Identification of CaV channel types expressed in muscle afferent neurons

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Ramachandra R, Hassan B, McGrew SG, Dompor J, Farrag M, Ruiz-Velasco V, Elmslie KS. Identification of CaV channel types expressed in muscle afferent neurons. J Neurophysiol 110: 1535–1543, 2013. First published July 10, 2013; doi:10.1152/jn.00069.2013.—Cardiovascular adjustments to exercise are partially mediated by group II/IV (small to medium) muscle afferents comprising the exercise pressor reflex (EPR). However, this reflex can be inappropriately activated in disease states (e.g., peripheral vascular disease), leading to increased risk of myocardial infarction. Here we investigate the voltage-dependent calcium (CaV) channels expressed in small to medium muscle afferent neurons as a first step toward determining their potential role in controlling the EPR. Using specific blockers and 5 mM Ba2+ as the charge carrier, we found the major calcium channel types to be CaV2.2 (N-type) > CaV2.1 (P/Q-type) > CaV1.2 (L-type). Surprisingly, the CaV2.3 channel (R-type) blocker SNX482 was without effect. However, R-type currents are more prominent when recorded in Ca2+ (Liang and Elmslie 2001). We reexamined the channel types using 10 mM Ca2+ as the charge carrier, but results were similar to those in Ba2+. SNX482 was without effect even though ~27% of the current was blocker insensitive. Using multiple methods, we demonstrate that CaV2.3 channels are functionally expressed in muscle afferent neurons. Finally, ATP is an important modulator of the EPR, and we examined the effect on CaV currents. ATP reduced CaV current primarily via G protein βγ-mediated inhibition of CaV2.2 channels. We conclude that small to medium muscle afferent neurons primarily express CaV2.2 > CaV2.1 ≥ CaV2.3 > CaV1.2 channels. As with chronic pain, CaV2.2 channel blockers may be useful in controlling inappropriate activation of the EPR. CaV2.2; CaV2.1; CaV2.3; dorsal root ganglia neurons; exercise pressor reflex

THE GROUP I (Aα) and II (Aβ) muscle afferents provide sensory information needed to guide motor activity (Houk 1974). The group III (Aδ) and IV (C) afferents transmit muscle pain signals and also mediate the exercise pressor reflex (EPR) (Kaufman and Hayes 2002), which is a critical neural mechanism that regulates the cardiovascular response to exercise (Kaufman and Hayes 2002). This reflex is clinically important because certain diseases, such as peripheral vascular disease and heart failure, can produce muscle ischemia that drives EPR activity, which can generate additional cardiac stress to increase the risk of myocardial infarction (Baccelli et al. 1999; Bakke et al. 2007; Smith et al. 2006). Here we investigate the voltage-dependent calcium (CaV) channels expressed in muscle afferent neurons as an initial step in understanding the role of these channels in controlling excitability of neurons that mediate the EPR.

CaV channels play a prominent role in neuronal excitability (Khosravani and Zamponi 2006). At synaptic terminals, they deliver the Ca2+ needed to induce the release of excitatory and inhibitory neurotransmitters (Lisman et al. 2007). There are 10 genes that encode CaV channels (Catterall et al. 2005). In dorsal root ganglia (DRG) neurons, the evidence supports the expression of L-type (CaV1.2), P/Q-type (CaV2.1), N-type (CaV2.2), R-type (CaV2.3), also called E-type or α1E), and T-type (CaV3.2) (Vanegas and Schaible 2000; Zamponi et al. 2009). The importance of CaV2.2 channels in chronic pain has been highlighted by the success of the specific blocker ziconotide (SNX-111 or ω-conotoxin MVIIA) in the treatment of chronic pain in many patients (Elmslie 2004; Molinski et al. 2009). This intrathecally delivered drug blocks transmission from primary to secondary nociceptors by blocking presynaptic CaV2.2 channels (Motin and Adams 2008). While the effect of blocking presynaptic CaV channels is to reduce central nervous system excitability, blocking CaV channels can also increase neuronal excitability through reduced activation of closely associated Ca2+-activated potassium channels (Marrion and Tavalin 1998; Yu et al. 2010). Indeed, blocking CaV channels increases excitability of sensory neurons (Lirk et al. 2008). Thus it is important that we understand the CaV channels expressed in muscle afferent neurons, along with the modulation of these channels by molecular activators of the EPR. ATP is one of these activators, which works by acting on P2X receptors (Cui et al. 2011; Hayes et al. 2008). However, ATP can inhibit CaV channels by activation of G protein-coupled P2Y receptors (Filippov et al. 2003; Gerevich et al. 2004). Here we identify the CaV channels that are functionally expressed by muscle afferent neurons and show that ATP can inhibit the CaV current by reducing activity of CaV2.2 (N-type) channels. As with chronic pain, we show that CaV2.2 channels could be an important target for the treatment of symptoms resulting from excessive EPR activity.

