Characterization of Voltage-Dependent Ca\textsuperscript{2+} Currents in Identified Drosophila Motoneurons In Situ

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Worrell JW, Levine RB. Characterization of voltage-dependent Ca\textsuperscript{2+} currents in identified Drosophila motoneurons in situ. J Neurophysiol 100: 868–878, 2008. First published June 11, 2008; doi:10.1152/jn.90464.2008. Voltage-dependent Ca\textsuperscript{2+} channels contribute to neurotransmitter release, integration of synaptic information, and gene regulation within neurons. Thus understanding where diverse Ca\textsuperscript{2+} channels are expressed is an important step toward understanding neuronal function within a network. Drosophila provides a useful model for exploring the function of voltage-dependent Ca\textsuperscript{2+} channels in an intact system, but Ca\textsuperscript{2+} currents within the central processes of Drosophila neurons in situ have not been well described. The aim of this study was to characterize voltage-dependent Ca\textsuperscript{2+} currents in situ from identified larval motoneurons. Whole cell recordings from the somata of identified motoneurons revealed a significant influence of extracellular Ca\textsuperscript{2+} on spike shape and firing rate. Using whole cell voltage clamp, along with blockers of Na\textsuperscript{+} and K\textsuperscript{+} channels, a Ca\textsuperscript{2+}-dependent inward current was isolated. The Drosophila genome contains three genes with homology to vertebrate voltage-dependent Ca\textsuperscript{2+} channels: Dmca1A, Dmca1D, and Dmax1G. We used mutants of Dmca1A and Dmca1D as well as targeted expression of an RNAi transgene to Dmca1D to determine the genes responsible for the voltage-dependent Ca\textsuperscript{2+} current recorded from two identified motoneurons. Our results implicate Dmca1D as the major contributor to the voltage-dependent Ca\textsuperscript{2+} current recorded from the somatodendritic processes of motoneurons, whereas Dmca1A has previously been localized to the presynaptic terminal where it is essential for neurotransmitter release. Altered firing properties in cells from both Dmca1D and Dmca1A mutants indicate a role for both genes in shaping firing properties.

\textbf{Introduction}

Voltage-dependent Ca\textsuperscript{2+} currents have a wide range of influence on neuronal function. In addition to their requirement in presynaptic terminals for neurotransmitter release (Kawasaki et al. 2004), voltage-dependent Ca\textsuperscript{2+} channels segregate to somatodendritic locations where they associate cellular activity to localized Ca\textsuperscript{2+} influx (Christie et al. 1995; Magee and Johnston 1995). Ca\textsuperscript{2+} channels in the somatodendritic processes of motoneurons may amplify postsynaptic current (Schwindt and Crill 1980; Heckman and Lee 1999; Hyngstrom et al. 2008; Johnson et al. 2003; Lee and Heckman 2000; Seamans et al. 1997; Simon et al. 2003) or regulate action potential firing frequency through Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (McManus 1991; Vergara et al. 1998). On a somewhat longer time scale, voltage-dependent Ca\textsuperscript{2+} influx may drive activity-dependent gene regulation (Catterall 2000; Hardingham et al. 1997), mediating, for example, adjustments in intrinsic excitability (Peng and Wu 2007). Thus determining the cellular mechanisms required for the appropriate localization of voltage-gated Ca\textsuperscript{2+} channels and the integrative consequences of Ca\textsuperscript{2+} channel activation is a necessary step in understanding how the activity of neural circuits is maintained at the level of the single cell.

Techniques available in Drosophila, such as cell-specific genetic manipulation and the ability to record in situ from identified neurons, make it an ideal system to study the influence of voltage-gated Ca\textsuperscript{2+} influx on neuronal function within an intact system. Thus the role of a particular channel type in determining the activity pattern of an identified neuron can be addressed rigorously through targeted genetic manipulation. Unfortunately, voltage-gated Ca\textsuperscript{2+} currents have not been well defined in Drosophila neurons in vivo. Our goal in the present study, therefore, was to verify that the somatodendritic processes of Drosophila motoneurons include these currents and to take advantage of genetic approaches to determine the genes responsible.

The Drosophila genome contains three genes with known homology to voltage-gated Ca\textsuperscript{2+} channel \( \alpha \) subunits in vertebrates; Dmca1A, Dmca1D, and Dmax1G (King 2007; Littleton and Ganetzky 2000; Smith et al. 1996; Zheng et al. 1995). Dmca1A, also known as cacophony (cac), shares sequence homology with vertebrate N, P, and Q-type channels and is expressed at the Drosophila neuromuscular junction where it contributes to the Ca\textsuperscript{2+} influx responsible for synaptic release (Kawasaki et al. 2000, 2002, 2004), synaptic growth (Rieckhof et al. 2003), and regulation of the neuromuscular junction (Xing et al. 2005). Additionally, in the Drosophila giant neuron culture system, derived from cytokinesis-arrested embryonic neuroblasts, cac contributes the major Ca\textsuperscript{2+} current and plays a role in the homeostatic regulation of the A-type K\textsuperscript{+} current (Peng and Wu 2007). While the role of cac has been well described at the neuromuscular junction, as well as in cell culture, the contribution of cac to voltage-dependent Ca\textsuperscript{2+} currents in the central processes of neurons in situ has not been determined. Dmca1D shares homology with vertebrate L-type channels (Zheng et al. 1995) and is responsible for the major dihydropyridine-sensitive current recorded from Drosophila larval muscle fibers (Ren et al. 1998). Whether Dmca1D plays a role in the CNS is not known. Dmca1A and Dmca1D appear to play nonredundant roles in Drosophila as null alleles of both genes are independently embryonic lethal (Eberl et al. 1998; Smith et al. 1996). Dmax1G shares homology with vertebrate...
LVA T-type channel. While the function of this gene has not been characterized in *Drosophila*, a current with steady-state inactivation at membrane potentials of $-30$ mV has been identified in embryonic motoneurons aCC and RP-2 (Baines and Bate 1998) as well as in larval body wall muscle (Gielow et al. 1995; Ren et al. 1998). These currents are sensitive to amiloride, a known blocker of vertebrate T-type currents.

