Myogenic tone is impaired at low arterial pressure in mice deficient in the low-voltage-activated Cav3.1 T-type Ca2+ channel


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Myogenic tone is impaired at low arterial pressure in mice deficient in the low-voltage-activated \( \text{Ca}_V3.1 \) T-type \( \text{Ca}^{2+} \) channel

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Abstract

Aim: Using mice deficient in the \( \text{Ca}_V3.1 \) T-type \( \text{Ca}^{2+} \) channel, the aim of the present study was to elucidate the molecular identity of non-L-type channels involved in vascular tone regulation in mesenteric arteries and arterioles.

Methods: We used immunofluorescence microscopy to localize \( \text{Ca}_V3.1 \) channels, patch clamp electrophysiology to test the effects of a putative T-type channel blocker \( \text{NNC} 55-0396 \) on whole-cell \( \text{Ca}^{2+} \) currents, pressure myography and \( \text{Ca}^{2+} \) imaging to test diameter and \( \text{Ca}^{2+} \) responses of the applied vasoconstrictors, and Q-PCR to check mRNA expression levels of several \( \text{Ca}^{2+} \) handling proteins in wild-type and \( \text{Ca}_V3.1^{-/-} \) mice.

Results: Our data indicated that \( \text{Ca}_V3.1 \) channels are important for the maintenance of myogenic tone at low pressures (40–80 mm Hg), whereas they are not involved in high-voltage-activated \( \text{Ca}^{2+} \) currents, \( \text{Ca}^{2+} \) entry or vasoconstriction to high KCl in mesenteric arteries and arterioles. Furthermore, we show that \( \text{NNC} 55-0396 \) is not a specific T-type channel inhibitor, as it potently blocks L-type and non-L-type high-voltage-activated \( \text{Ca}^{2+} \) currents in mouse mesenteric vascular smooth muscle cell.

Conclusion: Our data using mice deficient in the \( \text{Ca}_V3.1 \) T-type channel represent new evidence for the involvement of non-L-type channels in arteriolar tone regulation. We showed that \( \text{Ca}_V3.1 \) channels are important for the myogenic tone at low arterial pressure, which is potentially relevant under resting conditions \textit{in vivo}. Moreover, \( \text{Ca}_V3.1 \) channels are not involved in \( \text{Ca}^{2+} \) entry and vasoconstriction to large depolarization with, for example, high KCl. Finally, we caution against using \( \text{NNC} 55-0396 \) as a specific T-type channel blocker in native cells expressing high-voltage-activated \( \text{Ca}^{2+} \) channels.

Keywords arteriolar tone, \( \text{Ca}_V3.1 \) T-type channel, mesenteric artery, myogenic response, voltage-gated calcium channel.
Voltage-gated Ca$^{2+}$ channels are encoded by 10 different genes and are divided into the high-voltage-activated Ca$^{2+}$ channels, to which the L-type, P/Q-type, N-type and R-type belong, and the low-voltage-activated Ca$^{2+}$ channels to which the T-type channels belong (Catterall et al. 2003). In vascular smooth muscle cells (VSMCs), the L-type channels are important for excitation–constriction coupling and therefore have a dominant role in arteriolar tone regulation, blood pressure regulation, and hypertension (Nelson et al. 1990, Pratt et al. 2002, Moosmang et al. 2003). However, the contribution of non-L-type channels to vascular tone regulation is of potential clinical relevance because a proportion of vasospasms in the coronary and cerebral circulations are resistant to treatment with L-type Ca$^{2+}$ antagonists (Beltrame et al. 2004, Oshima et al. 2005, Fukumoto et al. 2008, Velat et al. 2011). Furthermore, coronary blood flow, endothelial function and renal function in hypertensive subjects show significant improvement after treatment with combined L- and T-type channel blockers (Beltrame et al. 2004, Oshima et al. 2005, Koh et al. 2007, Sasaki et al. 2008). Recently, the expression of CaV3.1 and CaV3.2 T-type Ca$^{2+}$ channels was demonstrated in small arteries and arterioles from the rat mesenteric (Gustafsson et al. 2001, Jensen et al. 2004, Braunstein et al. 2009), cremasteric (VanBavel et al. 2002), renal (Hansen et al. 2004) and cerebral (Kuo et al. 2010) circulations. Because the expression of L-type channels and the effect of L-type channel blockers were shown to be reduced in the most peripheral branches of the mesenteric circulation (Morita et al. 1999, 2002, Gustafsson et al. 2001, Jensen et al. 2004), it was suggested that the T-type channels are especially important for tone regulation in the most peripheral resistance vessels of the systemic circulation (Gustafsson et al. 2001, Jensen et al. 2004, Ball et al. 2009). Recombinant T-type channels are low-voltage-activated and undergo rapid voltage-dependent inactivation at depolarized potentials more positive than −50 mV (Perez-Reyes 2003). Nevertheless, previous studies in rat mesenteric resistance vessels have shown that high-voltage-activated whole-cell Ca$^{2+}$ currents and Ca$^{2+}$ entry to high KCl in VSMCs, which were resistant to L-type channel blockade using nifedipine, were highly sensitive to several T-type channel blockers, such as mibefradil, NiCl$_2$, pimozone, ethosuximide, NNC 55–0396 and R(-)-efonidipine (Morita et al. 2002, Jensen et al. 2004, Braunstein et al. 2009). In rat mesenteric arteries, P/Q-type channels were not expressed and the Ca$^{2+}$ entry to high KCl was not blocked by highly specific peptide toxin inhibitors of P/Q-type, N-type or R-type channels (Jensen et al. 2004). It was therefore speculated that a splice variant of T-type channels is expressed in mesenteric resistance vessels which activate and mediate Ca$^{2+}$ entry via non-inactivating window currents at membrane potentials more depolarized than the hyperpolarized potentials at which recombinant pore-forming subunits of the CaV3.1 channels are activated (Jensen & Holstein-Rathlou 2009).

