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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.

Keywords

Ryanodine receptor; Vascular smooth muscle; Vasoconstriction; Veins; Calcium; Sarcoplasmic reticulum

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

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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) 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 (Ref-Seq 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) was used to control the illuminator and photometer, and record all acquired data.

2.7. Intensiometric calcium imaging

RVC were dissected and cleaned of outer adipose tissue in PSS. Tissues were cut longitudinally, mounted *en face* in a custom imaging superfusion chamber, and incubated with the intensiometric fluorescent Ca^{2+} indicator Fluo 4. The dye solution was made in Ca^{2+}-replete PSS, containing: 10 μM Fluo 4-AM dye (Invitrogen, Carlsbad, CA, USA), 0.5% DMSO, and 0.01% Pluronic (Invitrogen, Carlsbad, CA, USA). Dye solution was applied to vena cava for 1 h at room temperature, and exchanged for fresh dye solution 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 and allow for dye de-

esterification and gradual temperature increase to 37 °C. After washout of non-esterified indicator dye, image sequences (500 frames at 30 frames/s) were recorded in the absence of agonist, and then in the presence of 10 μM norepinephrine. After 15-min washout of agonist, image sequences were then recorded in the presence of 20 mM caffeine. Vessels were imaged using a long working distance 63× water-immersion objective (N.A. 0.8, W.D. 3 mm; Leica, Wetzlar, Germany). Fluo 4-AM fluorescence at 526 nm was acquired at 30 frames per second using the CSU-10B spinning-disc confocal system (Solamere, Salt Lake City, UT, USA) with 488 nm laser illumination (Solamere, Salt Lake City, UT, USA) and an intensified CCD camera (XR Mega-10, Stanford Photonics, Palo Alto, CA). Images were recorded using Piper software (Stanford Photonics, Palo Alto, CA). A “calcium wave” was defined as an increase in fluorescence greater than 20% above background ($F/Fo > 1.20$).

2.8. Statistical analysis

All data from contractility experiments were normalized to the maximal tissue contraction during initial adrenergic challenge. Mean, standard error and variance was calculated from the normalized calculated data. For comparisons of two samples of equal variance, statistical significance between groups was established using two-tailed, unpaired Student’s *t*-tests ($\alpha = 0.05$). For samples of unequal variance, the Mann–Whitney U test was used ($\alpha = 0.05$). For multiple sample comparisons, two-way ANOVA was used followed by Bonferroni’s *post hoc* analysis to compare individual means. Calculations were performed using Microsoft Excel (Microsoft Corporation, USA) or GraphPad Prism (GraphPad Software Inc., USA).

3. Results

3.1. Presence of ryanodine receptor mRNA and protein

Real-time PCR was performed to measure mRNA for all 3 RyR subtypes in rat aorta and vena cava. Both aorta and vena cava expressed significantly more RyR2 mRNA as compared to RyR1 and RyR3, when normalized to β-2-microglobulin expression (Fig. 1a and b), suggesting the RyR2 was the most highly abundant RyR isoform in both tissues. Using an antibody against RyR1/RyR2 protein, the presence of RyR1 and/or RyR2 protein was then investigated using immunofluorescence and confocal microscopy in freshly dissociated RA and RVC smooth muscle cells (Fig. 2). Most of the freshly dissociated cells expressed RyR1/2 protein, as shown by positive red immunofluorescence (Fig. 2a and b). The fluorescence signal was significantly greater than with secondary antibody alone (Fig. 2g and h). The cells were also labeled with a FITC-conjugated smooth muscle alpha-actin antibody to distinguish between smooth muscle cells and other non-muscle cells (Fig. 2c and d). The cells with positive fluorescence for smooth muscle alpha-actin also had positive fluorescence for RyR1/2, indicating that smooth muscle cells from both aorta and vena cava express RyR1 and/or RyR2 (Fig. 2e and f).

3.2. Aorta and vena cava have sarcoplasmic calcium stores

As an indirect measurement of sarcoplasmic Ca^{2+} stores in aorta and vena cava, store-operated Ca^{2+} entry (SOCE) was measured in both tissues. SOCE is activated after intracellular Ca^{2+} stores depletion in smooth muscle, and thus the presence of SOCE is

indicative of the presence of sarcoplasmic Ca^{2+} stores [13]. To deplete intracellular Ca^{2+} stores, aorta and vena cava were placed in Ca^{2+}-free PSS and then exposed to vehicle or the irreversible sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) for 1 h. After 1 h, Ca^{2+}-replete PSS was reintroduced to the tissues and the resulting contraction was measured (Fig. 3a–d). Upon reintroduction of extracellular Ca^{2+}, aorta and vena cava exposed to thapsigargin (1 μM) exhibited a sustained contraction that was absent in vehicle-exposed tissues (Fig. 3a–d). This contraction was significantly greater in tissues exposed to thapsigargin (Fig. 3e), and was thus indicative of store-operated Ca^{2+} entry and the presence in intracellular Ca^{2+} stores in both tissues.

