The Role of L- and T-Type Calcium Channels in Local and Remote Calcium Responses in Rat Mesenteric Terminal Arterioles

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Key Words

Ca²⁺ channels • Conducted vascular responses • Endothelium • Gap junctions • Intercellular communication • Vascular smooth muscles

Abstract

Background/Aims: The roles of intercellular communication and T-type versus L-type voltage-dependent Ca²⁺ channels (VDCCs) in conducted vasoconstriction to local KCl-induced depolarization were investigated in mesenteric arterioles. **Methods:** Ratiometric Ca²⁺ imaging (R) using Fura-PE3 with micro-ejection of depolarizing KCl solution and VDCC blockers, and immunohistochemical and RT-PCR techniques were applied to isolated rat mesenteric terminal arterioles (n = 71 from 47 rats; intraluminal diameter: 24 ± 1 μm; length: 550–700 μm). **Results:** Local application of KCl (at 0 μm) led to local (ΔR = 0.54) and remote (ΔR = 0.17 at 500 μm) increases in intracellular Ca²⁺. Remote Ca²⁺ responses were inhibited by the gap junction uncouplers carbenoxolone and palmitleic acid. Ca_v1.2, Ca_v3.1 and Ca_v3.2 channels were immunolocalized in vascular smooth muscle cells and Ca_v3.2 in adjacent endothelial cells. Local and remote Ca²⁺ responses were inhibited by bath application of L- and T-type blockers [nifedipine, NNC 55-0396 and R(-)-efonidipine]. Remote Ca²⁺

responses (500 μm) were not affected by abolishing Ca²⁺ entry at an intermediate position on the arterioles (at 200–300 μm) using micro-application of VDCC blockers. **Conclusion:** Both L- and T-type channels mediate Ca²⁺ entry during conducted vasoconstriction to local KCl in mesenteric arterioles. However, these channels do not participate in the conduction process per se.

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Introduction

A vascular conducted response is the phenomenon whereby a locally initiated vasodilatation or vasoconstriction is conducted along the arteriolar wall independently of blood flow, diffusible factors or neural activity. Conducted vasodilatation and vasoconstriction play important roles in the local coordination of blood flow within tissues in response to changes in metabolic demand [1–3], and intercellular communication via gap junctions is believed to be crucial for conduction of these responses [4, 5]. Gap junction-forming connexins providing low-resistance electrical coupling were found to be expressed in rat mesenteric terminal arterioles [6]; however, functional evidence for the involvement of gap junc-

tions in the conduction of vasoconstrictor signals in this vascular bed is still lacking. In several arteriolar preparations, local depolarization is conducted along the vascular wall and is followed by both local and conducted vasoconstriction [7, 8]. As voltage-dependent Ca^{2+} entry is a central step in excitation-contraction coupling, we aimed to investigate Ca^{2+} signals underlying conducted vasoconstriction to local depolarization in arterioles. In rat mesenteric terminal arterioles, a role for a T-type voltage-dependent Ca^{2+} channel (VDCC) in Ca^{2+} entry and vasoconstriction has been proposed [9–11]. However, the expression and localization of T-type protein in mesenteric arterioles has not yet been demonstrated. In the present study, we examined the functional nature of conducted vasoconstriction initiated by local application of KCl in rat mesenteric terminal arterioles, and sought the potential role of VDCCs in this process. The main findings are that both L- and T-type channels are expressed at the protein level in mesenteric arterioles and are important for electromechanical coupling. However, they are not necessary for conduction of vasoconstriction to local KCl application.

Materials and Methods

Animals and Preparation

Male Sprague-Dawley rats (150–200 g; Charles River, Tokyo, Japan, or Taconic, Lille Skensved, Denmark) were sacrificed using pentobarbital sodium (Nembutal, 65 mg/kg i.p.). A mesenteric fat pad was excised along with a 5th-order mesenteric artery and its peripheral branches. The excised tissue was submerged in PSS containing 1% bovine serum albumin (low endotoxin) and placed under a stereomicroscope ($\times 30$ magnification) where terminal arterioles (intraluminal diameter, ID, 10–40 μm ; length 550–700 μm) were microdissected from their parent vessel.

Isolated arterioles (fig. 1) were made to adhere to the glass bottom of a perfusion chamber (model RC-22, Warner Instrument, Hamden, Conn., USA) by Cell Tak (Becton Dickinson Labware, Bedford, Mass., USA), and herein incubated for 90–120 min (at room temperature) with PSS containing (in mM): 140 NaCl, 5 KCl, 1.2 MgCl_2 , 2 CaCl_2 , 10 HEPES and 10 glucose (pH 7.4); to which 5 μM Fura-PE3/AM, 1% BSA, 0.1% DMSO, 0.02% Pluronic F127 and 0.06% Cremophore EL had been added. After abluminal loading of Fura, the vessels were rinsed with PSS and allowed to rest for ≥ 20 min before each experiment. In Ca^{2+} -free PSS, 2 mM CaCl_2 was replaced with 2 mM EGTA. In PSS used for preconstriction of arterioles, 25 mM NaCl was replaced mole for mole with KCl. In experiments using low $[\text{Na}^+]$, 126 mM NaCl was replaced with 126 mM N-methyl-D-glucamine-Cl. The recording chamber (~ 0.1 ml capacity) was mounted on the stage of an inverted epifluorescence microscope (Olympus IX70) placed on top of a vibration-free table (Newport, Irvine, Calif., USA). Solutions were added via continuous superfusion (flow rate ~ 1 ml/min), and excess solution was removed by suction. Experiments were per-

Fig. 1. **a** Bright-field image of isolated mesenteric terminal arteriole loaded with ratiometric Ca^{2+} indicator Fura-PE3 and mounted on a cover slide for measurement of $[\text{Ca}^{2+}]_i$. Pipette tip for micro-ejection of KCl is visible in the upper right corner. Direction of the superfusion flow is indicated by arrows. **b** Same arteriole shown with excitation of Fura-PE3 at 340 nm and with emission collected at 510 nm. Numbered circles indicate ROIs for Ca^{2+} measurement.

formed at room temperature (25–28°C). Rat anesthesia and euthanasia were done according to the local guidelines for animal research at Kyushu University and University of Copenhagen.

