

A method for noninvasive longitudinal measurements of $[Ca^{2+}]$ in arterioles of hypertensive optical biosensor mice

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Mauban JR, Fairfax ST, Rizzo MA, Zhang J, Wier WG. A method for noninvasive longitudinal measurements of $[Ca^{2+}]$ in arterioles of hypertensive optical biosensor mice. *Am J Physiol Heart Circ Physiol* 307: H173–H181, 2014. First published May 23, 2014; doi:10.1152/ajpheart.00182.2014.—We used two-photon (2-p) Förster resonance energy transfer (FRET) microscopy to provide serial, noninvasive measurements of $[Ca^{2+}]$ in arterioles of living “biosensor” mice. These express a genetically encoded Ca^{2+} indicator (GECI), either FRET-based exMLCK or intensity-based GCaMP2. The FRET ratios, R_{min} and R_{max} , required for in vivo Ca^{2+} calibration of exMLCK were obtained in isolated arteries. For in vivo experiments, mice were anesthetized (1.5% isoflurane), and arterioles within a depilated ear were visualized through the intact skin (i.e., noninvasively), by 2-p excitation of exMLCK (at 820 nm) or GCaMP2 (at 920 nm). Spontaneous or agonist-evoked $[Ca^{2+}]$ transients in arteriolar smooth muscle cells were imaged (at 2 Hz) with both exMLCK and GCaMP2. To examine changes in arteriolar $[Ca^{2+}]$ that might accompany hypertension, five exMLCK mice were implanted with telemetric blood pressure transducers and osmotic minipumps containing ANG II (350 ng·kg⁻¹·min⁻¹) and fed a high (6%) salt diet for 9 days. $[Ca^{2+}]$ was measured every other day in five smooth muscle cells of two to three arterioles in each animal. Prior to ANG II/salt, $[Ca^{2+}]$ was 246 ± 42 nM. $[Ca^{2+}]$ increased transiently to 599 nM on day 2 after beginning ANG II/salt, then remained elevated at 331 ± 42 nM for 4 more days, before returning to 265 ± 47 nM 6 days after removal of ANG II/salt. In summary, two-photon excitation of exMLCK and GCaMP2 provides a method for noninvasive, longitudinal quantification of $[Ca^{2+}]$ dynamics and vascular structure in individual arterioles of a particular animal over an extended period of time, a capability that should enhance future studies of hypertension and vascular function.

calcium; FRET; hypertension; two photon; vascular

STUDIES of experimental hypertension in animals are confronted with several fundamental technical difficulties. Determining how changes in arterial $[Ca^{2+}]$ might be involved in experimental hypertension is particularly challenging because measurements of $[Ca^{2+}]$ and artery contractile activation will change immediately upon removal of an artery for study. The processes controlling $[Ca^{2+}]$ are fast (ms), as is control of smooth muscle cross-bridge cycling, by Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Indeed $[Ca^{2+}]$ in smooth muscle cells of arterioles of hamster cheek pouches in vivo has been reported (~ 400 nM) (3) to be severalfold higher than that in smooth muscle cells of isolated arterioles (~ 55 nM or ~ 170 nM) (19, 28). Despite the key role of Ca^{2+} in controlling smooth muscle contraction, the extent to which changes in smooth muscle $[Ca^{2+}]$ are involved in artery narrowing in

hypertension remains unknown. Artery narrowing in hypertension is known to result also from structural “eutrophic” remodeling and to involve modulation of MLCP by ρ -kinase (4). Changes in vascular reactivity also occur (8–10), presumably the result of maintained molecular changes. Thus understanding the role of arterial smooth muscle Ca^{2+} in hypertension would clearly be benefited greatly by measurement of $[Ca^{2+}]$ in arterioles, over the time course of the disease. The advent of optical biosensor mice, which express genetically encoded calcium indicator molecules (GECIs) (15), has obviated some, but not all, of the difficulties in such measurements. The exMLCK biosensor mouse, which expresses the Förster resonance energy transfer (FRET)-based Ca^{2+} /calmodulin sensor molecule (exMLCK) based on smooth muscle myosin light chain kinase (MLCK) (7, 13), has enabled measurement of $[Ca^{2+}]$ in arteries of normal living animals (30) and in experimental hypertension (26). Measurement of a FRET ratio, rather than simple fluorescence changes, obviated problems related to artery contraction and blood flow, and permitted quantification of $[Ca^{2+}]$. Nevertheless, those studies required surgical exposure of the arteries to be visualized, were limited to large arteries (femoral), did not have subcellular resolution of the artery smooth muscle cells, and could not provide a measurement of inner artery diameter (as wide-field epifluorescence microscopy was used). Furthermore, because it is not feasible to perform serial surgeries of this type in an individual animal, the studies were cross-sectional and required large groups of animals to achieve the statistical power necessary to resolve small changes in $[Ca^{2+}]$. Surgical exposure of small arteries can induce undesirable changes in the tissue, due to difficulty in controlling tissue perfusion and physiological levels of gases (11).

The use of two-photon microscopy and near-infrared excitation of FRET type GECIs is expected to ameliorate some of these difficulties, particularly if blood vessels could be imaged noninvasively, and serially in a particular animal. Serial (i.e., longitudinal) measurements would facilitate time-course studies and provide increased statistical power, compared with cross-sectional studies. Two-photon fluorescence microscopy has already been used to image cells and blood vessels noninvasively and serially, through a thinned skull (6), and to image immune system functions noninvasively within mouse ear skin (16), salivary glands (1), and hind footpad (31).