3.3. Ryanodine receptor activation by caffeine

To test the relationship between RyR activation and smooth muscle function, isometric contraction to the RyR agonist caffeine was measured in aorta and vena cava (Fig. 4 a and b). Caffeine (20 mM) caused a significant and transient contraction in RA that was reproducible after a 45-min washout period (Fig. 4c). RVC exhibited no contraction; rather, caffeine caused a reproducible relaxation (Fig. 4c). In RA, the RyR antagonists ryanodine (10 μM) or tetracaine (100 μM) inhibited contraction to caffeine by ~50% and ~75%, respectively (Fig. 5).

To test if other veins were also unresponsive to 20 mM caffeine, caffeine-induced contraction was investigated in two other arteryvein pairs: [1] carotid artery and jugular vein; and [2] superior mesenteric artery and superior mesenteric vein. Unlike RVC, CA and JV contracted to 20 mM caffeine (Fig. 6a). However, the caffeine-induced contraction in jugular vein was significantly less than in carotid artery. In superior mesenteric artery and vein, 20 mM caffeine elicited equivalent contractions in both tissues (Fig. 6b). These data show that some veins do contract in the presence of caffeine, and thus the lack of response to caffeine in RVC is not representative of all veins.

3.4. Ryanodine receptor activation and intracellular calcium mobilization

RA contraction by caffeine (20 mM) correlated with an increase in global intracellular Ca^{2+}, as measured using the ratiometric Ca^{2+} indicator Fura-2 (Fig. 7a). However, no comparable increase in global Ca^{2+} was seen in response to caffeine (20 mM) in RVC. Instead, a small transient increase in Ca^{2+} was superimposed upon a fall in global Ca^{2+} (Fig. 7b). Consistent with the data presented in Fig. 4, caffeine again caused relaxation in vena cava, rather than contraction. These data indicate that RyR activation by caffeine was not able to cause contraction or a sustained increase in global cytosolic Ca^{2+} in RVC.

To determine if caffeine caused local, but not global, increases in intracellular Ca^{2+} in RVC, Fluo-4 fluorescence was measured in the presence of either NE (10 μM) or caffeine (20 mM). RVC exhibited spontaneous Ca^{2+}waves in the absence of agonist (supp. video 1). These Ca^{2+} waves were potentiated in the presence of 10 μM NE (supp. video 2), but absent the presence of caffeine (20 mM) (supp. video 3). These data suggest that localized Ca^{2+} waves exist in RVC, but are not elicited by caffeine.

Supplementary data related to this article found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2012.10.006.

4. Discussion

The principal and novel findings of this study are: [1] aorta and vena cava smooth muscle cells express ryanodine receptors; [2] RyR activation by caffeine neither contracts nor increases intracellular Ca^{2+} in vena cava; and [3] the lack of contraction to caffeine is unique to vena cava, since jugular vein and superior mesenteric vein contracted when exposed to caffeine. These findings were not because vena cava lacked intracellular Ca^{2+} stores, since both aorta and vena cava exhibited robust contraction after stores depletion and SERCA inhibition. This is consistent with SOCE and the presence of readily releasable intracellular Ca^{2+} stores. These findings imply that the absence of RyR-mediated contraction is specific to vena cava, is not indicative of the responses to caffeine in other veins or arteries, and is not because vena cava lacks intracellular stores of Ca^{2+}.

Study of venous smooth muscle is becoming increasingly more relevant since researchers linked changes in venous capacitance to changes in blood pressure [14]. Understanding the mechanisms governing contraction of the large, central veins is particularly important given that venous return to the heart is largely determined by central venous tone [14,15]. Impaired venous distensibility and decreased venous capacitance are seen in hypertensive patients, which can increase arterial blood volume by decreasing the storage capacity of veins [16,17]. Defects in RyR-mediated Ca^{2+} signals are also linked to multiple human cardiovascular pathologies, including congestive heart failure, hypertension and polymorphic ventricular tachycardia [2,18,19]. Thus, understanding how RyR regulate venous contractility may help clarify the role of the veins in hypertension and other vascular diseases where venous dysfunction is evident.