We used in situ whole cell patch-clamp techniques to record voltage-dependent $\text{Ca}^{2+}$ currents from identified motoneurons aCC and RP-2 in third instar *Drosophila* larvae. Motoneurons aCC and RP-2 were chosen based on their accessibility for recording and known influence on muscle function. We further recorded isolated voltage-dependent $\text{Ca}^{2+}$ currents from larvae carrying mutant alleles of *Dmca1A* and *Dmca1D*. In both aCC and RP-2, *Dmca1D* carried the major component of the voltage-dependent $\text{Ca}^{2+}$ current recorded from the cell body. To support these findings, we drove the expression of *Dmca1D* RNAi specifically in aCC and RP-2 and found a significant reduction in somatically recorded voltage-dependent $\text{Ca}^{2+}$ current. Whereas *Dmca1D* contributed the major voltage-sensitive current recorded at the cell body, mutations of both *Dmca1A* and *Dmca1D*, as well as RNAi knock-down of *Dmca1D*, had an influence on the firing properties of aCC and RP-2.

**METHODS**

*Drosophila* stocks

Wild-type strains used were Canton-s and w$^{1118}$. The GAL4 line, RRA, in which a transgene containing a region of the *even skipped* (eve) promoter drives GAL4 expression in specific neurons (Fujiooka et al. 2003), was used to drive expression of green fluorescent protein (GFP) and other transgenes in aCC and RP-2 motoneurons in each thoracic and abdominal hemisegment. A recombination was made that included RRA-GAL4 and UAS-GFP on the same chromosome [w$^{+}$; Sco/SM6a: RRA-Gal4, UAS-mCD8-GFP]. The two motoneurons were identifiable based on dendritic morphology and target innervation (Choi et al. 2004; Hoang and Chiba 2001) (Fig. 1). Due to a decrease in the level of *eve* expression by the end of third instar (Fujiooka et al. 2003), there was a mosaic pattern of GFP labeling, such that both cells were not always visible in each hemisegment. The homozygous viable allele of *Dmca1D*, AR66 (Eberl et al. 1998) (obtained from Dr. D. Eberl, University of Iowa, Iowa City, IA), contains a point mutation causing a hypomorphic phenotype (Ren et al. 1998). To obtain the AR66$^{w^{1118}}$ line, AR66 was crossed into a white background with a GFP-tagged balancer [w/w; L(2) 35fa/Cyo; Act:GFP; +/+ ] so that the wild-type strain w$^{1118}$ could be used as a control to examine the contribution of *Dmca1D* to somatically recorded $\text{Ca}^{2+}$ current. Additionally, AR66$^{w^{1118}}$ allowed for the selection of homozygous mutants by reversion to GFP in the gut of heterozygous animals. *Dmca1A* [cacophony (cac)] temperature-sensitive alleles cac$c^{at2}$, cac$c^{at3}$, and cac$c^{at5}$ (Kawasaki et al. 2000; Rieckof et al. 2003) and a hypomorphic, hemizygous viable allele, cac$c^{+}$ (Smith et al. 1996) (obtained from Dr. C. F. Wu, University of Iowa, Iowa City, IA) were used to determine the influence of *Dmca1A* on somatically recorded $\text{Ca}^{2+}$ current. The UAS-RNAi-S1491 line (RNAi91), targeted to *Dmca1D*, was obtained from the Vienna *Drosophila* RNAi Center (VDRC). Note that it was important to use appropriate control lines for each genotype because current densities varied among genotypes.

