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T-type voltage-gated Ca^{2+} channels do not contribute to the negative feedback regulation of myogenic tone in murine superior epigastric arteries

Brendan Mullan, Jessica Pettis & William F. Jackson

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824

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Correspondence
William F. Jackson, 1355 Bogue Street, Rm B429 Life Science, East Lansing, MI 48824.
Tel: 517 353 5476; Fax: 517 353 8915;
E-mail: jacks783@msu.edu

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Abstract

T-type voltage-gated Ca^{2+} channels (CaV3.2 VGCC) have been hypothesized to control spontaneous transient outward currents (STOCs) through large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), and contribute to the negative-feedback regulation of myogenic tone. We tested this hypothesis in superior epigastric arteries (SEAs) isolated from male C57BL/6 mice. SEAs were isolated and enzymatically dissociated to obtain single smooth muscle cells (SMCs) for whole-cell recording of paxilline-sensitive (PAX, 1 μmol/L) STOCs at −30 mV, or cannulated and studied by pressure myography (80 cm H_2O, 37°C). The CaV3.2 blocker Ni^{2+} (30 μmol/L) had no effect on STOC amplitude (20.1 ± 1.7 pA vs. 20.6 ± 1.7 pA; n = 12, P = 0.6), but increased STOC frequency (0.79 ± 0.15 Hz vs. 1.21 ± 0.22 Hz; n = 12, P = 0.02). Although Ni^{2+} produced concentration-dependent constriction of isolated, pressurized SEAs ($logEC_{50}$ = −5.8 ± 0.09; E_{max} = 72 ± 5% constriction), block of BK_{Ca} with PAX had no effect on vasoconstriction induced by 30 μmol/L Ni^{2+} (in the absence of PAX = 66 ± 4% constriction vs. in the presence of 1 μmol/L PAX = 65 ± 4% constriction; n = 7, P = 0.06). In contrast to Ni^{2+}, the nonselective T-type blocker, mibefradil, produced only vasodilation ($logEC_{50}$ = −6.9 ± 0.2; E_{max} = 74 ± 8% dilation), whereas the putative T-type blocker, ML218, had no significant effect on myogenic tone between 10 nmol/L and 10 μmol/L (n = 6–7, P = 0.59). Our data do not support a role for CaV3.2 VGCC in the negative-feedback regulation of myogenic tone in murine SEAs and suggest that Ni^{2+} may constrict SEAs by means other than block of CaV3.2 VGCC.

Abbreviations

PHE, phenylephrine; SEA, superior epigastric artery; SMC, smooth muscle cell; STOC, spontaneous transient outward current; TEA, tetraethylammonium; VGCC, voltage-gated Ca^{2+} channels.

Introduction

Resistance arteries control blood pressure and tissue perfusion in the cardiovascular system (Davis et al. 2011). Multiple stimuli regulate tone within these vessels by altering cytosolic $[Ca^{2+}]_i$ and the activities of myosin light-chain kinase and myosin light-chain phosphatase (Davis et al. 2011; Kim et al. 2008). The increase in $[Ca^{2+}]_i$ is, in turn, closely tied to changes in membrane potential and the subsequent activation of voltage-gated Ca^{2+} channels (VGCC). In particular, it is well established that CaV1.2 VGCC provide a major source of activator Ca^{2+} during pressure-induced myogenic tone in resistance arteries and arterioles (Davis et al. 2011). Studies in rat cerebral arteries and mouse mesenteric arteries have suggested that CaV3.2 VGCC, in contrast to CaV1.2 VGCC, participate in the negative-feedback regulation of myogenic tone by modulating the frequency of ryanodine

receptor-generated Ca^{2+} sparks from the sarcoplasmic reticulum (Harraz et al. 2014, 2015). These Ca^{2+} sparks control the activity of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) in the plasma membrane of resistance artery smooth muscle cells (SMCs) in the form of spontaneous transient outward K^+ currents (STOCs) (Harraz et al. 2014, 2015; Nelson et al. 1995). STOCs hyperpolarize the membrane, close VGCC, and limit vasoconstriction (Nelson et al. 1995). However, it remains unknown if this CaV3.2 VGCC-BK_{Ca} negative-feedback mechanism is operational in resistance arteries from other vascular beds.

If the CaV3.2 VGCC-BK_{Ca} negative-feedback hypothesis operates in all vessels, then: (1) Ni^{2+}, a CaV3.2 VGCC blocker (Perez-Reyes et al. 2009), should decrease the frequency of STOCs in whole-cell patch clamp experiments on isolated SMCs; (2) Ni^{2+} should constrict isolated, pressurized arteries with an EC_{50} similar to that for block of CaV3.2 VGCC; (3) Ni^{2+}-induced vasoconstriction should be similar in magnitude to that produced by block of BK_{Ca} with paxilline (Knaus et al. 1994); and (4) Ni^{2+}-induced vasoconstriction should be inhibited by blocking BK_{Ca} channels with paxilline prior to exposure to Ni^{2+}. Therefore, we tested these predictions using SMCs isolated from superior epigastric arteries (SEAs), which are feed vessels for the abdominal musculature of the mouse, and intact SEAs studied by pressure myography. Our results indicate that while Ni^{2+} constricts isolated pressurized SEAs, Ni^{2+} does not inhibit STOCs, Ni^{2+}-induced constriction is greater in magnitude than that produced by block of BK_{Ca}, and Ni^{2+}-induced constriction is not prevented by blockade of BK_{Ca} channels. These data do not support the hypothesis that CaV3.2 VGCC participate in the negative-feedback regulation of myogenic tone in all resistance arteries. In addition, our data suggest that Ni^{2+} may produce vasoconstriction by a mechanism that does not involve block of CaV3.2 VGCC.