MATERIALS AND METHODS

Isolation of muscle afferent neurons. Adult male Sprague-Dawley rats (weight 150—400 g) were obtained from Hill Top Laboratories (Scottsdale, PA). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and followed National Institutes of Health (NIH) guidelines. The labeling and neuronal isolation followed previously described procedures (Ramachandra et al. 2012). Briefly, muscle afferent neurons were labeled by retrograde transport of the lipophilic dye DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) [100 μl of 1.5% DiI in dimethyl sulfoxide (DMSO)] that was injected into both left and right gastrocnemius muscles of anesthetized rats (ketamine, xylazine, and acepromazine).
zine). Four to five days postinjection, the rats were killed with CO2 followed by decapitation. Neurons from the lumbar DRG L1 and L3 were isolated using an enzyme mixture of trypsin, collagenase, and DNase and plated onto polylysine-coated glass coverslips. The isolated neurons were maintained overnight at 37°C in a 5% CO2 incubator in minimal essential medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Expressed CaV channels. CaV2.3 channels (rL, β2A, and α2δ cDNA constructs, generous gifts from Dr. Henry L. Puhl, National Institutes of Health/National Institute on Alcohol Abuse and Alcoholism) were heterologously expressed in LN229 cells by intranuclear microinjection at a ratio of 1:1:2. The cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin. Following cDNA injection, the cells were incubated overnight, and electrophysiological recordings were performed the next day.

Solutions. Most DI-positive DRG neurons were initially recorded in an external solution containing (in mM) 45 NaCl, 100 N-methyl D-glucosamine (NMG)-Cl, 4 MnCl2, 10 Na-HEPES, 10 glucose, and 0.0003 tetrodotoxin (TTX), with pH = 7.4 and osmolality = 320 mosM, which was used to identify voltage-gated sodium (NaV) 1.8 expressing muscle afferent neurons (Ramachandra et al. 2012). When recording CaV current, the external solution was switched to a barium external solution medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Following cDNA injection, the cells were incubated over-night, and electrophysiological recordings were performed the next day.

RESULTS

We were interested in determining the CaV channels that are functionally expressed in muscle afferent neurons. Consistent with previous work, we used pharmacology to determine the percentage of total CaV current generated by each channel type. CaV2.2 (N-type) was determined from block by 10 µM GVIA, CaV2.1 (P/Q-type) from block by 0.2 µM AgaIVa, CaV1.2 from block by 3 µM Nif and CaV2.3 (R-type) from block by 0.3 µM SNX (Fuchs et al. 2007; Huang et al. 1997; Ikeda and Matsumoto 2003; Lu et al. 2010; Ōhnuma et al. 2011). Using our 5 mM Ba2+ external solution, we found that the largest block was produced by GVIA (47 ± 19%, n = 20) > AgaIVa (25 ± 14%, n = 16) > Nif (13 ± 14%, n = 16) (Fig. 1). The block produced by each of these three drugs was significant (P < 0.05). However, SNX produced no significant block (−7 ± 11%, n = 6). This was surprising since previous work had shown SNX-sensitive currents in DRG neurons (Fang et al. 2007; Fuchs et al. 2007). Perhaps muscle afferent neurons fail to express CaV2.3 channels. However, 22 ± 16% (P < 0.05, n = 11) of the total CaV current was resistant to all blockers (resistant current). As expected, only block by GVIA was irreversible (Boland et al. 1994; Liang and Elmslie 2002). Previous work has shown differences in the percentage CaV current blocked by various specific blockers in small vs. medium or large DRG neurons (Fuchs et al. 2007; Scroggs and Fox 1992). Thus it appears that expression of CaV channel types can vary across classes of DRG neurons. Unmyelinated C-type and thinly myelinated Aδ-type cutaneous afferents have cell body diameters < 35 µm, with C fibers having diameters < 23 µm (Djouhri et al. 2003). However, muscle afferents were found to have larger soma diameters relative to cutaneous afferents (Hu and McLachlan 2003; Ramachandra et al. 2012). Thus we include muscle afferent neurons up to 40 µm in the group III (Aδ) and define small group IV (C) afferent neurons as those with cell diameters < 30 µm (Ramachandra et al. 2012, 2013). While the CaV current was significantly blocked by Nif, AgaIVa, and GVIA (but not SNX) in both groups, we found no differences in percentage block by any CaV channel
blocker between small and medium neurons (Fig. 1B), which is consistent with previous findings from cutaneous afferents (Lu et al. 2010). In addition, the resistant current was not different between these two groups at 21 ± 12% (n = 7) of total CaV current in small neurons and 25 ± 24% (n = 4) in medium neurons. A more detailed examination of the CaV channel block data is shown using scatter plots (Fig. 1, C–E), which shows that there are no systematic differences among muscle afferent neurons ranging from 20 to 40 μm diameter. Thus neurons in both the group III (Aδ) and group IV (C) size range most prominently express CaV2.2 > CaV2.1 > CaV1.2 (Fig. 1).

