Identification of CaV channel types expressed in muscle afferent neurons

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ABSTRACT

Cardiovascular adjustments to exercise are partially mediated by group III/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 towards determining their potential role in controlling the EPR. Using specific blockers and 5 mM Ba$^{2+}$ 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 Ca$^{2+}$ (Liang and Elmslie 2001). We reexamined the channel types using 10 mM Ca$^{2+}$ as the charge carrier, but results were similar to those in Ba$^{2+}$. 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.

Key words: CaV2.2, CaV2.1, CaV2.3, dorsal root ganglia neurons, exercise pressor reflex
INTRODUCTION

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 (ERP) (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 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 Ca^{2+} 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 CNS excitability, blocking CaV channels can also increase neuronal excitability through reduced activation of
closely associated Ca\(^{2+}\)-activated potassium channels (Marrion and Tavalin 1998; Yu et al. 2010). Indeed, blocking Ca\(\text{v}\) channels increases excitability of sensory neurons (Lirk et al. 2008). Thus, it is important that we understand the Ca\(\text{v}\) 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 P\(_2\)X receptors (Cui et al. 2011; Hayes et al. 2008). However, ATP can inhibit Ca\(\text{v}\) channels by activation of G-protein coupled P\(_2\)Y receptors (Filippov et al. 2003; Gerevich et al. 2004). Here we identify the Ca\(\text{v}\) channels that are functionally expressed by muscle afferent neurons and show that ATP can inhibit the Ca\(\text{v}\) current by reducing activity of Ca\(\text{v}\)2.2 (N-type) channels. As with chronic pain, we show that Ca\(\text{v}\)2.2 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 (wt 150-400 gms) were obtained from Hill Top Laboratories (Scottdale, PA). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and followed 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',3'-tetramethylindocarbocyanine perchlorate) (100 μL of 1.5% DiI in DMSO) that was injected into both left and right gastrocnemius muscles of anesthetized rats (ketamine, xylazine and acepromazine). 4-5 days post injection, the rats were sacrificed with CO₂ followed by decapitation. Neurons from the lumbar DRG L₄ and L₅ 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 (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Expressed Caᵥ channels: Caᵥ2.3 channels (α1, β2A and α2δ cDNA constructs, generous gifts from Dr. Henry L. Puhl, NIH/NIAAA) 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 Medium (DMEM), supplemented with 10% fetal bovine serum, 1% glutamine and 1% penicillin-streptomycin. Following cDNA injection, the cells were incubated overnight and electrophysiological recordings were performed the next day.

Solutions: Most DiI 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, with pH = 7.4 and osmolarity = 320 mOsm, which was
used to identify Na\textsubscript{V}1.8 expressing muscle afferent neurons (Ramachandra et al. 2012). When
recording Ca\textsubscript{V} current, the external solution was switched to a barium external solution
containing (in mM) 145 NMG•Cl, 5 BaCl\textsubscript{2}, 10 NMG•HEPES and 5 glucose, with pH = 7.4 and
osmolarity = 320 mOsm. The pipet solution contained (in mM) 104 NMG•Cl, 14 Creatine•PO\textsubscript{4},
6 MgCl\textsubscript{2}, 10 NMG•HEPES, 5 Tris•ATP, 10 NMG\textsubscript{2}•EGTA, and 0.3 Tris\textsubscript{2}•GTP with pH 7.4 and
osmolarity = 300 mOsm.

For some experiments the Ca\textsubscript{V} currents in muscle afferent neurons were recorded in external
Ca\textsuperscript{2+}. Na\textsubscript{V}1.8 expressing muscle afferents were identified by expression of enhanced green
fluorescent protein (EGFP) (Puhl and Ikeda 2008) as previously described (Hassan and Ruiz-Velasco 2013). Briefly, DRG neurons were microinjected with a cDNA plasmid containing the
EGFP gene under control of the putative Na\textsubscript{V}1.8 promoter. The external solution for these
experiments contained (in mM) 145 tetraethanolamine (TEA)•CH\textsubscript{3}SO\textsubscript{3}H, 10 TEA•HEPES, 10
CaCl\textsubscript{2}, 15 glucose and 0.0003 TTX, pH 7.4, 325 mOsm. The internal solution contained (in
mM) 90 NMG•CH\textsubscript{3}SO\textsubscript{3}H, 25 TEA•CH\textsubscript{3}SO\textsubscript{3}H, 14 Creatine•PO\textsubscript{4}, 11 EGTA, 10 HEPES, 1 CaCl\textsubscript{2},
20 CsOH, 4 MgATP, 0.3 Na\textsubscript{2}GTP, pH 7.2, 300 mOsm. The expressed Ca\textsubscript{V}2.3 channels were
recorded in a solution containing (in mM): 140 NaCl, 5.4 KCl, 10 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose
and 10 HEPES, pH 7.4 with NaOH. The internal solution was the same at that used for muscle
afferent neurons recorded in Ca\textsuperscript{2+}.

