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Vascular Tone and Ca^{2+} Signaling in Murine Cremaster Muscle Arterioles *in Vivo*.

Mauban, Ca^{2+} Signaling in Arterioles in Vivo

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ABSTRACT

Objectives: We sought to determine some of the molecular requirements for basal-state ‘tone’ of skeletal muscle arterioles *in vivo*, and whether asynchronous Ca^{2+} waves are involved or not.

Methods: Cremaster muscles of anesthetized exMLCK and smGCaMP2 biosensor mice were exteriorized, and the fluorescent arterioles were visualized with wide-field, confocal or multi-photon microscopy to observe Ca^{2+} signaling and arteriolar diameter.

Results: Basal state tone of the arterioles was ~50%. Local block of Ang-II receptors (AT_1) or α_1 -adrenoceptors (α_1 -AR) had no effect on diameter, nor did complete block of sympathetic nerve activity (SNA). Inhibition of phospholipase C caused dilation nearly to the Ca^{2+} -free (passive) diameter, as did exposure to nifedipine or 2-APB. Arterioles also dilated when treated with SKF96365. High resolution imaging of exMLCK fluorescence (ratio) or GCaMP2 fluorescence in smooth muscle cells failed to reveal Ca^{2+} waves (although Ca^{2+} waves/transients were readily detected by both biosensors in small arteries, *ex vivo*).

Conclusions: Arterioles of cremaster muscle have vascular tone of ~ 50%, which is not due to α_1 -AR, AT_1 R or SNA. PLC activity, L-type Ca^{2+} channels, 2-APB- and SKF96365-sensitive channels are required. Propagating Ca^{2+} waves are not present. A key role for PLC and InsP_3 R in vascular tone *in vivo*, other than producing Ca^{2+} waves, is suggested.

Keywords: Calcium, Vascular tone, Smooth muscle, Arterioles, Sympathetic nerve activity

Abbreviations used: α_1 -AR , alpha 1 adrenoceptor; SNA, sympathetic nervous activity; AT₁, angiotensin Type 1 receptor; exMLCK, exogenous Myosin Light Chain Kinase biosensor; FRET, Forster Resonance Energy Transfer; InsP₃R, inositol 1,4,5-trisphosphate receptor; TRP, transient receptor potential channel; PE, phenylephrine; PLC, Phospholipase C enzyme; MT, myogenic tone; RyR, ryanodine receptor; smGCaMP2, circularly permuted, Ca/Calmodulin sensor based on green fluorescent protein; SR, sarcoplasmic reticulum; GPCR, G-protein coupled receptor; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; NE, norepinephrine; TTX, tetrodotoxin.

INTRODUCTION

Arterioles play a key role in controlling tissue perfusion, through the variation of their diameter or state of constriction ('vascular tone'). Arterioles and their contractile mechanisms are difficult to study *ex vivo* because of their small diameter. Even if isolated successfully however, the *ex vivo* arteriole would lack the physiological control systems (autonomic nerve activity, circulating vasoactive factors, blood flow, and others) that would influence vascular tone and the (underlying) Ca²⁺ signaling in the arteriolar smooth muscle cells. In this study of mechanisms of tone and Ca²⁺ signaling in arterioles *in vivo* we sought to 1) determine the extent to which the tone in arterioles of resting cremaster muscle might be neurogenic, myogenic, or involve certain GPCR, and 2) to determine whether asynchronous propagating Ca²⁺ waves are involved or not. Asynchronous propagating Ca²⁺ waves are routinely observed in arteries *ex vivo* and are usually attributed to sequential, propagating Ca²⁺ release from sarcoplasmic reticulum (SR) through

InsP₃ receptors. Ca²⁺ waves have been reported to be activated both in myogenic and neurogenic tone of isolated small arteries (15, 16, 18, 31). Despite intensive research using isolated tissues however, the actual function and importance of Ca²⁺ waves in small arteries and arterioles under physiological conditions is still unclear. In arteries that have not developed myogenic tone (MT), asynchronous propagating Ca²⁺ waves occur spontaneously and their frequency is increased greatly during contractions induced by activation of GPCRs, such as α₁-adrenoceptors. However, in arteries that had developed myogenic tone (a presumed 'physiological' condition), the probability of asynchronous propagating Ca²⁺ waves was reduced to relatively low levels, particularly in 'resting' arteries. Even after GPCR activation, the frequency of Ca²⁺ waves was relatively low (34). Nevertheless, in other types of small arteries and arterioles studied *ex vivo*, Ca²⁺ waves are thought to contribute to contractile activation (30) and, in some cases, to be dependent on RyR (22). Earlier reports have utilized transgenic mice expressing Ca²⁺ biosensors in endothelial cells to examine endothelial Ca²⁺ signaling *in vivo* (4, 5, 27). Here we used a similar approach of using transgenic mice that express genetically-encoded Ca²⁺ indicators (exMLCK and GCaMP2) in vascular smooth muscle combined with high resolution confocal microscopy, to define, for the first time, Ca²⁺ signaling in smooth muscle of skeletal muscle arterioles *in vivo*.

MATERIALS AND METHODS

Animals

Experiments and procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. We used two types of mice that express