The functional expression of CaV3 (T-type) channels in DRG neurons has been demonstrated (Ikeda and Matsumoto 2003; Jagodic et al. 2008; Scroggs and Fox 1992), but we failed to observe evidence of CaV3 currents in our recordings in Ba2+. Such currents are characterized by low-voltage activation (less than −30 mV) and rapid inactivation (Scroggs and Fox 1992). To quantitatively determine whether CaV3 currents contributed to the total CaV current, we calculated the percentage inhibition over a range of voltages for each of the three blockers that produced significant inhibitions. CaV3 currents would be revealed by the relatively smaller block at hyperpolarized voltages where CaV3 channels dominate (Ikeda and Matsumoto 2003; Jagodic et al. 2008; Scroggs and Fox 1992). Using our standard holding potential (−80 mV), we found no significant difference in the inhibition at −30 vs. +30 mV produced by any of our blockers (Fig. 2). It was not possible to do this analysis using more hyperpolarized voltages, since the most hyperpolarized voltage to produce measurable current. Under these conditions, it appears that CaV3 channels do not significantly contribute to total CaV current in muscle afferent neurons.

CaV2.1 (P/Q) currents are typically blocked using submicromolar concentrations of AgaIVa (Fuchs et al. 2007; Huang et al. 1997), since CaV2.2 channels have been shown to be blocked by 1 μM AgaIVa (Sidach and Mintz 2000). However, Q-currents are a CaV2.1 isoform that is less sensitive to AgaIVa block and is typically blocked using micromolar AgaIVa concentrations (Randall and Tsien 1995; Sidach and Mintz 2000). We wondered if our use of Ba2+ as the charge carrier was the problem, since it was previously demonstrated that R-like CaV current was enhanced when Ca2+ was the charge carrier (Boland et al. 1994; Liang and Elmslie 2001). Using the same blockers, we redid our study using a 10 mM Ca2+ external solution. In these neurons, GVIA blocked 50 ± 2% of current, and 0.2 μM AgaIVa blocked 26 ± 9% of current, but increasing AgaIVa to 1 μM blocked only an additional 4 ± 1% of current. Thus a weakly AgaIVa-sensitive CaV2.1 channel type (e.g., Q-current) does not substantially contribute to resistant current in muscle afferent neurons.

The absence of SNX-sensitive CaV current was puzzling. We wondered if our use of Ba2+ as the charge carrier was the problem, since it was previously demonstrated that R-like CaV current was enhanced when Ca2+ was the charge carrier (Boland et al. 1994; Liang and Elmslie 2001). Using the same blockers, we redid our study using a 10 mM Ca2+ external solution. In addition, we used a cumulative blocker application strategy to better gauge the resistant current (Fig. 4). It should be noted that,