Calcium Imaging and Diameter Measurements

A $\times 20$ quartz objective (Olympus UAp0/340) with numerical aperture = 0.75 was used for Ca^{2+} measurements. The non-presurized arterioles were visualized on a PC monitor using a CCD camera (Hamamatsu Hi SCA C6790, Hamamatsu City, Japan) and Aquacosmos software (Hamamatsu). Using a polychromator (Hamamatsu C7773) controlled by the software, arterioles were excited with light of alternating wavelengths (340 vs. 380 nm; 100-ms exposure for each wavelength; images acquired every 2 s). Regions of interest (ROIs, approximately 50 $\times$ 50 μm) for the mea-

surement of intracellular calcium concentration ($[Ca^{2+}]_i$) were placed next to each other covering the entire length of an arteriole (fig. 1b). The recorded 340- and 380-nm images and fluorescence intensities were stored on a PC hard disk. The field of view was $400 \times 535 \mu\text{m}$, and by arranging arterioles diagonally within the camera field, $[Ca^{2+}]_i$ could be measured simultaneously along $\sim 600 \mu\text{m}$ of an arteriole (fig. 1). Before data analysis, the length and diameter of an arteriole were measured using ImageJ software (version 1.34i; Wayne Rasband, National Institutes of Health, Bethesda, Md., USA) using stored 340-nm fluorescent and bright-field images. Then each ROI was assigned a distance (0–500 μm) from the site of local stimulation also using ImageJ. The Fura ratio ($R = F_{340}/F_{380}$) for each ROI was calculated after a constant background value had been subtracted from the intensity measured at each wavelength. Results presented in the text and illustrations are either R values or ΔR values (peak R – baseline R).

Images were focused inside the *media* layer, which could be visualized as brightly fluorescent bands of vascular smooth muscle cells (VSMCs) oriented perpendicularly to the length axis of the vessel, in order to record Ca^{2+} responses from the VSMCs only. In a subset of experiments, ID and R were tracked simultaneously during local application of KCl (fig. 2a). By adjusting the focal plane through the mid point of the vessel such that the lumen was clearly visible, ID was measured on each successive 340-nm image. Subsequently, R and ID were plotted for both local (0 μm) and remote sites (500 μm).

All estimates of Ca^{2+} concentrations were based on in vitro calibration of the Ca^{2+} imaging system. Calibration was performed as previously described [10] using Fura-PE3 K^+ salt and a K_d of 290 nM for Fura-PE3 in the presence of Mg^{2+} [12]. Fluorescence of bound versus unbound dye at 340 and 380 nm was determined using commercial calibration buffers (Invitrogen-Molecular Probes, Eugene, Oreg., USA) with low versus high $[Ca^{2+}]$ in the presence of EGTA, Mg^{2+} , and at constant ionic strength (pH 7.2) and temperature.

Localized Application of KCl and Drugs

Local depolarization was imposed using pressure micro-ejection of KCl (155 mM, 0.1% phenol red, 10 mM HEPES, pH 7.4). In brief, the tip of a micropipette (3–5 μm) pulled from 1.0-mm glass capillaries (Narishige, Tokyo, Japan) using a horizontal pipette puller (model P-97, Sutter Instrument, Novato, Calif., USA) was positioned $<10 \mu\text{m}$ from an arteriole. A short puff of KCl (1,500 ms) was given (at 30 psi) onto the arteriole using a Narishige IM 300 programmable microinjector. The pipette tip was positioned so that the ejected KCl was carried away perpendicularly to the arteriolar length axis. Before each experiment, it was visually confirmed that upon micro-ejection a ‘cloud’ of KCl/phenol red briefly covered a short segment ($<100 \mu\text{m}$) of an arteriole before being spread in the direction of the flow. While monitoring $[Ca^{2+}]_i$, KCl was ejected three times with 4-min intervals. Then a drug (inhibitor) was applied via superfusion for 3–10 min before repeating three KCl stimulations during exposure to the drug. In most experiments, the drug was then washed out for 10 min and the stimulation was repeated two or three times to assess reversibility.

In initial control experiments, ejection of KCl did not elicit a Ca^{2+} response when the micropipette tip was positioned 50–100 μm above an arteriole, whereas when it was lowered onto the arteriole (as shown in fig. 1a) local and remote Ca^{2+} responses were

readily observed at positions spatially separated by 500 μm ($n = 2$). In four arterioles, a small cut or crush was made using sharpened forceps at an intermediate position 200–300 μm distant to the micropipette tip. Prior to making a cut, local stimulation elicited a robust remote ($>500 \mu\text{m}$) Ca^{2+} response. After making the cut, local stimulation (at either end of the arteriole) resulted in remote Ca^{2+} responses up to the point of the lesion, but remote Ca^{2+} responses were never observed on the opposite side of the cut.

In some experiments, drugs were delivered only to a narrow region of an arteriole. This was done by applying a gentle pressure head (10–100 mm Hg) to a patch pipette (4–5 M Ω) pulled from 1.5-mm glass capillaries (Narishige). Again, strictly localized perfusion of a drug (dissolved in PSS + 0.1% phenol red, pH 7.4) was visually confirmed before each experiment. Micropipettes were secured with Narishige Hi-6 (1.5 mm) or Hi-7 (1.0 mm) injection holders connected to the pressure source with hard polyethylene tubing, and positioned using Narishige hydraulic manipulators.