Myogenic tone in CaV3.1 knockout mice

Studies were conducted using CaV3.1$^{-/-}$ mice and weight- and age-matched C57BL/6J mice (Taconic Farm Inc., Lille Skensved, Denmark). Mice knockout
for the Ca\textsubscript{v}3.1 T-type Ca\textsuperscript{2+} channel (Ca\textsubscript{v}3.1\textsuperscript{−/−}), originally developed in HS Shin’s laboratory (Kim et al. 2001), were backcrossed for more than 10 generations to the C57BL/6J genetic background. Genotyping was performed as described previously (Kim et al. 2001). The mice of both genotypes were 5–8-month-old males and weighed 26–39 g. There were no clinical signs or dysfunction due to Ca\textsubscript{v}3.1 deletion or age. Mice were killed immediately before use by cervical dislocation. The intestines were quickly removed and submerged in oxygenated (95\% O\textsubscript{2}/5\% CO\textsubscript{2}) Krebs buffer at room temperature. Second- or third-order mesenteric arteries (<200 \(\mu\)m lumen) were quickly removed using sharpened forceps and ophthalmic scissors under a stereo microscope (Leica M80; Leica Microsystems, Wetzlar, Germany) with cold light illumination (Leica microscope (Leica M80; Leica Microsystems, Wetzlar, Germany), and used for pressure myography, Q-PCR and immuno-fluorescence microscopy. Mesenteric terminal arterioles (<40 \(\mu\)m lumen) were excised from the fat pads adjacent to the border of the ileum as previously described (Braunstein et al. 2009) and used for Ca\textsuperscript{2+} imaging experiments. For whole-cell patch clamp electrophysiology, 10–11-week-old Sprague Dawley rats (\(N=10\)) and 9–12-week-old C57BL/6J mice (\(N=4\)) of either sex were used. These rats and mice were anaesthetized by exposure to sevoflurane and killed by cervical dislocation, and small mesenteric arterioles (<200 \(\mu\)m) were excised and used for isolation of single VSMCs. This study conforms to good publication practice in physiology (Persson & Henriksson 2011).

**Pressure myography**

Mouse mesenteric arteries were mounted in a pressure myograph system (120 CP, DMT A/S, Aarhus, Denmark) between two fire-polished glass pipettes with matching tip diameter (approx. 80 \(\mu\)m) for intraluminal perfusion and diameter measurement. The pressure myograph was placed on the stage of an inverted microscope (Olympus IX71; Olympus Danmark A/S, Ballerup, Denmark), and vessels were viewed through a 4X objective. The myograph chamber (5 mL) was continuously superfused (2 mL min\textsuperscript{−1}) with oxygenated (95\% O\textsubscript{2}/5\% CO\textsubscript{2}) Krebs buffer, while arteries were being slowly pressurized and heated to 37 \(^\circ\)C. To avoid bending of the artery at the maximal pressure of 120 mm Hg, the arteries were gently stretched to their approximate in situ length while maintaining the longitudinal force measurement between 0.1 and 0.35 mN. The luminal perfusion was blocked in the downstream direction to perform the measurements under no-flow conditions. It was checked that the pressure did not drop more than 1–2 mm Hg on the outflow side of the vessel to avoid vessels with side branches or leaks. Subsequently, the pressure was gently lowered to 40 mm Hg, and after resting for 5–10 min, the artery was constricted with Krebs buffer containing 75 mM KCl (K75) for 3–5 min. After washout of K75 and resting for 5–10 min, the artery was again constricted for 3–5 min by adding 1 \(\mu\)M noradrenaline (NA) to the superfusate. Only arteries without leaks and showing a uniform constriction to K75 and NA were included in the study. Arteries included in the study were equilibrated with Krebs buffer at 80 mm Hg for 30–40 min before commencing the myogenic pressure step protocol. The control pressure curve was performed by changing the pressure between 20 and 120 mm Hg in 20 mm Hg increments while letting the artery constrict for 5 min at each pressure. After the control pressure curve was constructed, the artery was rested for 30 min before repeating the pressure protocol in the presence of 1 \(\mu\)M nifedipine. Finally, after the second pressure curve, the myograph chamber was superfused with Ca\textsuperscript{2+} ‘-free Krebs’ buffer + 2 mM EGTA for at least 10 min before repeating the pressure step protocol to obtain the passive pressure curve. During experiments, the outer diameter of the artery was measured using Myo-view software (DMT A/S), and diameter, longitudinal force, temperature and pressure data were stored to a PC hard disk at 1 Hz. Offline analysis was used to calculate the mean steady-state diameters during the last 30 s at each pressure step. Mean diameters for K75 and NA measurements were measured at their steady-state diameter. There were no time-dependent diameter changes with K75 or NA after the steady-state diameter was reached.