4.1. Ryanodine receptor expression

While expression of RyR mRNA was similar in aorta and vena cava, RyR protein expression in these tissues could not be quantified reliably. Due to the large size of the RyR proteins (~550 kDa) [20], high molecular weight Western blotting techniques were required to reliably differentiate RyR protein from other proteins greater than ~300 kDa [21]. While we were able to consistently show RyR1 and RyR2 expression in positive controls (diaphragm and heart, respectively; data not shown), we were unable to show expression of any RyR protein in aorta or vena cava whole tissue homogenates. We were also unable to show RyR3 expression with any specificity in our controls due to the lack of an adequately selective RyR3 antibody. Also, many of the published results showing RyR protein by Western blot utilized enriched, purified RyR protein [22–25]. This technique was inappropriate for testing our hypotheses since reasonable comparisons between venous and arterial RyR expression would be impossible after enrichment and purification. Instead, immunofluorescence was used to provide evidence, albeit qualitative, that RyR mRNA in aorta and vena cava smooth muscle cells is translated into RyR protein. RyR fluorescence intensity (Fig. 2a and b) appears higher in aorta smooth muscle cells as compared to vena cava, but these experiments were analyzed under different conditions and on different days. Thus, it is impossible to know if the apparent differences in intensity were due to differences in protein expression or due to differences in detection parameters. Nonetheless, both tissues expressed positive immunofluorescence for RyR protein that was significantly greater than

control. This positive immunofluorescence for RyR protein in both tissues suggests that the lack of physiological response in vena cava was because RyR were incapable of adequate Ca^{2+} release for contraction in response to caffeine. Further immunofluorescent experimentation will be required to quantify the amount of RyR protein expressed in both tissues, to determine if the lack of contraction to caffeine in vena cava is due to decreased RyR expression and not RyR dysfunction.

4.2. RyR-mediated Ca^{2+} release and contraction

The RyR activator caffeine (20 mM) caused a transient but significant contraction in aorta that was absent in vena cava, but present in mesenteric and jugular vein. While these initial experiments implied that RyR activation is uncoupled from contraction in vena cava, these data did not measure changes in intracellular Ca^{2+} elicited by caffeine. Even though no contraction was evident, it was possible that caffeine was activating RyR-dependent Ca^{2+} release from the SR in a quantity that was insufficient to activate the contractile machinery of vena cava smooth muscle. When we investigated the global Ca^{2+} changes caused by caffeine in vena cava, caffeine did appear to cause a small, transient increase in global Ca^{2+} that was not associated with contraction. Instead, this increase occurred superimposed on a prolonged decrease in global intracellular Ca^{2+}that was associated with venorelaxation. We also investigated the localized changes in intracellular Ca^{2+} in vena cava, using a protocol that has consistently identified Ca^{2+} sparks and waves in smooth muscle [26,27]. The transient increase in Ca^{2+} was not associated with localized Ca^{2+} sparks and waves, as neither was apparent in response to caffeine in RVC. Taken together, these data are consistent with the idea that vena cava contain functional RyR, but these receptors are uncoupled from contraction due to insufficient Ca^{2+} release.

One possible explanation for the inactivity of RyR in vena cava is that the receptor is locked in an inactive state by any one of a number of RyR regulating proteins, such as FKBP12 or sorcin [2,28]. Caffeine is an *activator* – not an *agonist* – of RyR, and it elicits Ca^{2+} release by changing the Ca^{2+} sensitivity of RyR from micromolar to nanomolar (or even picomolar) concentrations [29,30]. Since several RyR regulatory proteins have been identified that act as allosteric inhibitors of RyR function, the receptors may be unresponsive to changes in Ca^{2+} sensitivity and thus remain closed [20]. Further investigation will be required to determine which of the known allosteric modulators of RyR function are expressed in vena cava smooth muscle and if these proteins are responsible for the lack of caffeine-induced Ca^{2+} release and contraction in vena cava.

Another explanation for the absence of contraction to caffeine in vena cava is that RyR are sparsely expressed on the SR membrane. For caffeine to cause contraction, RyR on the SR membrane need to be expressed in sufficient density and proximity to one another to cause CICR and subsequent contraction. If the spatial arrangement is such that CICR cannot occur, RyR activation may cause a small, transient release of SR Ca^{2+} that cannot propagate such that insufficient Ca^{2+} would be released to initiate contraction [31].