Materials and Methods

Preparation of SEAs and SMC isolation

The abdominal musculature containing SEAs was surgically removed from a euthanized mouse, placed in cold (4°C), Ca^{2+}-free physiological salt solution (PSS: 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4) containing 0.1% bovine serum albumin (# 10856 USB Corp., Cleveland, OH), sodium nitroprusside (10 μmol/L), and diltiazem (10 μmol/L) and SEAs were dissected from the abdominal muscles as described (Hayoz et al. 2014). Bovine serum albumin was included to reduce membrane protein digestion during enzymatic dissociation and to reduce vessel adhesion to surgical instruments, and isolated cells and vessels to glass surfaces. Sodium nitroprusside and diltiazem were included to maintain the SMCs in a relaxed state during the dissection and enzymatic dissociation process; their effects are readily reversible and without effect on subsequent reactivity and membrane currents as reported (Jackson et al. 1997). Isolated SEAs were either enzymatically dissociated to obtain single SMCs for patch clamp studies, or cannulated, intact, with glass micropipettes for pressure myography (Hayoz et al. 2014). For dissociation, SEAs were cut into ~800 μm segments, placed in 1 mL of dissociation solution (Ca^{2+}-free PSS plus 100 μmol/L $CaCl_2$, 10 μmol/L sodium nitroprusside, 10 μmol/L diltiazem, and 0.1% albumin) containing 1.24 mg/mL papain (P-4762 Sigma, St. Louis, MO) and 1 mg/mL dithioerythritol, and incubated at 37°C for 35 min. This solution was gently aspirated and replaced with 1 mL dissociation solution containing 1.5 mg/mL collagenase (C-8051 Sigma), 93 Units/mL elastase (#324682EMD Millipore: Calbiochem, San Diego, CA), and 1 mg/mL Type 1-S soybean trypsin inhibitor (T-9003, Sigma), and further incubated for 19 min at 37°C. This solution was aspirated and replaced with 4 mL of 4°C dissociation solution (to stop the enzymatic dissociation) and incubated for 10 min. This solution was then gently aspirated and replaced with 1 mL dissociation solution. This solution was gently triturated using a 1 mL Eppendorf-style pipette to release SMCs. The SMC-containing solution was stored at room temperature for up to 4 h.

Patch clamp methods

Whole-cell currents were recorded from isolated SMCs using the perforated patch technique as described (Hayoz et al. 2014). A 100 μL aliquot of SMC-containing solution was placed on the cover-slip bottom of a 500 μL recording chamber, allowed to settle (~5 min), and the chamber perfused with 5 mL of Ca^{2+}-PSS (PSS containing 1.8 mmol/L $CaCl_2$). Tips of patch pipettes (tip resistance: 3–4 MΩ) were filled with pipette solution (100 mmol/L K-aspartate, 43 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 1 mmol/L EGTA, 10 mmol/L glucose; pH 7 adjusted with NaOH, 295 mOsm) and back filled with pipette solution containing 120–180 μg/mL amphotericin. Gigaohm seals were made on single relaxed SMCs, and membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) controlled by a microcomputer running pClamp 10 software (Molecular Devices). After access resistance fell below 20 MΩ, cells were clamped at −30 mV (resting membrane potential of skeletal muscle resistance artery SMCs (Emerson and Segal 2000)), the chamber perfused

with Ca^{2+}-PSS, and STOCs were recorded. Membrane currents were filtered at 1 kHz, sampled at 5 kHz and recorded for 5 min. After control recordings, cells were exposed to solutions composed of Ca^{2+}-PSS-containing $NiCl_2$ (30 μmol/L hereafter referred to as Ni^{2+}), dimethysulfoxide (DMSO; 0.1%; vehicle for paxilline), paxilline (1 μmol/L), or Ni^{2+} and paxilline (30 μmol/L and 1 μmol/L, respectively) for 2 min prior to 5 min current recordings. The amplitude and frequency of STOCs were determined using Mini-analysis software (Synaptosoft, Decatur, GA). The 5 min recordings were low-pass filtered at 200 Hz using a Butterworth filter, and events with amplitudes greater than or equal to 10–15 pA measured, dependent on the high-frequency baseline noise levels of the recordings. Frequencies were computed as the total number of events during the 5 min recording divided by 5 min. Inhibition of STOCs by the BK_{Ca} blocker, paxilline (Knaus et al. 1994), was used to verify the identity of the events quantified as STOCs.

Pressure myography

Isolated, intact SEAs or second- to third-order mesenteric resistance arteries (MRAs) were cannulated with glass micropipettes (outer diameters ~100 μm), tied in place with 10–0 nylon suture (THR-G, Living Systems Instrumentation, Burlington, VT), superfused with Ca^{2+}-PSS (PSS composition as above), pressurized to 80 cm H_2O at 37°C, and allowed to develop myogenic tone as described (Hayoz et al. 2014). Vessels were studied with no flow through their lumens and were tested for leaks (undetected branches or around cannulating pipettes) at 80 cm H_2O prior to warming and superfusion with Ca^{2+}-PSS. Vessels with leaks were discarded and not studied. Prior to exposure to $NiCl_2$, paxilline, or other drugs, SEAs were exposed to 10 μmol/L phenylephrine, as a test for viability. Vessels were imaged with a CCD camera (DMK 41AU02; The Imaging Source, LLC; Charlotte, NC) coupled to an inverted microscope and vessel internal diameters were recorded and measured using MyoView software (DMT-USA, Inc., Ann Arbor, MI).

Diameter calculations

For each vessel, myogenic tone was calculated as: $[(D_{max} - D_{rest})/D_{max} \times 100\%]$, where D_{max} = maximum diameter of vessels exposed to 0 Ca^{2+} PSS at room temperature and D_{rest} = resting diameter of vessels after pressurization to 80 cm H_2O and development of stable myogenic tone. Vasodilation was expressed relative to D_{max} as: $(D_{drug} - D_{rest})/(D_{max} - D_{rest})$, where D_{drug} is the diameter observed during superfusion with a drug. Vasoconstriction induced by exposure of vessels to solution-containing Ni^{2+} was calculated as: $((D_{Ni2+} - D_{rest})/D_{rest})$, where D_{Ni2+} is the minimum diameter of the vessel in the presence of Ni^{2+} or paxilline + Ni^{2+}. Similar calculations were performed for constriction induced by paxilline or tetraethylammonium (TEA).

Data analysis and statistics

Summary data are expressed as means ± standard error (SE). Statistical significance was determined using Student's paired and unpaired *t*-tests, analysis of variance, or nonparametric Kruskal–Wallis test (due to nonhomogeneity of variances) using Prism 6.0 Software (Graphpad Software; San Diego, CA). Prism 6.0 also was used to perform nonlinear regression analyses to estimate EC_{50} and E_{max} values for Ni^{2+}, mibefradil, and ML218. For Ni^{2+}, the equation used was Relative Constriction = $E_{max} - E_{max}/(1 + 10^{((LogEC50 - Log\ [Ni2+])*HillSlope)})$, where Emax is the maximum relative constriction induced by Ni^{2+}, EC_{50} = concentration of Ni^{2+} that produced 50% of the maximal constriction, and the Hill-slope = the slope of the curve at the EC_{50} value. For mibefradil, the equation used was Relative Dilation = $E_{max}/(1 + 10^{((LogEC50 - [mibefradil])*HillSlope)})$, where E_{max} = maximum relative dilation produced by mibefradil, EC_{50} = concentration that produced 50% of the maximal dilation, and the Hill-slope as described above. The number of observations refers to the number of SMCs or SEA preparations studied from at least 3 mice, with no more than 4 SMCs or 2 SEAs studied from a given mouse. A value of $P < 0.05$ was considered statistically significant.