Despite the recent developments in two-photon in vivo imaging, no method existed for noninvasive quantification of $[Ca^{2+}]$ within resistance blood vessels of living mice. Thus we sought to develop such a method, with the additional capabilities to measure arterial blood pressure noninvasively (via implanted telemetric blood pressure transducers) and to produce hypertension via chronic infusion of ANG II (via implanted subcutaneous osmotic minipumps). Arterioles of the

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mouse ear were chosen, since they are accessible optically with two-photon microscopy. A current limitation is that these vessels are likely to have certain specialized functions and the changes they might undergo in hypertension are not presently known. Nevertheless, ear arterioles would be expected to be exposed, like all blood vessels, to circulating substances, and possibly, to altered sympathetic nerve activity that may occur in hypertension. In addition to the exMLCK biosensor mice, we also used GCaMP2sm biosensor mice (18), as the two biosensor molecules provide somewhat different capabilities in observing Ca^{2+} signaling. GCaMP2 is an improved version of GCaMP1 (20) that has proved useful in imaging Ca^{2+} in isolated arteries (14), Ca^{2+} transients in the beating heart (24), and in endothelium of cremaster muscle small arteries in vivo (23). Together, the two GECI-expressing animal models, combined with two-photon microscopy, have allowed a new non-invasive approach to quantify vascular smooth muscle Ca^{2+} in certain blood vessels of a given animal, over extended periods of time.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. The transgenic mouse lines, exMLCK (13) and smGCaMP2 (18, 23, 24), were the same as used previously. All mice were maintained on 12:12-h light/dark schedule at 22–25°C and 45–65% humidity and fed ad libitum on a standard rodent diet and tap water. Only male mice were used, at ages 12–20 wk.

Telemetric Recording of Arterial Blood Pressure

For implantation of aortic telemetric pressure transducers (DSI TA11PA-C10, Data Science International, Minneapolis, MN), mice were anesthetized with 2% isoflurane (IsoFlo, Abbott Animal Health, Abbott Park, IL) supplemented with 100% O_2 . The right common carotid artery was exposed and ligated via an anterior neck midline incision. The catheter, containing the sensor, was inserted into a small hole proximal to the ligature, the tip was passed to the origin of the carotid at the aortic arch, and the catheter was fixed in place with a suture and the hole sealed with adhesive (Vetbond, 3M, St. Paul, MN). The body of the sensor was passed through a subcutaneous tunnel to a subcutaneous pocket in the abdominal wall. Animals recovered from surgery for 7–10 days. DSI telemetric receivers and recording software were used.

Implantation of Subcutaneous Mini-Osmotic Pumps and Intraperitoneal Injections

Mice were implanted subcutaneously with an Alzet osmotic minipump (model 1004, Durect, Cupertino, CA) filled with ANG II. ANG II was delivered at 350 ng·kg⁻¹·min⁻¹ for 9 days. A 32-mm, 21-gauge flexible catheter (Abbott Laboratories, Abbott Park, IL) was installed in the abdominal cavity to enable intraperitoneal delivery of pharmacological agents. Phenylephrine (PE) was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile saline solution (0.9% NaCl).

Isolated Mesenteric Arteries for Ex Vivo Calibration Experiments

For ex vivo calibration experiments, mesenteric small arteries from both exMLCK and GCaMP2 mice were isolated as described previously (21, 27). Arteries were mounted isometrically, by being pulled over an appropriately sized glass cannula (see 29), and the recording chamber was placed on the stage of a Zeiss 710 NLO microscope.

These arteries were superfused continuously with a warmed solution containing (in mM) 112 NaCl, 25.7 NaHCO₃, 4.9 KCl, 2.0 CaCl₂, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, 11.5 glucose, and 10 HEPES (pH 7.4, equilibrated with gas of 5% O_2 , 5% CO_2 , 90% N_2 at 37°C). High (117 mM)-external K^+ ($[K^+]_o$) solution was made by replacing NaCl with equimolar KCl of normal PSS. Ca^{2+} -free solution was made by omitting Ca^{2+} and adding 2.0 mM EGTA (pH 7.4). exMLCK and GCaMP2 fluorescence was excited and recorded using the same wavelengths, optical filters, and objective lenses as used for the in vivo recordings (described in detail below).

Imaging the Mouse Ear

Three days before a mouse was to be used, the dorsal surface of an ear was carefully depilated using Nair hair removal lotion (Church and Dwight, Princeton, NJ). Two days were required for recovery of the skin, and the treatment lasted adequately for 10 days. A surface free of protruding hairs provided better optical access than one with hair intact. To immobilize mice during imaging, they were anesthetized with 1.5% isoflurane in 100% O_2 . To reduce environmental contamination by isoflurane, the exhaust gas from animals was passed through an activated charcoal canister (VaporGuard, VetEquip, Pleasanton, CA). After induction of anesthesia, mice were placed in a prone position on a temperature-controlled platform set to maintain the mouse core temperature at 37°C, which was monitored with a rectal thermoprobe. With the mouse in a prone position on the heated plate, one ear was affixed on its ventral surface to a small piece of sticky tape. This tape was then pinned to a silicone platform thus positioning the ear flat for imaging. A polypropylene ring (15-mm diameter, 3 mm high) was placed over the ear, close to the skull, and pinned to the silicone block (see Fig. 2A). This arrangement immobilized the ear, without directly pinning, and provided a fluid chamber above the dorsal surface of the ear into which the dipping objective lens would be placed. A small plastic block was placed against the head to limit any respiratory movements being transmitted into the ear. In a few imaging experiments, however (e.g., see Fig. 4), a rhythmic motion was present at the respiratory rate of the animal. This caused a portion of the arteriole being imaged to move out of focus for 1 frame (when being imaged at 1 frame/s). In this case, missing pixel values were obtained by simple linear interpolation of the values in the frames preceding and following the artifact.