Reverse Transcription Polymerase Chain Reaction

Isolation of total RNA was performed using RNeasy RNA stabilization reagent and the RNeasy micro kit (Qiagen, Hilden, Germany). In brief, 67 mesenteric terminal arterioles from 5 rats were microdissected and quickly submerged in RNeasy storage solution until dissection was finished. Arterioles were then submerged in 350 μl of lysis buffer containing guanidine-isothiocyanate and β -mercaptoethanol, and homogenized by repeated aspiration using a small-bore glass pipette. A total of 0.36 μg of RNA was recovered following the manufacturer’s instructions. Reverse transcription (RT) polymerase chain reaction (PCR) was performed twice (on pooled RNA from 2 vs. 3 rats) using a one-step RT-PCR kit (Qiagen). In brief, template RNA (in an amount corresponding to 3 mm of arteriole) and primer pairs (0.6 μM) were mixed with RT-PCR buffer, dNTPs, reverse transcriptase and HotStarTaq DNA polymerase in a PCR tube at 4°C. RT-PCR was

Fig. 2. a Simultaneous measurement of local (0 μm) and remote (500 μm) Ca^{2+} responses ($R = F_{340}/F_{380}$) and local and remote vasoconstriction ($n = 5$; $N = 5$). Note that the maximal constriction could be detected 2 s after the peak of the Ca^{2+} response at both locations (the image sampling frequency precluded higher time resolution of this delay). **b** Global mean Ca^{2+} responses ($\Delta R = \text{peak } R - \text{baseline } R$) as a function of the distance to the site of local KCl application in terminal arterioles ($n = 60$; $N = 42$; $ID = 25 \pm 1 \mu\text{m}$). The fitted line is a least-square regression analysis using first-order mono-exponential decay equation $Y = Y_0 + Ae^{-X/L}$ in which X is conducted distance, L is the empirical length constant, Y_0 is an asymptotic value and A is a constant. The values of constants obtained by fitting were: $Y_0 = 0.14$; $A = 0.42$; $L = 161 \mu\text{m}$ ($r^2 = 0.997$). When Ca^{2+} was removed from the superfusate, the baseline R decreased from 1.37 ± 0.07 to 1.19 ± 0.03 ($n = 3$; $N = 3$) and there were no detectable increases in R upon local KCl application. **c** At high rate of image acquisition (5 Hz), there was no detectable delay in the initiation or peak of Ca^{2+} increase to local KCl application. For the purpose of illustration, R was normalized with the value at 0 s (R_0).

performed sequentially using the following program: RT at 50°C for 30 min followed by 33 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min.

The following primer pairs were used: β -actin (GenBank accession No. NM031144), left primer: 5'-AGCCATGTACGTAGC-CATCC-3', right primer: 5'-CTCTCAGCTGTGGTGGTGAA-3' (product size 228 bp); $\text{Ca}_v1.2$ (Cagnalc, accession No. NM012517), left primer: 5'-CAGCAGGTCCTATGTCAGCA-3', right primer: 5'-CCGACAGCAGTGAATGAAGA-3' (product size 157 bp); $\text{Ca}_v1.3$ (Cacna1d, accession No. NM017298), left primer: 5'-AAGGCTAAAGCACGTGGAGA-3', right primer: 5'-CACC-AGAGACTTCCACAGCA-3' (product size 239 bp); $\text{Ca}_v3.1$ (Cag-nal1g, accession No. AF027984), left primer: 5'-CATTGCTGTGCCTTCTTCA-3', right primer: 5'-GCCCAGGTTG-TCAAAGTTGT-3' (product size 169 bp); $\text{Ca}_v3.2$ (Cagnalh, accession No. AF290213), left primer: 5'-ATCCTGGTCACTCT-GCTGCT-3', right primer: 5'-ACCTCCTCCGTCTGGTAGT-3' (product size 201 bp), and $\text{Ca}_v3.3$ (Cagnali, accession No. AF086827), left primer: 5'-ACAACCCCTGGATGCTACTG-3', right primer: 5'-GCATAGTAGGGCAGCCTCTG-3' (product size 205 bp).

Immunofluorescence Confocal Laser Scanning Microscopy

Mesenteric arterioles were excised as described above and immediately fixed in 2% paraformaldehyde dissolved in phosphate-buffered saline (PBS) for 15 min, followed by submersion in 50 mM NH_4Cl for 20 min to stop the fixation process. After rinsing with PBS, arterioles were mounted on metal stubs using Tissu-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and snap-frozen in liquid nitrogen. Sections (12 μm) were cut on a Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on Metzler microscope slides.

Tissue sections were permeabilized in 0.2% Triton X-100 in PBS with 1% goat serum for 15 min and blocked in PBS/1% goat serum. All incubations with antibody were carried out in the presence of 0.1% goat serum. Sections were incubated overnight (at 4°C) with primary antibodies to the following VDCC α_1 -subunits: $\text{Ca}_v1.2$ (1:25–1:50; Sigma-Aldrich; antigenic peptide from Alomone Labs, Jerusalem, Israel); $\text{Ca}_v3.1$ (1:300–1:500; [13]), or $\text{Ca}_v3.2$ (1:300; Alomone Labs). An antigen retrieval technique previously adapted for immunocytochemistry [14], which consisted of treatment with 1% SDS in distilled water (2 min), was essential for obtaining $\text{Ca}_v1.2$ -specific labeling in rat mesenteric arterioles. Peptide preincubation controls (1:1 w/w mixture; 30 min at room temperature) were included for both the $\text{Ca}_v1.2$ and $\text{Ca}_v3.2$ antibodies using their short epitopic peptides. After the primary incubation, sections were washed and incubated with goat anti-rabbit secondary antibody conjugated with Alexa-488 (1:800; Molecular Probes) and rhodamine-phalloidin (Molecular Probes) at room temperature for 45 min. After several washing steps, sections were mounted in ProLong Gold mounting medium (Molecular Probes) and imaged using a Leica TCS SP2 confocal laser scanning microscope. The same laser intensity and recording settings were used when comparing staining patterns or intensity between different sections.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo., USA), with the exception of tetrodotoxin (Wako Pure Chemical Industries, Osaka, Japan) and R(-)-efonidipine (Nissan

Chemical Industries, Tokyo, Japan). Stock solutions of nifedipine, R(-)-efonidipine and palmitoleic acid (*cis*-9-hexadecanoic acid) were prepared with DMSO. Stock solutions of other drugs were prepared with Millipore H_2O and stored at -20°C for use within 1 month.

