX-sensitive current on burst dynamics in these cells is beyond the scope of this study. Nevertheless, it does appear that despite its small magnitude, this current is present and plays a role in the output of these cells.

**Hyperpolarization-activated mixed cationic current.** We also employed hyperpolarized voltage steps in voltage clamp, as well as negative current inject in current clamp, to investigate the presence of hyperpolarization-activated mixed cationic current ($I_{\text{H}}$) in the somata of these cells. Although relatively abundant mRNAs for the HCN channel have been detected in these cells (Tobin et al. 2009), we did not see any evidence for the H current in the soma of these cells (data not shown) for voltages as low as $-120 \text{ mV}$ from a holding potential of $-40 \text{ mV}$.

**Immunohistochemical localization of voltage-gated calcium channels in LC motoneurons.** The crustacean CG LC burst potential is mediated by intrinsic conductances at least in part isolated to the LC somata. In many crustacean species this intrinsic burst, termed a driver potential, can be regularly elicited in an isolated LC somata with a short depolarizing current injection (Cooke 2002; Tazaki and Cooke 1983). However, in *C. borealis* CG the isolated LC soma will rarely fire an

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**Fig. 6.** TTX-sensitive persistent current in LC motoneurons. **A:** $I$-$V$ relationship in a LC motoneuron indicating the presence of a TTX-sensitive current. Inward currents were measured from a holding potential of $-40 \text{ mV}$ at depolarizing voltage steps under subsequent blocker conditions. Currents first were elicited in TEA + 4-AP, and then Cd$^{2+}$ was added to block calcium currents, revealing a residual inward current that was subsequently blocked with TTX. **B:** Representative recording of TTX-sensitive current elicited in saline containing TEA, 4-AP, and Cd$^{2+}$ with a $-80 \text{ mV}$ to $+20 \text{ mV}$ voltage ramp; then subtracted from this current was the same voltage ramp with the addition of TTX (top; $n = 3$). Step depolarizations also reveal a persistent TTX sensitive inward current. Gray trace, inward current in the presence of CdCl$_2$; black trace, current after the addition of $10^{-6} \text{ M} \text{ TTX}$ (bottom; $n = 4$). **C:** TTX-sensitive current is necessary for burst potential generation in LC motoneurons. Exaggerated driver potentials are elicited in 50 mM TEA and a short depolarizing current injection ($5 \text{ nA}, 50 \text{ ms}$; top). These driver potentials are eliminated in the presence of TTX, even though calcium currents remain intact (bottom), $n = 6$.  

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intrinsic burst without the application of potassium current blockers such as TEA. While outward currents are clearly present (Ransdell et al. 2012), our voltage clamp showed relatively low calcium current levels in the LC somata. This could be due to the fact that the calcium channels are localized more distant to the soma or reflect a low overall calcium current in the soma itself. Therefore, we sought to find whether and to what degree calcium channels are localized to the LC somata. We used a panspecies primary antibody selective for high-voltage-activated calcium channels (i.e., L type and P/Q type) combined with fluorescence microscopy to investigate the distribution of CbCaV1/CbCaV2 channels in the CG. The 19-amino acid residue epitope against which the primary antibody was raised is a 100% amino acid identity match to CbCaV1 and matches 17 of 19 amino acids in CbCaV2 (Fig. 7; see also Fig. 1C). No similarity of the epitope to CbCaV3 or full-length LCaV3 was detected via BLASTP comparison.

LC somata and their projections are stereotypically distributed and clearly identifiable in this preparation (see Fig. 1C), making rudimentary subcellular localization fairly straightforward. We found clear punctate staining localized to the LC somata and to a lesser degree staining in the neurites extending from the LC somata (Fig. 7). In all, nine whole-mount CG preparations were stained, and in each preparation we were able to detect punctate staining localized to the LC somata. Additionally, we often detected staining in the processes of the anterior branch point of the ganglion (Fig. 7C), where synaptic connectivity seems to be most prevalent among the cells of the C. borealis CG. We performed additional DAPI staining within these preparations to determine whether the punctate immunoreactivity on the somata may be the result of glia or other support cells positively staining. Immunofluorescence + DAPI overlays showed no evidence of overlapping fluorescence to indicate this was the case (e.g., see Fig. 7D2). We also performed secondary antibody-only incubations that showed no significant fluorescence indicative of nonspecific signal attributable to the secondary antibody. Finally, preabsorption of the primary antibody with the antigen peptide eliminated immunolabeling as well.