**Calcium imaging**

The intracellular [Ca\textsuperscript{2+}] measurements using Fura-PE3 ratiometric imaging of unpressurized mouse mesenteric terminal arterioles were performed at room temperature, as previously described (Jensen et al. 2004). The Ca\textsuperscript{2+} responses were evaluated as the peak Fura-PE3 Ratio to HB-PSS containing 60 mM KCl (K60). For each arteriole, normal HB-PSS was added first a couple of times to check for motion artifacts. Then, arterioles were challenged with K60 three times interrupted by washing steps for 5 min. Then arterioles were incubated with the Ca\textsuperscript{2+} antagonists for 10 min, followed by another series of challenges with K60. In most experiments, drugs were then washed away, and a third series of K60 challenge was performed.

**Whole-cell patch clamp electrophysiology**

Isolation of single VSMCs from mouse and rat mesenteric arteries (<200 \(\mu\)m) and the whole-cell patch clamp procedure was performed, as previously

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published (Morita et al. 1999). In brief, short segments of the distal part of the isolated arteries were transferred to PSS with 1 mM Ca	extsuperscript{2+} and 0.5 mM papain (Worthington) and 0.5 mM 1,4-dithioerythritol (Sigma; Sigma-Aldrich Denmark ApS, Brøndby, Denmark), and incubated at 37 °C for 15 min, followed by transfer to 1 mM Ca	extsuperscript{2+}-containing PSS with 2 mM collagenase (type I; Worthington Biochemical Corp., Lakewood, NJ, USA) and incubated at 37 °C for 45 min. Single cells were dissociated by triturating the digested segments with a large-bore Pasteur pipette in Ca	extsuperscript{2+}-free PSS and stored at 4 °C until use. A low-noise, high-performance patch clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA, USA) driven by a PC in conjunction with an A/D, D/A board (TL-1, Axon Instruments) was used to generate and apply voltage pulses to clamped cells and to record the corresponding membrane currents. Current signals were low-pass filtered at 1 kHz and digitized at 2 kHz before being stored to the PC hard disk. Linear leak subtraction was made using the hyperpolarizing P/4 or P/2 routine. Under the present experimental conditions, the series resistance of cells stayed almost constant throughout the experiment, and 50–70% of the series resistance was compensated. NNC 55-0396 was dissolved in water and added in increasing concentrations (10–10 μM) when steady-state inhibition was reached after approx. 100-s exposure. 100-s exposure. In initial experiments (N = 3) using only 1 μM NNC 55-0396 to block the total I_{Ba}, the remaining blocker-resistant current was totally abolished by the addition of Cd	extsuperscript{2+} (200 μM).

**Immunofluorescence microscopy**

Isolated second- and third-order mouse mesenteric arteries (3–5 from each mouse) were fixed in 2% paraformaldehyde dissolved in PBS for 1 h and subsequently cryo-protected in 30% sucrose for 2 h, or until they were submerged at the bottom of the sucrose solution. Cryo-protected arteries were embedded in Tissue-Tek®; Sakura Finetek Denmark ApS, Copenhagen, Denmark contained in a plastic mould and primary antibody towards the CaV3.1 epitope was synthesized by United Peptide Corp. (Bethesda, MD, USA). In parallel preabsorption experiments, the primary antibody diluted in PBS was incubated with the antigenic peptide at 40–50 μg/mL for 1 h at RT while shaking. The antibody–antigen mixture was vortexed and spun down shortly before applying it to the tissue sections and incubated as for primary antibody. In each experiment, a negative control with secondary antibody alone was also included. After incubation with primary antibody, the washing step consisted of wash with high-salt PBS for 5 min (to reduce non-specific staining) followed by normal PBS for 2 × 5 min. Secondary antibody (1 : 800; Alexa Fluor® 488 Donkey anti-rabbit IgG, Molecular Probes; Life Technologies Europe BV, Nærum, Denmark) and Rhodamin-Phalloidin (1 : 400, Molecular Probes; Life Technologies Europe BV, Nærum, Denmark) were diluted in PBS and added for 60 min at RT. The washing step was repeated. Sections were treated with DAPI (0.1 μg/mL) in PBS for 30 min at RT. Finally, sections were covered with Immersion Liquid Type F (Leica), and cover slip was mounted for inspection. Sections were imaged with a Leica DM RB epifluorescence microscope through a Leica PL Fluotar oil-immersion objective (40X/1.00-0.50) using an external light source (EL 6000), digital camera (DFC 350 FX) and software (LAS Vers. 2.8.1) from Leica. For each experiment, all sections were imaged under identical microscope and camera settings. There was no visible staining in the negative controls using secondary antibody alone. Using Adobe Photoshop CS4, captured images were cropped, rotated, and the brightness and contrast were enhanced equally in sections incubated with primary antibody and primary antibody + peptide preincubation.

