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Ryanodine receptors are uncoupled from contraction in rat vena cava

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ABSTRACT

Ryanodine receptors (RyR) are Ca^{2+}-sensitive ion channels in the sarcoplasmic reticulum (SR) membrane, and are important effectors of SR Ca^{2+} release and smooth muscle excitation–contraction coupling. While the relationship between RyR activation and contraction is well characterized in arteries, little is known about the role of RyR in excitation–contraction coupling in veins. We hypothesized that RyR are present and directly coupled to contraction in rat aorta (RA) and vena cava (RVC). RA and RVC expressed mRNA for all 3 RyR subtypes, and immunofluorescence showed RyR protein was present in RA and RVC smooth muscle cells. RA and RVC rings contracted when Ca^{2+} was re-introduced after stores depletion with thapsigargin (1 μM), indicating both tissues contained intracellular Ca^{2+} stores. To assess RyR function, contraction was then measured in RA and RVC exposed to the RyR activator caffeine (20 mM). In RA, caffeine caused contraction that was attenuated by the RyR antagonists ryanodine (10 μM) and tetracaine (100 μM). However, caffeine (20 mM) did not contract RVC. We next measured contraction and intracellular Ca^{2+} ($Ca^{2+}{}_i$) simultaneously in RA and RVC exposed to caffeine. While caffeine increased $Ca^{2+}{}_i$ and contracted RA, it had no significant effect on $Ca^{2+}{}_i$ or contraction in RVC. These data suggest that ryanodine receptors, while present in both RA and RVC, are inactive and uncoupled from Ca^{2+} release and contraction in RVC.

1. Introduction

Ryanodine receptors (RyR) are homotetrameric ion channels that are present in the sarcoplasmic reticulum (SR) of vascular smooth muscle which, in part, mediate SR Ca^{2+}release [1]. While most vascular smooth muscle expresses all 3 known RyR isoforms (RyR1–3), it is primarily the activation of RyR1 and RyR2 that regulates excitation–contraction coupling [2]. These receptors are activated by Ca^{2+}, and can act as amplifiers of smaller Ca^{2+} signals caused by Ca^{2+} influx or inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release [3]. This amplification, called "Ca^{2+} induced Ca^{2+} release" (CICR), mobilizes large amounts of SR Ca^{2+} into the cytosol and has been proposed to serve an important role in excitation–contraction coupling in smooth muscle [4]. CICR from RyR can also modulate voltage-dependent Ca^{2+} influx *via* small, localized releases of Ca^{2+}called Ca^{2+}sparks [5]. Ca^{2+} sparks activate Ca^{2+}-sensitive potassium channels, leading to membrane hyperpolarization and closure of voltage-gated Ca^{2+} channels [6]. Thus, RyR-mediated Ca^{2+} release from SR stores can serve as both a positive and negative regulator of agonist-induced excitation–contraction coupling in vascular smooth muscle.

In comparison to arteries, veins are extremely compliant and distensible, offering very little resistance to blood flow. This ability to stretch and expand easily allows the venous circulation to store approximately 70% of the body's total blood volume at a given moment [7]. This high distensibility does not mean that veins lack the contractile apparatus found in other vascular smooth muscle. While not as forceful as arteries, veins do exhibit significant vasoreactivity to many agonists [8]. Considering the large volume of blood in the venous circulation, even a small increase in venous tone could markedly increase the blood volume in the arterial circulation and cause unwanted increases in blood pressure [9]. However, very little is known about the mechanisms that govern contraction in venous smooth muscle beyond the general finding that venous contraction is regulated by Ca^{2+}[10,11].