Results

Paxilline inhibits STOCs

As we have reported previously (Hayoz et al. 2014), isolated SMCs from murine SEAs display STOCs at −30 mV (Fig. 1A). These STOCs arise from Ca^{2+} spark-dependent activation of BK_{Ca}, verified here by application of the BK_{Ca} antagonist, paxilline (1 μmol/L) (Knaus et al. 1994), which reduced STOC frequency to a value not significantly different from 0 ($P = 0.87$) in SMCs of SEAs at −30 mV holding potential (Fig. 1A, B, and C).

Ni^{2+} does not inhibit STOCs in murine SEAs at −30 mV

In contrast to what has been reported in rat cerebral and mouse mesenteric arteries (Harraz et al. 2015; Harraz and Welsh 2013), we found that the CaV3.2 VGCC blocker, Ni^{2+}, did not inhibit STOCs (Figs. 1D, E, and F). Instead, application of Ni^{2+} (30 μmol/L) increased STOC

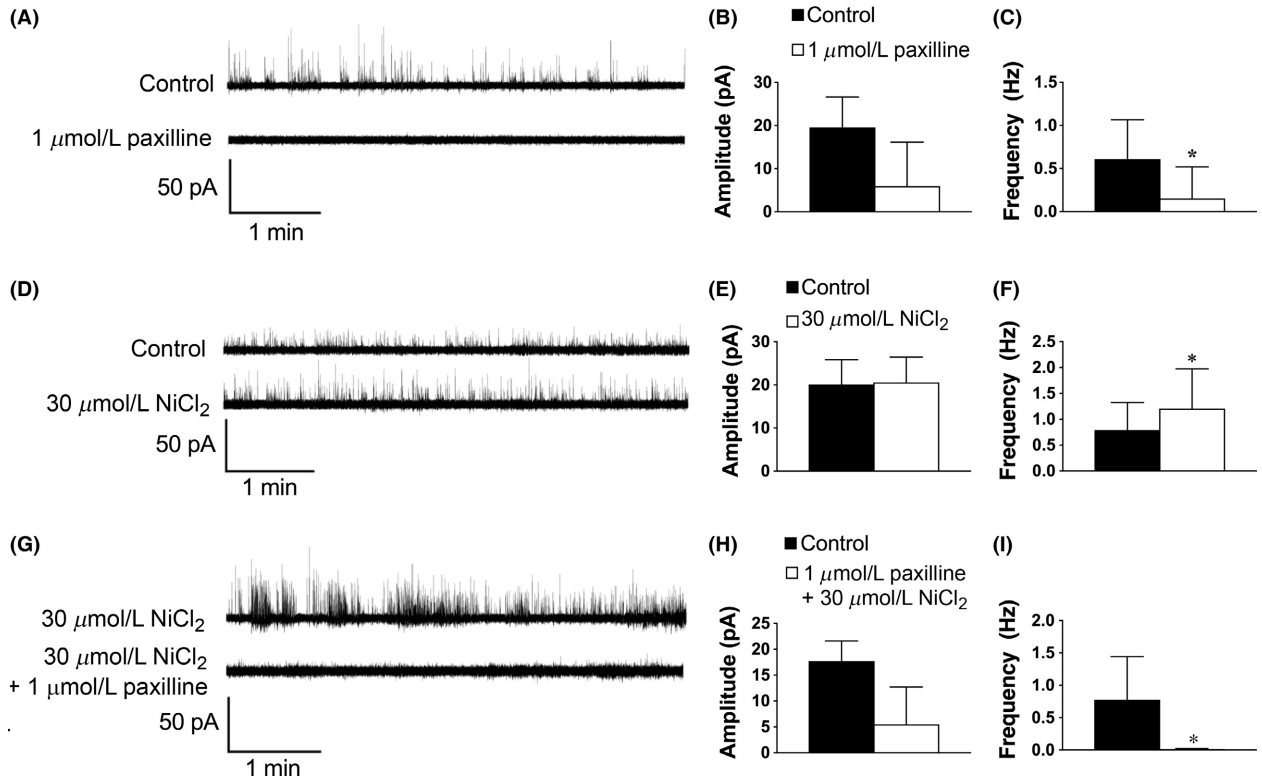

Figure 1. Paxilline reduces, whereas Ni^{2+} increases STOC frequency in SMCs of SEAs. (A) Typical 5 min whole-cell recording showing inhibition of STOCs by paxilline (1 μmol/L). (B and C) Summary data showing means ± SE (n = 6) for the amplitude (B) and frequency (C) of STOCs before (Control) and in the presence of paxilline, as indicated. (D) Typical 5 min whole-cell recording showing that 30 μmol/L Ni^{2+} increases the frequency of STOCs. (E and F) Summary data showing means ± SE (n = 12) for the amplitude (E) and frequency (F) of STOCs before and in the presence of Ni^{2+}. In pilot studies, we found that superfusion of cells with Ca^{2+}-PSS (the vehicle for Ni^{2+}) had no significant effect on the amplitude (amplitude after second application of Ca^{2+}-PSS = 103.4 ± 4.3% of amplitude after first application of PSS, n = 12; P = 0.43 vs. 100%) or frequency (frequency after second application of Ca^{2+}-PSS = 106.7 ± 12.5% of the frequency after the first application of Ca^{2+}-PSS, n = 12; P = 0.6 vs. 100%). These data suggest that the small, consistent increase in STOC frequency that we observed in the presence of Ni^{+} was not a vehicle effect or time-dependent run-up of STOC amplitude. (G) Typical 5 min whole-cell recording showing that 1 μmol/L paxilline effectively abolishes STOCs recorded in the presence of 30 μmol/L Ni^{2+}. (H and I) Summary data showing means ± SE (n = 5) for the amplitude (H) and frequency (I) of STOCs in the presence of Ni^{2+} and in the presence of Ni^{2+} + paxilline. *P < 0.05 by paired t-tests.

frequency, with no significant effect on STOC amplitude in SMCs of SEAs at −30 mV holding potential (Figs. 1D, E, and F). Paxilline (1 μmol/L) abolished STOCs that were recorded in the presence of Ni^{2+} (30 μmol/L) (Figs. 1G, H, and I), demonstrating that in the presence of Ni^{2+}, STOCs still arise from BK_{Ca}.