Fluorescence Recording

We used a Zeiss LSM 710 NLO microscope equipped with a femtosecond pulsed near-infrared (IR) laser (Chameleon Vision, Coherent, Santa Clara, CA) for two-photon microscopy. The objective lens was a Zeiss 20× water plan-apochromat with a working distance of 1.9 mm and numerical aperture of 1.0. Scanning was bidirectional, at frame rates varying from 0.125 to 4 Hz, and pixel sizes varied from 0.10 to 0.83 μ m, as required for a particular experiment. The microscope was inside a light-tight enclosure, and room lights were turned off to ensure little to no background signals.

exMLCK. Selective excitation of the CFP moiety of exMLCK was at 820 nm wavelength. Emitted light passed through an IR blocking filter before being separated into two channels (CFP and YFP) with a 510 nm LP mirror. CFP emission filter was band pass, 460–500 nm; YFP emission was band pass, 520–560 nm. Fluorescence emission detection was via Zeiss LSM BiG module, consisting of two binary GaAsP photodetector modules configured to collect fluorescence signals in a non-descanned manner. The gains of the two detector channels were fixed and held constant, for all experiments involving exMLCK, at levels that optimized the fluorescence from exMLCK (in a basal-state arteriole in vivo) in each emission channel. The use of polystyrene beads labeled with recombinant CFP or YFP revealed that direct excitation of YFP at 820 nm is negligible and yielded no emission signal, whereas excitation of CFP produced spectral overlap emission in the YFP channel (as expected), F_{YFP} , proportional to 32%

of that collected in the CFP channel, F_{CFP} . exMLCK fluorescence ratios (R) were therefore calculated as $R = F_{CFP}/(F_{YFP} - 0.32 \times F_{CFP})$.

GCaMP2. Excitation at 920 nm was found to produce an optimal signal, without excitation of artery intrinsic fluorescence, as measured in vivo in ear arterioles of mice not containing GCaMP2.

Data Analysis and Statistics

The data are expressed as means $\pm$ SE; n denotes the number of animals. Comparisons of data were made using ANOVA or Student's t -test, as appropriate. Differences were considered significant at $P < 0.05$. Image processing was via custom software routines using IDL (Exelisvis, CO).

Theory

The binding of Ca^{2+} /calmodulin to exMLCK causes a conformational change that 1) decreases FRET between the CFP and the YFP components of exMLCK, and 2) activates potential myosin light chain kinase activity (7). The amount of FRET (and the kinase activity) is directly proportional to the fraction of exMLCK molecules that are bound to Ca^{2+} /calmodulin. Since absolute quantification of fluorescence emission is not possible, fluorescence ratios (R) are used, defined as the CFP fluorescence emission divided by the YFP fluorescence emission. In exMLCK, maximum FRET occurs when no Ca^{2+} /calmodulin is bound. Under this condition, CFP fluorescence is minimal and YFP fluorescence is maximal. Thus there is a minimum ratio, R_{min} , when $[Ca^{2+}]$ is zero. A maximum ratio, R_{max} , occurs when all exMLCK are bound to Ca^{2+} /calmodulin. The fluorescence ratios, R_{max} and R_{min} , represent, respectively, 100% and 0% fractional occupancy of exMLCK by Ca^{2+} /calmodulin. The fractional occu-

pancy of exMLCK by Ca^{2+} /calmodulin (Y) can then be calculated as $Y = (R - R_{min})/(R_{max} - R_{min})$. The calcium concentration wherein $Y = 0.5$ is the EC_{50} . Free $[Ca^{2+}]$ is then calculated from Y , using the Hill equation, as $[Ca^{2+}] = [(Y \cdot EC_{50}^n)/(1.0 - Y)]^{1/n}$. The relationship between free $[Ca^{2+}]$ and exMLCK FRET ratio, R , as measured in α -toxin permeabilized mesenteric small arteries, is well fitted by the Hill equation, with an EC_{50} (K_A) of $0.892 \mu M$ (pCa, 6.05) and a Hill coefficient (n) of 1.4 (26). For this method to be valid, the values measured for CFP and YFP fluorescence emission must not contain fluorescence arising from any other sources, such as intrinsic fluorescence of smooth muscle that may arise from metabolic compounds such as NAD(P)H and flavins (12). Furthermore, the measured YFP fluorescence emission must arise entirely from FRET with CFP. These requirements were met acceptably under the conditions we used (pulsed IR laser tuned to 820 nm) as 1) direct excitation of YFP was not measurable (with labeled beads), 2) YFP fluorescence was corrected for spectral overlap (see above), and 3) tissue intrinsic fluorescence of the smooth muscle cells was found to be negligible for the purpose; smooth muscle cell cytoplasm was essentially not visible in arteries not containing exMLCK. Intrinsic fluorescence of basal lamina and perivascular nerve fibers was visible (Fig. 1A) but was excluded from the measurements by the focused excitation, optical sectioning that minimized its contribution, and appropriate masking of images to those regions in which exMLCK fluorescence was brightest.