For imaging purposes, crosses were performed to create homozygous AR66 [w$^{1118}$; l(2) 35fa/l(2) 35fa; RRA-GAL4, UAS-mCD8-GFP/+ ] and hemizygous male cac$c^{+}$ (cac$c^{+}$; +/+ ; RRA-GAL4, UAS-mCD8-GFP/+ ) animals carrying one copy of the RRA-GAL4 driver and UAS-GFP. Additionally, RNAi91 was crossed to RRA-GAL4/W; AR66; RRA/+; +/+ animals that was homozygous for the mutation [w$^{1118}$; l(2) 35fa/l(2) 35fa; RRA-GAL4, UAS-mCD8-GFP/+ ] Bottom right: an animal that expressed 1 copy of the RNAi91 transgene (+/+ ; +/+ ; RRA-GAL4, UAS-mCD8-GFP/+ ). Bottom left: a AR66 animal that was homozygous for the mutation [w$^{1118}$; l(2) 35fa/l(2) 35fa; RRA-GAL4, UAS-mCD8-GFP/+ ] Bottom right: an animal that expressed 1 copy of the RNAi91 transgene (+/+ ; +/+ ; RRA-GAL4, UAS-mCD8-GFP/UAS-RNAi91). In late 3rd instar, RRA-GAL4 is no longer expressed at high levels, leading to a fortuitous mosaic pattern of GFP-expressing cells. This minimized overlap among cells and allowed individual cell structure to be more readily resolved (see arrows indicating examples of aCC and RP-2). Cell body size, the characteristic locations of dendritic branches, and axonal projections were similar between control and experimental animals, although we did not perform a quantitative morphometric analysis for this study. Images shown are projections of the entire dorsal/ventral confocal series through the thoracic and abdominal neuromeres of the larval CNS. Anterior is to the top.
Preparation

All experiments were performed on wandering late third instar Drosophila larvae. Larvae were placed on ice for ~2–3 h prior to dissection. Larvae were pinned dorsal side up and bathed in Ca\(^{2+}\)-free A solution (which contained, in mM, 118 NaCl, 2 NaOH, 2 KCl, 4 MgCl\(_2\), 25 sucrose, 5 trehalose, and 5 HEPES, pH 7.1–7.2, 295 mosM) (Jan and Jan 1976). The CNS, segmental nerves, and body wall muscles were left intact. The preparation was visualized using an upright fixed stage Olympus microscope. To access motoneurons in the ventral nerve cord, Protease 14 (2 mg/ml extracellular solution-Sigma-Aldrich, St. Louis, MO) was focally applied to the ganglionic sheath by applying positive pressure to a recording electrode with the tip broken to a diameter of ~10 μM (adapted from Choi et al. 2004). Treatment of the sheath was performed with constant laminar superfusion, and the debris was removed by applying negative pressure to the electrode. In experiments requiring temperature regulation of the bathing solution, a Warner TC-324B in-line bath heater was added to the perfusion system. Motoneurons RP-2 and aCC in thoracic and anterior abdominal segments were targeted in all experiments.

Electrophysiology

Motoneurons aCC and RP-2 were identified though the expression of GFP under the control of RRA-GAL4 or, in wild-type or mutant strains that did not carry the driver and the UAS-GFP transgene, through intracellular dye fills using Rhodamine Dextran 3000 added to the intracellular recording solution. Recordings were obtained from abdominal segments and from thoracic segments T\(_3\) and T\(_5\). The extracellular recording solution contained (in mM) 118 NaCl, 2 NaOH, 2 KCl, 4 MgCl\(_2\), 1.8 CaCl\(_2\), 25 sucrose, 5 trehalose, and 5 HEPES (Jan and Jan 1976). The pH was adjusted to 7.1–7.2 and the osmolarity to 295 mmol/kg. Because the CNS was partially desheathed in our preparations, it was deemed appropriate to approximate intraganglionic Ca\(^{2+}\) concentrations as opposed to higher Ca\(^{2+}\) levels that are used in salines that are designed to mimic Ca\(^{2+}\) concentrations in the hemolymph. To facilitate measurements through voltage-dependent Ca\(^{2+}\) channels and to reduce Ca\(^{2+}\) activated K\(^+\) currents, 1.8 mM Ba\(^{2+}\) was exchanged for 1.8 mM Ca\(^{2+}\) in select experiments. The extracellular solution contained (in mM) 114 KCl, 1 MgCl\(_2\), 0.5 CaCl\(_2\), 5 EGTA, and 10 HEPES (Peng and Wu 2007). The pH was adjusted to 7.1–7.2 and the osmolarity to 290 mmol/kg. To isolate voltage-dependent Ca\(^{2+}\) currents, KCl was replaced with CsCl in the intracellular recording solution and 1 μM tetrodotoxin (TTX), 50 mM TEA, and 1.5 mM 4-aminopyridine (4-AP) were added to the recording solution (all ion channel blockers were purchased from Sigma-Aldrich). A small outward current remained under these conditions. Thin-walled borosilicate electrodes were pulled on a PP-83 (Narishige) to a resistance of 2.5–5 MΩ and was not well-corrected. The largest currents injected in current-clamp experiments (100 pA) would therefore have caused a voltage error of ~2.8 mV. The largest Ca\(^{2+}\)/Ba\(^{2+}\) currents measured in voltage-clamp experiments (~350 pA) would have caused a series resistance error of ~10 mV. In most cells, the maximal current was less (see Figs. 3–6).

Current amplitude was normalized to whole cell capacitance, and values were reported as current density in voltage versus current experiments. Whole cell capacitance was determined from the charging transient following a 20-mV hyperpolarizing voltage command and used to calculate current density.