Ion channel blockers were made up as stock solutions in either water or dimethyl sulfoxide
(DMSO) and diluted into the external solution to make the working concentration. All external
solutions contained the same DMSO concentration (max 0.03%) so that the only change was
presence or absence of the blocker. In experiments where Ca\textsuperscript{2+} was the charge carrier, 0.1 mg/ml
cytochrome C was added to all external solutions to minimize potential binding of the peptide toxins to the capillary columns used for drug delivery. Solutions were applied using a gravity fed perfusion system with a solution exchange time of 2 seconds.

**Measurement of ionic currents:** Dil labeled DRG neurons were identified using a Nikon Diaphot microscope with epifluorescence and voltage-clamped using the whole-cell configuration of the patch clamp technique. Pipettes were pulled from glass capillaries (King Precision Glass, Claremont, CA) on a Sutter P-97 puller (Sutter Instruments Co., Novato, CA). Currents were recorded using either an Axopatch 200A or 200B amplifier (Molecular Devices, Sunnyvale, CA) and digitized with an ITC-18 data acquisition interface (Instrutech Corporation, Port Washington, NY). Experiments were controlled using S5 data acquisition software written by Dr. Stephen Ikeda (NIH/NIAAA, Rockville, MD). Leak current was subtracted online using a -P/4 protocol. Recordings were carried out at room temperature and the holding potential was -80 mV.

**Data analysis:** Data were analyzed using IgorPro (WaveMetrics, Lake Oswego, OR) running on a Macintosh computer. Cell diameter was calculated using the cell capacitance as previously described (Ramachandra et al. 2012). Group data were calculated as mean ± standard deviation (SD) throughout the paper. Student’s T-test (unpaired, two-tailed) was calculated to determine significant differences (p < 0.05).

**Immunocytochemistry:** Neurons were fixed with 4% formaldehyde and permeabilized with 2% Tween 20 as previously described (Ramachandra et al. 2012). Neurons were labeled with primary antibodies for both NaV1.8 (mouse, Abcam, Cambridge, MA) and CaV2.3 (rabbit, Alomone Labs, Jerusalem, Israel) (1:500) and visualized using secondary antibodies Alexa Fluor 488 IgG goat anti-rabbit and Alexa Fluor 635 IgG goat anti-mouse (Invitrogen, Carlsbad, CA).
Images were captured using a Nikon ECLIPSE 80i epifluorescence microscope and neurons measured using ImageJ (rsbweb.nih.gov/ij/index.html). Cell size was calculated and positive fluorescent labeling was determined as described previously (Ramachandra et al. 2012).

**Chemicals:** DiI, MEM, DMEM, FBS, penicillin/streptomycin were obtained from Invitrogen. TTX citrate, nifedipine and SNX482 were obtained from Ascent Scientific (Princeton, NJ). ω-conotoxin GVIA (GVIA) and ω-agatoxin IVa (AgaIVa) were obtained from Bachem America (King of Prussia, PA). All other chemicals were from Sigma (St. Louis, MO).
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 nifedipine (Nif) and CaV2.3 (R-type) from block by 0.3 µM SNX482 (SNX) (Fuchs et al. 2007; Huang et al. 1997; Ikeda and Matsumoto 2003; Lu et al. 2010; Ohnami 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. 2013; Ramachandra et al. 2012). 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. 1C-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 (< -30 mV) and rapid inactivation (Scroggs and Fox 1992). To quantitatively determine if 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 -30 mV was the most hyperpolarized voltage to produce measureable 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 sub-micromolar concentrations of AgaIVa (Fuchs et al. 2007; Huang et al. 1997), since CaV2.2 channels have been shown to be blocked by 1µM 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 a component of our resistant current (22% of total CaV current) was contributed by Q-channels. Therefore, we compared block of muscle afferent CaV current in 0.2 vs. 1 µM AgaIVa after block by GVIA to remove the CaV2.2 component (Fig. 3). 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 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 Ca\(^{2+}\), but it is clear that Ca\(^{2+}\) did not enhance SNX-sensitive current as we predicted.