genetically-encoded fluorescent biosensor molecules specifically in smooth muscle cells. We refer to them here as 'exMLCK' and 'smGCaMP2'. *ExMLCK mice* were originally created by Dr. James T. Stull and his colleagues (17) specifically for monitoring MLCK activation by Ca^{2+} /Calmodulin in smooth muscle. Briefly, exMLCK contains a short form smooth muscle MLCK fused to enhanced cyan fluorescent protein (ECFP) and yellow fluorescent protein (EYFP) linked by the rabbit smooth muscle MLCK CaM-binding sequence. Binding of Ca^{2+} /Calmodulin to this sequence reduces FRET from the donor, ECFP to the acceptor, EYFP. In cells stably transfected with sensor MLCK, the FRET ratio (CFP/YFP) and phosphorylation of myosin regulatory light chains are similar functions of free $[\text{Ca}^{2+}]_i$ (10). Thus, binding of Ca^{2+} /Calmodulin to the sensor molecule both decreases FRET (increased CFP/YFP fluorescence emission ratio) and activates myosin light chain kinase activity. In exMLCK mice, the transgene is under control of the smooth muscle α -actin promoter (9) and the genetic background is ICR (Inbred Charles River). The exogenous MLCK (i.e. 'exMLCK') is expressed at a level of about 30% of the endogenous MLCK, specifically in smooth muscle, and does not affect force development of isolated arteries (26). Its presence did not affect the animal's weight, heart rate or arterial blood pressure (26). *SmGCaMP2 mice* contain the transgene for an enhanced form of the circularly permuted chromophore-modulating Ca^{2+} biosensor molecule, GCaMP (23) and were created originally by Dr. Michael I. Kotlikoff and his colleagues by methods similar to those they described in detail previously (28). Briefly, GCaMP2 contains several mutations that give it improved brightness, thermal stability and dynamic range compared to GCaMP; these properties have enabled monitoring of Ca^{2+} signaling in hearts (28) and vascular endothelium (27) of mice *in vivo*. To create the smGCaMP2 mice the GCaMP2 transgene was placed under transcriptional control of a ~ 16kb smooth muscle myosin heavy chain promoter (33). The

GCaMP2 construct was injected into oocytes and the lines with high expression were selected and maintained on a C57Bl/6 background.

Mice were anesthetized by 1.5-5% isoflurane (Baxter Pharmaceutical Products Inc., Deerfield, IL), immobilized on a heated (37°C) platform and the cremaster muscle was exteriorized and pinned to a silicone block. The dissection was similar to that described by Baez (3). A polyethylene ring was used to create a solution chamber with the cremaster muscle. The chamber allowed for superfusion of the tissue and established a minimum depth of solution necessary to enable use of long working distance dipping objectives. Care was taken to isolate the pinned cremaster muscle away from the body cavity where rhythmic breathing of the anesthetized animal would impart movement to the muscle. The chamber was perfused using a peristaltic pump, approximately 2 mL/minute at 34°C with a solution of the following composition: (in mmol/L) 112 NaCl, 4.9 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.7 NaHCO₃, 2.0 CaCl₂, 10 HEPES, and 11.5 glucose (pH 7.4 at 22°C, bubbled with (5% CO₂, 12% O₂, 83% N₂)). Heating of the solution is accomplished via an inline heater (HPRE2, Cell Microcontrols, Norfolk, VA). Ca²⁺-free solution was used to determine the full passive diameter of arteries. Ca²⁺-free solution is similar in composition to the normal superfusate, except Ca²⁺ is omitted and replaced with 2mmol/L EGTA. Pharmacological agents were prepared as stock solutions and dissolved in the perfusate to their final concentrations. For studies utilizing femoral arteries, steps to prepare animals for imaging were as described previously (35). For recording of arterial blood pressure, a small incision is made in the femoral artery, in between two ligature points, and a mouse femoral artery catheter (PE-10) (Braintree Scientific, Braintree, MA) was inserted into the artery, past the proximal ligature. The proximal suture was then tied tightly around the

catheter and the incision site was closed with 5mm EZ clips (Braintree). Prior to placement, the catheter was filled with a heparin sodium solution 1000 USP units/mL (American Pharmaceutical Partners, Inc., Los Angeles, CA). The catheter was connected to a fluid-filled pressure transducer (SP 844, Memscap, Skoppum, Norway). Arterial BP measurements were sampled at 1kHz with a PowerLab data acquisition system and LabChart Pro (ADInstruments, Colorado Springs, CO). Systemic administration of the autonomic ganglion blocker, hexamethonium, was by intra-peritoneal injection (30µg/g body weight).

Widefield Imaging of arteries/arterioles: diameter

The exteriorized cremaster preparation or pressurized mesenteric arteries were imaged with an Olympus MVX10 MacroView microscope (Olympus America, Center Valley, PA) (objective lens: 2X Plan Apochromat, 0.5 NA). Excitation illumination was via a xenon arc lamp (Lambda LS, Sutter Instrument, Novato CA). For measurements of arterial diameter, both biosensors (exMLCK, GCaMP2) were excited at 450-490nm. Emission was collected at 500-550nm with a charge-coupled device (ORCA ER, Hamamatsu, Bridgewater, NJ). Total optical zoom was set such that an effective imaging of 1.0 µm/pixel was established. Acquisition of images was set at 1.0 Hz. Image processing was performed with custom written software in IDL 8.1 (ITT Visual Information Solutions, Boulder, CO) and ImageJ. The diameter of arteries and arterioles can be readily derived from the widefield fluorescence images.

Imaging Ca²⁺ signaling dynamics in vivo

Arteries and arterioles of the exteriorized cremaster preparation were imaged using an (upright) Zeiss 710 multiphoton microscope or an inverted Zeiss 5 Live slit scanning microscope (Carl Zeiss MicroImaging, Gottingen, Germany). The anesthetized animal was placed on a raised

stage, with x y adjustments. The upright Zeiss 710 microscope utilized a 20X, W Plan-Apochromat, 1.0NA dipping objective. An objective inverter (400S M-27, LSM Tech, Etters, PA), with the same objective lens, was used to observe the cremaster vascular arcade on the inverted microscope. Two photon and single photon excitation of CFP (present in exMLCK) was at 820nm and 440nm, respectively. CFP and YFP fluorescence were detected at 455-500nm and 520-560nm, respectively. Sampling was set at 0.50-5.0Hz, 0.33-.43 μ m/pixel. Single photon excitation was 488nm for GCaMP2 and fluo-2, emission was long pass filtered at 505nm. Fluorescence data was acquired in third and fourth order cremaster arterioles (the same order arterioles used for diameter measurements). For studies using pressurized arteries, dissection and cannulation of mesenteric arteries followed methods described previously (19, 21).