**Quantitative real-time PCR (Q-PCR)**

After dissection, second- and third-order branches of mouse mesenteric artery (3–4 from each mouse) were stored in RNAlater® (Sigma) at 4 °C until used. Extraction of total RNA was performed using a TissueLyzer® homogenizer and RNEasy Micro kit (Qiagen; Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instruction. cDNA was produced from freshly extracted total RNA using a Promega kit according to the manufacturer’s instruction. Both RT+ and RT–samples were prepared for each mouse. cDNA from mouse whole brain was prepared in the same way and used as a calibrator sample. Primers were picked using...
Table 1 QPCR primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>Product size (BP)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casca1c</td>
<td>CGTTCTCATCCTCGCTCAACA</td>
<td>GGTGTACCTCGGTATTGCT</td>
<td>236</td>
<td>BC138031</td>
</tr>
<tr>
<td>Casca1h</td>
<td>CCTCTCTCAGGCTCCCAAC</td>
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<td>169</td>
<td>BC138026</td>
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<tr>
<td>Casca1a</td>
<td>TGCTGTGTCTACTGTTTTCC</td>
<td>TTCCTCCACAGCTCCTCTTTC</td>
<td>198</td>
<td>NM007578</td>
</tr>
<tr>
<td>Trpc1</td>
<td>GACATTCCAGTTTCTCTTGT</td>
<td>GTCAAGCTAATGTCTCAAGC</td>
<td>107</td>
<td>NM011643</td>
</tr>
<tr>
<td>Trpc3</td>
<td>GATCACGGATGACAGTGATAG</td>
<td>TCCTCATCGAAGTGGAGAGC</td>
<td>73</td>
<td>NM019510</td>
</tr>
<tr>
<td>Trpc6</td>
<td>TACAATCGTGCCAAGAATGGTG</td>
<td>CAGACGGTGTACATAAAGCCC</td>
<td>75</td>
<td>NM013803</td>
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<tr>
<td>Adra1a</td>
<td>ATGTGGTGGGATTGCTCTGT</td>
<td>ACGTCGTCGTTCGTTGAA</td>
<td>95</td>
<td>NM013461</td>
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<tr>
<td>Adra1b</td>
<td>GCAAAAACTTGGGATATTG</td>
<td>AAGTAGCAGACAGAAGAC</td>
<td>140</td>
<td>NM007416</td>
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<tr>
<td>Adra1d</td>
<td>CAAAGGCTACGCATCGTG</td>
<td>GAAGACGGCCTCTGATGGTT</td>
<td>112</td>
<td>NM013460</td>
</tr>
<tr>
<td>Acrb</td>
<td>AGCCATGTAGTAGGACATCC</td>
<td>CTTCAGCGTGTTGGA</td>
<td>228</td>
<td>NM007393</td>
</tr>
</tbody>
</table>

Primer Blast or Primer 3 (see Table 1). A Blast search confirmed that the primers did not contain perfect matches to unintended targets. Conventional PCR using mouse cDNA and intron-spanning primers did not reveal any genomic DNA contamination. Q-PCR was performed using a LightCycler 480® apparatus (Roche; Roche Diagnostics A/S, Hvidovre, Denmark) and SYBR® Green PCR Master Mix. The standard Q-PCR cycle protocol was annealing at 60°C for 10 s, and elongation at 72°C for 20 s. Production of a standard curve for each primer pair using calibrator cDNA showed efficiencies in the range [1.83–2.10] and slopes in the range [−3.13 to −3.67]. The melting curve analyses revealed single peaks, only. For each primer pair, the amplified product was of the expected size, and the RT– and H2O-loaded negative controls did not reveal any amplification. The reference gene β-actin (rActB) expression did not vary significantly between samples.

**Solutions and chemicals**

Krebs Buffer contained the following concentrations of salts (mM): NaCl 118; KCl 4.7; MgSO4 1.2; CaCl2 2; NaHCO3 25; KH2PO4 1.2. In the Krebs buffer with high-salt PBS (HS-PBS), the concentration of NaCl was increased to 411 mM. Glucose (5 mM) was added to each buffer (except PBS) immediately before use, pH was adjusted to 7.4 by equilibration with 5% CO2 for Krebs buffer(s), and by adding 1 N NaOH for HB-PBS, PBS and HS-PBS. For whole-cell patch clamp experiments, a HB-PSS was used as bath solution, except that 5 mM BaCl2 replaced Ca2+ as charge carrier and that pH was adjusted to 7.4 with Tris base. The pipette (internal) Cs+ solution contained (in mM) Cs+ 140, Mg2+ 2.0, Cl− 144, Phospho-creatine 5, Na2ATP 1, EGTA 10 and HEPES 10 (adjusted to pH 7.2 with Tris base). Nifedipine, NNC 55-0396, NiCl2, BaCl2, CdCl2, Pluronic F-127, Cremophore EL, DAPI and all other salts and chemicals were from Sigma Aldrich Co.