In arterial smooth muscle, localized Ca^{2+}release events from RyR, called Ca^{2+}sparks, activate large-conductance Ca^{2+}-activated K^+ (BK) channels to cause membrane repolarization and relaxation without increasing global cytosolic Ca^{2+} [5,6]. Ca^{2+} sparks

have also been identified in mesenteric vein, and inhibition of these sparks causes contraction [32]. This suggests that functional RyR are present and coupled to contraction in other tissues from the venous circulation. While sparks have not yet been identified in vena cava, functional BK channels are expressed in vena cava smooth muscle [33]. Our experiments only show that caffeine cannot significantly increase global cytosolic Ca^{2+}or cause Ca^{2+}sparks, but the possibility remains that RyR-mediated Ca^{2+} sparks could be activated by other agonists. Further investigation will be required to determine if other agonists cause Ca^{2+} sparks in vena cava smooth muscle, how these sparks are regulated and to determine their effect on venous tone.

Finally, it is possible that RyR in vena cava are incapable of forming functional channels in the SR membrane. In the rat myometrium, for example, RyR are expressed but the tissue is completely insensitive to caffeine [34,35]. This insensitivity is linked to the expression of an RyR variant that is incapable of associating into a functional tetrameric receptor [36]. Although we did not investigate the expression of RyR splice variants in vena cava, this is a possible explanation for the presence of RyR protein but the absence of a RyR-mediated response.

4.3. Conclusions

Our data suggest that ryanodine receptors, while present in vena cava, are uncoupled from contraction and incapable of causing a sustained increase in intracellular Ca^{2+}. In rat aorta, however, activation of RyR by caffeine causes intracellular Ca^{2+} release and contraction. The lack of RyR-mediated contraction also appears to be specific to vena cava, because both the jugular vein and superior mesenteric vein contract to caffeine. This suggests that regional heterogeneity of RyR function exists within the venous circulation. Consistent with this hypothesis, regional heterogeneity in the function and expression of RyR has previously been reported in other vascular tissue [26,27].

These data show that the vena cava is one of few – if not the only – blood vessel that is completely insensitive to caffeine, both in terms of contractile response and Ca^{2+} release. Furthermore, this insensitivity represents a fundamental difference between vena cava and aorta smooth muscle in terms of excitation–contraction coupling and Ca^{2+} mobilization during contraction. These studies also identify a new and important difference in SR Ca^{2+}release mechanisms present in vena cava, as compared to several other arteries and veins. Given the role of the vena cava in regulating venous return to the heart, this difference represents a novel area of research for potential therapeutic targets that can specifically alter vena cava smooth muscle tone to treat vascular diseases like hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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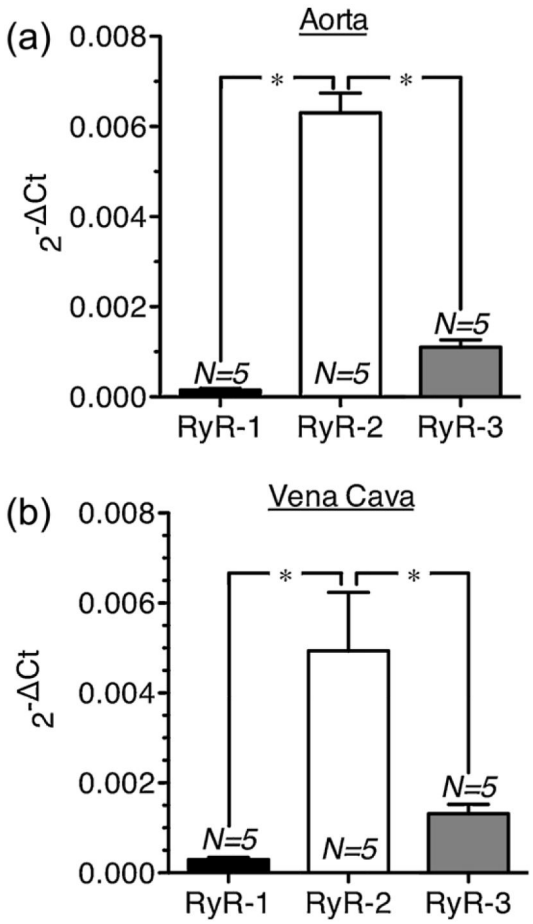

Fig. 1.

Summary graph of RyR1, RyR2 and RyR3 mRNA expression in rat aorta (a) and vena cava (b), measured using real-time RT-PCR. Black bars indicate RyR1 mRNA; white bars indicate RyR2 and gray bars indicate RyR3. All bars represent mean ± SEM for the number of animals indicated.