Fluorescence data was processed with IDL 8.1 and Image J. If movement artifacts are present in particular frames of the image stack, then those frames are either removed before signal analysis, or the image stack is rejected when artifacts are excessive. Fluorescence data was normalized (F/F_0) relative to the basal fluorescence value F_0 (fluo-2, GCaMP2) or as

$R/R_0 = (CFP/YFP)/(CFP_0/YFP_0)$ (exMLCK FRET biosensor), thus allowing grouping of data.

Data are presented as means $\pm$ SEM; n denotes the number of arterial sites (diameter measurements) or cells (Ca^{2+} fluorescence) used for the analysis. Data comparisons were performed using Student's t-test or ANOVA, as appropriate. A $P < 0.01$ denoted significant differences.

Results

Visualizing biosensor arterioles in vivo

The study of arterioles and small arteries *in vivo* presents several difficulties not encountered with isolated pressurized arteries. High resolution imaging *in vivo* was expected to be more difficult than *ex vivo* imaging for several reasons, 1) the arterioles are embedded within a scattering medium (cremaster muscle, in this case), 2) motion of the vessels can occur, as a result of the animal's respiration and blood flow, and 3) access of pharmacological substances to the arterioles may be more difficult, because the surrounding tissue may present a barrier to bulk solution flow and diffusion. These tissues were left intact in order to avoid perturbing the arterioles. Despite these potential difficulties however, the biosensor fluorescence within arterioles was readily visualized with satisfactory resolution both with confocal and 2-photon imaging (Figure 1). The restriction of fluorescent material from the central regions of the cell was evident in both single and multiphoton imaging. Both exMLCK (Figure 1A) and GCaMP2 (Figure 1C) were expressed in virtually all smooth muscle cells of both arterioles and small arteries, although some cells appeared substantially brighter than others. Despite potential tissue barriers, local application of GPCR agonists (e.g. phenylephrine, PE) caused rapid and large contraction (Figure 1A). When the optical section was 'radial' (Figure 1A), the biosensor fluorescence provided a highly accurate means of quantification of arteriole diameter. Finally, blood was flowing visibly within the arterioles, as could be seen from intrinsic fluorescence of cells in the blood (see movie 1, Data supplement). Superfusion of cremaster muscle arterioles expressing exMLCK FRET biosensor with 60mM KCl, *in vivo*, resulted in an increase in FRET ratio. The increase in normalized FRET ratio values (R/R_0) was from 1.0 to 1.96 ± 0.14 , $n=7$ Figure 1B. In arteries that express the Ca^{2+} biosensor GCaMP2, adrenergic stimulation also caused vasoconstriction and increase in Ca^{2+} -dependent fluorescence Figure 1D. These studies therefore demonstrate that biosensor arterioles and small arteries may be imaged with high spatial and temporal

resolution to resolve Ca^{2+} signaling *in vivo*, and access of locally applied solutions for pharmacological investigations is adequate.

Determinants of arteriolar tone in vivo

We first sought to determine how much vascular tone is present in the arterioles and what signaling systems might be the major determinants of this tone. The average outer diameters of the arterioles were $20 \pm 1.6 \mu\text{m}$ (n=15) and $15 \pm 1.1 \mu\text{m}$ (n=14) for 3rd and 4th order arterioles, respectively (Figure 2). Passive diameter was determined by exposure to the Ca^{2+} -free solution. Basal fractional diameter of 3rd and 4th order arterioles was 0.50 ± 0.04 (n=15) and 0.52 ± 0.04 (n=14) (relative to passive diameter), respectively (Figure 2C). Thus cremaster muscle arterioles have a high degree of vascular tone *in vivo* as also concluded previously (12, 13).

The possible involvement of particular GPCRs, channels, and PLC in maintenance of vascular tone *in vivo* was investigated by superfusing arterioles with specific antagonists (Figure 2). While treatment with the AT_1 receptor blocker, losartan (100nM, 12 minutes, n=6), or the α_1 -adrenergic receptor antagonist prazosin (100nM, 11-12 minutes, n=11), did not significantly affect arteriolar diameter, inhibition of PLC with $3 \mu\text{M}$ U73122 induced significant dilation of arterioles from 0.47 ± 0.01 to 0.87 ± 0.03 fractional diameter, n=10 (Figure 2 A-C). Treatment of the arterioles with $1.0 \mu\text{M}$ nifedipine n=3 or $50.0 \mu\text{M}$ 2-APB n=5 also caused significant dilation to 0.82 ± 0.03 and 0.79 ± 0.02 fractional diameter (Figure 2C). In addition, incubation of cremaster muscle arterioles with $10 \mu\text{M}$ SKF96365 (20) also resulted in significant dilation of arterioles, 0.49 ± 0.02 to 0.62 ± 0.04 , n=7 (Figure 2D).

To determine whether tonic sympathetic nerve activity (SNA) was contributing to high levels of vascular tone *in vivo*, we blocked autonomic ganglionic transmission with hexamethonium (intraperitoneally, 30.0

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 $\mu\text{g/g}$ body weight). This caused a significant drop in mean arterial pressure (MAP, $71.4 \pm 2.0 \text{ mmHg}$ to $45.4 \pm 4.3 \text{ mmHg}$, $n=7$) but without a change in heart rate (Figure 3A, B), indicating a likely decrease in systemic vascular resistance. In two of these animals, the diameter of the femoral artery was recorded simultaneously with blood pressure. In those arteries, hexamethonium treatment (5 minutes) resulted in an average dilation of $108 \mu\text{m}$ (Figure 3A, first column). In contrast, the diameter of 3rd and 4th order cremaster muscle arterioles did not significantly change after treatment with hexamethonium (Figure 3A, second column); 3rd $.53 \pm .01$ to $.52 \pm .03$, 4th $.51 \pm .04$ to $.45 \pm .02$, $n=3$ each) (Figure 3B, bottom graph). Thus, tonic SNA contributed highly to vascular tone in the femoral artery (neurogenic vasomotor tone), but not to that of cremaster muscle arterioles.

Ca²⁺ waves are not involved in tone of cremaster muscle arterioles in vivo.