Our present goal was to investigate if veins depend on RyR-mediated Ca^{2+} release, as is observed in arteries, to better understand the relationship between Ca^{2+} mobilization and changes in vascular tone. In this study, we first tested the hypothesis that RyR are present in rat aorta and vena cava, and are directly coupled to contraction. For comparison, we then examined the coupling of RyR activation and contraction in two other pairs of arteries and veins to see if our results in vena cava were recapitulated in veins from other vascular beds. While both aorta and vena cava expressed predominantly RyR2, the RyR agonist caffeine (20 mM)

Abbreviations: CA, carotid artery; CICR, calcium-induced calcium release; IP_3, inositol 1,4,5-trisphosphate; JV, jugular vein; NE, norepinephrine; PE, phenylephrine; RA, rat thoracic aorta; RVC, rat vena cava; RyR, ryanodine receptors; SMA, superior mesenteric artery; SMV, superior mesenteric vein; SOCE, store-operated calcium release; SR, sarcoplasmic reticulum.

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caused substantial Ca^{2+} release and contraction only in rat aorta. These data suggest that ryanodine receptors, while present in both tissues, are inactive and uncoupled from Ca^{2+} release and contraction in vena cava.

2. Methods

2.1. Animal care and use

All procedures that involved animals were performed in accordance with the Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* at Michigan State University. Normal male Sprague-Dawley rats (250–300 g) were used. Animals were euthanized with sodium pentobarbital (60 mg/kg i.p.).

2.2. Chemicals and compounds

Unless otherwise noted, all salts and reagents were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Thapsigargin was obtained from Tocris Biosciences (Bristol, UK). Ryanodine was obtained from Abcam Biochemicals (Cambridge, UK).

2.3. Real-time RT-PCR

Real-time RT-PCR was performed as previously described [12]. Briefly, rat aorta and vena cava were removed and placed in sterile water, then cleaned of fat and blood. Total RNA was isolated using the MELT Total Nucleic Acid Isolation System and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Standard real-time RT-PCR was performed using a GeneAMP 7500 Real-Time PCR machine (Applied Biosystems, Carlsbad, CA) and SYBR Green PCR Fast Master Mix (Applied Biosystems). Rat primers were purchased from Qiagen (Valencia, CA): RyR1 (RefSeq Accession #: XM_001078539; 131 bp amplicon), RyR2 (RefSeq Accession #: NM_001191043; 66 bp amplicon), and RyR3 (RefSeq Accession #: XM_001080527; 141 bp amplicon). Calibrator control was beta-2 microglobulin (RefSeq Accession #: NM_012512, 128 bp amplicon) (SA Biosciences, Frederick, MD, USA). PCR conditions were: 95 °C for 10 min followed by 40 cycles of (95 °C, 15 s; 60 °C, 60 s). A standard dissociation curve was run following the above cycle conditions. Each sample was run in duplicate.

2.4. Smooth muscle cell dissociation and immunofluorescence

Whole aorta and vena cava tissues were isolated, cleaned of perivascular fat, and cut into ~1 mm rings. Rings were transferred to 1.5 ml microcentrifuge tubes and incubated with dissociation solution (80 mM NaCl, 80 mM monosodium glutamate, 5.6 mM KCl, 20 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 1 mg/mL BSA, pH 7.3) with 1 mg/mL dithiothreitol and 0.3 mg/mL papain for 18 min in a 37 °C water bath. The solution was removed and replaced with fresh dissociation solution containing 100 μM $CaCl_2$ and 1 mg/mL collagenase and incubated 9 min in a 37 °C tissue bath. The solution was removed and cells were re-suspended in dissociation solution by gentle trituration. Cells were transferred to coverslips using a Shandon Cytospin 4 Centrifuge (Thermo Scientific, Waltham, MA, USA). Cells were then fixed in Zamboni's fixative for 20 min, permeabilized with 1% Triton X-100 in PBS for 20 min, and blocked with goat serum (1% diluted in PBS) for 1 h at 37 °C. Primary antibodies (mouse anti-RyR1/2, 1:500, Life Technologies, Grand Island, NY, USA; rabbit anti-α-actin, 1:100, ab5694, Abcam, Cambridge, MA, USA) diluted in blocker were added to the cover slips, and cells were incubated at 37 °C for 1 h. Coverslips were washed briefly 3 times with PBS, and cover slips were incubated in secondary antibodies (goat anti-mouse Alexa Fluor 568, 1:1000; goat anti-rabbit 568, 1:1000; and goat anti-rabbit 488, 1:1000, Life Technologies) for 1 h at 37 °C. Cover slips were washed 3 times with PBS and placed face down onto slides in Prolong Gold with DAPI (Life Technologies). Cells were then imaged using an Olympus® FV1000 confocal system mounted on an Olympus® inverted microscope.

