Downregulation of L-type Ca\textsuperscript{2+} channel in rat mesenteric arteries leads to loss of smooth muscle contractile phenotype and inward hypertrophic remodeling

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Voltage-dependent Ca\textsuperscript{2+} remodeling, such as atherosclerosis and hypertension.

The primary pathway for Ca\textsuperscript{2+} entry in phenotypically normal vascular smooth muscle cells (VSMCs). Membrane depolarization induces Ca\textsuperscript{2+} influx via LTCCs that is essential for excitation-contraction coupling and thus vascular tone. The crucial role of LTCCs in regulation of vascular resistance and consequently blood pressure and flow distribution is reflected in a substantial research interest focused on these channels as therapeutic targets in hypertension (54).

Moreover, LTCCs can influence VSMC gene transcription via activation of Ca\textsuperscript{2+}-dependent transcriptional factors. This excitation-transcription coupling (60) is important for VSMC phenotypic expression (26, 62). Changes in VSMC phenotype affect blood vessel function and structure. These changes can be adaptive (e.g., during vascular development and repair) but can also be associated with pathological processes [e.g., atherosclerosis (43) and hypertension (2)]. The importance of LTCCs for vascular structure is indicated by normalization of media-to-lumen ratio (MLR) of small arteries after long-term treatment of hypertensive humans (53) and rats (25) with LTCC blockers.

METHODS

Ethical approval. The study was performed with 12- to 16-wk-old male Wistar rats. All procedures complied with Danish animal welfare regulations. Animal facilities and experimental protocol were approved by the Danish Inspectorate for Experimental Animals and the Animal Welfare Officer of the Medical Faculty of the University of Aarhus.

In vivo siRNA transfection. Three different siRNAs directed against sequences in different exons of the α\textsubscript{1C} LTCC subunit with siRNA and transfected a single segment of rat mesenteric resistance arteries (33). We aimed to elucidate the role of LTCC expression for VSMC phenotype and for vascular structure in vivo independently of global changes in hemodynamic parameters.
Table 1. Sequences of primers and siRNAs

<table>
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<th>mRNA Accession Number and Primers</th>
<th>Target (Gene ID)</th>
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<td>Nonrelated siRNA-eGFP</td>
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siRNA, small interference RNA.

The α1A subunit of rat P/Q-type Ca<sup>2+</sup> channel gene CACNA1A was used (Table 1).

Transfection solution was prepared as described previously (33). In brief, 10 μl of each of the 10 μmol/l siRNA stock solutions was mixed with 330 μl of sterile 0.9% NaCl solution, containing 8.3% transfection reagent (TKO; Mirus) 10 min before the transfection procedure. Total concentration of siRNAs in the final transfection solution was 0.8 μM. Nonrelated siRNA directed against green fluorescence protein (eGFP; Eurofins MWG, Denmark) was used as control for side effects of the transfection procedure (sham transfection). In experiments addressing expression of contractile phenotype marker gene expression, a sham-transfection procedure using 0.9% NaCl solution without siRNA and transfection reagent was used as additional control.

Rats were anesthetized by a subcutaneous injection of combination of hypnorm (3 mg/100 g; VetaPharma, Leeds, UK) and midazolam (1.5 mg/100 g; Hameln Pharm, Langes Feld, Germany). Every 30 min one third of the initial dose of the anesthetic was supplied by a subcutaneous injection. siRNA transfection was performed as described previously (33). Shortly, a medial laparotomy was performed and a section of mesentery was extracted and spread on a sterile cloth, dampened with sterile salt solution (0.9% NaCl). A segment of first order branch of mesenteric artery (~1 to 2 mm) was gently cleaned, and a microfil cannula (World Precision Instruments) filled with the transfection solution was inserted into the artery. The transfection solution was injected into the lumen and kept there for 20 min, while downstream flow was occluded with a surgical clamp. The clamp was released and the arterial branches were flushed with a new portion of the transfection solution, each 2 to 3 min. Care was taken to avoid overdilatation of the arterial segment since this was seen to induce unrelated changes in protein expression in the arterial wall (unpublished observations). The operation field was covered by cloth dampened with sterile salt solution throughout the procedure. Body temperature was maintained at 37.5°C by a thermostatically controlled heating platform. Painkiller (0.2 ml/kg; Temgesic, Schering-Plough Europe, Belgium) was injected subcutaneously at the end of operation. Rats were euthanized by CO2-inhalation at 3 days or, where indicated, 10 days after operation, and mesenteric arteries were used for further studies. We have previously shown that this transfection procedure leads to uptake of siRNA into the VSMCs (32).
Wire myograph. The rats were euthanized, and 1st to 3rd order branches (100–350 μm passive inner diameter) of the mesenteric artery were dissected in ice-cold physiological salt solution (PSS) and cleaned from surrounding tissue. Segments of ~2 mm length were mounted in an isometric wire myograph (Danish Myo Technology, Denmark). Based on the passive tension-diameter relationship the artery diameter was normalized to a value where maximal active force is obtained (39). Force was recorded with a PowerLab 4/25 and Chart5 acquisition system (ADInstruments, New Zealand). The results were expressed as wall tension by dividing the force with two times the segment length. After 10 min of equilibration the arteries were exposed to 10 μmol/l norepinephrine (NE). Endothelial function was assessed by applying 10 μmol/l acetylcholine on top of a constriction evoked by NE.