Our prior studies documented some of the characteristics of exMLCK in isolated mesenteric small arteries (26, 32), but not with the high spatial resolution required to visualize sub-cellular Ca²⁺ signaling. In order to validate the ability of exMLCK to detect Ca²⁺ transients/waves, we first imaged isolated (*ex vivo*) pressurized mesenteric small arteries that expressed exMLCK. These arteries were treated with cytochalasin D and Y27632, both at $10 \mu\text{M}$ for 1 hour to prevent contraction and movement. For a standard reference, we also studied wild-type arteries that had been loaded with the conventional Ca²⁺ indicator fluo-2 under identical conditions (Figure 4A). Fluo-2 was used as a Ca²⁺ indicator because it is better retained by smooth muscle cells at physiological temperatures. Color coded AOIs in each xy image correspond to the fluorescence traces in each column. Under the conditions used, arteries loaded with fluo-2 readily revealed high amplitude, long duration Ca²⁺ waves (Figure 4A, blue arrow). Localized Ca²⁺ transients (green arrow), were observed with fluo-2 (Figure 4A) (see video 2, Online Supplement). Similarly, pressurized arteries expressing exMLCK readily revealed Ca²⁺ waves/transients with high signal to noise ratios (Figure 4B) (see video 3). The FRET ratio recordings in Fig 4B are typical of 26 cells, representing a total observation window of 26 minutes. The spontaneous Ca²⁺ waves recorded with

exMLCK were similar in time course and frequency of occurrence to those we recorded previously with fluo-4 in isolated pressurized mouse mesenteric small arteries (34). Since exMLCK is a FRET type biosensor, it is advantageous, particularly in confocal imaging, compared to fluo-2, because it is relatively less affected by small physical movements of the sample (e.g. peristaltic perfusion, blood flow, rhythmic breathing). However, exMLCK does not report lower amplitude localized Ca^{2+} signals and Ca^{2+} sparks, as compared to fluo-2 (Figure 4B and Online Supplement videos 2-3). *In vivo*, superfusion of cremaster muscle arterioles with 60mM KCl showed robust ability to detect FRET Ca^{2+} signals (see Figure 1B). We conclude that the FRET biosensor exMLCK is a robust tool to detect Ca^{2+} waves in vascular smooth muscle cells, regardless of the type of vascular bed and size of arteries.

We examined Ca^{2+} signaling in arterioles *in vivo* by imaging arterioles for a period of 1 minute, at a frame rate of 2s^{-1} (Figure 4C, see video 4 Online Supplement). During recording, animals were in the basal physiological (anesthetized) state and no spontaneous changes in arteriole diameter were observed. Slight respiratory motion ($\sim 1\text{s}^{-1}$) was present (see Online Supplement). Confocal images of mouse cremaster muscle arterioles expressing exMLCK biosensor, *in vivo*, show a distinct lack of high amplitude, long duration Ca^{2+} waves (Figure 4C). The FRET traces shown are typical of 36 cells representing a total observation window of 36 minutes from 11 separate acquisition runs. In order to compare *ex vivo* and *in vivo* exMLCK records directly, ratio values were normalized to basal values and a probability histogram was plotted (Figure 5). A normalized ratio value of $R/R_0=1.4$, for example, represents a 40% increase in FRET ratio from basal. Higher normalized values corresponding to elevated Ca^{2+} levels during a Ca^{2+} wave are readily observed *ex vivo* (Figure 4B, Figure 5 arrow). In contrast the higher R/R_0 values were virtually absent in the *in vivo* recordings (e.g. $R/R_0 > 1.6$) (Figure 4C, Figure 5 gray trace). Since the biosensor can positively report KCl-induced increases in Ca^{2+} *in vivo* (Figure 1B), the occurrence of Ca^{2+} waves in the same tissue would also be detected if and when they occur.

Thus, using the high signal to noise FRET biosensor exMLCK, the maintenance of high levels of vascular tone in 3rd and 4th order cremaster muscle arterioles, *in vivo*, is achieved in the virtual absence of Ca^{2+}

waves. If present, such Ca^{2+} waves must occur with an extremely low frequency, such that none were detected in 36 total minutes of recording.

Similar results to these were obtained with the GCaMP2 biosensor mice (Figure 6). Separate experiments using pressurized arteries (not shown) demonstrated that GCaMP2 also robustly detects Ca^{2+} waves in individual smooth muscle cells, similar to the exMLCK biosensor. Like exMLCK, fluorescence from individual smooth muscle cells in cremaster muscle arterioles can be resolved *in vivo* (Figure 6Aa). Figure 6Ab shows fluorescence traces from smooth muscle cells shown in (a). Utilizing GCaMP2 *in vivo*, F/F_0 values obtained from 50 cells during 30 minutes of recording were normally distributed around a mean of 1.0 (Figure 6B), indicating that the high F/F_0 values typical of Ca^{2+} waves (> 1.4) were not present.

DISCUSSION

Constriction (tone) of cremaster muscle arterioles of anesthetized mice in the basal state cannot be attributed to sympathetic nervous activity (SNA), Angiotensin II, or circulating α_1 -adrenoceptor agonists (as from NE 'spillover' elsewhere). The apparent lack of effective SNA to these arterioles may be attributable to anesthesia, as suggested previously to explain the lack of effect of TTX in this same preparation (13), although tonic SNA was present in the animal, as evidenced by the effects of autonomic blockade on diameter of femoral artery. Although the myogenic reactivity of the arterioles could not be examined directly, the data suggest a strong myogenic mechanism, since the arterioles remained constricted even when MAP fell after blockade of sympathetic nerve activity. With respect to Ca^{2+} signaling, high amplitude Ca^{2+} waves are virtually absent *in vivo*, as revealed by two different genetically encoded Ca^{2+} biosensor molecules. Because of limitations of the available signal-to-noise *in vivo*, the presence of lower amplitude Ca^{2+} transients cannot be ruled out and the roles of Ca^{2+} sparks could not be determined. Volatile anesthetics such as isoflurane have reported effects on smooth muscle reactivity,