As a positive control for SNX, we tested 0.3 µM SNX on Ca\(_{\text{V}2.3}\) channels heterologously expressed in LN229 cells. The recordings were done using a 10 mM Ca\(^{2+}\) 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 Ca\(_{\text{V}2.3}\) channels are not expressed by these neurons or the expressed Ca\(_{\text{V}2.3}\) channels are insensitive to SNX.

### Ca\(_{\text{V}2.3}\) in muscle afferent neurons

SNX has been used in many experiments to define Ca\(_{\text{V}2.3}\) current, but not all Ca\(_{\text{V}2.3}\) channels are sensitive to SNX (Tottene et al. 2000). To determine if Ca\(_{\text{V}2.3}\) channels were expressed in muscle afferent neurons, we exposed isolated DRG neurons to a rabbit Ca\(_{\text{V}2.3}\) antibody. For consistency with our electrophysiological recordings, we also tested for the presence of Na\(_{\text{V}1.8}\) using a specific mouse antibody. We found that all muscle afferent neurons were positively labeled by the Ca\(_{\text{V}2.3}\) antibody (Fig. 5A, B), and all but one of these neurons were also labeled by the Na\(_{\text{V}1.8}\) antibody (Fig. 5C). Most of the identified muscle afferent neurons had soma diameters between 20-40 µm, which overlaps with the electrophysiological recordings. Thus, muscle afferent neurons appear to express Ca\(_{\text{V}2.3}\) 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 Ca\(_{\text{V}2}\) specific agonist (Buraei et al. 2005; Buraei et al. 2007). While roscovitine affects many ion channels (Buraei et al. 2007; Ganapathi et al. 2009; Yarotskyy and Elmslie 2012; Yarotskyy and Elmslie 2007), it uniquely affects Ca\(_{\text{V}2}\) channels by slowing deactivation (Buraei et al. 2005; Buraei et al. 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. 6B, 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 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+ sensitive of the resistant current. It has been demonstrated in major pelvic ganglion (MPG) 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 carrier, we tested the dose-response for Ni2+ block of total Cav current and calculated an IC50 = 246 µM (n = 3-4 neurons), which is expected for Cav currents dominated by Cav2.2 and 2.1 channels (Liang and Elmslie 2001; Zamponi et al. 1996). However, following application of 1 µM GVIA to block the dominant Cav2.2 current, the Ni2+ block IC50 did not change with IC50 = 319 µM (n = 7-8 neurons), which was against our expectation of a decrease in IC50. It has been previously reported that the Ni2+ sensitivity of Cav2.3 current was ~300 µM when recorded in 10 mM Ba2+, but < 30 µM when recorded in 10 mM Ca2+ (Zamponi et al. 1996). In addition, the Ni2+-sensitive R-current of MPG neurons was
recorded in 10 mM Ca\(^{2+}\) (Won et al. 2006). Therefore, we reexamined the Ni\(^{2+}\) block of Ca\(\text{V}_{2.6}\) currents recorded in 10 mM Ca\(^{2+}\). We found that the Ni\(^{2+}\) block of the resistant current (nifedipine, GVIA and AgaIVa) recorded in Ca\(^{2+}\) was much more potent than when recorded in Ba\(^{2+}\) with IC\(_{50}\) = 4.4 µM (n = 2 to 5) and the maximum block = 64%. The potent Ni\(^{2+}\) block suggests that the majority of the resistant current (64%) was generated by the activity of Ca\(\text{V}_{2.3}\) channels.

\(\text{ATP modulates Ca}_{\text{V}2.2} \text{ channels}\)

ATP helps to generate the EPR by activating P\(_2\)X 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\(_2\)Y receptors. ATP has been shown to inhibit Ca\(\text{V}\) currents by activation of P\(_2\)Y receptors (Filippov et al. 2003; Gerevich et al. 2004), so we tested the effect on muscle afferent Ca\(\text{V}\) currents (Fig. 7). G protein-mediated inhibition of Ca\(\text{V}_{2}\) 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). 10 µM ATP produced an average 32 ± 15% (n = 13) (Fig. 7B) inhibition of Ca\(\text{V}\) 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 22-48 µm (Fig. 7B). Previous reports have demonstrated Ca\(\text{V}_{2.2}\) (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\(\text{V}_{2}\) 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\(\text{V}_{2.2}\) channels in the
same 5 neurons. CaV2.2 channels are the primary CaV channel target for ATP-induced inhibition in muscle afferent neurons.