Ni^{2+} constricts isolated, pressurized SEAs

In this study, SEAs developed myogenic tone when pressurized to 80 cm H_2O; for all vessels studied, resting diameters were 156 ± 3.9 μm, maximal diameters were 189 ± 1.6 μm yielding 18 ± 2% myogenic tone (n = 44; $P = 5.4 \times 10^{-13}$ vs. 0% tone by t-test). However, there was considerable variability in the magnitude of pressure-induced tone that ranged from 2 to 47%. Consistent with previous studies in other vessels (Harraz et al. 2015; Harraz and Welsh 2013), Ni^{2+} produced concentration-dependent vasoconstriction of pressurized SEAs (Fig. 2). However, in these cumulative concentration response experiments, the magnitude of the maximal effect of Ni^{2+} appeared to depend on the magnitude of the baseline SMC tone: vessels with little tone had a blunted maximal response (Fig. 2), whereas vessels with substantial myogenic tone or tone induced by exposure to phenylephrine (1–3 μmol/L) showed similar large maximal responses to Ni^{2+} (Fig. 2). The reduced magnitude of Ni^{2+}-induced constriction in SEAs with low myogenic tone did not appear to be due to a generalized loss of reactivity because constriction to 10 μmol/L phenylephrine (PHE) was similar in vessels that did not develop significant myogenic tone (28 ± 4% constriction to PHE, n = 7) to

PHE-induced tone in vessels that developed significant myogenic tone (40 ± 3% constriction to PHE, $n = 4$; $P = 0.37$ vs. vessels with no tone, unpaired t-test). Regardless of the magnitude of the maximal response to Ni^{2+}, no significant differences in the EC_{50} values for Ni^{2+} were detected between the three groups of vessels that were studied (Fig. 2).

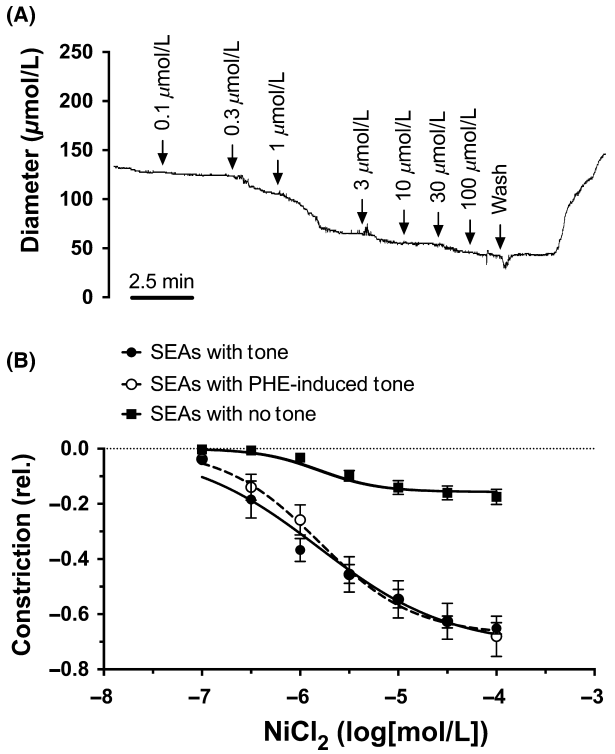

Figure 2. Ni^{2+} induces concentration-dependent constriction of SEAs. (A) Typical recording of a pressurized SEA showing concentration-dependent Ni^{2+}-induced vasoconstriction. This SEA had 40% myogenic tone with a maximum diameter = 192 μm. (B) Summary means ± SE for groups of vessels that developed 30 ± 3% myogenic tone (SEAs with Tone; $n = 5$ except for 0.1 and 0.3 μmol/L concentrations where $n = 3$), vessels that did not develop myogenic tone, but were preconstricted with phenylephrine (PHE; 1–3 μmol/L; 23 ± 5% PHE-induced tone; SEAs with PHE-induced Tone; $n = 7$), and vessels that did not develop significant myogenic tone (5 ± 1% myogenic tone; SEAs with no Tone; $n = 7$ except for 0.1 and 0.3 μmol/L concentrations where $n = 1$). Nonlinear curve fitting (see Methods) revealed the following: for SEAs with Tone, $E_{max} = -0.66 \pm 0.03$; Log $EC_{50} = -6 \pm 0.1$ (1 μmol/L); Hill-slope = 0.81 ± 0.15; for SEAs with PHE-induced Tone, $E_{max} = -0.68 \pm 0.06$; Log $EC_{50} = -5.8 \pm 0.17$ (1.7 μmol/L); Hill-slope = 0.87 ± 0.23; and for SEAs with no Tone, $E_{max} = -0.17 \pm 0.02$; Log $EC_{50} = -5.6 \pm 0.17$ (2.5 μmol/L); Hill-slope = −1.3 ± 0.6. The EC_{50} values for the three groups were not significantly different from one another ($P = 0.4$). However, the E_{max} value for SEAs with no Tone was significantly less than the other two groups ($P < 0.0001$ for each comparison), whereas the E_{max} value for SEAs with Tone was not different from the E_{max} value for SEAs with PHE-induced Tone ($P = 0.92$) by ANOVA and subsequent multiple comparison of means with Tukey's test.

L-type Ca^{2+}-mediate Ni^{2+}-induced constriction

Nickel-induced constriction of SEAs could be prevented (Fig. 3) or reversed by the L-type Ca^{2+} channel blocker, nifedipine (1 μmol/L) (diameter in 100 μmol/L Ni^{2+} = 125 ± 21 μm vs. diameter in 100 μmol/L Ni^{2+} + 1 μmol/L nifedipine = 173 ± 7 μm; $n = 7$, $P = 0.018$). These data suggest that CaV1.2 VGCC mediate the constriction induced by exposure of SEAs to Ni^{2+}.

Paxilline or TEA do not prevent Ni^{2+}-induced vasoconstriction

In vessels that developed only a minor degree of myogenic tone (9 ± 3%, $n = 6$), the BK_{Ca} blocker, paxilline (1 μmol/L) (Knaus et al. 1994), had little effect on resting diameter indicating that there was low activity of BK_{Ca} in