Measurements of intracellular Ca2+ concentration ([Ca2+]i). Isometric force was measured simultaneously with intracellular Ca2+ concentration ([Ca2+]i) in arterial segments mounted in a wire myograph as described previously (34). Arterial segments were incubated with 5 μmol/l Ca2+–fluorophore Fura2-AM (Invitrogen) for 1–1.5 h in darkness at room temperature. Fura 2-AM was dissolved in DSMO with addition of 0.2% Cremophor EL and 0.04% Pluronic F-127. The final concentration of DSMO in the myograph bath did not exceed 0.06%. The arteries were then washed two times with PSS and exposed to alternating excitation wavelengths of 340 nm and 380 nm from a 75-W xenon light source. The emission at 510 nm was collected at a sampling rate of 10 Hz and analyzed with Felix software (PTI). At the end of the protocol 0.25 mmol/l MnCl2 was added to quench Fura 2-AM and obtain background fluorescence. The ratio of emissions with excitations at 340 nm and 380 nm, respectively, was obtained (39). Force was recorded with a PowerLab 4/25 and Chart5 acquisition system (ADInstruments, New Zealand). The resistance to relaxing solution, containing 2 mmol/l EGTA, 130 mmol/l NaCl and thereafter with 10 μmol/l the receptor (IP3R) blocker, 2-APB (100 μmol/l), was assessed by applying 10 μmol/l acetylcholine on top of a constriction evoked by NE.

Resting [Ca2+]i, was obtained for 10 min at the beginning of experiments, and the arteries were then challenged with 10 μmol/l NE in PSS and thereafter with 10 μmol/l NE in Ca2+-free solution. For the latter experiments Ca2+-free PSS with 5 mmol/l EGTA was applied for 10 s before 10 μmol/l NE in Ca2+-free PSS was applied. The arteries were then exposed to 5 mmol/l caffeine in PSS. Each drug application lasted for 2 min and was followed by 10 min of washout. In a separate experiment effects of NE in Ca2+-free solution and caffeine were evaluated in the presence of the inositol triphosphate receptor (IP-R) blocker, 2-APB (100 μmol/l), and the ryanodine receptor (RYR) blocker ryanodine (10 μmol/l). 2-APB abolished responses to NE in Ca2+-free solution and ryanodine abolished response to caffeine (not shown).

Cumulative concentration-response curves to K+ (10, 20, 30, 50, 80, and 125 mmol/l) were obtained in the presence of the α-adrenergic receptor antagonist phenolamine (1 μmol/l) to exclude contribution from NE released from sympathetic nerves.

α-Toxin permeabilization. Arteries of interest were mounted in wire myograph and the endothelium removed. Arteries were then exposed to relaxing solution, containing 2 mmol/l ETGA, 130 mmol/l potassium methanesulphonate, 4 mmol/l MgCl2, 20 mM Tris maleate, 10 mmol/l creatine phosphate, 0.1 mg/ml creatine phosphokinase, 4 mM Na2ATP, and 1 μmol/l thapsigargin for 20 min. Permeabilization of VSMCs was performed by 1,000 U/ml α-toxin in the presence of 1 μM Ca2+ (24). After ~30 min α-toxin was washed out and relaxing solution added. Twenty minutes later the contractile response to 1 μmol/l Ca2+ was measured. Experiments were performed at 22°C, which is optimal for α-toxin efficiency (22).

Vascular responses in vivo. Studies of mesenteric small arteries 3 days after transfection in vivo were performed as described previously (12). Rats were anesthetized using ketamine (33 mg/100 g; Ketaminol vet; Intervet International, Holland) and xylazine (7.5 mg/100 g; Narcorex vet; Intervet International, Holland) administered subcutaneously and placed in a chamber heated to 33–37°C. A lateral laparotomy was performed, and a short segment (1.5–2 cm) of the intestine and mesentery was carefully pulled out. A single branch of the mesenteric artery was placed in a small reservoir with a volume of ~100 μl. The ends of the exposed arterial branch were passed through openings on opposing sides of the reservoir and sealed with high vacuum grease (Dow Corning, Wiesbaden, Germany). The artery segment in the reservoir was perfused in PSS containing 10 mM HEPES (HEPES-PSS) bubbled with 5% CO2 in N2. During the experiment, the extracted intestine and mesentery were kept moist using bandages soaked in HEPES-PSS.

Approximately 50% of the surrounding mesenteric fat was dissected from the top of the segment to enable visualization of the inner diameter of the artery. The arterial inner diameter was captured with a USB CCD Monochrome Camera (DMK 41AU02; Imaging Source, Germany) attached to the microscope (Motic PSM-1000) and processed using DMT Vessel Acquisition Suite software (Danish Myo Technology A/S, Denmark).

The diameter measured immediately after 3× washout was not different from the passive diameter measured in vivo in the presence of (in μmol/l) 10 papaverine hydrochloride, 1 phenolamine, 10 Y-27632, and 10 acetylcholine (data not shown). Therefore, changes in vascular tone are expressed as a percent reduction of the passive (washout) diameter.

RNA quantification and quantitative PCR. Total RNA was isolated using RNeasy microkit (Qiagen, VWR, Denmark), and cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Denmark). GenBank Accession numbers as well as the localization of each primer set used are shown in Table 1. Primers for tropomyosin 4 were purchased from Applied Biosystems (Rn0178617_m1). All other primers were purchased from Eurofins MWG Operon, Germany. Quantitative PCR (qPCR) was performed in Mx3005P Real-Time PCR system (Stratagene). SYBR Green (Qiagen, VWR, Denmark) assay was used for quantification of the α1C subunit of L-type Ca2+ channel (CACNA1C), α1G subunit of T-type Ca2+ channel (CACNA1G/Cav3.1), α1S subunit of T-type Ca2+ channel (CACNA1H/Cav3.2), α1A subunit of P/Q-type Ca2+ channel (CACNA1A/Cav1.2), smooth muscle myosin heavy chain 11 (Myh11), smooth muscle α-actin (Acta2), h-caldesmon (Cald1/l-cald), and l-caldesmon (Cald/l-cald). Dissociation curves confirmed that there was only one PCR product (data not shown). TaqMan assay was used for tropomyosin-4. Product size was verified by gel electrophoresis.