myogenic tone as well as calcium homeostasis (2, 7, 24, 25). We therefore cannot completely rule out the possibility that isoflurane has affected Ca^{2+} signaling under the conditions of our studies. Nevertheless, the mechanisms of vascular tone of cremaster muscle arterioles *in vivo* do appear to be different from those of isolated (i.e. *ex vivo*) cremaster muscle small arteries and arterioles. Ca^{2+} waves are reported involved in myogenic tone (MT) of isolated pressurized feed arteries and arterioles of murine cremaster muscle (30), pressurized cerebral arteries (22) and parenchymal arterioles (8). The mechanisms underlying the differences in Ca^{2+} signaling observed between earlier *ex vivo* studies and our present *in vivo* study are currently unresolved. However, the study of Jackson and colleagues 2012 (30) was conducted on pressurized murine cremaster muscle arteries (*ex vivo*). The report utilized cremaster feed arteries, as well as second order cremaster muscle arteries. The isobaric, no flow *ex vivo* preparation was therefore setup to only examine myogenically generated tone and calcium signals. The *ex vivo* experimental conditions therefore do not necessarily reflect the full myriad of factors experienced by the vasculature *in vivo*. These include blood flow, circulating vasoactive factors, just to name a few. Also, the source of vascular tissue and species used should be kept in mind since vascular bed heterogeneity/species differences can contribute to varied results. These differences can affect calcium signaling and thus may account for our results that high levels of vascular tone is maintained in the absence of calcium waves *in vivo*.

In advancing a possible mechanism of cremaster muscle arteriolar tone *in vivo*, we consider the evidence that Ca^{2+} , L-type Ca^{2+} channels, 2-APB- and SKF96365-sensitive channels and enzymic activity of PLC are required, but Ca^{2+} waves, SNA and certain GPCR (above) are not. Given the lack of specificity of 2-APB (6), we assume that the 2-APB-sensitive channels are InsP_3R and/or the receptor or store operated channels, composed of TRP family subunits (e.g. TRPC3, TRPC6, TRPM4, etc). Ca^{2+} waves in smooth muscle are the result of SR Ca^{2+} release through InsP_3Rs whose open probability is a bell-shaped function of cytoplasmic $[\text{Ca}^{2+}]$ (29). Thus, we have suggested previously (34) that the relative paucity of Ca^{2+} waves in isolated arteries that have developed MT may be related to the de-sensitization (decrease in open

probability) of InsP₃R caused by elevated cytoplasmic [Ca²⁺] (14, 29). The critical requirement for PLC activity *in vivo* suggests that [InsP₃] is elevated, but the absence of Ca²⁺ waves suggests that the sensitivity of InsP₃R is not sufficient to support propagating Ca²⁺ waves. In this case, the elevated [InsP₃] may lead to low-level SR Ca²⁺ release that activates inward current through TRP channels that depolarizes the membrane and leads to voltage-dependent Ca²⁺ entry (11) or to activation of a cation current through a direct interaction of the InsP₃R with a TRP channel (1).

PERSPECTIVES

We have provided the first observation of intracellular Ca²⁺ signaling in arterioles *in vivo*, by utilizing genetically-encoded Ca²⁺ biosensor molecules and high-resolution microscopy. Our data suggest that *ex vivo* Ca²⁺ recordings may not adequately represent what occurs in the intact animal. The results support a role for InsP₃ receptors and InsP₃ (since PLC activity is required) in the tone of the arterioles, but the role of these molecules does not appear to be the usually accepted one, of generating asynchronous propagating Ca²⁺ waves that activate contraction. The results support the possibility, raised in other studies (1, 11) that these molecules are required for generation of an inward current that causes depolarization and activation of L-type Ca²⁺ channels, thus elevating intracellular [Ca²⁺]. The use of transgenic ‘biosensor’ animals, that contain genetically-encoded biosensor molecules, together with classical intra-vital imaging methods, is a valuable technique for elucidating *physiological* mechanisms of cardiovascular regulation.

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Figure Legends

Figure 1. Arterioles *in vivo* have contractile ‘tone’, blood flow, and are responsive to G-protein coupled receptor agonists.

A, Two-photon microscopy images of cremaster arterioles *in vivo* under basal conditions and during contraction in response to local application of phenylephrine (PE, 10 μ M) (exMLCK biosensor, CFP excitation) are shown (see Video 1 Online Supplement). Arteriolar diameter was derived from the recording. **B**, Single photon confocal recording of normalized (R/R₀) spatially-averaged, exMLCK FRET signal of cremaster muscle small artery in response to superfusion with 60mM KCl, *in vivo*. **C**, Confocal microscopy allows optical sectioning and 3-dimensional reconstruction of a cremaster small artery, *in vivo*. The upper panel is a single optical section and the lower panel is the reconstructed image. GCaMP2 is expressed in all smooth muscle cells, although to different levels in different cells. **D**, Confocal images of a cremaster muscle arteriole expressing the Ca²⁺ indicator GCaMP2 under basal and PE stimulation, *in vivo*.

Figure 2. Ca²⁺, L-type Ca²⁺ channels, 2-APB- and SKF96365-sensitive channels, and enzymic activity of PLC contribute to arteriolar tone *in vivo*. **A**, Representative records of cremaster muscle arterioles under basal conditions and in the presence of a PLC antagonist (3 μ M U73122) indicate that arteriolar tone is dependent on PLC enzyme activity *in vivo*. **B**, Images in Figure 2A were used to measure the diameter during treatment with PLC inhibitor. Dotted white line in (A) shows the section of arteriole used to extract diameter measurements. **(C, D)**, Summary data showing the response of 3rd and 4th order arterioles to treatment with losartan (Los 100nM), prazosin (Prazo 100nM), U73122 (3 μ M), nifedipine (1 μ M), 2-APB (50 μ M) and SKF96365 (10 μ M) are illustrated. The number of experiments is indicated in parentheses. Fractional diameter= diameter/maximal diameter, *P<0.01.