In most cells (66%; 44/67 cells) from all genotypes examined except for the cac\(^{2+}\) mutants (see following text), the current amplitude increased gradually with increased depolarizing voltage command steps, indicating good voltage control. However, two observations suggested that the space clamp or voltage control was not adequate in some cells, which were not used for voltage versus current plots. In 4/67 cells, a current with delayed onset was evoked with moderate depolarization, suggesting that it arose from a poorly clamped region, whereas with larger depolarizing commands in the same cell the onset of current was immediate. In 5/67 cells, rather than having a gradual increase in current with increasing depolarizing command steps, the first current observed was the maximal current, suggesting inadequate control of the membrane potential. In 4/67 cells, both problems were observed. Both of these problems were observed more frequently in the cac\(^{2+}\) mutants. In these experiments, perhaps because of the temperature shift protocol, 10/13 cells had evidence of imperfect voltage control, as reflected in the voltage versus current plot of Fig. 4C.

Pharmacology

Nifedipine (Sigma) was dissolved in 95% ETOH and added to the extracellular solution for a final concentration of 10 μM (final concentration of ETOH was <0.01%). PLTX-II (Alomone Labs) was added to the extracellular solution for a final concentration of 40–300 nM. Bay K 8644 (Alomone Labs) was used at a concentration of 1 μM.

Statistical analysis

Statistical analyses were performed using a standard t-test with Excel software (Microsoft). Standard error values are reported. Significance was assumed when \( P \leq 0.05 \).

RESULTS

External Ca\(^{2+}\) influences the firing properties of identified motoneurons aCC and RP-2

Our first goal was to determine whether extracellular Ca\(^{2+}\) plays a role in shaping the firing properties of two identified Drosophila larval motoneurons; MN1-1b (aCC) and MN1SN-Is (RP-2) (Fig. 1). These cells were chosen based on their accessibility to recordings and because they have been well characterized in previous experiments (Baines and Bate 1998; Choi et al. 2004; Rohrbough and Broadie 2002). Whole cell current-clamp experiments were performed in situ on motoneurons aCC and RP-2.

In extracellular solution containing 1.8 mM Ca\(^{2+}\), the resting membrane potentials of aCC and RP-2 were ~58 ± 1.38 and ~66 ± 1.71 mV, respectively, and the input resistances were 699 ± 85.4 and 930 ± 91.0 MΩ, respectively (RRA-GAL4 line: aCC n = 10, RP-2 n = 9). Removing Ca\(^{2+}\) from the extracellular recording solution caused a reduction in the AHP between spikes and a decrease in the interspike interval.
Motoneurons aCC and RP-2 display voltage-dependent Ca\(^{2+}\) currents in late 3rd instar larvae

Whole cell voltage-clamp experiments were performed in situ to identify voltage-dependent Ca\(^{2+}\) currents within the central processes of aCC and RP-2 motoneurons in third instar larvae. Ca\(^{2+}\) currents were isolated by the addition of 1 \(\mu\)M TTX, 50 mM TEA, and 1.5 mM 4-AP to the extracellular recording solution and by replacing intracellular K\(^+\) with Cs\(^+\).

With 1.8 mM Ca\(^{2+}\) in the extracellular recording solution, a depolarizing voltage command to −10 mV, from a holding potential of −70 mV, elicited an inward current, which underwent rapid reduction over the initial 20 ms of a 200-ms voltage command (Fig. 3A, i and ii). In a subset of cells, no inward current remained after 20 ms (Fig. 3Ai). In another subset, however, a reduced inward current persisted throughout the 200-ms voltage command (Fig. 3Aii).

Replacing 1.8 mM Ca\(^{2+}\) with 1.8 mM Ba\(^{2+}\), known to have a higher conductance than Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels (Byerly and Leung 1988), caused a substantial increase in current amplitude and reduced current decrement (Fig. 3A, i and ii). The latter may reflect Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels. The addition of 500 \(\mu\)M Cd\(^{2+}\), a general blocker of voltage-gated Ca\(^{2+}\) currents, completely eliminated all inward current (Fig. 3Ai). The voltage-dependent Ca\(^{2+}\) current first became prominent at membrane potentials between −40 and −30 mV in both aCC and RP-2, and peak currents were elicited at approximately −10 mV (Fig. 3D).

Motoneuron aCC displayed a significantly larger current density than RP-2 (Fig. 3, C and D). RP-2 displayed a larger variability in peak current amplitude than aCC, but the variability was reduced when currents were normalized to cell size (Fig. 3C). Similar differences in current density between aCC and RP-2 were observed in all wild-type strains including w\(^{1118}\) and Canton-S and the RRA-GAL4 line. There was relatively little steady state inactivation of the Ba\(^{2+}\) current. In both aCC and RP-2, long depolarizing prepulses (1 s) caused partial reduction in current during a test pulse to −10 mV (Fig. 3E).