Determination of R_{max} and R_{min} in isolated exMLCK arteries. R_{max} and R_{min} are not constants, but depend on the optical apparatus used to measure them, just as for R , as the fluorescence emission in each channel is influenced by optical filters, detector gain, and other characteristics of the system. In previous studies in vivo (26, 30) that used widefield epifluorescence microscopy, R_{max} and R_{min} had to be

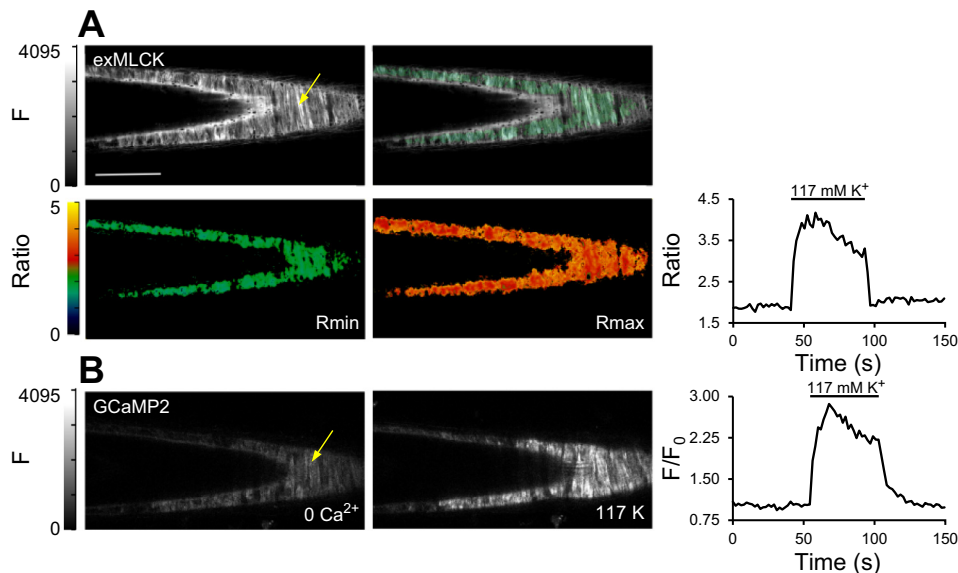


Fig. 1. Dynamic range of exMLCK (A) and GCaMP2 (B) with two-photon excitation determined in isolated mesenteric small arteries. Arteries were pulled over glass tubes to limit cell shortening during contraction. Arteries are tilted with respect to the optical section, providing a cross-sectional view that progresses to a longitudinal view through the smooth muscle cells, from left to right in each image. A: exMLCK mesenteric small artery. A, top left: CFP channel, 820-nm excitation. Scale bar is $100 \mu m$ and applies to all images. Pixel values range from 0 to 4,095, as indicated by the grayscale. A, top right: same image with a (green) mask superimposed. Mask, which excluded both very high and very low values, determined the pixels used in the calculation of exMLCK Förster resonance energy transfer ratios, R_{min} and R_{max} , during exposure to physiological solutions free of Ca^{2+} (A, bottom left) or high in K^+ (A, bottom right, at peak of response), respectively. The two ratio images, calculated as described in MATERIALS AND METHODS, are represented with color as indicated by the color scale bar. Graph at right shows ratio as a function of time in a region of interest (ROI) before, during, and after the switch from the R_{min} solution (0 $[Ca^{2+}]$) to the R_{max} solution (117 mM $[K^+]$). In this representative ROI (position indicated by arrow), R changed from ~ 1.9 to ~ 4.0 . The average values of R_{max} (3.9 ± 0.12) and R_{min} (1.8 ± 0.06), corresponding to the ratio metric range of the FRET change of exMLCK, were determined from analysis of 12 such ROIs in 3 arteries. B: GCaMP2sm mesenteric small artery subjected to the same procedure as above. GFP emission with two-photon excitation at 920 nm in the Ca^{2+} -free solution (B, left) and at the peak of the response in high K^+ solution (B, right). Fluorescence intensity is represented by grayscale. Graph at right shows F/F_0 from a selected ROI (position indicated by arrow), obtained by dividing F_{ROI} throughout by F_{ROI} during the initial exposure to 0 $[Ca^{2+}]$ solution. F/F_0 increased from 1.0 to ~ 2.9 , which defined the dynamic range of GCaMP2 in vascular smooth muscle, with two-photon excitation at 920 nm.

obtained, in each individual experiment, by superfusing a surgically exposed artery to high- $[K^+]$ and 0 $[Ca^{2+}]$ external solutions. This technique is neither possible nor necessary in the intact ear vasculature as imaged here. Here we obtained R_{max} and R_{min} from isolated mesenteric small arteries, imaged with exactly the same experimental conditions and setting as used for the in vivo experiments. Thus quantification of artery $[Ca^{2+}]$ with exMLCK is possible if fluorescence emission ratios obtained in the intact animal are exactly comparable to those obtained in separate, calibrating experiments using the identical optical conditions (viz. the same microscope and settings) to capture the data, as illustrated in Fig. 1. This condition is satisfied in two-photon excitation because of the intrinsic spatial resolution of the system, which eliminates intrinsic fluorescence from structures outside the focal point. In Fig. 1, the artery was tilted relative to the optical section, such that cells were viewed at the left in cross-section (or radial section), proceeding to tangential section at the right. As reported previously (21) the expression of exMLCK was variable among the smooth muscle cells. Because expression of exMLCK is low in some cells we used masks to exclude from ratio images regions of the arteries that were dim. Fluorescence of basal lamina and perivascular nerve fibers was present but did not change during the 0- Ca^{2+} /high- K^+ calibrating procedure and was therefore deemed intrinsic fluorescence, arising from unknown molecules. To

obtain images that excluded basal lamina and adventitia, a mask was constructed (Fig. 1A), which excluded these regions of the image from the ratio analysis. The shape of the ratio images therefore denotes the boundaries of the mask applied and used to calculate ratio values. The R_{min} and R_{max} images (Fig. 1) were then obtained from the masked CFP and YFP images in the 0- Ca^{2+} and high- K^+ solutions, respectively. Because the time course of fluorescence change in different regions of the artery was not exactly the same, the peak value of R in selected regions of the image was used to determine R_{max} , as shown in Fig. 1A. The time course of ratio change in a selected area-of-interest of the ratio image during the exposure is shown (Fig. 1A, right panel). From analysis of such recordings from three experiments, R_{max} was determined to be 3.9 ± 0.12 and R_{min} was determined to be 1.8 ± 0.06 .