**Statistics**

Data were presented as raw data or mean ± SEM with statistical significance tested using Student’s t-test, except for Fig. 2a,b where the Control and Nifedipine pressure curves were evaluated versus the passive curves using a two-way repeated measures ANOVA with Bonferroni post-test for comparing individual pressure levels (multiple comparisons). A P-value of <0.05 was considered statistically significant. In Fig. 3b, the pharmacological effects were calculated as the increase from baseline Fura-P3 R to K60 exposure (∆R) before and after addition of Ca2+ channel blockers and expressed as the % remaining Ca2+ entry in mesenteric arteries during drug exposure according to: (ARdrug/ARcontrol)*100. The vasocostriction to K75 and NA in mouse mesenteric artery was expressed as the difference in % vascular tone (PD – AD/PD)*100 between the resting diameter in normal Krebs buffer and Krebs buffer with high KCl or NA. Concentration-inhibition curves for NNC 55-0396 on IBa and NI-IBa were analysed by fitting data points averaged from 4 cells by the Hill equation for estimation of the 50% inhibitory concentration (IC50) and Hill coefficient (nH), respectively. Q-PCR data were evaluated either as Ratios of target gene to ActB cycle thresholds (Ct-values) in small mesenteric artery samples, or using the 2−ΔΔCt method of normalized
The latter was calculated by the formula: Normalized relative ratio \( = 2^{-\Delta C_t} \), where \( C_t \) (target) = \( C_t \) (rActB), and \( \Delta C_t = \Delta C_t \) (sample) – \( C_t \) (calibrator). The two calculation methods yielded similar results and statistical significance.

**Results**

We initially investigated by indirect immunofluorescence microscopy the expression and localization of CaV3.1 T-type Ca\(^{2+}\) channels in mouse mesenteric artery. As shown in a cross-section of an artery in Fig. 1 (Panel A), the green CaV3.1 staining is abundant in the arterial wall. CaV3.1 expression was not detectable in ECs, because the nuclei of ECs (white arrows) facing the lumen of the vessel are not covered by green CaV3.1 staining. In the longitudinal section through the tunica media (Panel C), it is clearly shown that CaV3.1 staining is abundant in VSMCs. We confirmed the specificity of the primary antibody for the CaV3.1 epitope by showing that the green staining is absent in sections of the same arteries labelled with primary antibody preincubated with its antigenic peptide (Panels B and D). Taken together, this expression pattern of CaV3.1 is consistent with our previous findings in rat mesenteric arterioles and small arteries (Braunstein et al. 2009).

We hypothesized that a CaV3.1 T-type channel window current could be involved in maintenance of myogenic tone at low pressures associated with relatively small myogenic depolarization in mouse mesenteric arteries. This was investigated by pressure myograph experiments in which the steady-state constriction to intraluminal pressures in the range from 20 to 120 mm Hg was recorded. As shown in Fig. 2a, arteries from age-matched wild-type mice were significantly constricted in the pressure range from 40 to 120 mm Hg in the control situation with Krebs buffer compared with the passive situation where the vessels were exposed to Ca\(^{2+}\)-free solution. In the wild-type mice, a concentration of nifedipine (1 \( \mu \)M) sufficient to abolish all L-type channels expressed in smooth muscle (Morel et al. 1998), resulted in a pressure curve that was not significantly different from the passive curve, except at low pressure (40 mm Hg). This indicated that a non-L-type Ca\(^{2+}\) channel might be important at low pressure. Interestingly, in arteries from CaV3.1\(^{-/-}\) mice, the myogenic tone was abolished at low pressures from 40 to 80 mm Hg, whereas the
CaV3.1 channels are inactivated at high pressures of genic tone at low pressures up to 80 mm Hg, that channels are involved in the maintenance of myogenic tone in small mesenteric arterioles of animals is shown in brackets. Nifedipine was used in the Krebs buffer to block all L-type channels. The passive pressure curve was obtained by superfusing arteries with a Ca2+-free (+EGTA) Krebs buffer. Nifedipine abolishes myogenic tone under control condition is not significant at pressures from 20 to 80 mm Hg, Nifedipine abolishes myogenic tone at 40 mm Hg. (a) Myogenic tone under control condition is not significant at pressures from 20 to 80 mm Hg, Nifedipine abolishes myogenic tone at 40 mm Hg, (b) Myogenic tone at 40 mm Hg, (**P < 0.01 control vs. passive diameter; ***P < 0.001 control vs. passive diameter; #P < 0.05 in nifedipine-treated vs. passive diameter). The number of vessels/animals is shown in brackets.

Figure 2 Myogenic response in small mesenteric artery of wild-type and CaV3.1−/− mice. (a) Control pressure curve shows diameter of artery when superfused with normal Krebs buffer. Nifedipine was used in the Krebs buffer to block all L-type channels. The passive pressure curve was obtained by superfusing arteries with a Ca2+-free (+EGTA) Krebs buffer. Note the development of myogenic tone at pressures from 40 to 120 mm Hg seen as a significant constriction compared with the passive curve. Note that nifedipine did not significantly block the myogenic tone at 40 mm Hg. (b) Myogenic tone at 40 mm Hg. (**) significant block of the myogenic tone at 40 mm Hg.

Arterial diameter and myogenic tone at 100 and 120 mm Hg were similar to the age-matched wild-type mice (Fig. 2b). These data strongly suggest that in wild-type mouse mesenteric artery, CaV3.1 T-type channels are involved in the maintenance of myogenic tone at low pressures up to 80 mm Hg, that CaV3.1 channels are inactivated at high pressures of 100–120 mm Hg, and that the L-type channels are strongly activated in this pressure range.