Two housekeeping genes were used for normalization of cDNA sample content: transferrin receptor and peptidylprolyl isomerase A in SYBR green assay, and transferrin receptor and GAPDH in TaqMan assay. All samples were amplified in duplicate, and the mean Ct was obtained for further calculations. The ΔΔCt method was applied to calculate relative mRNA quantities, using the following equation: Relative expression = (2^-ΔΔCt (transfected artery) − ΔΔCt (nontransfected artery)) × 100%. ΔCt for each artery was calculated as the difference between Ct of the target gene and average Ct of two housekeeping genes. The expression in nontransfected arteries was set to 100%, and relative expression in transfected arteries from the same rat was calculated.

Immunofluorescence of LTCC expression in freshly isolated VSMCs. Immunofluorescent staining is a semi-quantitative method for assessment of protein abundance that can be used in vascular preparations (11). Single VSMCs were isolated by overnight enzymatic digestion of arteries as described previously (30). VSMCs were transferred to tissue culture dishes and fixed with 4% paraformaldehyde. The cells were treated with 25 mmol/l glycine in PBS for 15 min to quench autofluorescence and then permeabilized with 0.1% Triton X-100 in PBS. VSMCs were incubated with the primary antibody for 2 h (1:100; against α1C subunit of L-type Ca2+ channel; Alomone) and then with Alexa-488 fluorescent-conjugated secondary antibody (Invitrogen) for 45 min. Fluorescence was detected with a confocal microscope (LSM-5 Pascal Exciter, Zeiss, Germany; excitation/emission at 488/530 nm). Detected protein was quantified using ImageJ program [version 1.37; National Institutes of Health (NIH)] using the following equation (16): whole cell fluorescence = whole cell integrated density − (cell area-mean background fluorescence).

From each artery 5–20 cells were used for quantification. The expression in nontransfected arteries was set to 100%, and relative
expression in transfected arteries from the same rat was calculated. Due to difficulties in setting absolute '0' and maximum (or 100%) values of expression from the images, we consider the quantification of the protein to be semiquantitative.

**Western blot.** Nontransfected arteries, sham-transfected arteries, and arteries transfected with siRNA targeting LTCC were isolated from rat, quick frozen in liquid N$_2$, and stored at $-80^\circ$C until further analyses. Arterial segments were lysed in 20 µL mixture of RIPA buffer (in mmol/L: 25 Tris·HCl, 150 NaCl, 1% nonyl phenoxypoly-ethoxylanol, 1% sodium deoxycholate, 0.1% SDS; Sigma Aldrich) and Halt Protease Inhibitor cocktail (100:1; Thermo Scientific). The homogenate was centrifuged at 13,000 g for 10 min, and the supernatant was collected. The protein-containing supernatant was adjusted to a homogenate concentration and arteries transfected with siRNA targeting LTCC were isolated from nontransfected arteries, sham-transfected arteries, and arteries transfected with siRNA targeting LTCC and stained with antibody (ab) against α$_{1C}$ subunit protein. Omission of primary antibody was used as a negative control. Bars represent 20 µm. C: protein expression of α$_{1C}$ subunit of LTCC in sham-transfected arteries (n = 4) and arteries transfected with siRNA targeting LTCC (n = 4) measured by immunofluorescence on isolated VSMCs. The results are shown relative to the level of LTCC protein in nontransfected arteries from the same rat (100%). D: Western blot control demonstrated that pre-incubation of LTCC antibody with control peptide antigen omits the lower band at $\sim$200 kDa. E: representative Western blots against α$_{1C}$ subunit of LTCC (arrow indicates the expected band size $\sim$200 kDa) and for pan-actin (arrow indicates the expected band size $\sim$47 kDa) in various transfection groups. F: averaged semiquantitative results from Western blots shown in E for expression of α$_{1C}$ subunit of LTCC in sham-transfected arteries (n = 6) and arteries transfected with siRNA targeting LTCC (n = 6). The expression was normalized to pan-actin. The results are shown relative to the LTCC protein level in nontransfected arteries (100%). *P < 0.05, **P < 0.01, and ***P < 0.001.

**Table 2.** Vascular smooth muscle nuclei density and internal elastic lamina integrity

<table>
<thead>
<tr>
<th></th>
<th>Nontransfected</th>
<th>Sham-transfected</th>
<th>LTCC siRNA</th>
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<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Number luminal vascular smooth muscle cell propidium iodide labeled nuclei per $10^3$ μm$^2$</td>
<td>27.8 ± 2.9</td>
<td>27.6 ± 3.3</td>
<td>26.8 ± 0.7</td>
</tr>
<tr>
<td>Proportion of arteries with intact internal elastic lamina, %</td>
<td>100</td>
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<td>100</td>
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</tbody>
</table>

Values are means ± SE. LTCC, L-type Ca$^{2+}$ channel.
water immersion lens), as previously described (40). Wires were brought in the position when they touched the arterial walls. Adventitia and media thickness, as well as the distance between the wires, were measured at three different points on either side of the vessel. Adventitia and media thickness were then normalized to the lumen diameter obtained during the normalization procedure in the wire myograph (39). Media area was calculated from measured media thickness, using the following equation: media area = \( \pi m_t(d_{int} + m_t) \), where \( m_t \) is the media thickness obtained during measurements in the wire myograph and \( d_{int} \) is internal artery diameter, calculated as \( 2(d_{wire} + \pi d_{wire} + 2 \times \text{distance between wires})/\pi \). The analysis of VSMC size and number was made using dissector as previously described (37).