To determine whether the voltage-dependent Ca\(^{2+}\) currents recorded somatically from aCC and RP-2 were sensitive to...
FIG. 3. Larval aCC and RP-2 motoneurons express voltage-dependent Ca\(^{2+}\) currents. A): recordings were generated in situ from 3rd instar larvae using a whole cell voltage-clamp protocol. Voltage-dependent Ca\(^{2+}\) currents were isolated by the addition of TTX, TEA, and 4-aminopyridine (4-AP) to the recording solution. Furthermore, Cs\(^{+}\) replaced K\(^{+}\) in the patch pipette. With 1.8 mM Ca\(^{2+}\) as the charge carrier, an inward current underwent rapid rundown during depolarizing voltage commands. Replacing Ca\(^{2+}\) with 1.8 mM Ba\(^{2+}\) prevented the rapid rundown in current and increased the current amplitude. Addition of 500 \(\mu M\) Cd\(^{2+}\), a general blocker of voltage-gated Ca\(^{2+}\) channels, completely eliminated inward currents. Aii: same as A) but records from a different cell. The time base has been expanded to better show the time course of current activation. B): representative Ba\(^{2+}\) current traces from aCC and RP-2 after blockade of Na\(^{+}\) and K\(^{+}\) currents. C): the range of peak current densities in RP-2 and aCC. RP-2 displayed smaller Ba\(^{2+}\) current densities than aCC \((w^{118}; aCC n = 5, RP-2 n = 7)\). D): voltage vs. current (V-I) plot. aCC and RP-2 displayed a significant difference in the peak current density \([w^{118}; P < 0.05 (\text{-10-mV command}); aCC n = 5, RP-2 n = 7]\). Similar results were obtained with the other control lines used in this study (RRA and Canton-S). E): steady-state inactivation. One-second prepulses ranging from -90 to -40 mV were administered in 10-mV increments followed by a 200-ms test pulse to -10 mV. Ba\(^{2+}\) was the charge carrier. \((w^{118}; aCC n = 4, RP-2 n = 5)\).

traditional pharmacological modifiers of voltage-dependent Ca\(^{2+}\) channels, we applied nifedipine, Plectreurys toxin-II (PLTX-II), and Bay K 8644 to the recording solution. Nifedipine has been shown to block the voltage-dependent Ca\(^{2+}\) current carried by Dmca1D in larval Drosophila muscle (Gielow et al. 1995; Ren et al. 1998) but did not block the Ca\(^{2+}\) current in aCC or RP-2, whether the holding potential was set at -70 or -40 mV. Similarly, Bay K 8644 enhances tail current amplitude of the Dmca1D-independent Ca\(^{2+}\) current in larval muscle (Gielow et al. 1995) but did not have an effect on currents recorded in aCC and RP-2. PLTX-II blocks synaptic transmission at the Drosophila neuromuscular junction where cac channels support synaptic release (Branton et al. 1987; Kawasaki et al. 2000, 2004), suggesting that cac is PLTX-II sensitive. PLTX-II did not reduce the voltage-dependent current recorded somatically in aCC and RP-2.

Mutant alleles of cacophony (Dmca1A) did not reduce the voltage-dependent Ba\(^{2+}\) current recorded in either aCC or RP-2.

To determine which genes contribute to the voltage-dependent Ca\(^{2+}\) current recorded from the cell bodies of aCC and RP-2, mutant alleles of Dmca1A (cac) and Dmca1D were examined. cac mutants have been well characterized at the larval neuromuscular junction and include temperature-sensitive alleles cac\(^{es2}\), cac\(^{es3}\), and cac\(^{es5}\) (Kawasaki et al. 2002; Rieckof et al. 2003) and a hypomorphic, hemizygous viable allele cac\(^{e}\) (Peng and Wu 2007; Smith et al. 1998). Whole cell voltage-clamp recordings were generated in situ from cac\(^{e}\) third instar larvae using 1.8 mM Ba\(^{2+}\) as the charge carrier. In hemizygous cac\(^{e}\) mutant males, the peak current density generated was not significantly different from those generated in Canton-S control (Fig. 4A). Furthermore, the kinetics and voltage sensitivity of the current were similar in both cac\(^{e}\) and control (Fig. 4B).

To confirm the results generated from the cac\(^{e}\) mutants, recordings were obtained from cac\(^{es2}\) temperature-sensitive mutants. At permissive temperatures (<26°C) cac\(^{es2}\) mutant channels conduct Ca\(^{2+}\)/Ba\(^{2+}\) current similar to wild-type channels; however, at restrictive temperatures (>32°C), cac\(^{es2}\) mutant channels no longer conduct Ca\(^{2+}\)/Ba\(^{2+}\) current (Kawasaki et al. 2002). In hemizygous male cac\(^{es2}\) larvae, we recorded voltage-dependent Ba\(^{2+}\) currents from the same aCC and RP-2 motoneurons at permissive and restrictive temperatures. Threshold for current activation, peak Ba\(^{2+}\) current density, and reversal potential were indistinguishable between record-
FIG. 4. The cacophony mutation does not significantly reduce voltage-dependent Ba\textsuperscript{2+} currents recorded from the cell body in situ. A: peak Ba\textsuperscript{2+} current densities generated in hypomorphic mutants (cac\textsuperscript{c}) were not significantly different from Canton-S control in either aCC or RP-2 [cac\textsuperscript{c}: RP-2 (n = 5), aCC (n = 5); Canton-S: RP-2 (n = 5), aCC (n = 6)]. Note that there was a nonsignificant reduction at \(-20\) mV for aCC. B: representative current traces from Canton-S (top) and cac\textsuperscript{c} (bottom) in response to a \(-10\) mV voltage command. (RP-2). C: peak currents generated at permissive (<26°C) and restrictive (>32°C) temperatures, in the same aCC motoneurons, were not significantly different in cac\textsuperscript{c} mutants [cac\textsuperscript{c}: <26°C (n = 3), >32°C (n = 3)]. Note the abrupt transition from zero to maximum current, indicative of inadequate voltage control in these temperature shift experiments. D: overlay of current traces generated from the same RP-2 motoneuron at permissive (<26) and restrictive (>32) temperatures in cac\textsuperscript{c} (voltage command to \(-10\) mV). To better show the similarity in time course, the capacitive artifacts have been subtracted off-line using the artifact evoked by a hyperpolarizing voltage command. Similar results were obtained with other temperature-sensitive alleles.