Intrinsic fluorescence. The intrinsic fluorescence of basal lamina and adventitia could be a confounding factor, contributing an unknown fluorescence to the measurement of exMLCK fluorescence. Such contribution will be minimal when optical sections are through the luminal center (cells then in cross-section), or tangential, through only smooth muscle cells. The inherent high spatial resolution, due to the small excitation volume of 2-p microscopy, is highly advantageous in this regard, as this fluorescence is not present when the laser is focused inside the smooth muscle cells. Analysis of wild-type

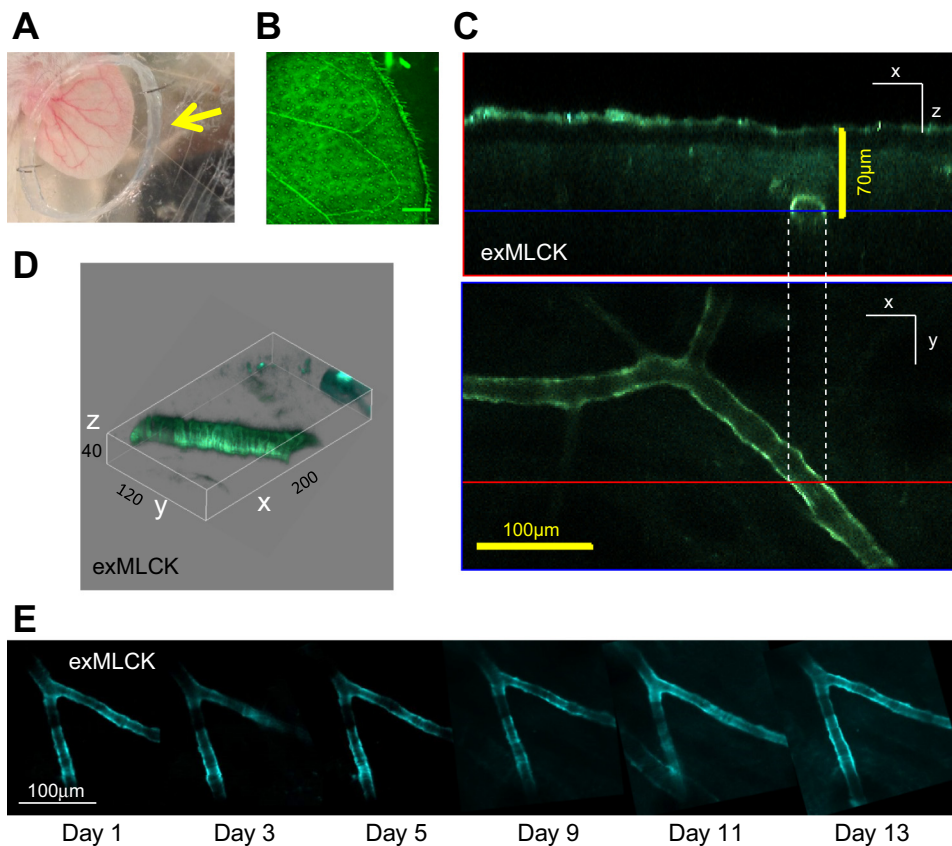


Fig. 2. Preparation of exMLCK mouse ear vasculature for in vivo, longitudinal, 2-photon imaging. **A:** dorsal surface of depilated ear of anesthetized mouse. The ear is positioned within a recording chamber constructed of a polypropylene ring (arrow) pinned to a silicone block. Veins are readily visible and provided consistent reference points for imaging during longitudinal studies. Ear is adhered via sticky tape, and the tape is pinned to a silicone block. The polypropylene ring retains water for the 20 $\times$ dipping objective lens and has a cut-out region on its lower surface to ensure that blood flow is not occluded. The ear is thus positioned firmly without any direct impalement or perturbation. **B:** confocal image of the ear vasculature reveals the architecture of arteries and arterioles (single-photon excitation of YFP, YFP emission). Low-magnification fluorescence images are used to precisely map the location of arterial sections used for longitudinal measurements (bar = 800 μ m). **C:** multiphoton excitation of CFP allows for FRET imaging of arterioles beneath light-scattering tissue and 3D reconstruction of arterial sections. **C, top,** is an orthogonal view reconstruction (xz) (Z stack). The blue and red lines denote the optical section shown in the paired panel. **D:** 3D reconstruction of a separate arteriole, in vivo, shows resolution of single smooth muscle cells (numbers denote scale bar in μ m). **E:** CFP channel images of the same arterioles, $\sim 18 \mu$ m in diameter, which were located and imaged on 6 different occasions in a 13-day period. Scale bar = 100 μ m. The data demonstrate the ability to locate particular sites in an individual animal, as required for longitudinal studies.

arteries showed that the intrinsic fluorescence of smooth muscle cytoplasm itself is negligible. This may reflect the fact that two-photon excitation of NAD(P)H is small at wavelengths greater than 800 nm (12), although flavoproteins, present in an unknown amount in smooth muscle, may contribute fluorescence with 820 nm excitation.