Data Analysis and Presentation

Data are presented as raw data or means $\pm$ SE (n = number of arterioles; N = number of rats) for Fura ratio and diameter measurements. Local and remote responses were evaluated as the difference between baseline and peak R (ΔR). Statistical analysis was done using SigmaStat 3.0, and statistical significance ($p < 0.05$) was determined by either paired Student's t test or Wilcoxon's rank sum test. Non-linear curve fitting was performed with Origin 6.1 using a least-square fit of the single exponential function shown in figure 2b.

Results

Initial Characterization of Remote Ca^{2+} Responses

In 71 arterioles from 47 rats with ID = $24.1 \pm 1.0 \mu\text{m}$, the overall local response at the site of local KCl micro-ejection (0 μm) was an increase in R from 1.25 ± 0.02 to 1.79 ± 0.04 (corresponding to an increase in $[\text{Ca}^{2+}]_i$ from 88 to 283 nM). The simultaneously measured Ca^{2+} response at a remote site (500 μm) was an increase in R from 1.32 ± 0.02 to 1.49 ± 0.02 (increase in $[\text{Ca}^{2+}]_i$ from 110 to 167 nM). Simultaneous tracking of ID and $[\text{Ca}^{2+}]_i$ (R) was performed in 5 arterioles from 5 rats during local KCl stimulation (fig. 2a). We observed a local increase in $[\text{Ca}^{2+}]$ followed by a local constriction, and, at a distance of 500 μm to the site of stimulation, a smaller remote Ca^{2+} response was also followed by vasoconstriction. At both locations, peak constriction could be detected 2 s after peak R.

In figure 2b, mean ΔR values are plotted versus distance from the local site of KCl application (0–500 μm). The data points exhibit a monotonic decay suggesting that the remote Ca^{2+} responses decayed exponentially with distance. Indeed, the responses (0–500 μm) could be approximated by a first-order exponential equation $Y = Y_0 + Ae^{-X/L}$, in which X is distance, Y is ΔR , Y_0 is an asymptote, and A and L are constants (see legend of fig. 2b). When extracellular Ca^{2+} was replaced by EGTA, all Ca^{2+} responses were abolished, and the Ca^{2+} responses returned to the control level when extracellular Ca^{2+} was reintroduced via the superfusate.

Even with the fastest acquisition rate attainable (5 Hz) we could not detect any time lag in activation or peak of Ca^{2+} responses obtained at 0, 200 or 500 μm (fig. 2c). The estimated 90% rise time (τ_{90}) from the onset of the

Table 1. Lack of effect of tetrodotoxin (TTX), low [Na⁺] or vehicle (DMSO) on local and remote Ca²⁺ responses

$\Delta R \pm SE$	Distance					
	0 μm	100 μm	200 μm	300 μm	400 μm	500 μm
Control (n = 5; N = 4)	0.53 $\pm$ 0.09	0.36 $\pm$ 0.07	0.25 $\pm$ 0.06	0.20 $\pm$ 0.05	0.17 $\pm$ 0.04	0.18 $\pm$ 0.06
TTX (1–5 μM)	0.51 $\pm$ 0.07	0.37 $\pm$ 0.07	0.28 $\pm$ 0.07	0.24 $\pm$ 0.05	0.21 $\pm$ 0.05	0.23 $\pm$ 0.07
ID, μm	22 $\pm$ 4					
Control (n = 4; N = 2)	0.67 $\pm$ 0.12	0.57 $\pm$ 0.18	0.37 $\pm$ 0.11	0.30 $\pm$ 0.06	0.25 $\pm$ 0.04	0.24 $\pm$ 0.07
Low [Na ⁺] (14 mM)	0.74 $\pm$ 0.11	0.59 $\pm$ 0.10	0.39 $\pm$ 0.09	0.33 $\pm$ 0.08	0.31 $\pm$ 0.10	0.17 $\pm$ 0.02
ID, μm	23 $\pm$ 3					
Control (n = 5; N = 4)	0.31 $\pm$ 0.06	0.20 $\pm$ 0.05	0.16 $\pm$ 0.03	0.14 $\pm$ 0.03	0.13 $\pm$ 0.03	0.11 $\pm$ 0.03
DMSO (0.1%)	0.33 $\pm$ 0.07	0.23 $\pm$ 0.07	0.17 $\pm$ 0.04	0.16 $\pm$ 0.04	0.14 $\pm$ 0.03	0.11 $\pm$ 0.03
ID, μm	20 $\pm$ 3					

local Ca²⁺ response to the peak of the local or the remote Ca²⁺ responses were 1.82 $\pm$ 0.21 s at 0 μm , 1.83 $\pm$ 0.24 s at 200 μm and 1.90 $\pm$ 0.22 s at 500 μm (n = 4; N = 2). As there was no difference between remote responses at 200 versus 500 μm , the conduction velocity was faster than 300 μm per 200 ms (= interval between images), i.e. >1.5 mm/s.

Effects of Blockade of Na⁺ Channels

A velocity of this magnitude suggests electrical conduction along the vascular wall, and to investigate this possibility we tested the effects of voltage-gated Na⁺ channel inhibition. In 5 arterioles tetrodotoxin at a concentration sufficient to inhibit neuronal Na⁺ channels (1–5 μM) did not affect local or remote responses (table 1). In addition, we tested the effect of substituting 90% of Na⁺ ions in the superfusate with N-methyl-D-glucamine (126 mM). There were no effects on local or remote Ca²⁺ responses in the presence of low [Na⁺] PSS (table 1).

Effects of Gap Junction Uncouplers

Next, we tested whether gap junctions mediate the remote response to local KCl. Palmitoleic acid is a 16C fatty acid capable of blocking gap junctional intercellular communication in blood vessels [15, 16]. Palmitoleic acid (50 μM) irreversibly abolished remote Ca²⁺ responses to local KCl with no effect on the baseline R or local Ca²⁺ responses (fig. 3a). Carbenoxolone is a water-soluble molecule derived from β -glycyrrhetic acid, and it is capable of blocking gap junctional intercellular communication in blood vessels [15, 17]. When applied at 30 μM (n = 6; p = 0.066; data not shown) or 100 μM (fig. 3b), carben-

oxolone reversibly reduced the remote Ca²⁺ responses in a concentration-dependent manner with no effect on the baseline R or local Ca²⁺ responses. In addition, we tested the effect of the highest concentration of vehicle DMSO (0.1%) used. There were no effects of DMSO on the baseline R (not shown) or the local and remote Ca²⁺ responses to local KCl application (table 1).