In previous studies, it has been suggested that a splice variant of T-type channels is expressed in mesenteric resistance vessels and contribute to sustained Ca2+ entry at membrane potentials normally found in VSMCs (Jensen & Holstein-Rathlou 2009). To investigate this possibility, we compared the Ca2+ entry and vasoconstriction to high KCl in age-matched wild-type and CaV3.1−/− mice. As shown in Fig. 3a, the Ca2+ increase to high KCl in mouse mesenteric terminal arterioles (<40 μm) was similar in wild-type and CaV3.1-deficient mice. Furthermore, the vasoconstriction measured as the increase in vascular tone from baseline to high KCl (ΔTone) in small mesenteric arteries (<200 μm) was also similar in wild-type and CaV3.1-deficient mice (Fig. 3c). These data clearly show that CaV3.1 channels are not involved in the nifedipine-insensitive responses to high-voltage activation with KCl. How does this comply with the pronounced effects of NNC 55-0396, which was recently shown to potently inhibit recombinantly expressed CaV3.1 channels with no effect on high-voltage-activated Ca2+ currents (Huang et al. 2004). To investigate this, we next tested the pharmacology of KCl-induced Ca2+ entry in mesenteric arterioles from CaV3.1−/− mice (Fig. 3b). About 40% of the Ca2+ entry was insensitive to nifedipine, and when adding the CaV3.2-specific blocker NiCl2 (30 μM) on top of nifedipine, there was no further inhibition of the Ca2+ entry. These data show that neither CaV3.1 nor CaV3.2 T-type Ca2+ channels mediate nifedipine-insensitive Ca2+ entry to KCl. Furthermore, NNC 55-0396 (10 μM), irrespective of whether it was added on top of nifedipine or NiCl2, abolished all Ca2+ entry to high KCl in mesenteric arterioles from CaV3.1-deficient mice. These data clearly demonstrate that NNC 55-0396 is not a specific T-type channel blocker.

The question still remained whether NNC 55-0396 can be used to distinguish between high-voltage-activated Ca2+ channels of the L-type vs. non-L-type in native mesenteric artery VSMCs. To investigate this further, we tested the effects of NNC 55-0396 on high-voltage-activated whole-cell barium (Ba2+) currents (Iba) before or after addition of 10 μM nifedipine to block all L-type channel activity. Fig. 4a,b show original recording and mean Ba2+ currents found in mouse mesenteric VSMCs. It is seen that the total Iba is activated by stepping from a holding potential of −80 mV to depolarized potentials in the range from −30 mV to +60 mV with a peak of the I/V-curve at 0 to +10 mV. The current remaining after addition of nifedipine (NI-Iba) amounted to approx. 20% of the total Iba. As the Ba2+ currents in mouse VSMCs were rather small, we continued investigating the effects of NNC 55-0396 in rat mesenteric artery VSMCs. In Fig. 5a–c is shown that NNC 55-0396 dose dependently blocks both total Iba and NI-Iba in rat mesenteric artery VSMC with IC50 values of 0.59 μM and 0.96 μM, respectively. These data clearly show that NNC 55–0396 blocks high-voltage-activated Ca2+ channels of both L-type and non-L-type with similar high potency and should therefore not be used in native VSMCs to distinguish between different high-voltage-activated Ca2+ channels.
We also tested the vasoconstrictor effects of a submaximal concentration of noradrenaline (NA 1 μM) in mouse small mesenteric arteries pressurized at 40 mm Hg. Surprisingly, NA elicited a significantly stronger vasoconstriction (increase in vascular tone from baseline) in mice deficient in the CaV3.1 channel than in age-matched wild-type mice (Fig. 3d). We speculated that this could be due to an upregulation in the expression of different Ca2+ handling proteins and therefore tested by Q-PCR the mRNA expression of several isoforms of voltage-gated Ca2+ channel, transient receptor potential channel subfamily C, and α1-adrenergic receptor in mouse small mesenteric arteries. As shown in Table 2, these Ca2+ handling proteins were not differentially expressed in mesenteric arteries from age-matched wild-type mice and CaV3.1-deficient mice.

Discussion

The involvement of T-type channels in the regulation of arteriolar tone has long been controversial for several reasons. Firstly, the membrane potentials at which the T-type channels are activated and inactivated are more hyperpolarized than what has been measured in VSMCs of pressurized arteries and arterioles (Perez-Reyes 2003, Jensen & Holstein-Rathlou 2009). Secondly, despite the fact that many studies have shown the existence of a non-L-type Ca2+ channel, which contributes to arteriolar tone in mesenteric, cremasteric, renal and cerebral vessels (Gustafsson et al. 2001, Hansen et al. 2001, Morita et al. 2002, VanBavel et al. 2002, Jensen et al. 2004, Kuo et al. 2010), these studies have relied on mibefradil and other non-specific T-type channel blockers with a known effect on high-voltage-activated Ca2+ channels (Bezprozvanny & Tsien 1995, Aczel et al. 1998, Jimenez et al. 2000, Moosmang et al. 2006). In an attempt to clarify the contribution of T-type channels to arteriolar tone regulation, we employed mice deficient in the CaV3.1 subtype of T-type channels and tested the following hypotheses: (i) Do CaV3.1 T-type channels contribute to myogenic tone at low arterial pressure, under which the membrane potential of VSMCs in pressurized arteries was previously measured to be in the range from −60 to −50 mV (Knut & Nelson 1998, 2001).