The contribution of Dmca1D to the voltage-dependent Ca\textsuperscript{2+} current in Drosophila neurons has not been determined. We used a homozygous viable mutant allele of Dmca1D, AR66, to determine the role played by Dmca1D in generating the somatic voltage-dependent Ca\textsuperscript{2+} current recorded in aCC and RP-2. AR66 is a hypomorphic mutation of Dmca1D that reduces the ability of the channel to carry current (Ren et al. 1998). Viability was reduced in AR66 homozygous mutants as evident by the reduced number of larva reaching the wandering third instar stage. Nevertheless, in third instar larvae that could crawl from the food, both aCC and RP-2 displayed healthy membrane potentials, were of normal size (as determined by whole cell capacitance), and maintained the ability to generate action potentials in response to current injection. Additionally, dye filling or GFP labeling of the cells did not indicate gross changes in cell morphology (Fig. 1), although we did not perform a quantitative analysis of dendritic branching complexity for this study.

Larvae homozygous for the AR66 mutation displayed a significant reduction in voltage-dependent Ba\textsuperscript{2+} or Ca\textsuperscript{2+} current density compared with wild-type control (w\textsuperscript{118b}) in both aCC and RP-2 (Fig. 5, A and B). Further, larvae heterozygous for the mutation displayed a current phenotype intermediate to that of the homozygote and wild-type (Fig. 5C), consistent with recordings generated from body wall muscle of AR66 mutants (Ren et al. 1998). The voltage sensitivity of steady-state inactivation was similar for AR66 mutants and wild-type larvae (Fig. 5D). To summarize, the AR66 mutation of Dmca1D significantly reduced the ability of aCC and RP-2 to generate a Ba\textsuperscript{2+} or Ca\textsuperscript{2+} current, implicating Dmca1D as a major contributor to the voltage-dependent Ca\textsuperscript{2+} current recorded from the central processes of aCC and RP-2 in situ.

RNAi knockdown of Dmca1D expression reduced the voltage-dependent Ba\textsuperscript{2+} current recorded at the cell body.

The GAL4-UAS system available in Drosophila allows for the expression of RNAi transgenes in a cell-specific manner. The expression of Dmca1D RNAi was driven in aCC and RP-2 motoneurons to determine if it would reduce the Ba\textsuperscript{2+}/Ca\textsuperscript{2+} current recorded somatically in these cells. Drosophila lines carrying UAS-RNAi-51491 (RNAi91), an RNAi to Dmca1D, were crossed to the RRA-GAL4 line, driving expression of RNAi91 in aCC and RP-2 motoneurons. The expression of RNAi91 in aCC and RP-2 did not affect the development or the
viability of the animals, and both aCC and RP-2 maintained normal resting membrane potentials. The overall morphology of these neurons within the CNS was normal (Fig. 1). In aCC and RP-2 motoneurons expressing RNAi91, isolated Ba\(^{2+}\)/Ca\(^{2+}\) currents were significantly reduced compared with control (RNAi91 not crossed to RRA-GAL4) (Fig. 6A). This result further implicates Dmca1D as the major contributor to voltage-dependent Ca\(^{2+}\) current recorded somatically in aCC and RP-2 motoneurons. The range for current activation was similar between RNAi91 expressing cells and control.

**Hypomorphic mutations of Dmca1D and Dmca1A, as well as RNAi91 expression, alter the firing properties of aCC and RP-2 in response to somatic current injection**

To determine whether Dmca1D and Dmca1A (cac) play a role in shaping the firing properties of aCC and RP-2, somatic current injections were administered to AR66 and cac\(^{c}\) hypomorphic mutants and to cells expressing the RNAi91 transgene. Action potential shape was variable in both hypomorphic mutants (Fig. 7). A, B, and D). The mean spike amplitude was reduced significantly in AR66 mutants as compared with motoneurons from the control line (AR66, 5.54 ± 1.3 mV; n = 5; w1118, 10.24 ± 1.2 mV; n = 5; P = 0.03). The AHP amplitude was not significantly different (AR66, 11.28 ± 1.9 mV; n = 5; w1118, 14.67 ± 1.7; n = 5; P = 0.22). Spike amplitude in the cac\(^{c}\) line was not significantly different from the Canton-S control line (cac\(^{c}\), 13.89 ± 1.6 mV; n = 4; Canton-s, 11.22 ± 1.1 mV; n = 4; P = 0.2). Similarly, the AHP amplitude was not significantly different (cac\(^{c}\), 12.43 ± 2.0 mV; n = 4; Canton-s, 13.73 ± 1.0 mV; n = 4; P = 0.59). Spike frequency was increased in both AR66 and cac\(^{c}\) mutants compared with their respective controls and there was a reduc-