Expression of VDCC α_1 -Subunits in Mesenteric Terminal Arterioles

RT-PCR amplification with subtype-specific primers successfully detected the messages of Ca_v1.2, Ca_v3.1 and Ca_v3.2 channels (fig. 4). Marginal expression of Ca_v1.3 subtype was also suggested by a faint band; however, this might reflect contamination from perivascular nerve endings. In contrast, no evidence was obtained for the expression of the brain T-type isoform Ca_v3.3. These data suggest that both L- and T-type channels are expressed in mesenteric terminal arterioles.

We next attempted to verify the expression of the VDCC subtypes described above at the protein level, and to characterize their localization by immunofluorescence laser confocal scanning microscopy. Our initial experiments failed to reveal consistent labeling of Ca_v1.2 in mesenteric terminal arterioles. However, with the use of an epitope-unmasking technique consisting of short SDS treatment of tissue sections [14], we consistently obtained immunostaining of Ca_v1.2 protein in VSMC of mesenteric arterioles (n = 3; N = 2; fig. 5a). This was not due to some nonspecific action of the antibodies used after SDS treatment, since the staining was blocked by preincubation with epitopic peptide (N = 2; data not shown). In addition, positive staining was also observed without SDS

Fig. 3. **a** Summary of results obtained with the gap junction uncoupler palmitoleic acid (* $p < 0.05$; ** $p < 0.01$; $n = 5$; $N = 4$; $ID = 26 \pm 3 \mu\text{m}$). **b** Summary of results obtained with the gap junction uncoupler carbenoxolone (* $p < 0.05$; $n = 8$; $N = 4$; $ID = 22 \pm 3 \mu\text{m}$).

treatment in VSMC from renal afferent arterioles ($N = 2$; fig. 5b) and in cardiac myocytes ($N = 3$; fig. 5c).

Staining for the $\text{Ca}_v3.1$ α_1 -subunit was observed in the media of mesenteric terminal arterioles ($n = 18$; $N = 3$; fig. 6a, c). The $\text{Ca}_v3.1$ -specific staining was intense and clearly confined to the single layer of VSMC (fig. 6c), but not found in endothelial cells (ECs): observe lack of staining around endothelial nuclei (fig. 6b). The same staining pattern was observed in small mesenteric arteries ($n = 6$; $N = 2$; fig. 6e, f).

Figure 7 depicts the results ($n = 8$; $N = 2$) for the $\text{Ca}_v3.2$ α_1 -subunit. While intense staining was observed in EC, more diffuse cytoplasmic staining was detected in VSMC (fig. 7). The staining was eliminated by preincubation of the antibody with its epitopic peptide or with secondary antibody alone ($N = 2$; data not shown).

Effects of Calcium Channel Blockers

To quantify the contribution of T-type VDCCs to local and remote Ca^{2+} responses, we tested the effects of a classical T-type blocker mibefradil [18], and the newly developed T-type-specific blockers NNC 55-0396 [19] and (R-)-efonidipine [20, 21]. As shown in table 2, mibefradil inhibited both local and remote Ca^{2+} responses in a concentration-dependent manner. These effects were partially reversible and use dependent, as previously observed [10]. Similar dose-dependent inhibition was also

Fig. 4. Agarose gel electrophoresis showing RT-PCR products in bands corresponding to amplification of Ca^{2+} channel genes expressed in mesenteric terminal arterioles. Bands visible for the housekeeping gene β -actin or VDCC α_1 -subunits $\text{Ca}_v1.2$ (L-type), $\text{Ca}_v3.1$ (T-type) and $\text{Ca}_v3.2$ (T-type) are of the expected sizes. At the limit of detection, a band appears at the expected product size for the $\text{Ca}_v1.3$ (L-type) channel. No bands were detected with primer specific for the $\text{Ca}_v3.3$ (T-type) channel and in the absence of template RNA (β -actin). Results are representative of two RT-PCR reactions on pooled RNA obtained with 67 arterioles from 5 rats.

observed for NNC 55-0396 and R(-)-efonidipine (table 2). Due to the limited solubility of R(-)-efonidipine in PSS, this drug could not be tested at concentrations $>33 \mu\text{M}$. The classical L-type blocker nifedipine (table 2) also

Fig. 5. Immunolocalization of Cav1.2 L-type Ca^{2+} channels (green) in mesenteric terminal arterioles (**a**), renal afferent arterioles (**b**) and cardiac myocytes (**c**). Actin staining (red) is shown as overlay (green + red; **c**). To unmask the Cav1.2 epitope, short SDS treatment was used for the mesenteric arterioles. The observed staining patterns suggest expression of L-type channels in VSMC of mesenteric and afferent arterioles, and in T-tubules of cardiac myocytes. **a** Representative of 3 terminal arterioles from 2 rats.

Fig. 6. Immunolocalization of Cav3.1 T-type Ca^{2+} channel (green; **a**) in transverse section of a mesenteric arteriole. Staining of actin (red) and nuclei (blue) is included in the overlay image (**b**). Cav3.1 protein is exclusively localized to VSMC with no green staining surrounding nuclei of ECs (**b**). Cav3.1 localization in a longitudinal section of another mesenteric arteriole is clearly visible in the single layer of VSMC (green; **c**) with actin staining (red) shown as overlay (**d**). Cav3.1-specific staining (green) is also shown in a small mesenteric artery (**e**) with multiple VSMC layers (overlay of Cav3.1 and actin staining, **f**). Images are representative of Cav3.1 staining in 18 terminal arterioles and 6 small arteries from 3 rats.

caused a reversible and concentration-dependent inhibition, but even at the high concentration of $10 \mu\text{M}$, complete inhibition could not be attained, indicating the presence of a nifedipine-resistant component in KCl-induced Ca^{2+} entry. The concentration-response curves shown in figure 8 summarize the effects of bath-applied VDCC antagonists on the local Ca^{2+} responses evoked by KCl micro-ejection.