Fig. 1. Voltage-dependent calcium (CaV) 2.2 channels generate the dominant CaV current in muscle afferent neurons. CaV current in 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) labeled muscle afferent neurons was measured using 5 mM Ba2+ external solution. A: example currents showing the effect of 0.3 μM SNX482 (SNX), 0.2 μM ω-agatoxin IVa (AgaIVa), 3 μM nifedipine (Nif), and 10 μM ω-conotoxin GVIA (GVIA). The voltage protocol is shown below the current traces. Cntl, control. B: comparison of mean ± SD calcium current block in small (S; 20–30 μm) vs. medium (M; 30–40 μm) muscle afferent neurons by the indicated blockers. C–E: the distribution of percentage block vs. neuron diameter for 3 μM Nif (C), 0.2 μM AgaIVA (D), and 10 μM GVIA (E). Note the different y-axis scale for GVIA. The dashed line indicates average block.
all but one of these neurons were also labeled by the NaV1.8 block in Ca2+ (14%). We do not understand the reasons for the smaller AgaIVa on muscle afferent neurons are that CaV2.3 channels are not sensitive current, as we predicted. Thus two possible explanations for the absence of a SNX effect (Tottene et al. 2000). To determine whether CaV2.3 channels were expressed in muscle afferent neurons, we exposed isolated DRG neurons to a rabbit CaV2.3 antibody. We found that all muscle afferent neurons were insensitive to SNX. For consistency with our electrophysiological record-nings, we also tested for the presence of NaV1.8 using a specific mouse antibody. We found that all muscle afferent neurons were positively labeled by the CaV2.3 antibody (Fig. 5, A and B), and all but one of these neurons were also labeled by the NaV1.8 antibody (Fig. 5C). Most of the identified muscle afferent neurons had soma diameters between 20 and 40 μm, which overlaps with the electrophysiological recordings. Thus muscle afferent neurons appear to express CaV2.3 channels.

We also wanted to test if these channels were functional, but our blocker (SNX) did not work. One strategy was to use roscovitine, which is a CaV2-specific agonist (Buraei et al. 2005, 2007). While roscovitine affects many ion channels (Buraei et al. 2007; Ganapathi et al. 2009; Yarotsky and Elmslie 2007, 2012), it uniquely affects CaV2 channels by slowing deactivation (Buraei et al. 2005, 2007), which produces slow tail currents (Fig. 6). For these experiments, we tested the effect of 100 μM roscovitine after blocking CaV2.1 and CaV2.2 with 0.2 μM AgaIVa and 10 μM GVIA, respectively. Figure 6A shows the effect of roscovitine to inhibit step current and slow deactivation of total CaV current (before block of CaV2.1 and 2.2). After toxin application, the tail current is still reversibly slowed by roscovitine (Fig. 6, B and C). The roscovitine-induced inhibition was similar before and after applications of the toxins, with average reductions of 39 ± 15% and 52 ± 18% for control and in toxins, respectively. The slowed deactivation shows that functional CaV2 channels produce current in the presence of AgaIVa and GVIA. The similar inhibition before and after toxin application suggests that roscovitine did not reverse toxin block to reveal CaV2.2 or 2.1 channels, which would have been caused either by an increase in step current or a decrease in inhibition. The most likely conclusion is that SNX-resistant CaV2.3 channels comprise a large fraction of the resistant CaV current in small to medium muscle afferent neurons.

As a second test, we examined the Ni2+ sensitivity of the resistant current. It has been demonstrated in major pelvic ganglion neurons that SNX-resistant R-current is blocked by Ni2+ with an IC50 (22 μM) similar to that of expressed CaV2.3 channels (21 μM) (Won et al. 2006). Using Ba2+ as the charge under these recording conditions (HP −80 mV), we observed T-type currents, but only in ~10% of muscle afferent neurons. The percentage block by Nif, GVIA, and SNX was statistically similar (P > 0.05) to that in Ba2+, and the resistant current was also similar between Ca2+ and Ba2+ (P > 0.05). However, the block by AgaIVa was smaller in Ca2+ (13 ± 2%) vs. Ba2+ (25 ± 14%). We do not understand the reasons for the smaller AgaIVa block in Ca2+, but it is clear that Ca2+ did not enhance SNX-sensitive current, as we predicted.

As a positive control for SNX, we tested 0.3 μM SNX on CaV2.3 channels heterologously expressed in LN229 cells. The recordings were done using a 10 mM Ca2+ external solution, and SNX blocked 93 ± 8% (n = 3) of the expressed current. Thus two possible explanations for the absence of a SNX effect on muscle afferent neurons are that CaV2.3 channels are not expressed by these neurons, or the expressed CaV2.3 channels are insensitive to SNX.