**FIG. 5** The AR66 mutant allele of Dmca1D significantly reduces voltage-dependent Ba\(^{2+}\) current recorded from the cell body of aCC and RP-2. A: peak current density was significantly reduced in AR66 homozygotes in both aCC and RP-2 motoneurons compared with w1118 controls [AR66: RP-2 (n = 6), aCC (n = 7); w1118: RP-2 (n = 7), aCC (n = 5)]. *, regions of significant difference: P ≤ 0.05. B: representative current traces from AR66 and w1118 in aCC and RP-2. C: AR66 heterozygotes displayed an intermediate peak current amplitude relative to control and homozygotes on a −10 mV voltage command from a holding potential −70 mV (AR66 heterozygotes: aCC n = 2, RP-2 n = 3). D: steady-state inactivation did not differ in AR66 mutants. One-second prepulses to −90, −70, and −40 mV were administered to w1118 control and AR66. Prepulses were followed by a 200-ms test pulse to −10 mV (aCC: w1118 n = 4, AR66 n = 4).
tion in spike threshold in both mutants (Fig. 8). Unlike control lines (see Fig. 2), removing Ca\(^{2+}\) from the recording solution did not cause a significant increase in spike frequency in either AR66 or cac\(^{c}\) mutants (Fig. 8).

Motoneurons expressing RNAi91 also displayed altered action potential shapes (Fig. 7C). RNAi91 expressing motoneurons displayed spontaneous, spike-like, events that were not influenced by current injection and were clearly of a different shape from the spikes evoked by depolarizing current injected into the same cells (Fig. 7C). The spontaneous spike-like events did not increase in frequency or change amplitude with increased membrane potential depolarization (Figs. 7C and 8B). As current injection depolarized the membrane potential beyond −30 mV, however, the spontaneous events were replaced by evoked spikes with a shape and frequency resembling those of control motoneurons (Figs. 7C and 8B). Perhaps the spontaneous spike-like events are initiated at a more distant site that is not within the influence of current injected into the cell body and are blocked by evoked spikes traveling orthodromically.

There was a significant reduction in the AHP in the evoked spikes of RNAi91 expressing cells (RNAi91, 6.49 ± 0.4 mV; n = 6; control, 12.41 ± 1.6 mV; n = 5; P = 0.02), and a reduction, although not significant, in the amplitude of evoked spikes when compared with control (RNAi91, 3.42 ± 0.4 mV; n = 6; control, 6.71 ± 1.6 mV; n = 5; P = 0.1). The frequency of evoked spikes was not altered by RNAi91 expression (Fig. 8). In the absence of Ca\(^{2+}\) in the extracellular solution, motoneurons expressing RNAi91 displayed a high-frequency of spontaneous spike-like events (Fig. 7C), such that it was difficult to obtain frequency versus current relationships for the evoked spikes.

**Discussion**

Multiple genes encode the α1 subunit of voltage-dependent Ca\(^{2+}\) channels, and each may experience alternative splicing (Lipscombe et al. 2002) and posttranslational modifications. In addition, the voltage sensing and current conducting α1 subunit is usually found in association with auxiliary subunits (Dickman et al. 2008). These processes ensure that each cell is equipped with the assortment of voltage-dependent Ca\(^{2+}\) currents needed to function appropriately. The **Drosophila** genome contains three genes with homology to each class of voltage-dependent Ca\(^{2+}\) channel found in vertebrates (King 2007; Littleton and Ganetzky 2000; Smith et al. 1996; Zheng et al. 1995). While the **Drosophila** Ca\(^{2+}\) channel genes share sequence homology with their vertebrate counterparts, classification of currents by voltage threshold and pharmacology is not as clear-cut. In **Drosophila**, all three gene products generate whole cell currents that activate at membrane potentials approximating −40 mV (Gielow et al. 1995; Leung and Byerly 1991) and vertebrate Ca\(^{2+}\) channel blockers have varied effects (King 2007). Genetic tools available in **Drosophila** have been used to dissect voltage-dependent Ca\(^{2+}\) currents in larval muscle (Ren et al. 1998), at the neuromuscular junction (Kawasaki et al. 2000, 2002, 2004), and in the giant neuron culture system (Peng and Wu 2007).

To determine which of the genes encoding voltage-dependent Ca\(^{2+}\) channels was responsible for the current observed in
not completely eliminate Dmca1D protein, it was not possible to
determine whether the residual current was through
Dmca1D-encoded channels or through channels encoded by
Dmca1A or Dmca1G. The residual current in AR66 mutants was
relatively insensitive to voltage-dependent inactivation. This
suggests that the residual current may not reflect Dmca1G
expression, as this putative T-type current displays rapid volt-
dependent inactivation (Gielow et al. 1995; Ren et al.
1998). As mentioned in the preceding text, the pharmacologi-
cal tools used to classify vertebrate Ca\textsuperscript{2+} channels have varied
effects in Drosophila (King 2007). Nifedipine was ineffective
in our experiments even though it has been reported to block
the current carried by Dmca1D in larval Drosophila muscle
(Gielow et al. 1995; Ren et al. 1998). Different splice variants
of Dmca1D may be expressed in neurons and muscle (Zheng
et al. 1995). Alternatively, the alpha subunits may be associ-
ated with different auxiliary subunits or cytoskeletal proteins
in the different cell types.