Determination of dynamic range of GCaMP2 in isolated arteries. Mesenteric small arteries were isolated from GCaMP2 mice, mounted as above, and exposed to high- $[K^+]$ and 0- $[Ca^{2+}]$ external solutions. This caused a 2.9-fold change in fluorescence (similar time course to that observed for the exMLCK ratio) (Fig. 1B). Intrinsic fluorescence in vascular smooth muscle of WT (C57BL) arteries in vivo at 920 nm was virtually undetectable. Thus the images are entirely composed of GCaMP2 fluorescence. GCaMP2 has characteristics useful for observing large rapid Ca^{2+} transients as a consequence of its known kinetic characteristics. The K_D for Ca^{2+} GCaMP2 in cardiac cytosol is 146 nM, with high cooperativity of Ca^{2+} activation (Hill coefficient, 3.8) (24). The kinetics of GCaMP2 have been assessed in the rapidly beating mouse heart; with an excitation cycle length of 200 ms (i.e., a heart rate of 5 Hz), GCaMP2 fluorescence rose (and fell, after $[Ca^{2+}]$ decreased) with a time constant of ~ 38 ms (24). Thus the affinity, sensitivity, and kinetics are clearly adequate to record Ca^{2+} waves and oscillations in vascular smooth muscle, but not Ca^{2+} sparks. Similar detailed kinetic data are not yet available for exMLCK, but the exMLCK FRET ratio follows fast Ca^{2+} signaling in urinary bladder smooth muscle with comparable speed to indo-1; both exMLCK FRET ratio and indo-1 ratio followed similar time courses and reached half-maximal values after neural stimulation in ~ 250 ms (5).

RESULTS

Locating Visible Blood Vessels and Mapping

Since our goal was to observe the same blood vessels repeatedly over days or weeks, an experiment began by mapping the blood vessels in a region of the ear, such that imaging sites could be recognized on all subsequent days of the experiment. Veins were readily visible and provided a large-scale map (Fig. 2A). Locating arterioles was aided by the use of a low-power objective lens ($2.5\times$) and direct excitation of YFP (Fig. 2B). Imaging through the intact skin of the ear (Fig. 2B) was confined to the lower horizontal vascular plexus (2) consisting of small arteries and arterioles, from which nutritive capillaries and metarterioles branch upward. The arterioles imaged were typically at a depth of ~ 200 μ m below the skin surface, at or below the level of the deepest portion of the hair bulbs. Multiphoton imaging allowed reconstruction of arterial segments with high spatial resolution (Fig. 2, C and D).

Noninvasive, Serial Blood Vessel Imaging

Serial imaging of the same small arteriole and arterial regions was performed, in some cases for up to 2 wk (Fig. 2E). Blood vessels appeared in slightly different orientations on different days, reflecting slightly different orientation of the ear, but vessels could easily be identified unequivocally by