CaV2.3 in muscle afferent neurons. SNX has been used in many experiments to define CaV2.3 current, but not all CaV2.3 channels are sensitive to SNX (Tottene et al. 2000). To determine whether CaV2.3 channels were expressed in muscle afferent neurons, we exposed isolated DRG neurons to a rabbit CaV2.3 antibody. For consistency with our electrophysiological recordings, we also tested for the presence of NaV1.8 using a specific mouse antibody. We found that all muscle afferent neurons were positively labeled by the CaV2.3 antibody (Fig. 5, A and B), and all but one of these neurons were also labeled by the NaV1.8 antibody (Fig. 5C). Most of the identified muscle afferent neurons had soma diameters between 20 and 40 μm, which overlaps with

**Fig. 2. Voltage-independent block by CaV channel blockers. A: current-voltage relationships from a muscle afferent neuron in the presence of either 3 μM Nif, 0.2 μM AgaIVa, or 10 μM GVIA. CaV currents were measured in 5 mM Ba2+ at the end of 25-ms voltage steps to the indicated voltage. B: the mean inhibition (±SD) for each CaV channel blocker is shown over voltages ranging from −30 to 30 mV. The currents were measured as described for A. There was no statistical difference between the block at −30 mV vs. that at 30 mV for each blocker (n = 5 for each blocker).**
carrier, we tested the dose response for Ni\textsuperscript{2+} block of total Ca\textsubscript{V} current and calculated an IC\textsubscript{50} = 246 μM (n = 3–4 neurons), which is expected for Ca\textsubscript{V} currents dominated by Ca\textsubscript{V}2.2 and 2.1 channels (Liang and Elmslie 2001; Zamponi et al. 1996). However, following application of 1 μM GVIA to block the dominant Ca\textsubscript{V}2.2 current, the Ni\textsuperscript{2+} block IC\textsubscript{50} did not change with IC\textsubscript{50} = 319 μM (n = 7–8 neurons), which was against our expectation of a decrease in IC\textsubscript{50}. It has been previously reported that the Ni\textsuperscript{2+} sensitivity of Ca\textsubscript{V}2.3 current was ~300 μM when recorded in 10 mM Ba\textsuperscript{2+}, but < 30 μM when recorded in 10 mM Ca\textsuperscript{2+} (Won et al. 1996). In addition, the Ni\textsuperscript{2+}-sensitive R-current of major pelvic ganglion neurons was recorded in 10 mM Ca\textsuperscript{2+} (Zamponi et al. 1996). Therefore, we reexamined the Ni\textsuperscript{2+} block of Ca\textsubscript{V} currents recorded in 10 mM Ca\textsuperscript{2+}. We found that the Ni\textsuperscript{2+} block of the resistant current (Nif, GVIA, and AgaIVa) recorded in Ca\textsuperscript{2+} was much more potent than when recorded in Ba\textsuperscript{2+} with IC\textsubscript{50} = 4.4 μM (n = 2 to 5) and the maximum block = 64%. The potent Ni\textsuperscript{2+} block suggests that the majority of the resistant current (64%) was generated by the activity of Ca\textsubscript{V}2.3 channels.

**ATP modulates Ca\textsubscript{V}2.2 channels.** ATP helps to generate the EPR by activating P\textsubscript{2X} ionotropic receptors (Cui et al. 2011; Hayes et al. 2008), but ATP modulation of the reflex is also possible by activation of G protein-coupled P\textsubscript{2Y} receptors. ATP has been shown to inhibit Ca\textsubscript{V} currents by activation of P\textsubscript{2Y} receptors (Filippov et al. 2003; Gerevich et al. 2004), so we tested the effect on muscle afferent Ca\textsubscript{V} currents (Fig. 7). G protein-mediated inhibition of Ca\textsubscript{V}2 currents is often found to be voltage dependent in that the inhibition is temporarily reversed by brief, strong depolarization (Elmslie et al. 1990; Ikeda 1991). ATP (10 μM) produced an average 32 ± 15% (n = 13) (Fig. 7B) inhibition of Ca\textsubscript{V} current that was reversed following strong depolarization (Fig. 7A). In addition, there was no clear difference in the percentage inhibition observed among neurons with diameters ranging from 22 to 48 μm (Fig. 7B). Previous reports have demonstrated Ca\textsubscript{V}2.2 (N-type) to be a major target of G protein-mediated inhibition (Elmslie et al. 1990; Gerevich et al. 2004; Ikeda 1991), but other Ca\textsubscript{V}2 channels can be modulated as well (Colecraft et al. 2000; Meza and Adams 1998). Thus we determined the effect of GVIA on ATP modulation (Fig. 7D). Prior to GVIA application, ATP inhibited current (prepulse) by an average of 19 ± 8%, while the inhibition was reduced to an average of 3 ± 4% following GVIA block of Ca\textsubscript{V}2.2 channels in the same five neurons. Ca\textsubscript{V}2.2 channels are the primary Ca\textsubscript{V} channel target for ATP-induced inhibition in muscle afferent neurons.