Figure 3. **Sympathetic nerve activity controls tone of femoral artery in anesthetized mouse, but not arterioles of the cremaster muscle.**

A, The effects of intraperitoneal injection of hexamethonium (arrows, 30 μ g/g body weight) on blood pressure and diameter of femoral and cremaster artery are illustrated. The drop in MAP is associated with dilation of femoral arteries, but not in cremaster muscle arterioles. **B**, Summary data showing the response to hexamethonium treatment (Hexa); (upper panel) mean arterial pressure MAP, heart rate, n=7, (lower panel) diameter of 3rd (■) and 4th (▒) order cremaster arterioles, (n=3 each). * P<0.01

Figure 4. **Ca²⁺ waves can be detected by exMLCK biosensor, but are absent in cremaster muscle arterioles *in vivo*.**

A, Typical confocal fluorescence recordings from mesenteric artery smooth muscle cells loaded with fluo-2 calcium indicator. Green arrow=localized transient, Blue arrow=Ca²⁺ wave. **B**, Mesenteric arteries expressing the FRET biosensor exMLCK also demonstrate robust ability to detect Ca²⁺ waves in smooth muscle cells, *ex vivo*. **C**, Typical confocal fluorescence recordings from individual smooth muscle cells expressing exMLCK in cremaster muscle arterioles, *in vivo*, are illustrated. The dashed lines indicate F/F₀=1 or a normalized ratio value (R/R₀)=1. Colored fluorescence traces correspond to colored AOIs indicated. (see videos 2-4 Online Supplement) Ca²⁺ waves were not detected in 3rd and 4th order cremaster muscle arterioles, *in vivo*.

Figure 5. **Vascular tone in murine cremaster muscle arterioles *in vivo* is maintained in the absence of Ca²⁺ waves.**

Plots of the probability of normalized exMLCK FRET value ratios from isobaric mesenteric arteries and cremaster muscle arterioles (*in vivo*) are represented. FRET ratio values (R) are normalized to basal ratio R₀. Probability is defined as n/total number of observations for each group, where n is total number of

occurrence of a ratio value $R/R_0 = (CFP/YFP)/(CFP_0/YFP_0)$. Isobaric *ex vivo* trace, n=26 cells, 26 minute duration; *in vivo* trace, n=36 cells, 36 minute total duration. Higher normalized ratio values correspond to values associated with elevated Ca^{2+} levels of Ca^{2+} waves. Ca^{2+} waves were not detected in 3rd and 4th order cremaster muscle arterioles, *in vivo*.

Figure 6. **GCaMP2 Ca^{2+} biosensor fluorescence shows absence of Ca^{2+} waves in cremaster muscle arterioles *in vivo*.**

A Typical confocal xy images (a) and fluorescence recordings from individual smooth muscle cells (b) in cremaster muscle arterioles, *in vivo*, are illustrated (GCaMP2, see video 5). Regions of interest in individual cells (a) were marked to yield fluorescence traces shown in (b). The dashed lines indicate $F/F_0=1$. **B**, Population histogram of GCaMP2 fluorescence in smooth muscle cells of cremaster muscle arterioles, (n=50 cells, total observation >2000 seconds). Ca^{2+} waves were not detected in 3rd and 4th order cremaster muscle arterioles, *in vivo*.