Adventitial collagen staining. Picro-sirius red staining was used to identify collagen fibers in adventitia. Longitudinal paraffin sections of the arteries (3 \( \mu \text{m} \)) were deparaffinized, stained with Weigert’s hematoxylin for 8 min and then with 0.1% Sirius Red in saturated aqueous picric acid for 60 min. The sections were then treated with 0.5% acetic acid, dehydrated in 99% ethanol, treated with xylene, and mounted. Sirius red staining was quantified using ImageJ program (version 1.37; NIH) as described elsewhere (http://tsweb.mh.gov/sj/docs/examples/stained-sections/index.html). From each artery 2-10 regions of interests (ROIs) were used. The amount of collagen in adventitia was normalized to ROI area and to ROI length. The expression in nontransfected arteries was set to 100%, and relative expression in transfected arteries from the same rat was calculated.

Propidium iodide staining and internal elastic lamina examination. Rats were anesthetized with pentobarbital (50 mg/kg; Aarhus University hospital pharmacy, Denmark) and perfused via the left ventricle with PSS containing 0.1% BSA, 0.1% NaNO\textsubscript{3}, and 10 U/ml heparin to fully dilate the arteries and empty them of blood, and then with 4% formalin for fixation (11). The arteries were stored in PBS containing 0.02% sodium azide at 4°C. Arterial segments of 3-5 mm length were cut longitudinally and pinned to a Sylgard-coated dish using 25 and 50 \( \mu \text{m} \) tungsten pins, as ‘flat’ sheets, and further fixed in 2% formalin for 10 min. Tissue was then rinsed for 3 \( \times \) min and mounted in oil buffered glycerol containing propidium iodide (PI; 0.002% in buffered glycerol anti-fade mounting medium).

PI stained nuclei were acquired on an Olympus FV100 using same settings for all arteries at 600 nm. The internal elastic lamina (IEL) was imaged at 488 nm. Intensity of IEL was assessed by its appearance: with normal being a uniform flat sheet with scattered holes in contrast with a broken or cracked appearance, which is indicative of damage (Dr. Shaun Sandow, personal communication). The density of VSMC nuclei in the media and the IEL integrity were not different between nontransfected arteries, sham-transfected arteries, and arteries downregulated for LTCCs (Table 2).

**Fig. 2.** The effect of LTCC downregulation on resting [Ca\textsuperscript{2+}]i, and on contractile responses to norepinephrine (NE) and extracellular [K\textsuperscript{+}].

A: representative traces of [Ca\textsuperscript{2+}]i responses (expressed as fura-2 340/380 emission ratio) to 10 \( \mu \text{mol/l} \) NE in sham-transfected artery and artery transfected with siRNA targeting LTCC. B: averaged resting [Ca\textsuperscript{2+}]i, presented as fura-2 340/380 emission ratio, measured in nontransfected arteries (\( n = 7 \)), sham-transfected arteries (\( n = 14 \)), and arteries transfected with siRNA targeting LTCC (\( n = 15 \)). C: averaged [Ca\textsuperscript{2+}]i, presented as fura-2 340/380 emission ratio, measured in the presence of 10 \( \mu \text{mol/l} \) NE (\( n = 15 \) for sham-transfected arteries and \( n = 12 \) for arteries transfected with siRNA targeting LTCC). D: maximal contractile responses to 10 \( \mu \text{mol/l} \) NE of nontransfected arteries (\( n = 8 \)), sham-transfected arteries (\( n = 15 \)), and arteries transfected with siRNA targeting LTCC (\( n = 12 \)). E: effect of cumulative elevation of extracellular [K\textsuperscript{+}]i on [Ca\textsuperscript{2+}]i, in sham-transfected arteries (\( n = 7 \)) and arteries transfected with siRNA targeting LTCC (\( n = 5 \)). F: concentration-dependent contractions of sham-transfected arteries (\( n = 7 \)) and arteries transfected with siRNA targeting LTCC (\( n = 5 \)) to cumulative elevation of extracellular [K\textsuperscript{+}]i. **P < 0.05 and ***P < 0.001. A.u., arbitrary units.
digestion solution had the following composition (in mmol/l): 110 NaCl, 5 KCl, 0.16 CaCl₂, 2 MgCl₂, 10 NaHEPES, 10 NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 10 glucose, 0.49 EDTA, 10 taurine, 1.5 mg/ml papain, 1.6 mg/ml albumin, and 0.4 mg/ml DL-dithiothreitol, adjusted to pH 7.0. Low Ca²⁺ solution for cell isolation had the following composition (in mmol/l): 135 NaCl, 6 KCl, 10 NaHEPES, 1 MgCl₂, and 0.1 CaCl₂, adjusted to pH 7.4.

Data analyses. All expression data are given relative to nontransfected control. All data are given as means ± SE, and n represents the number of arteries used. In each instance only one transfected artery of each type was used from a single rat. Student’s t-test was used for single comparisons between sham-transfected arteries and LTCC-downregulated arteries. One-way ANOVA with Dunnet’s post-test was used for comparisons between nontransfected arteries, sham-transfected arteries, and LTCC-downregulated arteries. Sham-transfected arteries were used as a reference group for Dunnet’s post-test. For comparisons of the concentration-response curves an F-test was used (GraphPad Prism v. 2.0; GraphPad Software). P < 0.05 was considered statistically significant.