**DISCUSSION**

The crustacean LC is a valuable model for studying motoneuron burst organization. The robust nature of the LC

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**Fig. 7.** High-voltage-activated calcium channels (HVACC) are localized to the somata of LC motoneurons. We used an Anti-CaV Pan-α1 primary antibody to detect putative HVACC immunoreactivity in the cardiac ganglion. The alignment of the antibody epitope with the corresponding amino acid sequence in CbCaV1 and CbCaV2 is shown at top. The entire cardiac ganglion is shown based on assembled tiled images taken at x4 magnification. Insets: magnified images of the corresponding portion of the ganglion marked with dashed boxes. We detected immunoreactivity for HVACC in the somata of all 5 LC motoneurons. A and B: HVACC immunoreactivity in anterior LCs appears to be localized to the somata, consistent with the ability to measure these currents in isolated LC somata. C: immunoreactivity was also detected in processes of neurons in the cardiac ganglion, particularly in the anterior branch point. D: the paired posterior LC neurons also showed immunoreactivity apparently localized to the somata (D1), which is not the result of glial or support cell staining as revealed by colocalization of immunoreactivity (red) and DAPI counterstain (blue) (D2). Scale bars in insets all represent 200 μm.
somata in electrophysiology experiments and segregation from action potential conductances as well as other network components make these cells ideal in experiments (Cooke 2002; Hartline 1967). However, a detailed understanding of the ionic conductances and their underlying ion channels, particularly depolarizing conductances, is lacking in these cells. Here we report the results of sequence from three voltage-gated calcium channel α-subunit genes in the crab *C. borealis* and then characterize their expression as well as the depolarizing currents present in LC motoneurons of the CG. While we detect mRNA for three different channel types in individual LCs, we measured and can distinguish only two distinct voltage-activated calcium currents in LC somata. We additionally identified a calcium-activated nonspecific cationic (CAN) current as well as a TTX-sensitive persistent inward current in these cells. Finally, we performed immunostaining that demonstrates that the high-voltage-activated calcium channels described are at least partially localized to the LC somata.

Sequence analysis reveals that the three calcium channel α-subunits in this study, namely, CbCaV1, CbCaV2, and CbCaV3, correspond very closely to identified calcium channel subunits of the snail *L. stagnalis* (LCaV1, LCaV2, and LCaV3, respectively). Thorough sequence and expression analyses of these snail channels have been performed and the resulting current properties and kinetics of each channel reported (Senatore and Spafford 2010; Spafford et al. 2003, 2006). On the basis of the extensive similarities between these expressed snail currents and the native crab currents characterized in our study, we are confident that we can use this analysis to infer the identity of these channels and currents. The characteristics of crab LC $I_{CaS}$, a slowly inactivating high-voltage-activated calcium current, are very similar to those of the current carried by the LCaV1 channel (Spafford et al. 2006), which has been classified as an invertebrate L-type calcium channel and current. Therefore, based on the similarities in sequence between CbCaV1 and LCaV1, and the similarities between crab $I_{CaS}$ and current carried by LCaV1, we suggest that CbCaV1 encodes an L-type calcium current with the properties described here as the slow calcium current $I_{CaS}$. Similarly, the characteristics of crab $I_{CaT}$, a transient inactivating high-voltage-activated calcium current, are very similar to those of the current carried by the LCaV2 channel (Spafford et al. 2003), which has been classified as an invertebrate calcium channel with biological characteristics found in mammalian P/Q- and N-type channels. On the basis of the similarities in sequence between CbCaV2 and LCaV2, and the similarities between crab $I_{CaT}$ and current carried by LCaV2, we suggest that CbCaV2 encodes a P/Q-type calcium current with the properties described here as the transient calcium current $I_{CaT}$.

Both of the two voltage-gated calcium currents ($I_{CaS}$ and $I_{CaT}$) were present in experiments where the LC soma was isolated, indicating that these channels are present in the LC somata. This result is supported by immunohistochemistry experiments in which high-voltage-activated calcium channel antibody was used to stain the entire CG, resulting in immunostaining largely localized to the LC soma, although some staining also could be seen on neuronal processes in different regions of the ganglia. These results are also consistent with previous immunolocalization of calcium channel subunits in the lobster STG; Ca1A (P/Q type) and Ca1D (L type) immunoreactivity both were localized to STG somata, as well as primary neurites (French et al. 2002).

Calcium currents previously have been studied in crustacean motoneurons, particularly in the STG. Total calcium current has been characterized in STG neurons of two species of lobster (Johnson et al. 2003; Turrigiano et al. 1995; Zhang and Harris-Warwick 1995) as well as crab (Golowasch and Marder 1992; Hurley and Graubard 1998). However, across these studies there are species- and cell-specific differences in the calcium current types reported. For example, Hurley and Graubard (1998) specifically searched for, but failed to find, two distinct calcium currents with differing kinetics in the somata of STG neurons of the crab *Cancer productus*. However, Turrigiano et al. (1995) measured calcium currents in isolated STG neurons of the lobster *Panulirus interruptus* in culture and noted both transient and persistent components to these currents, ultimately deciding to model calcium current in these cells as two separate components. Many of these discrepancies also may be explained by the fact that these inward currents have been, and continue to be, difficult to characterize in voltage clamp (Golowasch and Marder 1992; Turrigiano et al. 1995). Here, with the additional insight of molecular biology and a scaffolding of comparison provided by work in *L. stagnalis*, we believe we have made substantial progress toward a better understanding of these complex native currents in crustacean motoneurons.