**DISCUSSION**

We have identified the Ca\textsubscript{V} channels that generate Ca\textsubscript{V} current in muscle afferent neurons along with examining the modulation of that current by ATP. The results demonstrate that, regardless of using external Ba\textsuperscript{2+} or Ca\textsuperscript{2+}, Ca\textsubscript{V}2.2 channels dominate by generating 40–50% of the total Ca\textsubscript{V} current. Ca\textsubscript{V}2.1 channels generated between 13 and 25% of the current, while Ca\textsubscript{V}1.2 channels generated ~15% of the total current. The Ca\textsubscript{V}2.3 channel blocker SNX had no effect on muscle afferent Ca\textsubscript{V} current, while the portion of the current that was resistant to all blockers averaged ~25% of the total current. Using roscovitine and Ni\textsuperscript{2+}, we demonstrated that the majority of resistant current is generated by Ca\textsubscript{V}2.3, which shows that the current generated by this channel type is roughly equal to that generated by Ca\textsubscript{V}2.1. In the small to medium neurons recorded in our study, there was no difference in Ca\textsubscript{V} channel expression between these two groups. ATP inhibited Ca\textsubscript{V} current by activation of G protein-coupled receptors, and that inhibition primarily targeted Ca\textsubscript{V}2.2 channels. Based on the neuronal size and the expression of Na\textsubscript{V}1.8 by these neurons (Ramachandra et al. 2012), we conclude that group IV (C) and group III (Aδ) neurons primarily express Ca\textsubscript{V}2.2 channels that are inhibited by extracellular ATP.

**Ca\textsubscript{V} channels in sensory neurons.** The percentage of Ca\textsubscript{V} channel types comprising the total current in our work matches well with most studies on small- and medium-diameter sensory neurons with Ca\textsubscript{V}2.2 > Ca\textsubscript{V}2.1 = Ca\textsubscript{V}2.3 > Ca\textsubscript{V}1.2 (Huang et al. 1997; Lu et al. 2010). However, Scroggs and Fox (1992) found a much larger block by 2 μM nimodipine (53%) in small sensory neurons (20–27 μm) and a much smaller block (7%) in medium-sized neurons (33–38 μm) than we and others have observed. The source of this difference is unclear, but it is possible that there is a population of DRG neurons in which Ca\textsubscript{V}1.2 channels dominate.

One important issue when using blockers to identify component channels is the specificity and concentration of the chosen compounds. The blockers used in this study were the same as used in many previous studies. GVIA is a highly specific blocker of Ca\textsubscript{V}2.2 channels, but the concentration we used was higher than some other studies (Fuchs et al. 2007; Huang et al. 1997; Lu et al. 2010; Scroggs and Fox 1992). Early studies of Ca\textsubscript{V}2.3 showed this channel was reversibly blocked by 5 μM GVIA when expressed in Xenopus oocytes.
is highly specific for CaV2.1 channels at submicromolar concentrations (Randall and Tsien 1995). The identification of CaV2.3 (α1β) channel blockers fit the characteristics of R-current, and this channel type was called R-type (Zhang et al. 1993). SNX was identified as a blocker of CaV2.3 channels, but only a subpopulation of those channels was sensitive to this blocker (Tottene et al. 2000). We found that 22–27% of our CaV current was resistant to the classic CaV channel blockers, but that current was also insensitive to SNX. Two obvious possibilities were that CaV2.3 channels were not expressed in muscle afferent neurons, or that some other channel was insensitive to SNX. Thus, we were able to achieve fast block of L-type channels using this Nif concentration. AgaIVa is highly specific for CaV2.1 channels at submicromolar concentrations, but CaV2.2 channels are blocked at concentrations ≥ 1 μM (Sidach and Mintz 2000). Thus, like other studies, we utilized 0.2 μM AgaIVa for most of our experiments. However, the Q-type variant of CaV2.1 is less sensitive to AgaIVa than the P-type variant (Bourinet et al. 1999; Randall and Tsien 1995). Thus, when determining if lower affinity CaV2.1 channel variants were expressed in muscle afferents, we preblocked CaV2.2 channels with GVIA, which allowed us to show that only 4% of the total CaV current could be attributed to Q-like CaV2.1 channels. Since resistant current comprises ~25% of CaV current, our results suggest that Q-like CaV2.1 channels generate ~16% (4%/25%) of resistant current.