Figure 3 (a) Measurements of intracellular Ca2+ increase (Fura-PE3 Ratio) to high KCl (60 mM) in mesenteric terminal arterioles (<40 μm) from wild-type and CaV3.1-deficient mice. (b) Remaining Ca2+ increase (% of control response) to K60 in terminal arterioles from CaV3.1−/− mice during exposure to L-type channel inhibitor nifedipine (10 μM), CaV3.2 T-type Ca2+ channel inhibitor NiCl2 (30 μM) and putative T-type channel blocker NNC 55-0396 (10 μM). N.D. = not detectable. (c, d) Vasoconstriction (ΔTone) to high KCl (75 mM) and noradrenaline (1 μM) in mesenteric small arteries (<200 μm) pressurized to 40 mm Hg. The number of vessels/animals is shown in brackets.
Kotecha & Hill 2005). (ii) Do CaV3.1 T-type channels partially mediate Ca2+ influx and vasoconstriction to high-voltage activation using high KCl concentration in mouse mesenteric arteries (<200 µm) and arterioles (<40 µm)? (iii) Is NNC 55-0396 a specific T-type channel blocker?

With respect to the first hypothesis, it was clearly shown that the myogenic tone in CaV3.1−/− mice was abolished in the pressure range from 40 to 80 mm Hg, as compared with age-matched wild-type mice (Fig. 2). We did not measure the membrane potential of pressurized mouse mesenteric arteries to confirm that it was in the range required for sustained activation of CaV3.1 channels through window currents. However, our data indicate that L-type channels were not crucial for myogenic tone at low arterial pressure, because nifedipine (1 µM) was ineffective at blocking myogenic tone at 40 mm Hg in the wild-type mice. Furthermore, the fact that the myogenic response at 100–120 mm Hg was very powerful in CaV3.1−/− mice and constricted the arteries to attain the same diameter as in the wild-type mice, suggests that the mechanosensor involved in the development of spontaneous myogenic tone is intact in mice deficient in the CaV3.1 channels. In addition, nifedipine (1 µM) was effective in blocking myogenic tone at 100–120 mm Hg in wild-type and CaV3.1−/− mice, which lends further support to the notion that L-type channels are crucial for maintenance of myogenic tone at high arterial pressure. The role of CaV3.1 channels in myogenic tone is supported by the presence of the CaV3.1 protein in the smooth muscle layer of mouse mesenteric arteries (Fig. 1). It is therefore suggested that CaV3.1 channels are activated and contribute to myogenic tone at low arterial pressure and that they are inactivated at high arterial pressure where L-type channel activation is responsible for the maintenance of myogenic tone. It remains to be established whether T-type channels are important for basal tone in vivo, but as the pressure in mesenteric...
arteries in conscious rats was in the range from 60 to 80 mm Hg (Fenger-Gron et al. 1995), we suggest that T-type channels may to some extent contribute to the basal tone under resting conditions.

Our finding that the noradrenergic tone measured at 40 mm Hg in mesenteric arteries was augmented in CaV3.1−/− mice compared with their wild-type controls (Fig. 3d) deserves attention. None of the Ca2+ channels, TRPC channels or z1-adrenergic receptor subtypes present in mouse mesenteric arteries were differentially expressed at the mRNA level in the wild-type vs. knockout animals (Table 2). Although we cannot be sure that these channels and receptors are not upregulated at the protein level in the CaV3.1−/− mice, our data suggest that the augmented noradrenergic tone is not explained by a simple upregulation of these mechanisms. However, the increase in noradrenergic tone could be a natural compensation for mice born with a deletion of the CaV3.1 channels, as the myogenic tone was abolished at the lower arterial pressures, which would necessitate a secondary mechanism to compensate for the loss in vascular tone. Furthermore, the CaV3.1−/− animals are bradycardic, due to a slowing of the sinoatrial node pacemaker rhythm and the A-V conduction, but they have normal mean arterial blood pressure (Mangoni et al. 2006). It is tempting to speculate that the noradrenaline sensitivity of the resistance vessels is upregulated in CaV3.1−/− animals to increase the peripheral resistance enough to keep the animals normotensive under resting conditions.