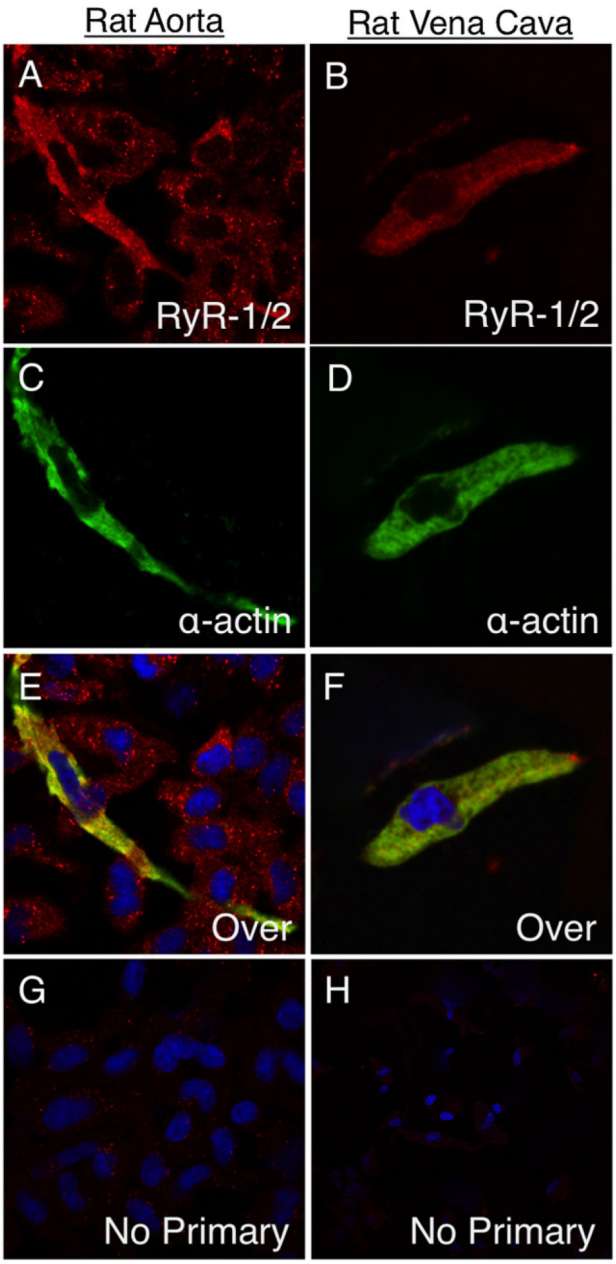

Fig. 2.

Representative immunohistochemical staining, using an antibody against RyR1/2, in freshly dissociated smooth muscle cells from aorta and vena cava. (a, b) Red fluorescence indicates the presence of RyR1/2 protein. (c, d) Green fluorescence indicates staining for smooth muscle α-actin. (e, f) Overlay of RyR1/2 (red), smooth muscle α-actin (green) and DAPI nuclear stain (blue). (g, h) Negative controls, where primary antibodies were absent. Representative of 3 experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