Current that is resistant to the classic CaV channel blockers (e.g., GVIA, AgaIVa, and Nif) was originally termed R-current (Randall and Tsien 1995). The identification of CaV2.3 (α1β) channel blockers fit the characteristics of R-current, and this channel type was called R-type (Zhang et al. 1993). SNX was identified as a blocker of CaV2.3 channels, but only a subpopulation of those channels was sensitive to this blocker (Tottene et al. 2000). We found that 22–27% of our CaV current was resistant to the classic CaV channel blockers, but that current was also insensitive to SNX. Two obvious possibilities were that CaV2.3 channels were not expressed in muscle afferent neurons, or SNX-insensitive variants were expressed in these neurons. We conclude that CaV2.3 channels are expressed in muscle afferent neurons, but these channels are insensitive to SNX, regardless of whether CaV2.3 was used to record current. This conclusion is based on three lines of evidence. First, muscle afferent neurons were labeled by a CaV2.3 antibody. Second, the deactivation of CaV current in the presence of GVIA and AgaIVa was slowed by roscovitine. GVIA and AgaIVa block CaV2.3 and 2.1, respectively, and roscovitine has been shown to specifically slow deactivation of CaV2 channels, including CaV2.3 channels (Buraei et al. 2007). A small component (~16%) of this effect can be attributed to the Q-like current,
but the robust roscovitine response supports expression of CaV2.3 in muscle afferent neurons. Finally, the potent Ni2+/H11001 block of the resistant current recorded in 10 mM Ca2+/H11001 (IC50/4.4 M) is consistent with the majority (64%) of that current being generated by the activity of CaV2.3 channels (Won et al. 2006). The difference in Ni2+/H11001 blocking affinity in Ca2+/H11001 vs. Ba2+/H11001 external solutions is also consistent with the current being generated by CaV2.3 channels (Zamponi et al. 1996).

ATP modulation of CaV2.2 channels. ATP is an important regulator of the EPR (Cui et al. 2011; Hanna and Kaufman 2003; Gerevich et al. 2004). Since the majority of neurons recorded for this study had small to medium diameters and all expressed NaV1.8, these neurons are likely group III and IV neurons that participate in the EPR (Kaufman and Hayes 2003). The number of muscle afferent neurons tested is indicated in the middle bar.

Fig. 6. Roscovitine (Rosc)-induced slowed deactivation reveals functional CaV2 channels in the presence of GVIA and AgaIVA. All currents were recorded in 5 mM Ba2+. A: a muscle afferent neuron shows the effect of Rosc on CaV2 currents (no toxins present). Rosc (100 μM) slowed deactivation (black trace) compared with Cntl and recovery (Recover; gray traces). B: current traces from the same muscle afferent neuron as shown in A recorded in 10 μM GVIA and 0.2 μM AgaIVA. Rosc (100 μM) (black trace) slowed deactivation compared with Cntl (Toxin) and Recover (gray traces). C: a single exponential equation was fit to the deactivating currents at −40 mV to determine the deactivation τ in presence of toxin (GVIA and AgaIVA) and toxin + 100 μM Rosc. The average deactivation τ (±SD) is shown. *Significant slowing of deactivation induced by Rosc. The number of muscle afferent neurons tested is indicated in the middle bar.