RESULTS

Downregulation of LTCCs in vivo. In vivo transfection of rat mesenteric artery segments with specific siRNAs resulted in significant downregulation of the pore-forming α₁C subunit of LTCCs at both mRNA (by 87.3 ± 2%, n = 13; Fig. 1A) and protein levels (by 43.8 ± 9.8%, n = 4; measured by immunofluorescence; Fig. 1, B and C; and by 50.9 ± 10.5%, n = 6; measured by Western blot, Fig. 1, E and F) three days after the transfection. Molecular analyses of other voltage-gated Ca²⁺ channels by qPCR showed significant reduction in mRNA for T-type Ca²⁺ channel subunits [Ca₃.1 by 77.9 ± 4.8% (n = 5) and Ca₃.2 by 72.1 ± 6.7% (n = 8)] and P/Q type Ca²⁺ channels [Ca₂.1 by 80.1 ± 10.1% (n = 4)] in the arteries downregulated for LTCCs compared with sham-transfected arteries.

Sham-transfected arteries relaxed to acetylcholine similarly to nontransfected arteries [86.2 ± 2.7% (n = 9) and 91.9 ± 0.9% (n = 7), respectively].

LTCC-downregulated arteries have increased resting [Ca²⁺], but reduced contractility to NE and high extracellular K⁺. Arteries downregulated for LTCCs had significantly elevated resting [Ca²⁺] (Fig. 2, A and B). In LTCC-downregulated arteries the increase of [Ca²⁺] in response to 10 μmol/l NE was significantly less than in the controls (Fig. 2A), and the steady state [Ca²⁺], in the presence of 10 μmol/l NE was not significantly different between three groups of arteries (Fig. 2C). Despite apparently similar [Ca²⁺], arteries downregulated for LTCCs developed significantly less tension in response to NE compared with nontransfected and sham-transfected arteries (Fig. 2D). No difference between nontransfected and sham-transfected arteries was seen.

Elevation of extracellular K⁺ caused a concentration-dependent increase in [Ca²⁺] (Fig. 2E) and wall tension (Fig. 2F) in sham-transfected arteries. Both [Ca²⁺] and tension responses were due to Ca²⁺ influx via LTCCs since they were inhibited by 1 μmol/l nifedipine (not shown). The arteries downregulated for LTCC had markedly reduced tension responses to high extracellular K⁺ (Fig. 2F). Accordingly, the increase of [Ca²⁺] in arteries downregulated for LTCC was significantly less in comparison with the sham-transfected arteries (the rise from resting to maximally stimulated levels was 0.48 ± 0.20 arbitrary units (n = 5) for LTCC-downregulated arteries vs. 1.46 ± 0.25 arbitrary units (n = 7) for sham-transfected arteries, P < 0.05; Fig. 2E). Importantly, with high K⁺ (80–125 mmol/l) in the bath solution [Ca²⁺] (80–125 mmol/l) in the bath solution [Ca²⁺], reached the same level in both experimental groups (Fig. 2E) despite different tension responses (Fig. 2F).
LTCC downregulation suppresses \([\text{Ca}^{2+}]\), response to NE but not to caffeine. Stimulation with NE in extracellular \([\text{Ca}^{2+}]\)-free conditions increases \([\text{Ca}^{2+}]\), primarily through activation of IP$_3$R and partially through RYR via \([\text{Ca}^{2+}]\) release (18). In \([\text{Ca}^{2+}]\)_free conditions, arteries downregulated for LTCCs had a significantly reduced increase in \([\text{Ca}^{2+}]\), and tension in response to 10 \(\mu\text{mol/l}\) NE in comparison with sham-transfected arteries that had responses similar to non-transfected arteries (Fig. 3, A and B).

In contrast, the increase in \([\text{Ca}^{2+}]\), during stimulation with caffeine was not significantly different between the groups (Fig. 3C), but the contractile response was significantly sup-
pressed in arteries downregulated for LTCCs in comparison with sham-transfected arteries (Fig. 3D).

**Downregulation of LTCC reduces NE-induced arterial contraction in vivo.** To test whether functional changes observed in LTCC-downregulated mesenteric small arteries in vitro are also present in vivo, we studied the function of these arteries in anesthetized rats and compared them with the neighboring sham-transfected arteries in the same rat (Fig. 4, A–C). We found that unstimulated arteries downregulated for LTCC have slightly but significantly reduced inner diameter in comparison with sham-transfected arteries (Fig. 4D). Arteries downregulated for LTCC also had a significantly suppressed concentration-dependent decrease in diameter in response to NE (Fig. 4, A–C). The response to 80 mmol/l K⁺ depolarization was also significantly reduced in arteries downregulated for LTCC in comparison with sham-transfected arteries (Fig. 4E).

**Ten days after transfection LTCC mRNA and arterial contractile responses to NE and high extracellular K⁺ are normalized.** Ten days after transfection the expression of LTCC mRNA was not different between sham-transfected arteries (96 ± 15%; n = 5) and arteries transfected with siRNA targeting LTCC (110 ± 13%; n = 5). The LTCC protein levels were also not different between sham-transfected arteries and arteries downregulated for LTCC 10 days post-transfection (Fig. 5, A and B). Furthermore, 10 days after the transfection, arteries transfected with siRNA targeting LTCC exhibited a stronger contractile response to elevated K⁺ than sham-transfected arteries (Fig. 5C). The contractile responses to 10 µmol/l NE were not different between sham-transfected and LTCC siRNA transfected arteries (Fig. 5D). The normalization of expression and functional parameters of arteries transfected with LTCC-siRNA suggests a transient action for siRNA targeting LTCC.