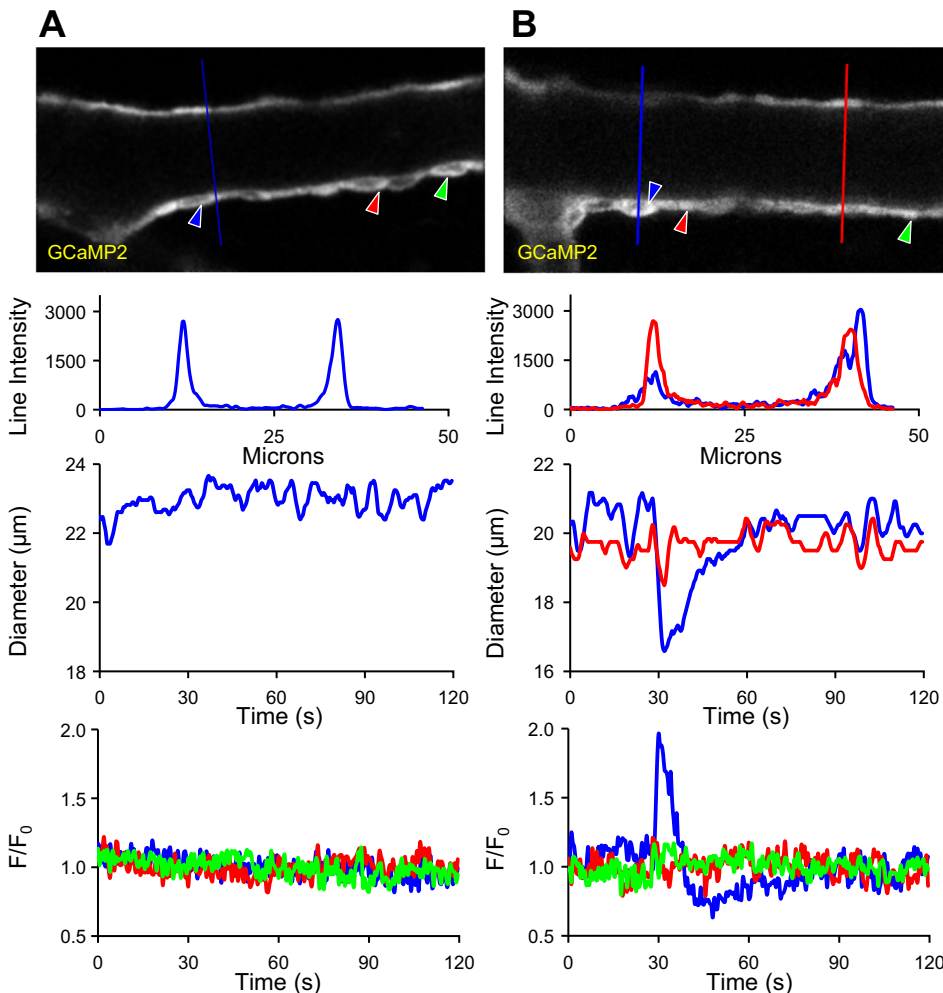


Fig. 3. $[Ca^{2+}]$ signaling in GCaMP2sm mouse arterioles. GCaMP2sm arterioles imaged at 2 Hz^{-1} for 2 min with animal in basal state. Shown is the signal-to-noise achievable with GCaMP2 fluorescence, which is used in measuring arteriolar diameter, spontaneous Ca^{2+} transients, and local contractions (rarely observed). Measurements of GCaMP2 F/F_0 were made using ROI of $1.1\text{ }\mu\text{m}^2$, in the cells indicated by the arrowheads. Videos of the 2 min of recording in A and B, with the ROIs shown in place, are in the Data Supplement, available with the online version of this article. ROIs were adjusted manually in each frame to adjust for any motion of the cell. A: single frame image of arteriole in GCaMP2sm mouse ear. Diameter was derived from the profile of fluorescence at the line shown and graphed below. Background fluorescence is negligible, compared with the peak GCaMP2 fluorescence in the arteriole. Little change in diameter occurred during these 2 min. F/F_0 was obtained from 3 ROIs indicated by arrowheads and plotted (A, bottom). Photobleaching or other time-dependent processes are not apparent during 2 min of recording. The standard deviation of F/F_0 for the 3 ROIs was 0.07. B: a GCaMP2sm arteriole with a spontaneous Ca^{2+} transient during the 2 min recording, but otherwise identical to A. At the site at which the spontaneous Ca^{2+} transient occurred, the diameter decreased by $4.8\text{ }\mu\text{m}$ (blue trace). F/F_0 increased from 1.0 to nearly 2.0.

their unique branches and other recorded landmarks. If an altered orientation sacrificed optical quality, new sites were acquired with optimum focus. In general, when animals were followed over 2–3 wk, less than 20% of sites had to be dropped and were replaced.

Ca^{2+} Dynamics Recorded with GCaMP2

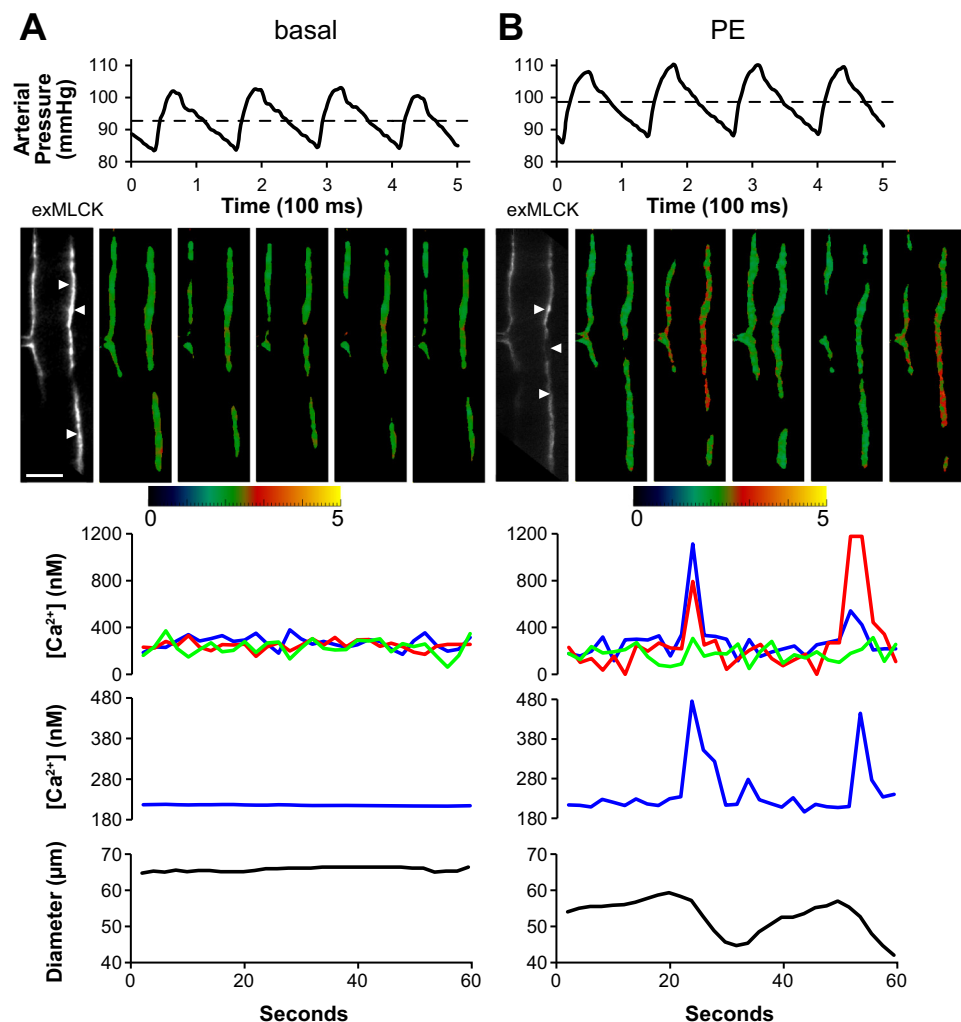
When observation of Ca^{2+} transients was sought, imaging was typically at 2 frames/s for 2 min. GCaMP2 has the dynamic range and kinetic properties required to record Ca^{2+} waves in smooth muscle. In GCaMP2 arterioles in vivo, excited at 920 nm, individual smooth muscle cells were readily identifiable in single frames and the walls of arterioles were clearly defined (Fig. 3), reaching peak values of $\sim 3,000$ fluorescence units (maximum 4,095) against a background of virtually zero. Arteriole diameter was obtained at a selected line, drawn at 90° to the luminal axis, as the difference between the peaks of the fluorescence in the arteriole wall. The measured diameter did not vary by more than approximately ± 0.5 μm during 2 min of recording (Fig. 3; see videos in Data Supplement, available with the online version of this article). Fluorescence was measured in regions of interest (ROIs), typically, ~ 1.0 μm^2 (5×5 pixels, 0.21 μm /pixel). Because some motion in the arteriole wall was present, or when con-