2.5. Isometric contraction

RA, RVC, carotid artery (CA), jugular vein (JV), superior mesenteric artery (SMA) and superior mesenteric vein (SMV) from Sprague-Dawley rats were dissected and cleaned of outer adipose tissue in physiological salt solution (PSS) containing (mM): NaCl, 130; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4{\cdot}7H_2O$, 1.17; $NaHCO_3$, 14.8; dextrose, 5.5; $Na_2EDTA{\cdot}2H_2O$, 0.03; $CaCl_2$, 1.6; (pH = 7.2), and then cut into ~5 mm long rings. Endothelium-intact rings were then mounted in isolated tissue baths (20 ml) containing warmed, aerated PSS (37 °C; 95/5% O_2/CO_2) for measurement of isometric contractile force using a 750 TOBS Tissue Organ Bath System (Danish Myo Technology, Aarhus, Denmark) and Power Lab for Windows (AD Instruments, Colorado Springs, CO, USA). The tissues were placed under optimum resting tension (4 g for RA; 1 g for RVC, CA and JV; 1.5 g for SMA; and 0.1 g for SMV), as determined previously. Tissues were initially challenged with 10 μM phenylephrine (PE) (RA), 10 μM norepinephrine (NE) (RVC, SMA, and SMV) or 60 mM KCl (CA and JV) to test for tissue viability. Average initial contractions for each acceptable tissue were as follows: 2427 ± 191 mg (RA), 56 ± 4 mg (RVC), 965 ± 110 mg (CA), 344 ± 30 mg (JV), 3941 ± 343 mg (SMA) and 289 ± 132 (SMV). Endothelium viability was then confirmed by relaxation to 1 μM acetylcholine after plateau of initial contractile challenge. Tissues were washed every 15 min until they returned to resting tension. Cumulative concentration response curves or responses to single concentrations of agonists were recorded. Antagonists, inhibitors, or their vehicles were incubated with the tissues for 1 h prior to addition of agonists. The specific agonists and antagonists (and corresponding solvents) were: caffeine (PSS), NE (dH_2O), PE (dH_2O), thapsigargin [dimethyl sulfoxide (DMSO)], ryanodine (ethanol) and tetracaine (dH_2O).

2.6. Ratiometric calcium imaging

Using a custom-fabricated wire myograph imaging chamber, RA or RVC were loaded with the ratiometric Ca^{2+} indicator Fura 2-AM (Life Technologies, Grand Island, NY, USA) by bath incubation, after application of resting tension as described above. The dye solution was made in Ca^{2+}-replete PSS and contained: 5 μM Fura 2-AM dye, 0.5% dimethyl sulfoxide, and 0.01% Pluronic (Life Technologies, USA). The dye solution was applied to RVC for 1 h and RA for 1.5 h at room temperature, and exchanged once during that time. Before imaging, a 30-min superfusion with aerated PSS (95/5% O_2/CO_2) was performed to wash any extracellular dye from the bath, allow for dye de-esterification, and gradual temperature increase to 37 °C. Isometric contraction and fluorescence ratio were measured simultaneously during exposure to 20 mM caffeine. To isolate changes in smooth muscle cell Ca^{2+} from changes in endothelial cell Ca^{2+}, a focal plane was chosen in the cell layer immediately above the endothelium. Fura 2 fluorescence ratio (excitation: 340 and 380 nm; emission: 510 nm) was recorded using a DeltaRam-X multi-wavelength illuminator and an 814 photomultiplier system (Photon Technologies Int'l (PTI), Birmingham NJ, USA) mounted on a Nikon TE-300 inverted microscope (Nikon Instruments, Melville, NY, USA) equipped with a 40× (N.A. 0.75) Plan-Fluor long working-distance objective. FeliX software (PTI)