**α-Toxin permeabilization revealed reduced Ca²⁺-responsiveness in LTCC-downregulated arteries.** The reduced contractile responses to high extracellular K⁺ and NE seen in arteries 3 days after transfection with siRNA targeting LTCC (Fig. 2, D and F) suggest a reduced function of the contractile machinery in these arteries. We tested this hypothesis in α-toxin permeabilized arteries by assessing the response to 1 µmol/l Ca²⁺ (applied at arrow) of a sham-transfected artery and artery transfected with siRNA targeting LTCC permeabilized with α-toxin. B: contraction of permeabilized arteries (from protocol shown in A) was significantly reduced in arteries downregulated for LTCC (n = 7) in comparison with sham-transfected arteries (n = 9). ***P < 0.001.

Expression of contractile protein genes is reduced by LTCC downregulation. Expression of contractile machinery proteins in VSMCs 3 days post-transfection was assessed by qPCR and Western blotting (Fig. 7). Expression of mRNAs for smooth muscle myosin heavy chain, smooth muscle α-actin, and h-caldesmon was significantly lower in the arteries downregulated for LTCCs in comparison with sham-transfected arteries (Fig. 7A). Transfection using 0.9% NaCl saline was used as an additional control to exclude any potential effect of transfection reagent on the gene expression of the contractile marker genes. No significant difference between saline-transfected and sham-transfected arteries was observed. Reduction in the expression of genes important for contraction in the arteries downregulated for LTCCs was not, however, associated with changes in the noncontractile phenotype marker genes l-caldesmon and tropomyosin 4 (44, 55) (Fig. 7B).

Consistent with the mRNA data, the protein expression of smooth muscle myosin heavy chain was significantly reduced in the arteries downregulated for LTCCs in comparison with sham-transfected arteries and nontransfected arteries (Fig. 7, C and D). Consistent with the tone development to NE and K⁺ the reduction of myosin heavy chain was transiently reduced and 10 days post-transfection was normalized (Fig. 7E).

**Downregulation of LTCCs is associated with altered arterial structure.** Three days after transfection with siRNA targeting LTCCs, morphological changes in the arterial wall were observed (Fig. 8A). Although lumen diameters did not differ significantly between the LTCC-downregulated, sham-transfected, and nontransfected arteries (Fig. 8B), the media thickness (Fig. 8, A and C) and the adventitia thickness (Fig. 9A) were increased in LTCC-downregulated arteries. Additionally, the media area (Fig. 8D) was significantly increased. Finally, the media-to-lumen ratio was increased in arteries downregulated for LTCCs (Fig. 8E), suggesting that downregulation of LTCCs led to hypertrophic remodeling of media.

Stereological analysis revealed a significant increase in the number of VSMCs per unit length (Table 3) in arteries downregulated for LTCCs. There was, however, no significant change in the size of VSMCs between the groups. The amount of collagen per adventitia length (Fig. 9, B and C) and the relative content of collagen in the adventitia (Fig. 9D) were not significantly affected in arteries downregulated for LTCCs.

**siRNA targeting P/Q-type Ca²⁺ channels has no major effects on arterial structure and function.** Because downregulation of LTCC was also associated with reduced mRNA expression of other voltage-gated Ca²⁺ channels, we tested...
whether downregulation of P/Q-type Ca\textsuperscript{2+} channels has structural and functional consequences. Three days after transfection with siRNA targeting P/Q-type Ca\textsuperscript{2+} channels, mRNA encoding this protein was significantly reduced in comparison with nontransfected and sham-transfected arteries (Fig. 10A). No changes in mRNA encoding \(\alpha_{1C}\) subunit of LTCCs, as well as proteins associated with contractile function (smooth muscle myosin heavy chain and \(\alpha\)-actin), were found. Arteries downregulated for P/Q-type Ca\textsuperscript{2+} channels contracted similarly to NE as sham-transfected arteries (Fig. 10B). P/Q-type Ca\textsuperscript{2+} channel-downregulated arteries contracted slightly stronger to the maximal K\textsuperscript{+}-induced depolarization in comparison with sham-transfected arteries (Fig. 10C). No change on arterial morphology (relaxed lumen diameter, media thickness and area, and media-to-lumen ratio) was found (Fig. 10D).

**DISCUSSION**

It is well known that LTCCs are important for VSMC contraction by being the major Ca\textsuperscript{2+} influx pathway. It is also well known that change in VSMC phenotype from a contractile to a proliferative or synthetic phenotype is associated with downregulation of LTCCs (17, 60). However, a causal relationship between loss of LTCCs and loss of VSMC cell contractile phenotype is not established. In the current study we have provided evidence that this association is causal (i.e.,
that downregulation of LTCC leads to a noncontractile VSMC phenotype.

We downregulated LTCCs in vivo in a single segment of rat mesenteric small artery using siRNA transfection. Several important observations were made. We found that arteries downregulated for LTCCs have 1) increased resting VSMC [Ca$^{2+}$], 2) reduced contractility to [Ca$^{2+}$], and 3) reduced mRNA for VSMC contractile genes and corresponding protein, and underwent inward, hypertrophic remodeling. These results provide evidence that LTCCs are not only important for VSMC contractility but also contribute to resting [Ca$^{2+}$], level and maintenance of a VSMC contractile phenotype and have complex effects on vascular structure in vivo.

**LTCCs can be effectively downregulated in vivo.** Transfection with siRNA specifically targeting LTCC led to marked reduction of LTCC mRNA and protein in the arterial wall. Moreover, staining of freshly isolated VSMCs with a LTCC-specific antibody indicated an effective downregulation of LTCC protein by specific siRNAs. The effectiveness of the LTCC downregulation in vivo was further supported by the expected reduction in [Ca$^{2+}$], increase and contraction in response to K$^+$-induced depolarization and stimulation with NE. We compared LTCC-downregulated arteries with arteries transfected with siRNA directed against eGFP and nontransfected arteries. Importantly, arteries transfected with siRNA targeting eGFP (which is not present in the rat genome) had unchanged contractile function, Ca$^{2+}$ homeostasis, expression of contractile phenotype marker genes, and vascular structure in comparison with nontransfected arteries. This indicates that the sham-transfection procedure did not have unspecific effects consistent with our previous reports (9, 33). Furthermore, transfection procedure did not affected the endothelium-specific relaxation.