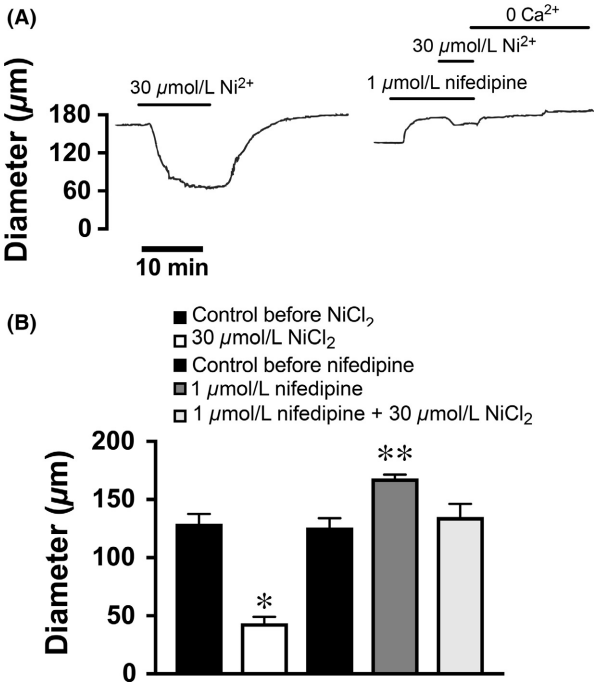

Figure 3. Nifedipine inhibits Ni^{2+}-induced constriction of SEAs. Panel A shows a representative tracing of the response of a SEA to 30 μmol/L Ni^{2+} before (left panel) and in the presence of the CaV1.2 blocker, nifedipine (1 μmol/L; right panel). The break between the traces represents ~30 min interval after washout of Ni^{2+}. Nifedipine substantially inhibited constriction of the SEA by Ni^{2+}. Panel B shows summary mean diameters ± SE ($n = 7$). As in Panel A, nifedipine substantially reduced the magnitude of the Ni^{2+}-induced constriction such that it did not attain statistical significance ($P = 0.18$). *Significantly different from Control before Ni^{2+} ($P = 0.0004$); **Significantly different from Control before nifedipine ($P = 0.015$). Data analyzed by repeated measures ANOVA with subsequent multiple comparison of means using Tukey's test.

these preparations (Figs 4A and B). If Ni^{2+} were producing constriction by inhibiting the activity of BK_{Ca}, then Ni^{2+} should produce constriction similar in magnitude to that produced by paxilline. Instead, we found that Ni^{2+} (30 μmol/L), at a concentration that selectively blocks CaV3.2 VGCC (Harraz et al. 2014, 2015), produced a much larger constriction (Fig. 4A and B). Furthermore, in the presence of paxilline, the constriction induced by

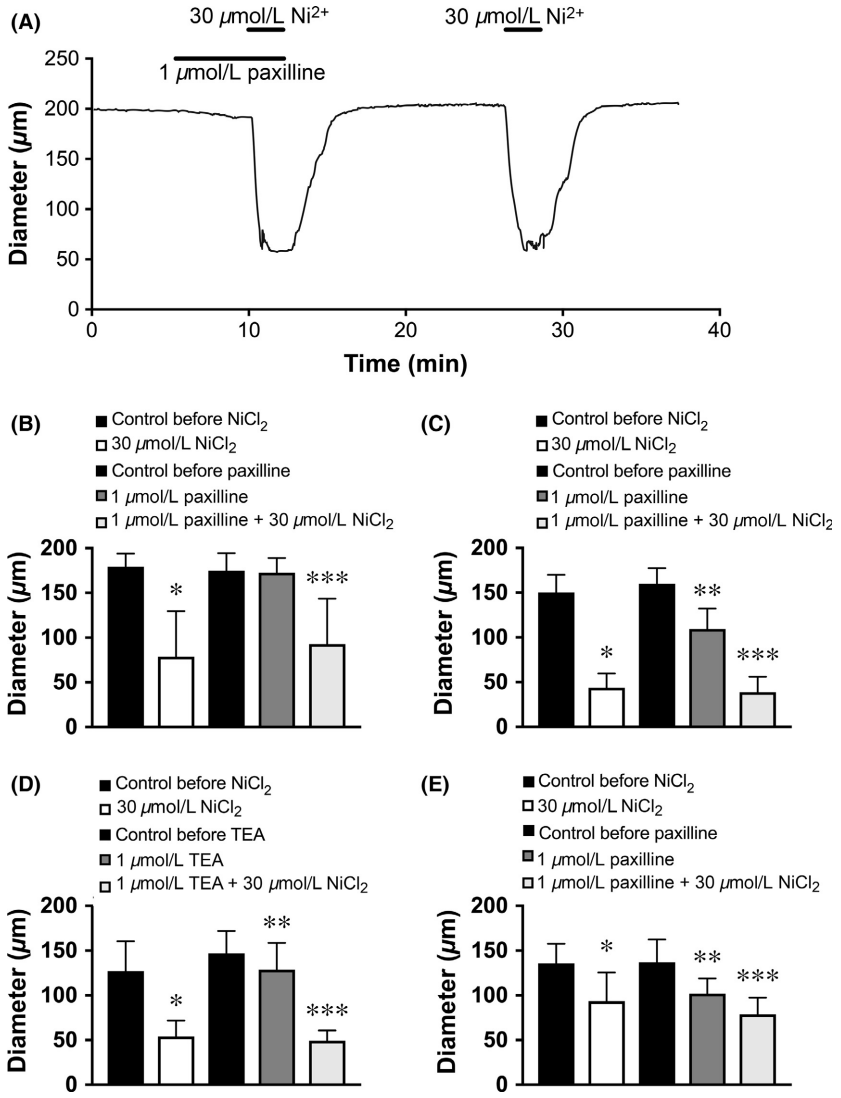

Figure 4. Paxilline does not block Ni^{2+}-induced constriction. (A) Typical recording of a pressurized SEA with little myogenic tone, illustrating that the magnitude of Ni^{2+}-induced constriction is greater than that produced by paxilline, and that paxilline does not prevent Ni^{2+}-induced vasoconstriction. (B) Summary mean diameters ± SE (n = 6) for Ni^{2+}-induced constriction in the absence and presence of 1 μmol/L paxilline in SEAs with little myogenic tone. In this series of experiments, the order of treatments was randomized such that in three experiments, SEAs were exposed to paxilline first as shown in Panel A. Due to concerns about the lack of reversibility of the effects of paxilline, subsequent experiments (Panels C–E) were not randomized. Paxilline produced 3.3 ± 1.4% constriction (n = 6; P = 0.07 vs. 0% constriction by t-test), in contrast, Ni^{2+} produced 55 ± 11% constriction, n = 6, P = 0.007 by paired t-test vs. paxilline-induced constriction). In the presence of paxilline, Ni^{2+} still produced 47 ± 11% constriction (P = 0.39 vs. prepaxilline response). (C) Summary mean diameters ± SE (n = 7) for Ni^{2+}-induced constriction in the absence and presence of 1 μmol/L paxilline in SEAs with 18 ± 3% myogenic tone. D: Summary mean diameters ± SE (n = 6) for Ni^{2+}-induced constriction in the absence and presence of 1 mmol/L TEA in SEAs with 24 ± 5% myogenic tone. (E) Summary mean diameters ± SE (n = 6) for Ni^{2+}-induced constriction in the absence and presence of 1 μmol/L paxilline in MRAs with 14 ± 3% myogenic tone. *Significantly different from Control before $NiCl_2$; **Significantly different from Control before paxilline or TEA, and significantly different from $NiCl_2$; ***significantly different from paxilline or TEA + $NiCl_2$, but not significantly different from $NiCl_2$. All $P < 0.05$ as assessed by repeated measures ANOVA followed by Tukey's test for multiple comparison of means.