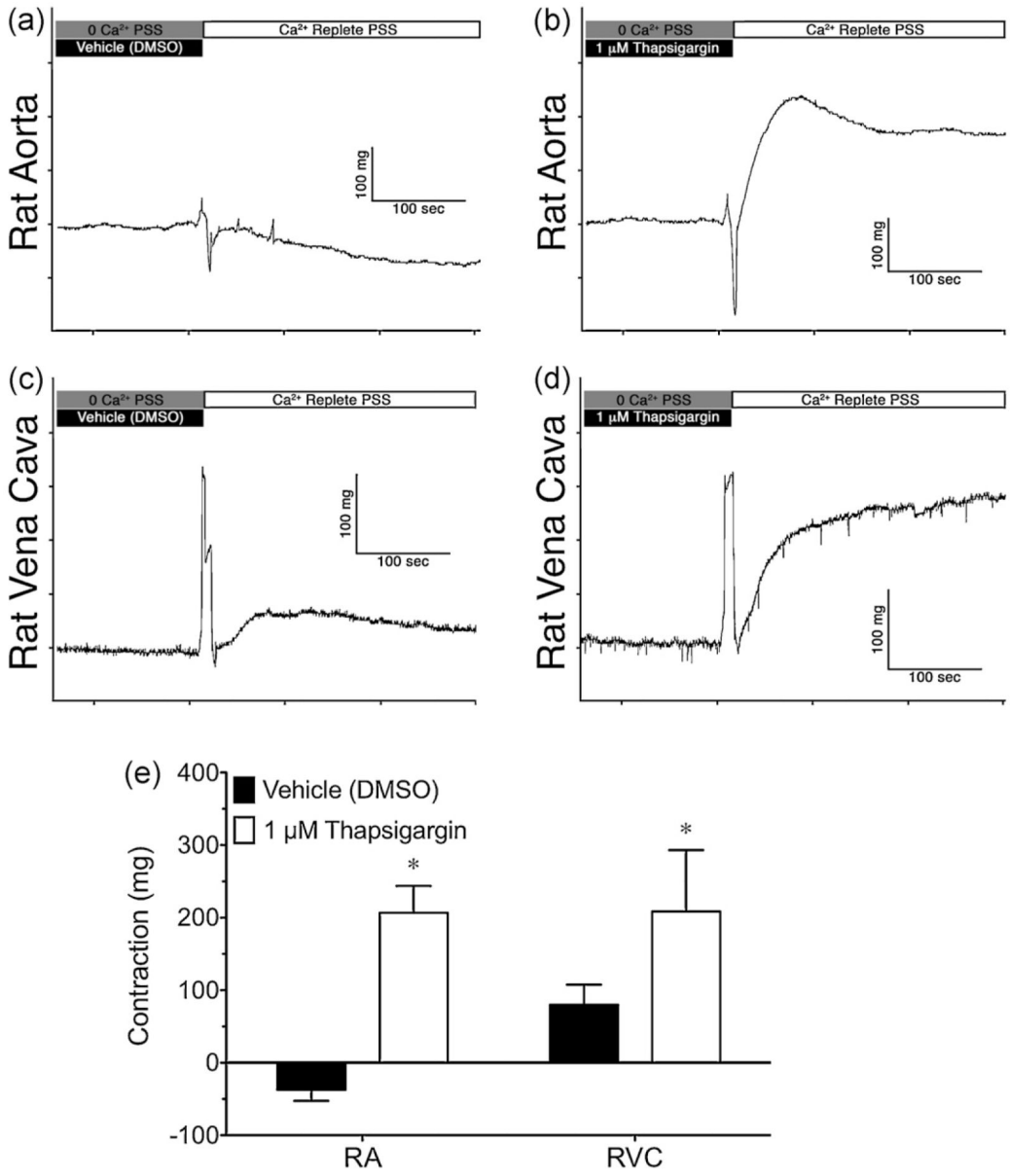

Fig. 3.

(a–c) Representative tracings of rat aorta and vena cava contraction, after sarcoplasmic Ca^{2+} stores depletion and upon exposure to Ca^{2+}-replete physiological salt solution. Tissues were incubated in Ca^{2+}-free buffer for 15 min before addition of thapsigargin (1 μM) to inhibit SERCA-mediated Ca^{2+} reuptake. Tissues were then incubated for 1 h before reintroduction of Ca^{2+}. Shown are responses from tissues incubated with vehicle (a, c) and 1 μM thapsigargin (b, d). (e) Summary bar graphs indicating the maximum contractile response after reintroduction of Ca^{2+}-replete physiological salt solution in aorta (RA) and vena cava (RVC). Black bars represent vehicle-exposed tissues. White bars represent tissues exposed to thapsigargin (1 μM). N = 4; $*p < 0.05$ *versus* vehicle.