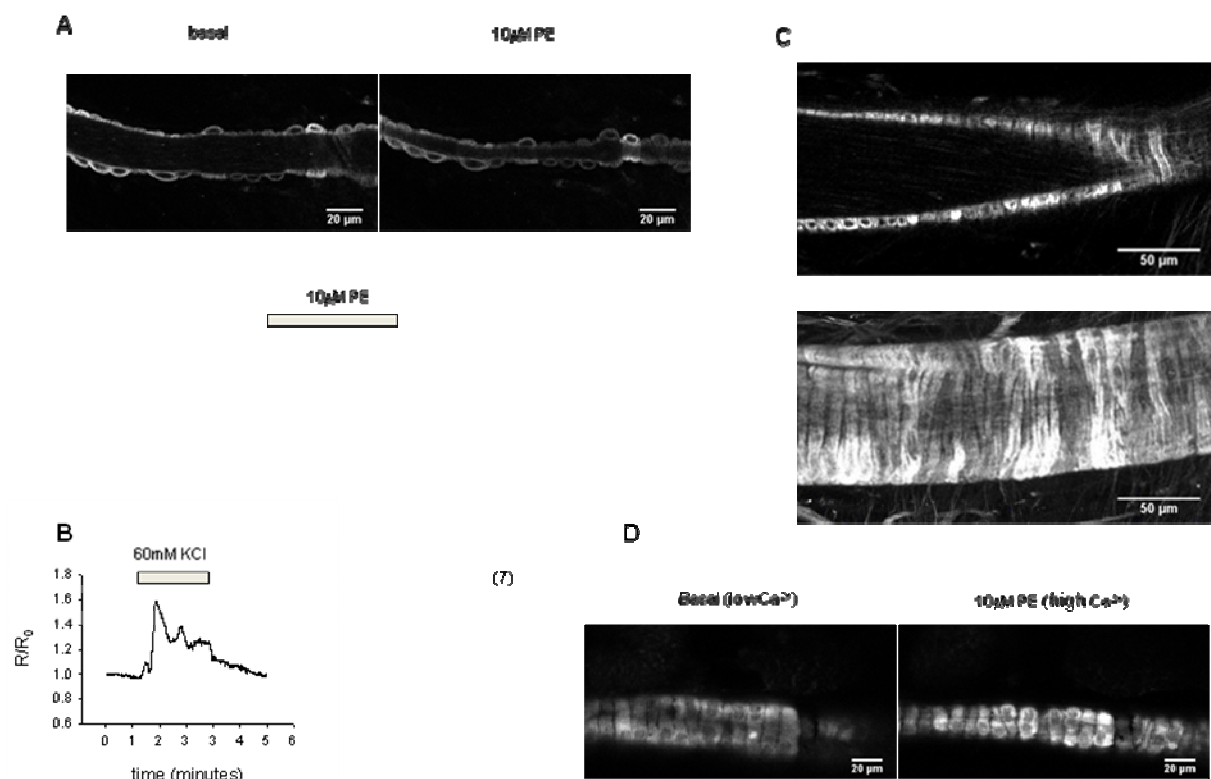


Figure 1

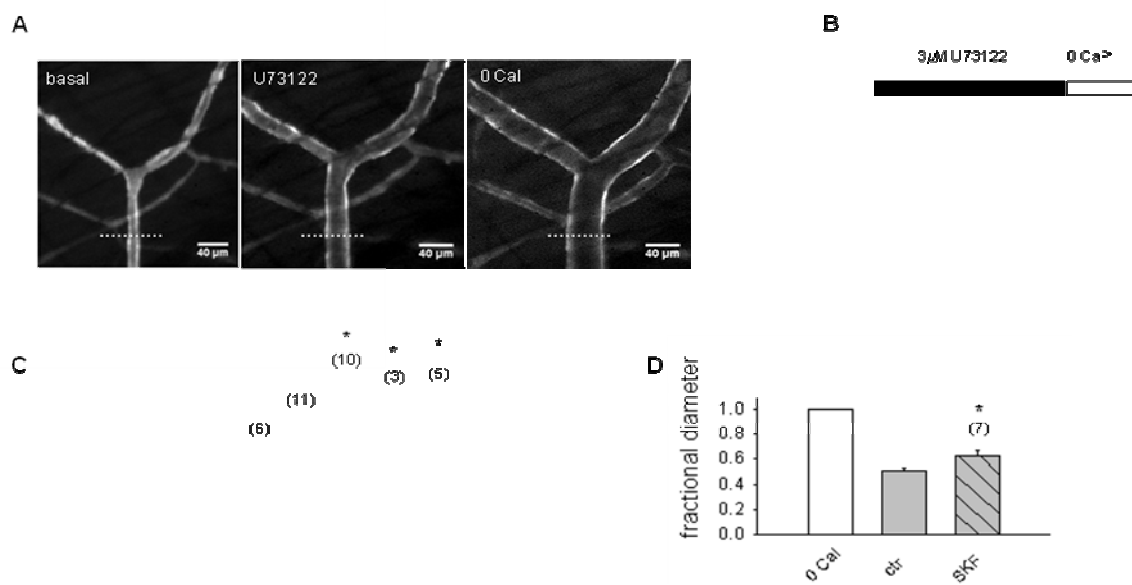


Figure 2

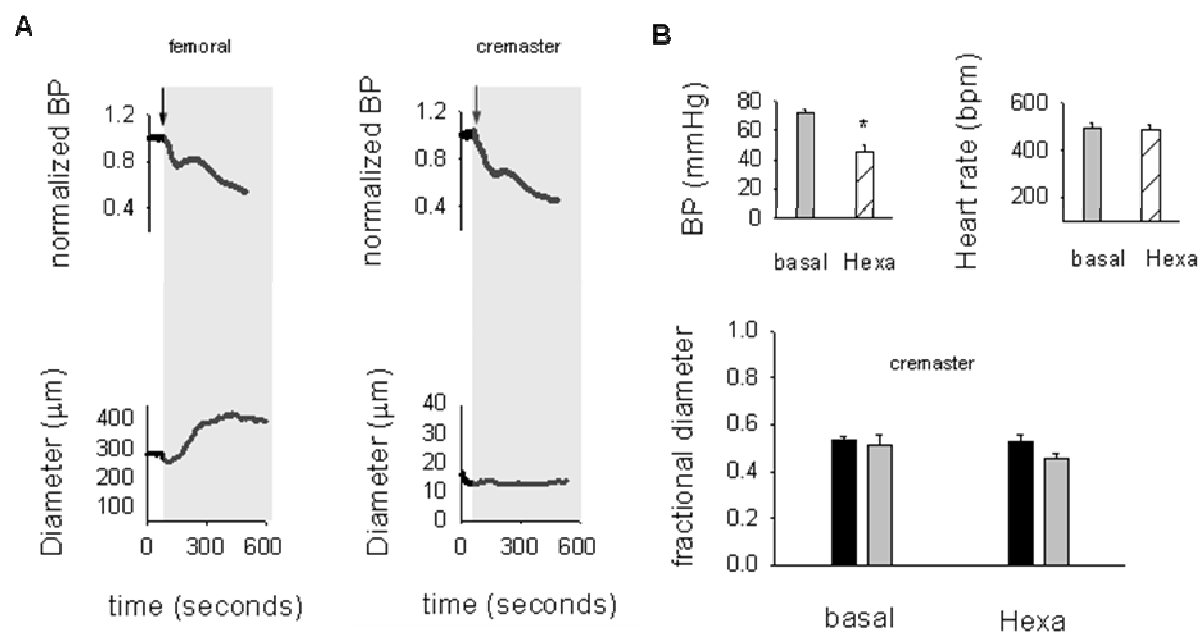


Figure 3