Fig. 7. ATP inhibits CaV2.2 channels in muscle afferent neurons. All currents were recorded in 10 mM Ca2+. A: the inhibition of CaV current induced by 10 μM ATP (black trace) compared with Cntl (gray trace) recorded from a muscle afferent neuron. The inhibition is transiently reversed by strong depolarization (+80 mV), which can be seen by comparing the prepulse (before the +80-mV step) and postpulse (following the +80-mV step) currents. B: there was no clear differences in ATP (10 μM) induced inhibition in small (<30 μm) vs. medium (30–40 μm) vs. large (>40 μm) muscle afferent neurons. The percent inhibition of prepulse current measured from 13 muscle afferent neurons is plotted vs. neuron diameter. C: the ATP (10 μM) inhibition is blocked by preapplication of 10 μM GVIA. This time course shows the inhibition induced by ATP prior to GVIA application and little or no ATP response in the presence of GVIA. The prepulse (solid circle) and postpulse (open circle) current amplitudes are plotted. D: the average (±SD) inhibition induced by 10 μM ATP is shown before (ATP) and during (GVIA + ATP) application of 10 μM GVIA. *ATP response in GVIA is significantly different from that in Cntl. The number of muscle afferent neurons tested is indicated.
2002). Thus we were interested in the effect of ATP on CaV current in our muscle afferent neurons. We found that ATP inhibited CaV current, and this inhibition primarily involved CaV2.2 channels. While we did not specifically test the involvement of P2Y receptors, there is no doubt that the inhibition is mediated via activation of G protein-coupled receptors. G protein-mediated inhibition of CaV2 channels is characterized by rapid reversal following strong depolarization (Elmslie 1992; Ikeda 1991; Meza and Adams 1998), which was exhibited by the ATP-induced inhibition of CaV current in our study. In addition, this type of inhibition is specifically mediated by the G protein βγ-subunit (Ikeda 1996). Thus ATP activation of G protein-coupled receptors (likely P2Y) inhibits CaV2.2 channels in muscle afferent neurons by G protein βγ-subunits binding directly to the channels (Elmslie and Jones 1994; Zamponi and Snutch 1998).

CaV2.2 channels are one of the primary CaV channel types that trigger excitatory neurotransmitter release from nociceptor synaptic terminals in the dorsal horn (Elmslie 2004; Vanegas and Schaible 2000; Zamponi et al. 2009), which is the reason for the clinical effectiveness of the CaV2.2 channel blocker ziconotide in controlling pain in chronic pain patients (Elmslie 2004; Snutch 2005; Zamponi et al. 2009). Activation of P2Y receptors in the dorsal horn of the spinal cord was shown to inhibit nociceptor synaptic transmission by inhibition of CaV2.2 (N-type channels), which was associated with reduced pain responses (Gerevich et al. 2004). Thus activation of P2Y receptors on group III and/or IV muscle afferent synaptic terminals could inhibit CaV2.2 channel activity to reduce cardiovascular effects of the EPR. These results also suggest that intrathecal application of ziconotide could be used to treat excessive EPR activity resulting from peripheral vascular disease or heart failure (Baccelli et al. 1999; Bakke et al. 2007; Smith et al. 2006).

CaV channel control of neuronal excitability. CaV channel activity can produce either an increase or decrease in neuronal excitability, depending on the Ca2+-sensitive proteins activated by the Ca2+ influx. As discussed above, inhibition of CaV channels in excitatory presynaptic terminals (e.g., primary sensory neurons) inhibits excitability. On the other hand, inhibition of CaV channel activity can also lead to enhanced neuronal excitability through reduced activation of Ca2+-activated potassium channels (Marrion and Tavalin 1998; Yu et al. 2010). In a animal models of chronic pain, a reduction of CaV2.2 currents in small DRG neurons (Fuchs et al. 2007), and small to medium cutaneous afferent neurons (Lu et al. 2010) has been demonstrated. This reduction leads to enhanced excitability, which may help to produce chronic pain (Hogan et al. 2008; Lirk et al. 2008). The prominent expression of CaV2.2 channels by group III and IV neurons suggests that peripheral application of ziconotide or GVIA could enhance action potential activity in these neurons to increase EPR-induced effects on the cardiovascular system. However, natural activators could achieve the same effect. The ATP level in muscle has been demonstrated to depend on muscle contraction (Li et al. 2003; Mortensen et al. 2011), and the concentration (10 μM) is consistent with that used in this study (Li et al. 2003). If P2Y receptors and CaV2.2 channels are expressed on afferent endings in skeletal muscle, muscle released ATP could enhance the EPR via inhibition of CaV2.2 channels.

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

AUTHOR CONTRIBUTIONS

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