Ni^{2+} (30 μmol/L) was unabated (Fig. 4A and B). Despite the low level of tone in this group of SEAs, 30 μmol/L Ni^{2+} produced constriction greater than predicted from the cumulative concentration response experiments displayed in Figure 2B. We have no explanation for this difference in reactivity: myogenic tone (9 $\pm$ 3% vs. 6 $\pm$ 1%, $P = 0.32$, by separate variance *t*-test) and PHE-induced constriction (27 $\pm$ 5% constriction vs. 28 $\pm$ 4% constriction, $P = 0.84$, by *t*-test) were similar between the two groups.

In vessels that developed higher levels of myogenic tone (18 $\pm$ 3%; $n = 7$), paxilline produced 31 $\pm$ 4% constriction ($n = 7$; Fig. 4C). However, Ni^{2+} again produced constrictions of greater magnitude before (70 $\pm$ 4%) and in the presence of paxilline (65 $\pm$ 4%) (Fig. 4C). Similar results were obtained using another BK_{ca} blocker, TEA (1 mmol/L) (Hayoz et al. 2014) (Fig. 4D): Ni^{2+}-induced constrictions were larger than those produced by TEA, in the absence or presence of this BK_{ca} blocker. Thus, consistent with our patch clamp data (Figs. 1D, E and F), the data presented in Fig. 4A–D do not support the hypothesis that the constriction of SEAs induced by Ni^{2+} is due to inhibition of BK_{Ca}-dependent STOCs.

Our results (Fig. 1 and 4A–D) deviate from those reported in other vessels (Harraz et al. 2014, 2015). Therefore, we also assessed the effects of paxilline and Ni^{2+} on mouse MRAs (Fig. 4E). In our hands, these vessels developed myogenic tone when pressurized to 80 cm H_2O (14 $\pm$ 3%; $n = 7$; $P = 0.005$ vs. 0%). However, in contrast to our observations in murine SEAs, we found that Ni^{2+} produced constriction of these vessels (32 $\pm$ 6%; $n = 7$) that was comparable to that produced by paxilline (25 $\pm$ 2%; $n = 7$; $P = 0.31$ vs. constriction to Ni^{2+} by paired *t*-test) (Fig. 4E). In MRAs, Ni^{2+} still produced additional constriction in the presence of paxilline (Fig. 4E), but this response was attenuated relative to that which we observed in SEAs (Fig. 4A–D).

Mibefradil only dilates murine SEAs

We also examined the effects of the putative CaV3.X VGCC blocker, mibefradil (Perez-Reyes et al. 2009). In contrast to Ni^{2+}, mibefradil only dilated pressurized SEAs (Fig. 5), with a $\log EC_{50} = -6.87 \pm 0.18$. The IC_{50} for block of CaV3.X VGCC is reported to be 0.25 μmol/L (Log $(0.25 \times 10^{-6}$ mol/L) $= -6.6$) (Perez-Reyes et al. 2009).

Lack of effect of the T-type Ca^{2+} channel blocker, ML218, on murine SEAs

Given the difference in activity between Ni^{2+} (Figs. 2 and 4) and mibefradil (Fig. 5), we also assessed the effects of another small molecule blocker of CaV3.2 VGCC, ML218 (Xiang et al. 2011). In contrast to Ni^{2+} or mibefradil, we found that over the range of concentrations where ML218 should block CaV 3.2 VGCC (IC_{50} = 10 nmol/L) (Xiang et al. 2011), ML 218 had no significant effect on myogenic tone (Fig. 6): in SEAs with control diameters of 174 $\pm$ 8 μm (9 $\pm$ 3% myogenic tone), diameters in 10 μmol/L ML 218 were 169 $\pm$ 9 μm ($n = 6$, $P = 0.30$ by paired test). In contrast, in this same group of SEAs, 30 μmol/L Ni^{2+} constricted the vessels to 99 $\pm$ 21 μm (45 $\pm$ 10% constriction, $n = 6$, $P = 0.02$ by paired *t*-test).

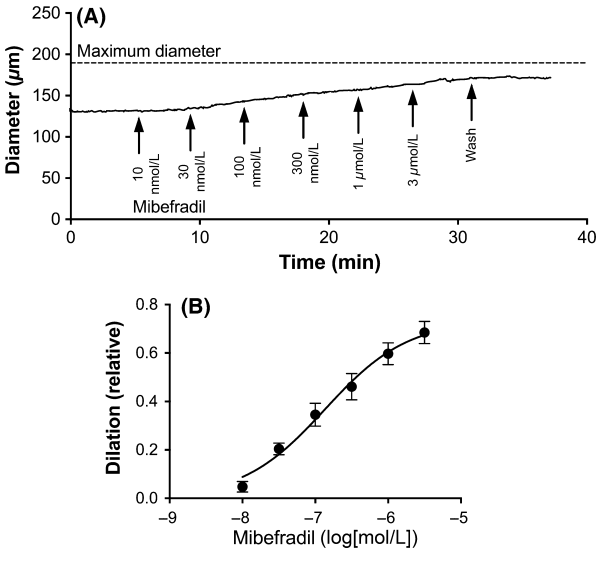

Figure 5. Mibefradil only dilates SEAs. (A) Typical recording of a pressurized SEA showing that mibefradil induces vasodilation in a concentration-dependent manor. (B) Summary means $\pm$ SE ($n = 7$) for mibefradil-induced dilation in SEAs with 19 $\pm$ 4% myogenic tone. $E_{max} = 0.74 \pm 0.08$; Log $EC_{50} = -6.9 \pm 0.7$ (0.13 μmol/L); Hill-slope = 0.8 $\pm$ 0.2 by nonlinear curve fitting (See Methods).

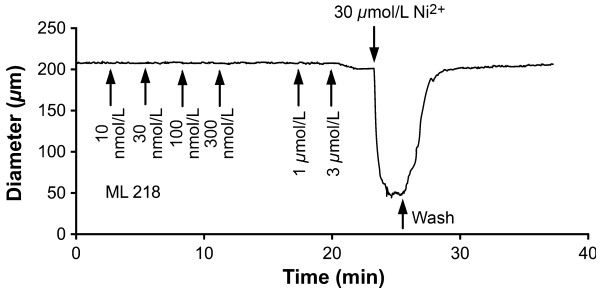

Figure 6. Lack of effect of ML 218 on the diameter of SEAs. Typical recording of a pressurized SEA showing that cumulative addition of ML 218 has no significant effect on myogenic tone over a concentration range where it should block CaV3.2 VGCC, whereas Ni^{2+} produced robust vasoconstriction in the same vessel.