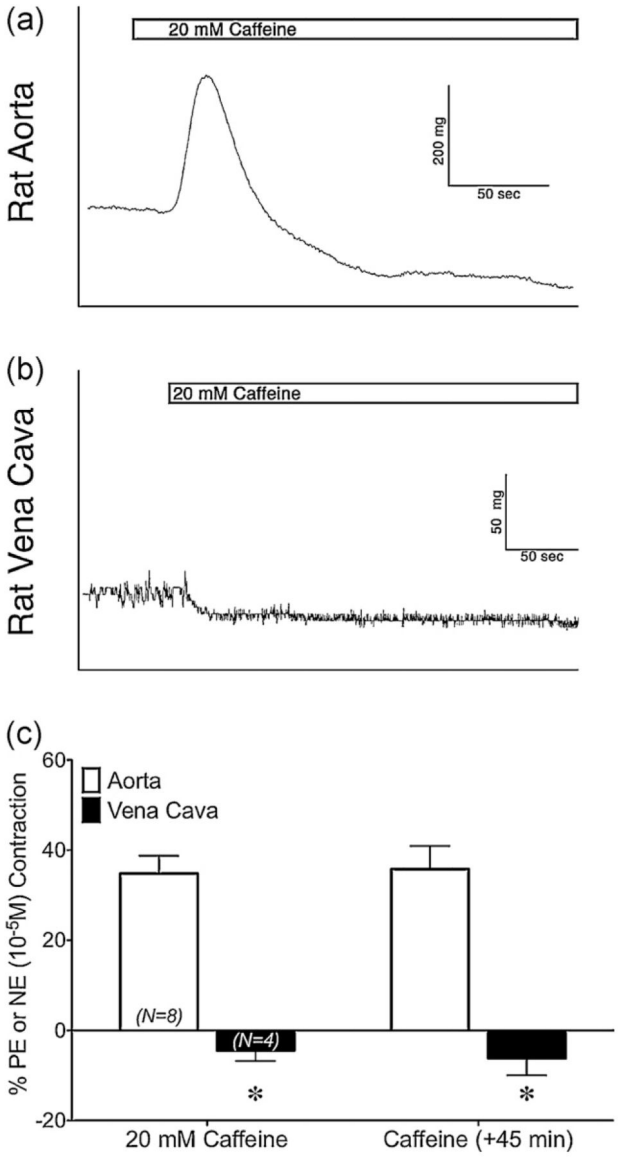

Fig. 4.

(a, b) Representative tracings of the response to 20 mM caffeine in rat aorta (a) and rat vena cava (b). (c) Summary bar graphs indicating the maximum response to initial caffeine exposure, and then to a second exposure after 45 min of washout. White bars represent aorta. Black bars represent vena cava. $N = 4–8$; $*p < 0.05$ *versus* aorta.

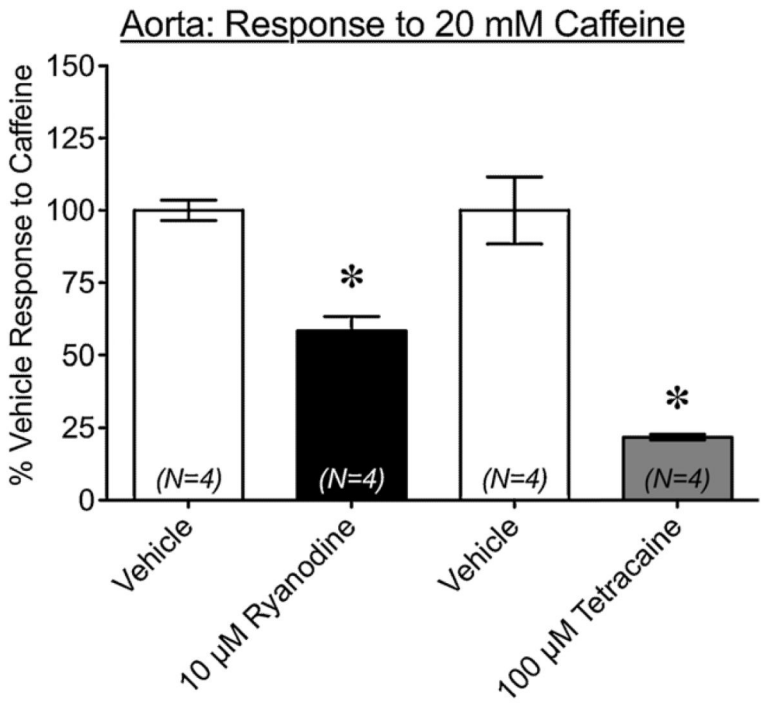

Fig. 5.

Measurement of 20 mM caffeine-induced contraction of rat aorta, in the presence of the ryanodine receptor antagonists ryanodine (10 μM) or tetracaine (100 μM). Vehicle or antagonists were incubated with tissue for 1 h prior to caffeine exposure. White bars represent vehicle-exposed aorta. Black bars represent ryanodine-exposed aorta. Gray bars represent tetracaine-exposed aorta. Bars represent mean ± SEM for the number of animals indicated in parentheses. *$p < 0.05$ *versus* vehicle.