With respect to the second hypothesis, our data clearly showed that KCl-induced Ca2+ entry and vasoconstriction in mesenteric arteries and arterioles was not different in wild-type and CaV3.1-deficient mice (Fig. 3a,c). Furthermore, the nifedipine-insensitive Ca2+ increase to high KCl in arterioles from CaV3.1−/− animals was not sensitive to a low concentration of Ni2+ (30 μM), which is considered to be specific for CaV3.2 channels (Fig. 3b). Taken together, our data from the wild-type and knockout mice clearly shows that CaV3.1 and CaV3.2 T-type Ca2+ channels do not mediate depolarization-induced Ca2+ entry and vasoconstriction to high KCl. We must assume that this is due to the fact that the depolarization induced by high KCl is so large that the T-type channels are inactivated. This conclusion is supported by a patch clamp study in rat mesenteric artery VSMCs showing that nifedipine-insensitive whole-cell Ca2+ currents with T-type-like pharmacology was not reminiscent of recombinant CaV3.1 T-type Ca2+ channels expressed in HEK cells (Morita et al. 2002). Previous studies argue against the possibility of several high-voltage-activated Ca2+ channels as candidates for the nifedipine-insensitive Ca2+ entry pathway in mesenteric arteries and arterioles, because specific peptide toxins targeting P/Q-type, N-type, or R-type channels were not effective (Morita et al. 2002, Jensen et al. 2004). In addition, the mesenteric nifedipine-insensitive Ca2+ currents did not match those of recombinant CaV2.3 R-type channels expressed in HEK cells (Morita et al. 2002). One hypothetical explanation for our observations could be that depolarization per se triggers Ca2+ store release (Urena et al. 2007), and indirectly activates plasma membrane Ca2+ channels by emptying of these stores. However, the Ca2+ currents measured with Ba2+ as charge carrier in the presence of 10 μM nifedipine in mesenteric artery VSMCs (Morita et al. 1999, 2002) are not similar to any of the storage-operated Ca2+ permeable channels of the TRPC family, so the identity of the high-voltage-activated nifedipine-insensitive Ca2+ channel awaits further elucidation.

With respect to the third hypothesis, NNC 55-0396 potently inhibited high-voltage-activated Ca2+ influx in CaV3.1−/− mice and Ba2+ currents in the presence and absence of nifedipine with similar potency. These data are supported by a recent study in which 1 μM NNC 55-0396 abolished the total high-voltage-activated Ba2+ currents in rat basilar artery myocytes (Kuo et al. 2004). This suggests that NNC 55-0396, which is known to inhibit the CaV2.3 R-type channels (Kuo et al. 2004), blocks the high-voltage-activated Ca2+ channel in arteries.

### Table 2: Q-PCR data

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Protein symbol</th>
<th>WT mice: target gene expression (N)</th>
<th>CaV3.1−/− mice: target gene expression (N)</th>
<th>P-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cacna1c</td>
<td>Cav1.2 (L-type)</td>
<td>0.225 ± 0.052 (7)</td>
<td>0.231 ± 0.037 (6)</td>
<td>0.93</td>
</tr>
<tr>
<td>Cacna1h</td>
<td>Cav3.2 (T-type)</td>
<td>0.202 ± 0.024 (7)</td>
<td>0.155 ± 0.049 (6)</td>
<td>0.39</td>
</tr>
<tr>
<td>Cacna1a</td>
<td>Cav2.1 (P/Q-type)</td>
<td>0.024 ± 0.003 (7)</td>
<td>0.034 ± 0.010 (3)</td>
<td>0.26</td>
</tr>
<tr>
<td>Trpc1</td>
<td>TRPC1</td>
<td>0.177 ± 0.011 (7)</td>
<td>0.181 ± 0.022 (3)</td>
<td>0.87</td>
</tr>
<tr>
<td>Trpc3</td>
<td>TRPC3</td>
<td>0.078 ± 0.017 (7)</td>
<td>0.051 ± 0.025 (3)</td>
<td>0.41</td>
</tr>
<tr>
<td>Trpc6</td>
<td>TRPC6</td>
<td>0.137 ± 0.017 (7)</td>
<td>0.149 ± 0.022 (6)</td>
<td>0.66</td>
</tr>
<tr>
<td>Adra1a</td>
<td>z1α-R</td>
<td>0.363 ± 0.028 (7)</td>
<td>0.279 ± 0.064 (3)</td>
<td>0.32</td>
</tr>
<tr>
<td>Adra1b</td>
<td>z1δ-B-R</td>
<td>0.020 ± 0.003 (7)</td>
<td>0.024 ± 0.008 (3)</td>
<td>0.67</td>
</tr>
<tr>
<td>Adra1d</td>
<td>z1δ-D-R</td>
<td>0.102 ± 0.013 (7)</td>
<td>0.077 ± 0.012 (3)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Normalized mRNA expression data are presented as 2−ΔΔCt-value (see Methods). N equals the number of mice studied. For each mouse studied, RNA from three to four small mesenteric arteries was pooled and used for the Q-PCR analysis.
2010). These findings demonstrate that NNC 55-0396, like its analogue mibefradil, cannot be considered as a specific T-type blocker in the concentration range from 1 to 10 \( \mu \text{M} \). It is therefore crucial for any study making use of mibefradil and its derivatives as well as any other putative T-type channel blocker, to perform the necessary positive and negative control experiments to verify the specificity of the drugs.

**Conclusions**

Our data using mice deficient in the CaV3.1 T-type channel represent new evidence for the involvement of non-L-type channels in arteriolar tone regulation. We showed that CaV3.1 channels are important for maintenance of myogenic tone at an arterial pressure in the range from 40–80 mm Hg, which is found under resting conditions in vivo. We also showed that, contrary to earlier findings in the literature, CaV3.1 channels are not involved in Ca\(^{2+}\) entry and vasoconstriction to large depolarization such as during stimulation with high KCl. Finally, we caution against using NNC 55-0396 as a specific T-type channel blocker in native cells expressing high-voltage-activated Ca\(^{2+}\) channels. A candidate for the non-L-type high-voltage-activated Ca\(^{2+}\) entry channel in mesenteric artery myocytes is still missing.

**Conflicts of interest**

The authors declare no conflicts of interest.

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