Beyond the voltage-activated calcium currents, LCs also possess a calcium-activated inward current represented by $I_{CAN}$. The calcium-activated nonspecific cationic current is found in a variety of excitatory cell types (Partridge and Swandulla 1988) but is often implicated in neuronal bursting (Rubin et al. 2009; Swandulla and Lux 1985; Wilson et al. 1998). This current in invertebrates has been best characterized in crab STG (dorsal gastric) neurons. In these cells, the current is important in coordinating bistability of output; voltage-gated calcium currents allow calcium influx that activates $I_{CAN}$-inducing plateau potentials indicative of the output of these cells (Zhang and Harris-Warrick 1995; Zhang et al. 1995). LC motoneurons of the CG do not undergo plateau potentials during normal network output, so this is unlikely to be the role of this current in the cardiac output. While we do not yet know the role of $I_{CAN}$ in the cardiac motoneurons, a model for similar rhythm generation in mammalian pre-Bötzinger complex invokes $I_{CAN}$ as vital in triggering networkwide bursts after recurrent synaptic excitation—a role that may have parallels to $I_{CAN}$ in LCs (Del Negro and Hayes 2008).

Finally, we also report for the first time in crab LCs a largely noninactivating TTX-sensitive current that is necessary for driver potential generation in these cells, which could indicate the presence of a persistent sodium current ($I_{nap}$). $I_{nap}$ are often implicated in burst potential generation. In mammalian pre-Bötzinger neurons, $I_{nap}$ presence is known to be important in burst generation and its relationship with leak conductances is thought to dictate whether the neuron spontaneously bursts or requires excitatory input (Del Negro et al. 2002a, 2002b). There is evidence that DG neurons of the STG contain $I_{nap}$ important to plateau potential generation (Elson and Selverston 1997), and a TTX-sensitive persistent current has been voltage clamped in cultured STG cells (Turrigiano et al. 1995). While there is no channel gene known to directly mediate this current (indeed, there is only one voltage-gated sodium channel known in invertebrates; Loughney et al. 1989; Olson et al. 2008), there
is the possibility that the \( I_{\text{NaP}} \) is mediated by the same channel population as the fast-inactivating sodium current as seen in rat tuberomammillary neurons (Taddese and Bean 2002). However, because there are no spike-mediating sodium currents in these crustacean motoneuron somata (Golowasch and Marder 1992), it seems unlikely that these persistent sodium currents represent a window in fast-spiking sodium currents. Rather, it seems more likely that a distinct channel isomorph could mediate these currents, perhaps as a result of a splice variant (Lin et al. 2009) or RNA editing event (Liu et al. 2004) of the previously identified CbNaV channel (Dai et al. 2010).

The specific roles of the inward currents in these cells, especially those that are calcium mediated, are still to be experimentally determined. The crustacean cardiac motoneurons share extensive similarities with other excitatory central pattern generator circuits. For example, >90% of rat pre-Bötzinger complex neurons elicit burst potentials only when driven by excitation, and like LC motoneurons these neurons also express \( I_{\text{CAN}} \), \( I_{\text{NaP}} \), and intrinsic calcium currents (Del Negro et al. 2002b; Morgado-Valle et al. 2008; Pena et al. 2004). Whether the CG network LC burst potential is a result of summed excitatory postsynaptic potentials (EPSPs) or requires some or all of these intrinsic inward components to generate its intrinsic burst potential is currently under investigation (Ball et al. 2010, Franklin et al. 2010).

The depolarizing components found in the LC bursting neurons are prevalent across animal phyla, especially in those neurons that are also bursters. The experimental advantages of the crustacean CG and the simplicity of the network make it an ideal system to study how these components interact with each other and other network characteristics to shape and maintain output. In characterizing the inward components of *C. borealis* LC motoneuron, we obtain critical information to now move forward in investigating how the LC intrinsic components function within the rest of the cardiac network, and how this model can be applied broadly to understand neural network function in general.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: J.L.R. and D.J.S. conception and design of research; J.L.R., S.T., N.L.W., and M.L.L. performed experiments; J.L.R. and D.J.S. analyzed data; J.L.R. and D.J.S. interpreted results of experiments; J.L.R. and D.J.S. prepared figures; J.L.R. and D.J.S. drafted manuscript; J.L.R., S.T., and D.J.S. edited and revised manuscript; J.L.R., S.T., N.L.W., M.L.L., and D.J.S. approved final version of manuscript.

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