In addition to VSMCs, several cell types present in the arterial wall can contribute to overall mRNA and protein signals. T-type and P/Q-type Ca$^{2+}$ channels were shown to be expressed in VSMCs too (1, 7, 21). When the P/Q-type Ca$^{2+}$ channels were downregulated in mesenteric small arteries, there was no change in the expression of LTCC and contractile proteins. P/Q-type Ca$^{2+}$ channel-downregulated arteries had normal structure and normal contractile response to NE. Although there was a slight increase in the sensitivity to K$^+$ of P/Q-type Ca$^{2+}$ channel-downregulated arteries, these findings support the specificity of the approach used to downregulate LTCCs.

**LTCC downregulation elevates resting [Ca$^{2+}$].** Stimulation of VSMC with either increased extracellular K$^+$ or NE did not lead to an increase of [Ca$^{2+}$], in LTCC-downregulated arteries. This might be expected since LTCCs are known to play a dominant role for the [Ca$^{2+}$] increase following G protein-coupled receptor stimulation of depolarization of VSMCs. However, resting [Ca$^{2+}$]-in arteries downregulated for LTCCs was higher than resting [Ca$^{2+}$] in sham-transfected arteries. This is in contrast with data from mice with a VSMC-specific knock-down of LTCCs where [Ca$^{2+}$] was reported reduced (13) or unaffected (14). However, blocking LTCCs with dihydropyridines induces an increase in [Ca$^{2+}$] in VSMCs from pulmonary hypertensive patients via activation of the Ca$^{2+}$-sensing receptor (61). A high resting [Ca$^{2+}$], is also reported in noncontractile VSMCs in culture (5). High resting [Ca$^{2+}$], is
Fig. 9. Adventitia thickness (A) of nontransfected arteries (n = 9), sham-transfected arteries (n = 23), and arteries transfected with siRNA targeting LTCC (n = 24) 3 days post-transcription. Collagen content in adventitia was measured using Picro-sirius red staining (B) per unit length (C) and per unit area (D) in sham-transfected arteries (n = 4) and arteries transfected with siRNA targeting LTCC (n = 3). The results are shown relative to the level of collagen in nontransfected arteries from the same rat (100%). Bars indicate 50 μm. *P < 0.05.

unlikely to be a result of compensatory upregulation of other voltage-dependent Ca\(^{2+}\) channels since we found a reduction at the mRNA level of Ca\(^{3,1}\), Ca\(^{3,2}\), and Ca\(^{2,1}\) in LTCC-downregulated arteries. Another possibility is that store-operated Ca\(^{2+}\)-entry (SOCE) is responsible for the high resting \([Ca^{2+}]_i\). It has previously been shown that change from contractile to a noncontractile VSMC phenotype is associated with the shift from voltage-dependent to voltage-independent SOCE-mediated Ca\(^{2+}\) influx (49). SOCE components (e.g., transient receptor potential channels, stromal interaction molecule 1, and Orai1) are markedly upregulated during proliferation of rat aortic VSMCs in culture (5). It will be relevant in future studies to assess whether SOCE proteins of functions changes in arteries downregulated for LTCCs. A third possibility is that reduced Ca\(^{2+}\) removal is responsible for the elevated resting \([Ca^{2+}]_i\) (5, 31). The loss of contractile phenotype in VSMCs in culture is associated with downregulation of SERCA (SERCA2a to SERCA2b) pumps (8), which may contribute to increased \([Ca^{2+}]_i\). It will be of interest to see whether this is also the case in the LTCCs-downregulated arteries.

Sarcoplasmic reticulum Ca\(^{2+}\) handling in LTCC-downregulated arteries. In LTCC-downregulated arteries NE-induced IP\(_3\)-dependent Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) was reduced. The reduction was not due to modification in RYR-mediated Ca\(^{2+}\) release since Ca\(^{2+}\) release by caffeine was preserved. This is consistent with the findings in mice with VSMC-specific LTCC knock-down (14). It is possible that IP\(_3\)R are deactivated by high VSMC \([Ca^{2+}]_i\), (35) or that upstream NE-induced signaling including the adrenergic receptor function is compromised. Altered SR Ca\(^{2+}\) handling is typically observed during phenotypic modulation (59). However, the current findings differ from previous studies on noncontractile VSMCs in culture where IP\(_3\)-mediated Ca\(^{2+}\) release is enhanced (5, 59).

Downregulation of LTCC leads to shift from contractile to noncontractile phenotype of VSMCs. Ca\(^{2+}\) influx through LTCCs activates Ca\(^{2+}\)-dependent transcription factors. Genes regulated by these factors in VSMCs include those involved in control of VSMC proliferation and phenotypic expression, namely early response genes such as c-fos and egr-1 (47) and genes characteristic for contractile (41) and noncontractile phenotypes (56). It is consistently reported that phenotypic