As the arterioles used above were non-pressurized, their cell membrane potentials might have been hyperpolarized compared to in vivo pressurized conditions. In order to re-examine the effects of VDCC blockers under depolarized conditions where classical T-type channels should be almost inactivated, we preconstricted the arterioles for ≥ 10 min prior to Ca^{2+} measurements by superfusing with PSS containing 25 mM KCl. Baseline R in-

Fig. 7. Immunolocalization of $\text{Ca}_v3.2$ protein is clearly visible in EC (green; **a**) in a longitudinal section of a mesenteric terminal arteriole. Overlay of actin and $\text{Ca}_v3.2$ staining (red + green) is shown (**b**). In a close-up overlay image of an arteriolar wall (**c**; lumen of vessel indicated by asterisk), $\text{Ca}_v3.2$ -specific staining (green) is clearly visible in an EC showing no actin staining, and scattered green staining is visible in the *media* (M) identified by intense actin staining in red. A similar staining pattern was observed in a small mesenteric artery; note that EC nuclei (blue) were surrounded by green $\text{Ca}_v3.2$ -specific staining (**d**). Images are representative of $\text{Ca}_v3.2$ staining in 8 arterioles and 1 small artery from 2 rats.

creased from 1.27 ± 0.02 (resting) to 1.39 ± 0.03 (pre-constricted; $p < 0.01$; $n = 8$), but the local and remote Ca^{2+} responses evoked by KCl micro-ejection were not significantly different from those seen under resting conditions (table 2). NNC 55-0396 and nifedipine exerted similar effects in resting versus precontracted arterioles with the exception of $0.1 \mu\text{M}$ NNC 55-0396, which was not effective in precontracted arterioles (fig. 8).

Micro-Application of VDCC Blockers

Finally, we questioned whether VDCCs are involved in the intercellular conduction process per se. To separate the possible effects of VDCC blockers on the local Ca^{2+} response from those on the conduction process, we applied either NNC 55-0396 or nifedipine (both $10 \mu\text{M}$) by pressure micro-ejection onto the middle part of arteriolar segments (at $200\text{--}300 \mu\text{m}$). Both blockers severely impaired the Ca^{2+} responses at the site of application ($300 \mu\text{m}$), but were without effect on local ($0 \mu\text{m}$) and remote ($500 \mu\text{m}$) Ca^{2+} responses (fig. 9).

Discussion

The results of the present study show that local Ca^{2+} responses evoked by local KCl micro-ejection were rapidly conducted to remote sites along the arteriolar wall and decayed mono-exponentially with distance. These responses were independent of neuronal Na^+ channels, abolished by gap junction uncouplers and disappeared in Ca^{2+} -free solution. These observations are compatible with the notion that local KCl application leads to a conducted depolarization, which in turn elicits vasoconstriction via an increase in intracellular Ca^{2+} . Conducted endothelial Ca^{2+} responses to local acetylcholine application were recently demonstrated in hamster feed arteries [22], and a previous study from our laboratory investigated remote Ca^{2+} responses in VSMCs to local current application in renal interlobular arteries [23]. The present investigation extends the latter study [23] using a different arteriolar preparation in which T-type channels have been suggested to play an important role.

Table 2. Effect of bath application of organic VDCC blockers on local and remote Ca^{2+} responses

$\Delta R \pm SE$	Distance	
	0 μm	500 μm
Control (n = 5; N = 3; ID = $23 \pm 4 \mu\text{m}$)	0.48 ± 0.09	0.18 ± 0.03
0.1 μM mibefradil	0.46 ± 0.12	$0.13 \pm 0.02^*$
1.0 μM mibefradil	$0.14 \pm 0.05^*$	$0.02 \pm 0.006^{***}$
10.0 μM mibefradil	$-0.005 \pm 0.01^{***}$	$0.00 \pm 0.00^{***}$
Control (n = 5; N = 5; ID = $36 \pm 2 \mu\text{m}$)	0.35 ± 0.05	0.08 ± 0.02
0.1 μM NNC 55-0396	$0.24 \pm 0.06^*$	$0.04 \pm 0.01^*$
1.0 μM NNC 55-0396	$0.08 \pm 0.04^{***}$	$0.01 \pm 0.003^*$
10.0 μM NNC 55-0396	$0.01 \pm 0.004^{**}$	0^{**}
<i>Resting arterioles</i>		
Control (n = 4; N = 2; ID = $26 \pm 4 \mu\text{m}$)	0.74 ± 0.11	0.22 ± 0.03
<i>Precontracted arterioles</i>		
Control (n = 4; N = 2)	0.68 ± 0.11	0.19 ± 0.01
0.1 μM NNC	0.67 ± 0.11	0.21 ± 0.02
1.0 μM NNC	$0.25 \pm 0.10^*$	$0.05 \pm 0.01^{***}$
10.0 μM NNC	$0.00 \pm 0.00^*$	$0.00 \pm 0.00^*$
Control (n = 5; N = 3; ID = $16 \pm 4 \mu\text{m}$)	0.37 ± 0.07	0.15 ± 0.04
1 μM (R-)-efonidipine	0.31 ± 0.08	0.12 ± 0.03
10 μM (R-)-efonidipine	$0.20 \pm 0.04^*$	$0.08 \pm 0.02^*$
33 μM (R-)-efonidipine	$0.15 \pm 0.03^*$	$0.05 \pm 0.01^*$
Control (n = 5; N = 4; ID = $25 \pm 4 \mu\text{m}$)	0.44 ± 0.06	0.13 ± 0.02
0.1 μM nifedipine	$0.30 \pm 0.05^*$	$0.05 \pm 0.01^{**}$
1.0 μM nifedipine	$0.10 \pm 0.03^{**}$	$0.02 \pm 0.01^{**}$
10.0 μM nifedipine	$0.07 \pm 0.03^{**}$	$0.01 \pm 0.005^{**}$
<i>Resting arterioles</i>		
Control (n = 4; N = 2; ID = $25 \pm 4 \mu\text{m}$)	0.90 ± 0.14	0.25 ± 0.03
<i>Precontracted arterioles</i>		
Control (n = 4; N = 2)	0.54 ± 0.12	0.25 ± 0.06
0.1 μM nifedipine	0.40 ± 0.15	0.09 ± 0.03
1.0 μM nifedipine	$0.17 \pm 0.07^*$	$0.01 \pm 0.008^*$
10.0 μM nifedipine	$0.09 \pm 0.04^*$	$0.01 \pm 0.003^*$

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, drug vs. control.