Discussion

The major findings of this study are that: (1) Ni^{2+} (30 μmol/L), at a concentration that should selectively block CaV3.2 VGCC (Harraz et al. 2014, 2015; Kang et al. 2006), increased rather than decreased the frequency of STOCs (Figs. 1D, E, and F); (2) Ni^{2+} produced vasoconstriction that was substantially larger than constriction induced by block of BK_{Ca} channels with paxilline or TEA; (3) Ni^{2+} produced robust vasoconstriction in the presence of paxilline; and (4) the selective CaV3.2 blocker, ML 218, had no effect on SEA myogenic tone in vessels that constricted when exposed to 30 μmol/L Ni^{2+}. Taken together, these data do not support the hypothesis that CaV3.2 VGCC are involved in the negative-feedback regulation of myogenic tone through control of the activity of BK_{Ca} channels in murine SEAs. Our data also indicate that the vasoconstriction induced by micromolar concentrations of Ni^{2+} likely does not involve inhibition of CaV3.2 VGCC in murine SEAs.

We do not know the mechanism by which Ni^{2+} increased STOC frequency. However, we could inhibit or reverse constriction induced by Ni^{2+} with the CaV1.2 blocker, nifedipine (Fig. 3). Activation of CaV1.2 VGCC is known to increase STOC frequency in other systems (Jaggar et al. 1998). Thus, it is possible that the Ni^{2+}-induced stimulation of STOCs that we observed resulted from activation of CaV1.2 VGCC in SEA SMCs. This is unlikely to be a direct effect because Ni^{2+} at higher concentrations is known to block CaV1.2 VGCC (Fox et al. 1987). Millimolar concentrations of Ni^{2+} are known to activate TRPV1 channels in neurons and expression systems (Luebbert et al. 2010). Activation of these channels in SEA SMCs could increase Ca^{2+} influx resulting in increased Ca^{2+} uptake into intracellular Ca^{2+} stores, which is known to increase the frequency of Ca^{2+} sparks and STOCs (Essin et al. 2007). Additional research will be required to define the mechanism by which Ni^{2+} increased the frequency of STOCs in murine SEAs. Regardless, the finding that Ni^{2+} did not inhibit STOCs at −30 mV does not support the hypothesis that CaV3.2 VGCC are involved in the negative-feedback regulation of myogenic tone in murine SEAs. Paxilline eliminated STOCs in the presence of Ni^{2+} (Figs. 1G, H and I). These data confirm that STOCs observed in the presence of Ni^{2+} originated from BK_{Ca}, and that the concentration of paxilline (1 μmol/L) used was effective.

Contrary to our findings, Harraz et al. (2014, 2015) reported that 30 μmol/L Ni^{2+} significantly reduced the frequency of STOCs recorded from both cerebral (Harraz et al. 2014) and mesenteric artery (Harraz et al. 2015) SMCs when the cells were held at −40 mV, with no effect observed when SMCs were held at −20 mV. We studied SMCs held at −30 mV because this more depolarized potential has been reported for skeletal muscle feed artery SMCs studied under conditions similar to those used in the present pressure myography study (Emerson and Segal 2000). Thus, it is possible that the use of this more depolarized potential might explain the lack of inhibition of STOC frequency that we observed in this study. However, given that skeletal muscle feed arteries, like SEAs, appear to be more depolarized under physiological conditions (Emerson and Segal 2000), our findings suggest that the regulation of STOCs by CaV3.2 VGCC may not be universally applicable.

The EC_{50} for constriction of SEAs by Ni^{2+} (1–2.5 μmol/L; Fig. 2) is consistent with block of CaV3.2 VGCC (4.5 μmol/L; (Kang et al. 2006)). However, the magnitude of the constriction induced by a maximally effective concentration of Ni^{2+} (30 μmol/L) was greater than the constriction produced by a maximally effective concentration of the BK_{Ca} blocker, paxilline (1 μmol/L) (Fig. 4). In addition, neither paxilline nor TEA prevented constriction induced by Ni^{2+} (Fig. 4). Consistent with our patch clamp observations (Figs. 1D, E and F), these data do not support the hypothesis that CaV3.2 VGCC contribute to the negative feedback regulation of myogenic tone in murine SEAs through modulation of BK_{Ca} STOCs. Our observations do not agree with the findings of Harraz et al. (2014, 2015), and suggest that there are regional differences in the function of CaV3.2 VGCC and control of BK_{Ca} activity. Our data also indicate that a large portion of Ni^{2+}-induced constriction in murine SEAs involves a mechanism that is independent from inhibition of CaV3.2-BK_{Ca} negative feedback.

As a test of the regional heterogeneity hypothesis, and to see if we could replicate the findings of (Harraz et al. 2015), we also performed pressure myography studies on mouse MRAs under the same conditions as for SEAs. Consistent with the CaV3.2 negative-feedback hypothesis and the findings of Harraz et al. (2015), we found that 30 μmol/L Ni^{2+} produced constriction that was similar in magnitude to that produced by paxilline (1 μmol/L). However, in our hands, in the presence of a concentration of paxilline that should maximally inhibit BK_{Ca}, Ni^{2+} (30 μmol/L) still produced significant constriction. This observation suggests that even in mouse MRAs, in addition to inhibition of CaV3.2-BK_{Ca} negative feedback, Ni^{2+} likely produces vasoconstriction by an additional mechanism.

In contrast to Ni^{2+}, mibefradil induced only vasodilation (Fig. 3), whereas ML218 had no significant effect on myogenic tone (Fig. 4) over concentration ranges where they should selectively block CaV3.2 VGCC (Perez-Reyes et al. 2009; Xiang et al. 2011). Whereas mibefradil has been shown to block CaV3.2 VGCC in studies with

cloned channels (Perez-Reyes et al. 2009), this drug has no effect on vascular tone in CaV1.2 VGCC knockout mice (Moosmang et al. 2006). Thus, it seems likely that the vasodilation induced by mibefradil in this study (Fig. 4) arose from block of CaV1.2 VGCC, which are essential for myogenic tone (Davis et al. 2011). The complete lack of effect of ML218 was surprising, and may indicate that CaV3.2 VGCC are not functional in SEAs under the conditions of this study. Regardless, the data suggest that the Ni^{2+}-induced vasoconstriction may not involve effects on CaV3.2 VGCC. We found that Ni^{2+}-induced vasoconstriction could be prevented or reversed by nifedipine (1 μmol/L; Fig. 3). This observation indicates that CaV1.2 VGCC are involved in the mechanism of action of Ni^{2+}. Thus, either Ni^{2+} is causing membrane depolarization (or activating CaV1.2 VGCC by some other unknown means) or Ni^{2+} is entering SMCs via CaV1.2 VGCC to produce smooth muscle contraction. It is known that Ni^{2+} cannot substitute for Ca^{2+} to activate myosin light-chain kinase and stimulate smooth muscle contraction (Evans et al. 1993), so a membrane delimited effect would seem most probable. Our finding of a major role for CaV1.2 VGCC in the mechanism of action of Ni^{2+} is supported by earlier studies in the dog coronary circulation, where the CaV1.2 blocker, verapamil, was shown to inhibit Ni^{2+}-induced vasoconstriction (Koller et al. 1982). However, Ni^{2+}-induced contraction of dog coronary artery strips was not inhibited by verapamil, in vitro (Rubanyi et al. 1982). Additional research will be required to identify the precise mechanism by which extracellular Ni^{2+} induces vasoconstriction of isolated murine SEAs.