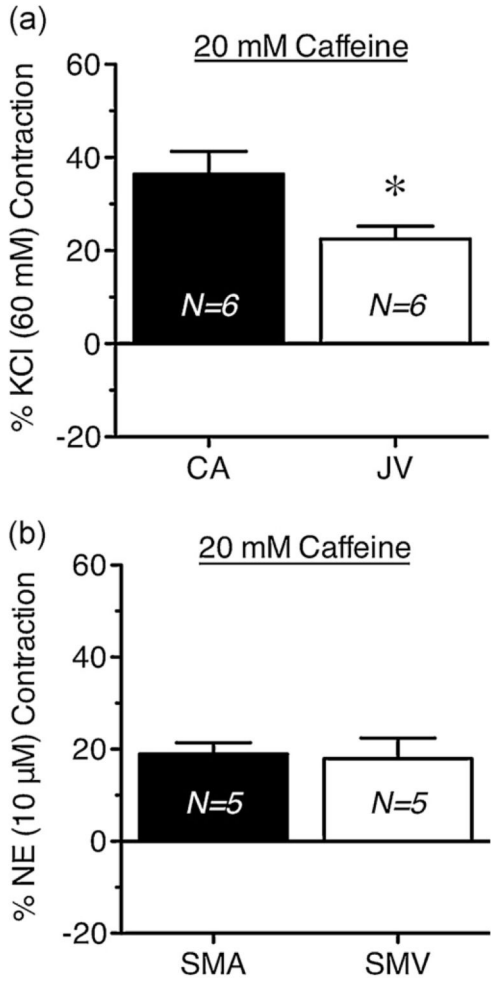

Fig. 6.

Responses to 20 mM caffeine in two other pairs of arteries and veins: (a) carotid artery and jugular vein; and (b) superior mesenteric artery and vein. Summary bar graphs indicating the maximum response to caffeine exposure as a percent of initial contractile stimuli. Black bars represent carotid artery (CA) and superior mesenteric artery (SMA). White bars represent jugular vein (JV) and superior mesenteric vein (SMV). $N = 5–6$; $*p < 0.05$ *versus* artery.

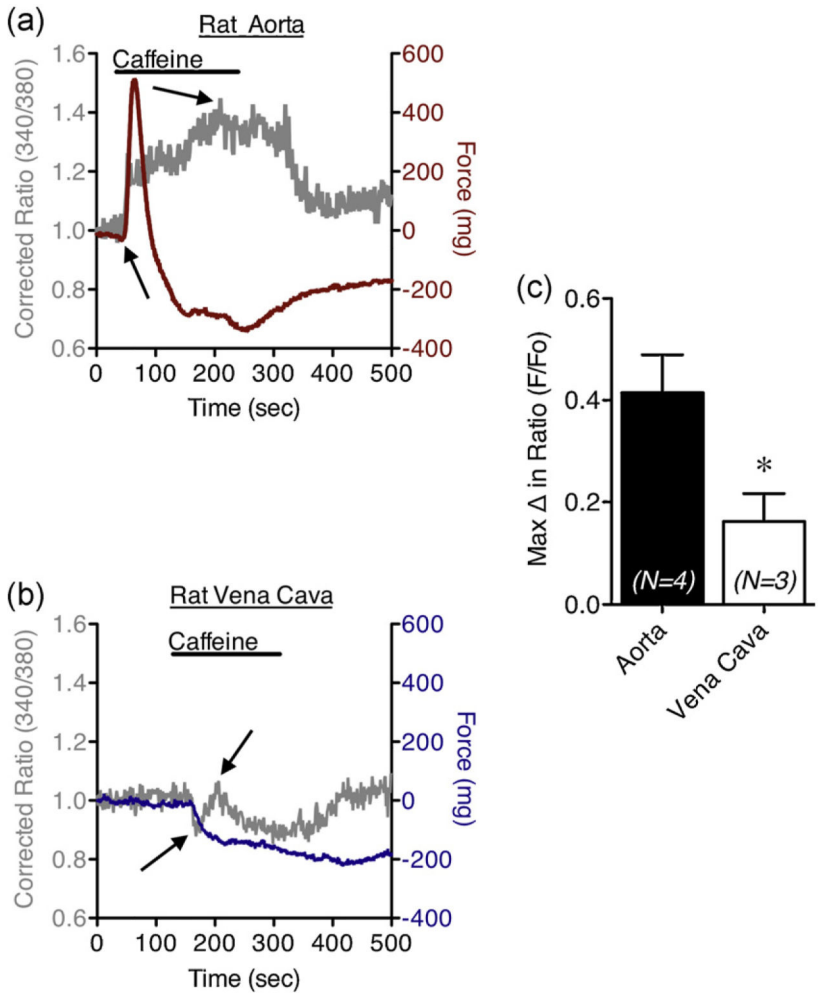

Fig. 7.

(a, b) Representative tracings of Fura2-AM fluorescence ratio (gray, left axis) and caffeine response (red/blue, right axis), measured simultaneously, in vena cava (a) and aorta (b). Arrows indicate the minimum and maximum values used to calculate the maximum change in fluorescence ratio. Representative of 3–4 experiments. (c) Summary bar graph indicating the mean maximum change in fluorescence ratio in aorta (black bar) and vena cava (white bar). Bars represent mean ± SEM for the number of experiments indicated. $N = 3–4$; $*p < 0.05$ *versus* aorta. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)