DISCUSSION

We have identified the CaV channels that generate CaV current in muscle afferent neurons along with examining the modulation of that current by ATP. The results demonstrate that, regardless of using external Ba\(^{2+}\) or Ca\(^{2+}\), CaV2.2 channels dominate by generating 40-50\% of the total CaV current. CaV2.1 channels generated between 13-25\% of the current, while CaV1.2 channels generated ~15\% of the total current. The CaV2.3 channel blocker SNX had no effect on muscle afferent CaV current, while the portion of the current that was resistant to all blockers averaged ~25\% of the total current. Using roscovitine and Ni\(^{2+}\), we demonstrated that the majority of resistant current is generated by CaV2.3, which shows that the current generated by this channel type is roughly equal to that generated by CaV2.1. In the small to medium neurons recorded in our study, there was no difference in CaV channel expression between these two groups. ATP inhibited CaV current by activation of G protein-coupled receptors and that inhibition primarily targeted CaV2.2 channels. Based on the neuronal size and the expression of NaV1.8 by these neurons (Ramachandra et al. 2012), we conclude that group IV (C) and group III (A\(\delta\)) neurons primarily express CaV2.2 channels that are inhibited by extracellular ATP.

CaV channels in sensory neurons

The percentage of CaV channel types comprising the total current in our work matches well with most studies on small and medium diameter sensory neurons with CaV2.2 > CaV2.1 = CaV2.3 > CaV1.2 (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
csmaller 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 Cav1.2 channels dominate.

One important issue when using blockers to identify component channels is the specificity and
cconcentration 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 Cav2.2 channels, but the
cconcentration 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 Cav2.3 showed this channel was
reversibly blocked by 5 µM GVIA when expressed in Xenopus oocytes (Zhang et al. 1993), but
we did not observe a reversible component to the GVIA block in our studies. In addition, GVIA
has been shown to be specific for Cav2.2 in neurons at concentrations up to 100 µM (Boland et
al. 1994; Elmslie 1997). Finally, the percentage CaV current blocked by GVIA in our study was
similar to that in studies using lower concentrations (Huang et al. 1997; Lu et al. 2010). The
higher GVIA concentration in our study was used to ensure we could obtain data from multiple
blockers applied to the same neuron. GVIA is a very high affinity blocker, but the access to the
blocking site is highly sensitive to divalent cation concentration (Boland et al. 1994; Liang and
Elmslie 2002; Wagner et al. 1988). Thus, the blocking rate is much lower in higher divalent
cation concentrations (Boland et al. 1994; Liang and Elmslie 2002; Wagner et al. 1988) and also
much slower in Ca²⁺ vs. Ba²⁺ (Liang and Elmslie 2001). Thus, our data collection when using 10
mM Ca²⁺ was greatly enhanced by the fast block achieved using a higher GVIA concentration.
Unlike GVIA, there is evidence that Nif and AgalIVa loose selectivity at higher concentrations.
Nif has been shown to block non-L-type CaV currents at 10 µM (Jones and Jacobs 1990), which
was one reason for our use of 3 μM. We were also 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 $\geq 1$ 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 ($\alpha_{1E}$) 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 Ca$^{2+}$ or Ba$^{2+}$ 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 CaV2.2 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⁺ blocked the resistant current recorded in 10 mM Ca²⁺ (IC₅₀ = 4.4 μM) 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⁺ blocking affinity in Ca²⁺ vs. Ba²⁺ 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; Hayes et al. 2008) primarily via activation of ionotropic P₂X receptors (Cui et al. 2011; Hanna and Kaufman 2004; Hayes et al. 2008). While it is clear that activation of G protein-coupled P₂Y receptors cannot generate the EPR (Hayes et al. 2008), it is possible that activation of these receptors could modulate the reflex. One potential mechanism for that modulation is via CaV channels. ATP has been shown to inhibit CaV2.2 channels by activation of P₂Y receptors (Filippov et al. 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 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 Ca\(^{2+}\)-sensitive proteins activated by the Ca\(^{2+}\) 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 Ca\(^{2+}\)-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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The authors declare no conflicts of interest.
AUTHOR CONTRIBUTIONS