Limitations

We did not directly measure the activity of CaV3.2 in SEA SMCs, and only infer their role (or lack thereof) through the use of Ni^{2+} at concentrations known to block CaV3.2 VGCC. We do know that mRNA for both CaV1.2 and CaV3.2 are expressed in murine SEAs (Stein, Kelly and Jackson, unpublished observations), but we have not directly measured currents through CaV3.2 VGCC in SEA SMCs.

Second, although we did demonstrate that Ni^{2+} caused concentration-dependent constriction, we only studied a single concentration of Ni^{2+} in the presence of paxilline and TEA. We cannot exclude subtle effects of BK_{Ca} channel blockade on the Ni^{2+}-induced constriction.

Finally, we only studied the effects of Ni^{2+} on STOCs at a single membrane potential (−30 mV). Thus, we cannot exclude a positive role for CaV3.2 in the regulation of BK_{Ca} at more negative membrane potentials. Additional research will be required to resolve these limitations.

In summary, our data do not support a role for CaV3.2 VGCC in the negative-feedback regulation of myogenic tone in murine SEAs. These findings indicate that there are regional differences in the function of CaV3.2 VGCC. Our data also suggest that vasoconstriction induced by micromolar concentrations of Ni^{2+} may not involve inhibition of CaV3.2 VGCC. Thus, caution is urged in the interpretation of data from the effects of this metal ion on vascular SMC contractile activity.

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Disclosures

None.

References

Davis MJ, Hill MA, Kuo L (2011). Local Regulation of Microvascular Perfusion. Pp 161–284 in Comprehensive Physiology. John Wiley & Sons, Inc., Hoboken, New Jersey.

Emerson GG, Segal SS (2000). Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: role in vasomotor control. Circ Res 87: 474–479.

Essin K, Welling A, Hofmann F, Luft FC, Gollasch M, Moosmang S (2007). Indirect coupling between Cav1.2 channels and ryanodine receptors to generate Ca^{2+} sparks in murine arterial smooth muscle cells. J Physiol 584: 205–219.

Evans DH, Chipouras E, Toop T, Donald JA (1993). The effect of Ca^{2+}, Cd^{2+} and Ni^{2+} on detergent-permeabilized vascular smooth muscle from the shark, Squalus acanthias. Toxicology 83: 1–8.

Fox AP, Nowycky MC, Tsien RW (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. J Physiol 394: 149–172.

Harraz OF, Welsh DG (2013). T-type Ca(2)(+) channels in cerebral arteries: approaches, hypotheses, and speculation. Microcirculation 20: 299–306.

Harraz OF, Abd El-Rahman RR, Bigdely-Shamloo K, Wilson SM, Brett SE, Romero M, et al. (2014). CaV3.2 Channels and the Induction of Negative Feedback in Cerebral Arteries. Circ Res 115: 650–661.

Harraz OF, Brett SE, Zechariah A, Romero M, Puglisi JL, Wilson SM, et al. (2015). Genetic Ablation of CaV3.2 Channels Enhances the Arterial Myogenic Response by

Modulating the RyR-BKCa Axis. Arterioscler Thromb Vasc Biol 35: 1843–1851.

Hayoz S, Bradley V, Boerman EM, Nourian Z, Segal SS, Jackson WF (2014). Aging increases capacitance and spontaneous transient outward current amplitude of smooth muscle cells from murine superior epigastric arteries. Am J Physiol Heart Circ Physiol 306: H1512–H1524.

Jackson WF, Huebner JM, Rusch NJ (1997). Enzymatic isolation and characterization of single vascular smooth muscle cells from cremasteric arterioles. Microcirculation 4: 35–50.

Jaggar JH, Stevenson AS, Nelson MT (1998). Voltage dependence of Ca^{2+} sparks in intact cerebral arteries. Am J Physiol 274: C1755–C1761.

Kang HW, Park JY, Jeong SW, Kim JA, Moon HJ, Perez-Reyes E, et al. (2006). A molecular determinant of nickel inhibition in CaV3.2 T-type calcium channels. J Biol Chem 281: 4823–4830.

Kim HR, Appel S, Vetterkind S, Gangopadhyay SS, Morgan KG (2008). Smooth muscle signalling pathways in health and disease. J Cell Mol Med 12: 2165–2180.

Knaus HG, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LM, et al. (1994). Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. Biochemistry 33: 5819–5828.

Koller A, Rubanyi G, Ligeti L, Kovach AG (1982). Effect of verapamil and phenoxybenzamine on nickel-induced coronary vasoconstriction in the anaesthetized dog. Acta Physiol Acad Sci Hung 59: 287–290.

Luebbert M, Radtke D, Wodarski R, Damann N, Hatt H, Wetzel CH (2010). Direct activation of transient receptor potential V1 by nickel ions. Pflugers Arch 459: 737–750.

Moosmang S, Haider N, Bruderl B, Welling A, Hofmann F (2006). Antihypertensive effects of the putative T-type calcium channel antagonist mibefradil are mediated by the L-type calcium channel Cav1.2. Circ Res 98: 105–110.

National Research Council (U.S.), Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.), & National Academies Press (U.S.) (2011). Guide for the care and use of laboratory animals. 8th ed. National Academies Press, Washington, D.C.

Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ, et al. (1995). Relaxation of arterial smooth muscle by calcium sparks. Science 270: 633–637.

Perez-Reyes E, Van Deusen AL, Vitko I (2009). Molecular pharmacology of human Cav3.2 T-type Ca2 + channels: block by antihypertensives, antiarrhythmics, and their analogs. J Pharmacol Exp Ther 328: 621–627.

Rubanyi G, Kalabay L, Pataki T, Hajdu K (1982). Nickel induces vasoconstriction in the isolated canine coronary artery by a tonic Ca2 + -activation mechanism. Acta Physiol Acad Sci Hung 59: 155–159.

Xiang Z, Thompson AD, Brogan JT, Schulte ML, Melancon BJ, Mi D, et al. (2011). The discovery and characterization of ML218: a novel, centrally active t-type calcium channel inhibitor with robust effects in STN neurons and in a rodent model of Parkinson's Disease. ACS Chem Neurosci 2: 730–742.