**Table 3. Number and size of smooth muscle cells determined by the three-dimensional disector technique**

<table>
<thead>
<tr>
<th></th>
<th>Sham-transfected</th>
<th>LTCC siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Cell volume, μm(^3)</td>
<td>2,173 ± 201</td>
<td>1,691 ± 118</td>
</tr>
<tr>
<td>Cells per unit length, μm(^{-1})</td>
<td>8.3 ± 1.3</td>
<td>12 ± 1*</td>
</tr>
<tr>
<td>Cell length, μm</td>
<td>49.3 ± 6.6</td>
<td>36.8 ± 4.6</td>
</tr>
<tr>
<td>Cell cross section, μm(^2)</td>
<td>46.1 ± 4.2</td>
<td>49.9 ± 6.2</td>
</tr>
<tr>
<td>Cell layers</td>
<td>3.0 ± 0.3</td>
<td>3.9 ± 0.7</td>
</tr>
</tbody>
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Values are means ± SE. *P < 0.05 compared with sham-transfected.
modulation of VSMCs is associated with loss of LTCCs (17, 23, 48). Downregulation of LTCC currents are observed in dedifferentiated Ar75 cell and rat aortic VSMCs after balloon catheter injury (17, 48). Migratory and proliferative VSMCs of newly formed neointima demonstrate no LTCC current (48). Connection between LTCC and VSMCs phenotype is underlined by high coordination between expression of LTCCs and contractile phenotype marker genes (17, 48). However, the precise role of LTCCs in VSMC phenotypic expression has not been established. Our findings strongly suggest that the expression of LTCCs plays a key role in maintaining the VSMC contractile phenotype.

The loss of contractility was directly confirmed in arteries permeabilized with α-toxin. This is consistent with reduced mRNA expression of several genes (smooth muscle myosin heavy chain, smooth muscle α-actin, and h-caldesmon) important for contractile function and associated with the contractile phenotype of VSMCs (45) as well as reduced expression of smooth muscle myosin heavy chain protein. Interestingly, downregulation of LTCCs was associated with VSMC hyperplasia. The stereological analysis demonstrated a significant increase in the number of VSMCs per segment length in arteries downregulated for LTCCs. The mechanism of this hyperplastic response is unclear. It could be due to inhibition of VSMC degradation, although a low VSMC turnover in the normal media makes this possibility unlikely. A more likely possibility is increased VSMC proliferation. On the other hand, tropomyosin 4 and l-caldesmon, which are considered to be markers for a noncontractile phenotype associated with cell proliferation (44, 55), were not increased. It will therefore be important in future work to test other indexes of VSMC proliferation, possibly at earlier time points than 3 days used in this study.

Arteries downregulated for LTCCs demonstrated hypertrophic vascular remodeling. In arteries downregulated for LTCCs inward hypertrophic remodeling (38) was found. Although changes in normalized lumen diameter did not achieve significance in vitro, a significantly reduced inner diameter in LTCC-downregulated arteries was seen in vivo. This finding contrasts with the reduction in media-to-lumen ratio after chronic treatment with LTCC blockers of spontaneously hypertensive rats (25) and patients with essential hypertension.
The plasticity of VSMCs in vivo. LTCCs in control of VSMC phenotype and the dramatic LTCC downregulation further supports the powerful role of vascular remodeling and VSMC phenotype (3). Finally, chronic pharmacological blockade of vascular LTCCs reduced high media-to-lumen ratio in hypertensive individuals together with a reduction of transmural pressure, which is not seen after LTCC downregulation in the present study. Very few studies have addressed the effect of dihydropyridines on the structure of small arteries under normotensive conditions. No change in lumen diameter and media-to-lumen ratio was seen in mesenteric small arteries from normotensive rats after chronic treatment with LTCC blockers (28, 42). Small arteries from other vascular beds under normotensive conditions were also unaffected by LTCC blockers. This is in contrast with one study with large arteries showing that pharmacological blockade under normotensive conditions leads to reduction in inner diameter and wall thickness and, consequently, reduced media-to-lumen ratio (58). Interestingly, the change in inner diameter under normotensive conditions was consistent with our findings. A few other studies report, however, no structural changes in large arteries of normotensive rats after chronic treatment with LTCC blockers (15).

The rapidity (3 days) of arterial remodeling in the arteries downregulated for LTCCs was remarkable. However, this is consistent with the findings that remodeling of resistance arteries can occur already after two days after application of a stimulus (10, 29). Interestingly, arterial remodeling induced by loss of LTCCs was observed not only in media but also in adventitia. Adventitia thickness and area were increased in the arteries downregulated for LTCCs, indicating that the modification of the VSMCs phenotype had consequences for the adventitia layer. However, we were not able to demonstrate a significant increase in collagen content in the adventitia.

Reversal of the effect of LTCC downregulation. The dramatic change, with almost complete disappearance of contractility and expression of contractile proteins, after in vivo LTCC downregulation could suggest irreversible damage of VSMC in these arteries. However, the naked siRNA used has only a transient effect in cells (4, 63), and it was possible to test the reversibility of the changes. Ten days after transfection the expression of LTCC mRNA and protein in the arteries transfected with LTCC-siRNA was similar to that in control arteries as was the reactivity and contractility to NE. The arteries transiently downregulated for LTCCs had an increased response to extracellular K⁺ compared with controls 10 days after transfection. It is possible that this reflects a compensatory increase in other Ca²⁺ influx pathways. This reversibility of the effect of LTCC downregulation further supports the powerful role of LTCCs in control of VSMC phenotype and the dramatic plasticity of VSMCs in vivo.

Conclusion
In the current study we have demonstrated that rat mesenteric resistance arteries essentially lacking LTCCs have increased resting [Ca²⁺], but greatly impaired contractile responses associated with decreased expression of contractile phenotype marker genes. Structural changes corresponding to inward, hypertrophic remodeling with VSMC hyperplasia were observed in the arteries downregulated for LTCCs. The results suggest that LTCCs are essential not only for VSMC contractility but also for maintaining the contractile phenotype and normal vascular structure.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
Author contributions: O.K., K.M.H., M.S.S., D.M.B.B., V.V.M., and C.A.

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