Differential distribution of voltage-dependent Ca\textsuperscript{2+}
channels has been established in a variety of cell types including
vertebrate hippocampal neurons and motoneurons (Cristie
The results of the present study suggest that Dmca1A and Dmca1D
are differentially distributed in Drosophila motoneurons. Al-
though our recordings were from the cell body, Ca\textsuperscript{2+} channels
that reside in dendritic processes probably contributed to the
whole cell current. The presence of poorly clamped and de-
layed onset currents in some records (see METHODS) suggests
that the Ca\textsuperscript{2+} channels are located, at least in part, in dendritic
processes that are somewhat distal to the cell body (Heckman
et al. 2008; Johnson et al. 2003). Dendritic Ca\textsuperscript{2+} currents have
been revealed in other insect motoneurons using both voltage
clamp and Ca\textsuperscript{2+} imaging (Duch and Levine 2002). Additional
Ca\textsuperscript{2+} channels, such as those in presynaptic terminals, proba-
bly lie outside the range for adequate space clamp and did not
contribute to the voltage-dependent current we recorded from
the cell body. It is well established that cac is expressed in
motoneurons and localizes to the neuromuscular junction (Ka-
may serve as a favorable model for investigating the mecha-
nisms that determine the distribution of distinct Ca\textsuperscript{2+} channel
types.

In contrast to our findings from in situ recordings, Dmca1A
was found to be the major contributor to voltage-dependent
Ca\textsuperscript{2+} currents in the Drosophila giant neuron culture system
(Peng and Wu 2007). Three separate mutant alleles for
Dmca1A (cac\textsuperscript{a}, cac\textsuperscript{ts2}, and HC129) reduced Ca\textsuperscript{2+} current
recorded from the cell body of these cell-division-arrested
embryonic neuroblasts. This may reflect differences in Ca\textsuperscript{2+}
channel gene expression among cell types, although it is not
clear what neuronal types the giant neurons represent. Alter-
natively, there may be a shift in the contributions of different
channel types during embryonic and postembryonic develop-
ment, with cac providing the dominant contribution at embry-
onic stages. It is also likely that the contribution of Dmca1A to
the Ca\textsuperscript{2+} current in axon terminals is more readily measurable
from the cell body in the more compact cultured cells.

Whereas mutant alleles of cac did not significantly reduce
whole cell voltage-dependent Ca\textsuperscript{2+} currents in our experi-
ments, they did alter cellular firing properties. This may rep-
resent a role for distant cac-encoded Ca\textsuperscript{2+} channels in action.
potential initiation or shape, or a low-density population of this channel type within the dendritic membrane, that would not have been prominent in our voltage-clamp analysis or in images of fluorescently tagged channels (Kawasaki et al. 2004). The firing frequency was increased in cac motoneurons. This may reflect reduced activation of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. Alternatively, there may have been a compensatory developmental reduction in the expression of K\textsuperscript{+} channels in cac mutants (Peng and Wu 2007). It may be possible to test this possibility using cac\textsuperscript{ac} lines or by expressing an appropriate RNAi transgene in the motoneurons during a restricted temporal window (Nicholson et al. 2008).

The same hypotheses apply to the increased firing frequency that was observed in AR66 mutants and is consistent with the effect of removing external Ca\textsuperscript{2+} from the extracellular solution. Furthermore, the reduction in spike amplitude in AR66 mutants may reflect a contribution of somatic or dendritic Ca\textsuperscript{2+} channels in these regions. Although in most insect motoneurons the action potential is initiated where the axon leaves the dendritic region and is thought to invade the cell body passively (Gwilliam and Burrows 1980), somatodendritic Ca\textsuperscript{2+} channels can boost this depolarization (Duch and Levine 2002). The spontaneous spike-like events observed in motoneurons expressing RNAi91 may reflect spike initiation in a second, more distal, location. Initiation at this site may have been allowed by a reduction in K\textsuperscript{+} channel activation or expression.

An important next step will be to determine whether the Ca\textsuperscript{2+} current described in this study reflects the dendritic compartment of the motoneurons. Ca\textsuperscript{2+} imaging will help to resolve this question. Voltage-dependent Ca\textsuperscript{2+} currents localize to the dendrites of vertebrate motoneurons where they are proposed to increase the sensitivity to both excitatory and inhibitory inputs (Heckman 2003; Heckman et al. 2008; Hultborn et al. 2004; Hyngstrom et al. 2008; Lee and Heckman 2000). Furthermore, modulation of voltage-gated Ca\textsuperscript{2+} channels in dendrites may shape motoneuron recruitment patterns during locomotion (Heckman et al. 2008) or other types of patterned motor activity (Johnson et al. 2003). Whereas it is challenging to investigate the contribution of Ca\textsuperscript{2+} channels and their modulation to the activation patterns of specific neurons in the intact mammalian nervous system, these important mechanisms can be addressed readily in Drosophila. With the development of in situ whole cell recording techniques and the availability of cell-specific genetic manipulations that are possible in Drosophila, this system will allow rigorous examination of mechanisms contributing to motoneuron recruitment in diverse systems.

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