Experimental design: KSE, VRV, RR
Electrophysiology: RR, BH, MF, VRV
Immunocytochemistry: SM, JD
Writing of manuscript: KSE, VRV, RR
Editing of manuscript: KSE, RR, SM, JD, BH, MF, VRV
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FIGURE LEGENDS

Figure 1  CaV2.2 channels generate the dominant CaV current in muscle afferent neurons.  
CaV current in DiI labeled muscle afferent neurons was measured using 5 mM Ba^{2+} external solution.  
A. Example currents showing the effect of 0.3 μM SNX, 0.2 μM AgaIVa, 3 μM Nif, and 10 μM GVIA.  
The voltage protocol is shown below the current traces.  
B. A comparison of mean ± SD calcium current block in small (20-30 μm) vs. medium (30-40 μm) muscle afferent neurons by the indicated blockers.  
The blocker concentrations are the same as indicated for panel A.  
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.

Figure 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 Ba^{2+} 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 panel 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).

Figure 3  Little or no Q current in muscle afferent neurons.  
CaV current from muscle afferent neurons was recorded in 5 mM Ba^{2+} external solution.  
A. Example currents from a muscle afferent neuron in control (Cntl), 10 μM GVIA, 0.2 μM AgaIVa, and 1 μM AgaIVa.  
The mean (± SD) percentage block by GVIA and the two concentrations of AgaIVa tested on the
same neurons. The number of cells tested is indicated in the GVIA bar. The asterisks indicate that the CaV current was significantly blocked.

**Figure 4**  
CaV current block in external Ca$^{2+}$ is similar to that in Ba$^{2+}$. A. An example time course for CaV current block in 10 mM external Ca$^{2+}$. This experiment utilized cumulative blocker application to better determine the size of the resistant current (Resistant). The blocker concentrations are indicated and were the same as those used when recording in external Ba$^{2+}$. B. The average (± SD) percentage block by each CaV channel blocker. The asterisk indicates that CaV current was significantly blocked and the number of cells tested is indicated in each bar.

**Figure 5**  
Immunocytochemistry shows CaV2.3 channels expressed in muscle afferent neurons. A. The upper four images show sensory neurons stained with both NaV1.8 and CaV2.3 antibodies. The brightfield (left) image shows the three neurons and the DiI panel (right) shows the one labeled muscle afferent neuron in this field. All three neurons were positive for both NaV1.8 and CaV2.3. The white bar in the brightfield image indicates 50 µm. The lower four images show NaV1.8 and CaV2.3 controls along with brightfield (left) image and the DiI image showing one labeled muscle afferent neuron. B. There is no correlation between CaV2.3 label intensity and muscle afferent neuron size. The threshold line was determined from measurement of control muscle afferent neurons (e.g. lower images in panel A). The units for the Y-axis are arbitrary fluorescence units. C. A comparison of CaV2.3 label intensity vs. that of NaV1.8 in muscle afferent neurons. The threshold lines were determined from control muscle afferent neurons. Only one CaV2.3 positive muscle afferent neuron was negative for NaV1.8.
Figure 6  Roscovitine-induced slowed deactivation reveals functional CaV2 channels in the presence of GVIA and AgaIVa. All currents were recorded in 5 mM Ba\(^{2+}\). A. A muscle afferent neuron shows the effect of roscovitine (Rosc) on CaV2 currents (no toxins present). 100 µM Rosc slowed deactivation (black trace) compared with control (Cntl) and recovery (Recov, gray traces). B. Current traces from the same muscle afferent neuron as shown in panel A recorded in 10 µM GVIA and 0.2 µM AgaIVa. 100 µM Rosc (black trace) slowed deactivation compared to control (Toxin) and recovery (Recov, gray traces). C. A single exponential equation was fit to the deactivating currents at -40 mV to determine the deactivation \(\tau\) in presence of toxin (GVIA and AgaIVa) and toxin + 100 µM Rosc. The average deactivation \(\tau\) (±SD) is shown and the asterisk indicates significant slowing of deactivation induced by Rosc. The number of muscle afferent neurons tested is indicated in the middle bar.

Figure 7  ATP inhibits CaV2.2 channels in muscle afferent neurons. All currents were recorded in 10 mM Ca\(^{2+}\). A. The inhibition of CaV current induced by 10 µM ATP (black trace) compared to 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 pre-application 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. The asterisk shows that the ATP response in GVIA is significantly different from that in control. The number of muscle afferent neurons tested is indicated.