The key findings with respect to the involvement of T-type channels in local and remote Ca^{2+} responses are: (1) T-type channels do not mediate the conduction of depolarizing signals per se, but contribute to local electromechanical coupling via Ca^{2+} entry. Furthermore, our immunohistochemical as well as functional data show that: (2) two isoforms of T-type VDCCs, $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$, are expressed at the protein level in vascular smooth muscle and/or endothelium of rat mesenteric arterioles. (3) In addition to the L-type VDCC blocker nifedipine, NNC 55-0396 and (R-)-efonidipine, two T-type VDCC-specific blockers, also effectively block depolarization-induced Ca^{2+} entry in rat mesenteric terminal arterioles.

Although loading of Fura into VSMCs of intact arterioles was achieved by abluminal exposure to the dye, it cannot be excluded that some loading of ECs will take place. However, the loaded cells showed up as brightly fluorescent bands oriented perpendicular to the length axis of the vessel, and the images were obtained by focusing on the media of the vessel. Finally, there was a strict correlation between increases in the $[\text{Ca}^{2+}]$ signal and vasoconstriction (fig. 2a). It is therefore highly likely that the recorded fluorescence predominantly originated from VSMCs. The estimated resting $[\text{Ca}^{2+}]_i$ of $\sim 100 \text{ nM}$ in our study compare well with previously reported estimates (70–110 nM) in non-pressurized arterioles [10, 24]. However, estimates from pressurized arterioles are slightly higher (130–170 nM), most likely due to pressure-induced depolarization of VSMC [25, 26].

Role of Intercellular Communication

The remote Ca^{2+} responses were inhibited by the gap junction uncouplers carbenoxolone and palmitoleic acid, with no significant effects on local Ca^{2+} responses (fig. 3). In previous studies, both palmitoleic acid and carbenoxolone were shown to inhibit gap junctional communication in a concentration-dependent manner without extra-junctional effects at the concentrations used in the present study [17, 27–29]. The role of gap junctions for conducted vasomotor responses was originally proposed by Segal et al. [2] and Segal and Duling [5] based on studies performed in hamster cheek pouch arterioles. During the last 2 decades, a large body of evidence has confirmed the central role of gap junctions for conducted vasomotor responses [for a recent review, see ref. 1]. The cellular pathway for the conducted response to KCl was not resolved in the present study. However, connexins 37, 40 and 43 were previously shown to be expressed exclusively in the endothelium of rat mesenteric arterioles [6]. Whether the local depolarization was conducted via the endothelium or smooth muscle is not critical for the conclusions of the present study.

Role of VDCCs

Voltage-dependent L-type Ca^{2+} channels provide a crucial link between excitation and contraction in vascular smooth muscle. Recently, additional roles of T-type and P/Q-type channels in excitation-contraction coupling have been demonstrated in renal resistance vessels [30, 31]. In rat mesenteric small arteries and arterioles, our previous investigations have suggested the presence of T-type channels and the absence of N-, P/Q- and R-type channels [9–11]. Our RT-PCR results (fig. 4)

Fig. 8. Concentration-response curves for the effects of nifedipine ($n = 5$; $N = 4$) and the T-type blockers mibefradil ($n = 5$; $N = 3$), NNC 55-0396 ($n = 5$; $N = 4$) and R(-)-efonidipine ($n = 5$; $N = 3$)

on local Ca^{2+} responses to local KCl application in arterioles maintained under control ('resting') or depolarized ('preconstricted') conditions. See table 2 for corresponding ΔR data.

indicated that one L-type isoform ($\text{Ca}_v1.2$) and two T-type isoforms ($\text{Ca}_v3.1$ and $\text{Ca}_v3.2$) were expressed in rat mesenteric arterioles. Furthermore, L- and T-type proteins ($\text{Ca}_v1.2$, $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$) were localized in VSMCs, and $\text{Ca}_v3.2$ was also clearly present in ECs (fig. 5–7). To our knowledge, these results are the first direct demonstration of endothelial expression of $\text{Ca}_v3.2$ in arterial tissues.

Our previous investigations showed that L-type channels were not expressed in rat mesenteric arterioles [9, 10]. However, in those studies, we used terminal arterioles

(ID $\sim 15 \mu\text{m}$) isolated from the transparent mesenteric connective tissue, whereas in the present study we used terminal arterioles (ID $\sim 24 \mu\text{m}$) from the fat pad adjacent to the ileum. Whether such regional differences may explain this discrepancy is unknown, but it is possible that the expression of L-type channels gradually disappears and conversely the expression of T-type channels predominates in the smallest and most peripheral branches of the rat mesenteric arteriolar tree, as reported previously for the guinea pig [32].

Fig. 9. a Summary of results obtained for micropipette delivery of the T-type blocker NNC 55-0396 (10 μM) to a narrow region of the arteriole at ~ 300 μm from the site of local KCl application (* $p < 0.05$; $n = 5$; $N = 4$; $\text{ID} = 23 \pm 3$ μm). **b** Summary of results

obtained for micropipette delivery of nifedipine (10 μM) at 200–300 μm from the site of local KCl application (* $p < 0.05$; $n = 4$; $N = 4$; $\text{ID} = 21 \pm 3$ μm).