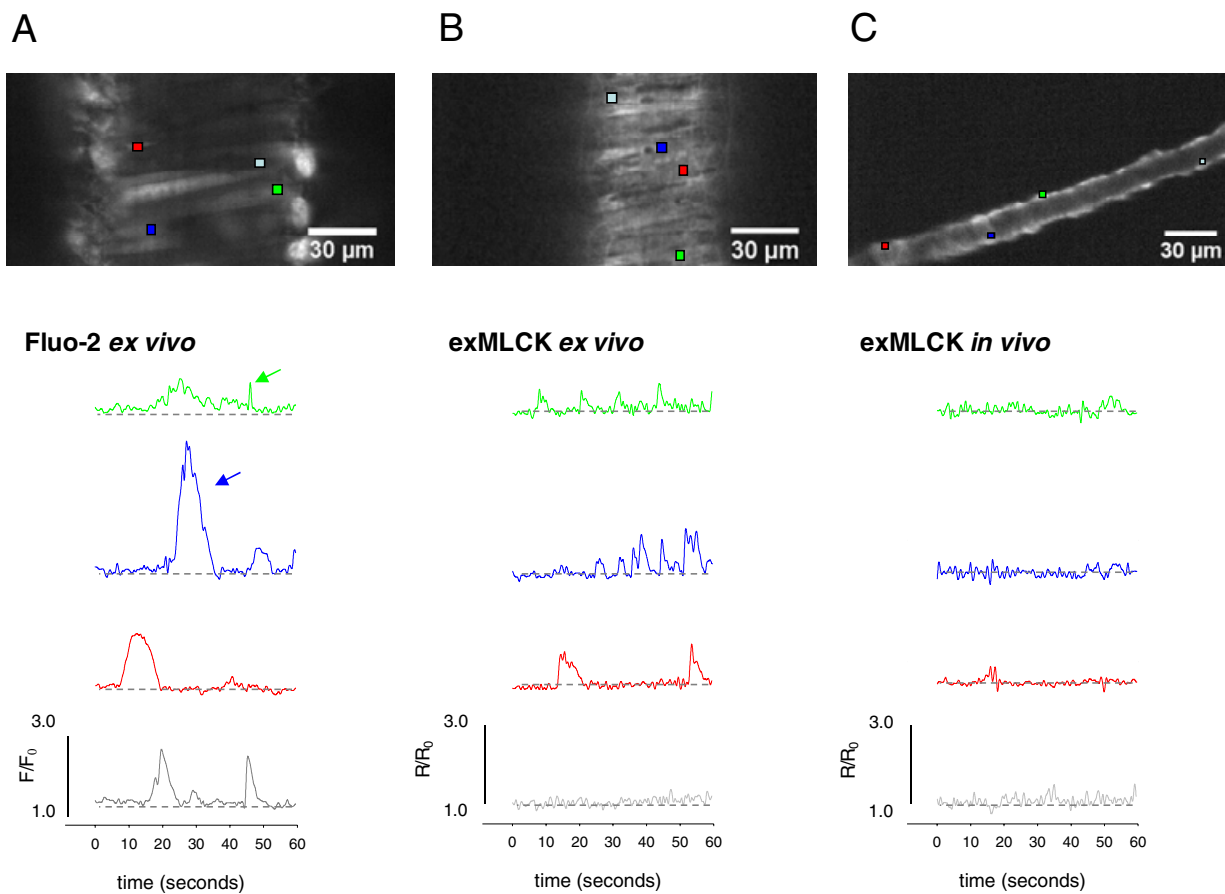


Figure 4

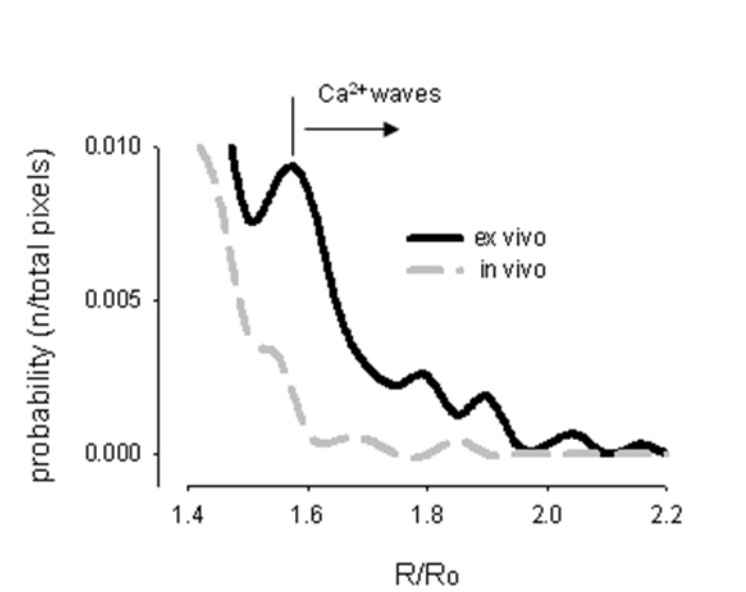


Figure 5

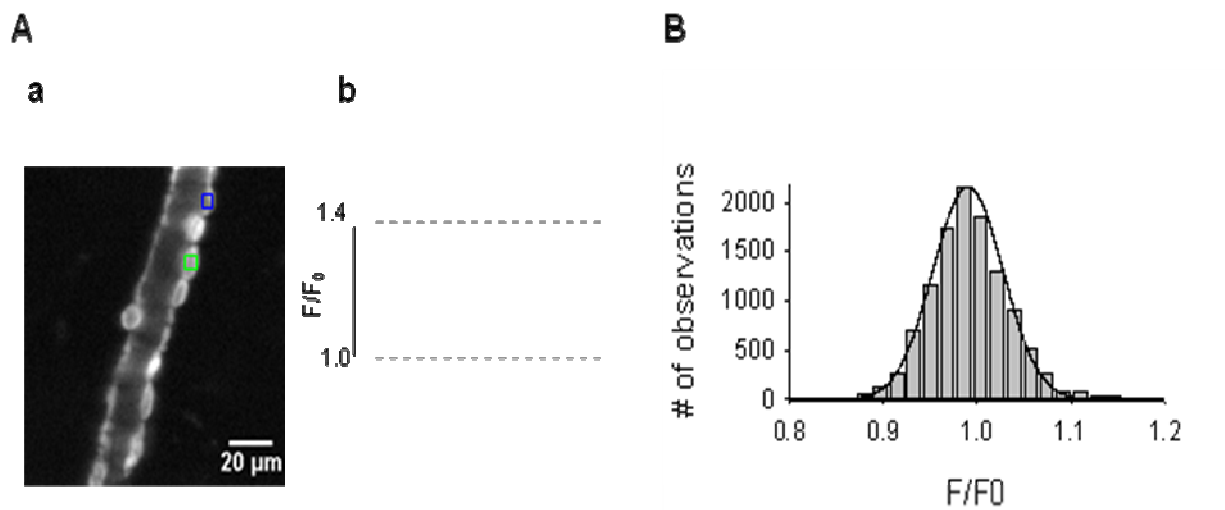


Figure 6