The antibodies used in the present study were specific for the respective Ca^{2+} channel epitopes. The $\text{Ca}_v1.2$ antibody produced positive control staining in both renal afferent arterioles and cardiac myocytes, two types of native tissue known to abundantly express L-type channels. The $\text{Ca}_v3.1$ antibody, which generated highly reproducible results in mesenteric arterioles, is well characterized in brain tissue where the channel localization is consistent with that revealed by in situ hybridization [13]. In the same study, this antibody detected $\text{Ca}_v3.1$ expression in rat aorta and coronary artery, and in peripheral vasculature in rat brain, kidney, lung, liver and testes [13]. The $\text{Ca}_v3.2$ antibody specifically recognizes a protein with the expected molecular weight in a $\text{Ca}_v3.2$ -expressing cell line (manufacturer's product information), and incubation with the epitopic peptide eliminated the staining in rat mesenteric arterioles (present study). The observed endothelial localization of $\text{Ca}_v3.2$ -specific staining is not consistent with a role in vasoconstriction. Interestingly, a recent report has postulated a role for $\text{Ca}_v3.2$ channels in conducted vasodilatation to local electrical stimulation in mouse cremaster arterioles [33], and it has been shown that genetic elimination of $\text{Ca}_v3.2$ results in marked attenuation of endothelial- and NO-dependent vasodilatation in coronary arteries [34]. T-type channels are also suggested to participate in differentiation and prolifera-

tion [35] as well as control of von Willebrand factor secretion and coagulation in lung microvascular endothelium [36].

Mibefradil has previously been used as a selective blocker of T-type channels at ≤ 1 μM [18]. However, its antihypertensive effects were recently shown to be mediated via the block of $\text{Ca}_v1.2$ L-type channels [37]. Instead we have used NNC 55-0396 and R(-)-efonidipine, two new organic T-type blockers. NNC 55-0396 is a new non-hydrolyzable analogue of mibefradil, which is not metabolized in the cytoplasm to an active form responsible for block of L-type channels [19]. NNC 55-0396 did not block high voltage-activated currents in insulin-secreting INS-1 cells at a high concentration (100 μM), whereas it blocked $\text{Ca}_v3.1$ T-type currents expressed in HEK293 cells with an IC_{50} of ~ 7 μM [19]. Thus, the fact that 10 μM NNC 55-0396 abolished the local and remote Ca^{2+} responses indicates that T-type channels are functionally important in mesenteric terminal arterioles. At 1 μM , NNC 55-0396 blocked both local and remote Ca^{2+} responses by $>50\%$, which indicates a higher efficacy than that reported for inhibition of $\text{Ca}_v3.1$ T-type currents [19]. This may be caused by a different drug sensitivity of native T-type channels expressed in mesenteric arterioles. The R(-)-enantiomer of efonidipine inhibited T-type currents in mammalian cells or amphibian oocytes

with IC_{50} values ranging from 0.1 to 10 μM , with little or no effect on high voltage-activated currents [20, 21]. Although we were not able to estimate the maximum effect of R(-)-efonidipine, its concentration-dependent effects in mesenteric arterioles are consistent with a role for T-type channels.

Nifedipine is a potent L-type blocker with an IC_{50} of $3.7 \cdot 10^{-9}$ M for voltage-dependent block ($V_H = -50$ mV) of the vascular isoform of $Ca_v1.2$ (α_{1C-b}) [38]. In the present study, a much higher concentration of nifedipine (0.1–1 μM) was required to block 50% of the KCl-induced Ca^{2+} responses (fig. 8). Nimodipine has long been recognized as a partial blocker of T-type channels [32, 39], and it has been reported that nifedipine blocks both native and recombinant T-type currents, with the highest sensitivity ($IC_{50} = 1.2$ – 5 μM) displayed by currents carried via $Ca_v3.2$ channels [40, 41]. Thus, the moderate efficacy obtained with nifedipine in mesenteric terminal arterioles may suggest that its effects are due to a predominant expression of T-type channels in this preparation.

To test if the potent effect of NNC 55-0396 could be attributed to the hyperpolarized membrane potential of isolated, non-pressurized arterioles, we performed another set of experiments in which the arterioles were partially depolarized and preconstricted using PSS containing 25 mM KCl (fig. 8). Under these conditions, the membrane potential should be sufficiently depolarized ($E_K \sim -45$ mV) to inactivate classical T-type channels [42]. The results, however, indicated that, except for a very low concentration (0.1 μM), the potency of NNC 55-0396 (1–10 μM) remained almost unchanged (fig. 8). This result may indicate unusually high activation and inactivation thresholds of T-type channels expressed in mesenteric terminal arterioles, as previously reported for mesenteric small arteries [11, 32].

The potent effects of either nifedipine or NNC 55-0396 on the Ca^{2+} responses in mesenteric terminal arterioles deserve attention. These results are not compatible with an additive effect of NNC 55-0396 and nifedipine, and may suggest a functional coupling between L- and T-type channels. A functional coupling might be the consequence of a co-localization and interaction between L- and T-type channels in membrane microdomains, such as caveolae. Such non-additive effects of L- and T-type blockers have previously been observed in preglomerular resistance arterioles [31].

The finding that inhibition of Ca^{2+} entry by micro-application of nifedipine or NNC 55-0396 between the local and the remote sites did not affect local and remote Ca^{2+}

responses (fig. 9) suggests that the (inward) Ca^{2+} currents through VDCCs do not contribute to the conducted depolarization initiated by local KCl application. If this was the case, we would have expected the Ca^{2+} response at the most remote site (500 μm) to be diminished by blocking Ca^{2+} entry at a site half-way along the arteriolar preparation. Thus, the role of VDCCs seems to be limited to providing local electromechanical coupling rather than supporting conduction during a locally initiated vasomotor response.

In summary, we have shown that local application of a depolarizing KCl solution leads to local and remote Ca^{2+} responses in terminal arterioles isolated from the rat mesenteric fat pad. The conduction mechanism most likely involves intercellular electrical communication via gap junctions, as remote Ca^{2+} responses were inhibited by two chemically distinct gap junction uncouplers. We detected mRNA and protein expression of $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ VDCCs, and we provided evidence that both L- and T-type VDCCs are involved in electromechanical coupling, but are not necessary for the conduction of vasoconstrictor signals per se.

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