traction occurred because of a Ca^{2+} transient (Fig. 3B), an ROI was positioned in an individual cell manually in each frame, with the aid of custom software. In the example of Fig. 3A, the mean fluorescence in each frame from three cells changed little during the 2 min of recording. The distribution of F/F_0 values, where F_0 is the basal fluorescence value, was narrow compared with the dynamic range of GCaMP2 (Fig. 1B). Most importantly, it is apparent from these records that there was no appreciable photobleaching or photoinactivation of GCaMP2 fluorescence during the standard 2 min recording period. Spontaneous Ca^{2+} transients were occasionally recorded from such cells (Fig. 3B, and video in Data Supplement, available with the online version of this article). In the case illustrated, the maximum F/F_0 was 1.8, indicating a large $[Ca^{2+}]$ transient, corresponding to a large fraction of the available dynamic range (F/F_0 from 1.0 to 2.9). This $[Ca^{2+}]$ transient was associated with an inward movement of that cell of just less than 4.0 μm .

Simultaneous Recording of Arterial Blood Pressure and Quantification of Ca^{2+} with exMLCK

Clearly, GCaMP2 can be used for visualizing large Ca^{2+} transients in individual smooth muscle cells in vivo, but it cannot be used for quantifying $[Ca^{2+}]$ without making assump-

Fig. 4. Simultaneous telemetric recording of arterial blood pressure and exMLCK arteriolar $[Ca^{2+}]$ and diameter during action of a systemic vasoconstrictor. **A:** basal state. **B:** during the action of phenylephrine (PE) 1 min after intraperitoneal (ip) administration. From top to bottom: traces are mean arterial blood pressure (MAP), raw CFP fluorescence followed by 5 images of arteriolar $[Ca^{2+}]$ during a 1 min recording window, $[Ca^{2+}]$ derived from selected ROIs in the images, average $[Ca^{2+}]$ derived from the entire arteriole image, and arteriole diameter at a selected, representative region. White arrowheads indicate the position of the ROIs (5×5 pixels). A breathing artifact (see MATERIALS AND METHODS) propagated through the image, resulting in the disappearance (as a result of intensity masking) of regions of the arteriole in some frames. When this occurred in an ROI, values were obtained by interpolation from preceding and following frames. During the action of PE, MAP rose by 6.2 mmHg, and pulse pressure rose by 6.3 mmHg. Synchronized $[Ca^{2+}]$ transients appeared in some, but not all, cells. $[Ca^{2+}]$ in individual regions rose to levels approaching 1.0 μM , prior to the decrease in diameter. The peak spatial average $[Ca^{2+}]$ was less than that reached in some individual cells because of the heterogeneity of the $[Ca^{2+}]$ transients.



tions about the $[Ca^{2+}]$ corresponding to F_0 . GCaMP2, as expressed in smooth muscle in our test animals, is a nonratio-metric Ca^{2+} indicator. By contrast, exMLCK is ideal for quantification of $[Ca^{2+}]$, particularly when a high signal-to-noise can be obtained by reducing temporal resolution. To demonstrate the feasibility of simultaneously measuring $[Ca^{2+}]$ noninvasively in an arteriole and simultaneously recording arterial blood pressure, an exMLCK mouse was implanted with an intra-aortic telemetric pressure transducer 2 wk before the experiment (Fig. 4). For simultaneous imaging and recording of blood pressure, the telemetry receiver was positioned to the side of the microscope stage, within 6 in. of the mouse. This mouse was also implanted with an intraperitoneal catheter for injection of phenylephrine (PE). In the basal state, the mean arterial blood pressure (MAP) was 97.9 mmHg. Imaging was performed at 0.5 Hz, for 1 min during a control period (Fig. 4A) and again for 1 min (Fig. 4B) after 1 min following intraperitoneal injection of PE (10 μ g/g). $[Ca^{2+}]$ was calculated from the mean ratio value within ROIs (5×5) as described previously. During control, $[Ca^{2+}]$ was ~ 200 nM in all regions, and no inhomogeneity of ratio or $[Ca^{2+}]$ was evident. After injection of PE, MAP and pulse pressure increased by 6.3 and 6.2 mmHg, respectively. Vasomotion became evident, and $[Ca^{2+}]$ rose periodically in some ROIs from 200 to $\sim 1,000$ nM. The spatial average $[Ca^{2+}]$, calculated from all arteriole pixels, rose from 200 to >400 nM. This lower, spatial-average, value resulted from the fact that some regions of the arteriole did not produce Ca^{2+} transients. The Ca^{2+} transients peaked before any significant constriction, and $[Ca^{2+}]$ returned to the baseline in all the regions by the time the minimum diameter was reached, in accord with our previous in vitro studies (17).

Longitudinal Measurement of Spatial Average $[Ca^{2+}]$

A primary goal was to develop a method for measuring, noninvasively, vascular smooth muscle $[Ca^{2+}]$ in individual arterioles in experimental hypertension. For these experiments, $[Ca^{2+}]$ was measured in five cells of two to three arterioles on every other day in five exMLCK mice. The square area of interest was 5×5 pixels and $1.975 \mu\text{m}$ on a side, thus enclosing a region of $\sim 4 \mu\text{m}^2$, within an individual smooth muscle cell. Recording was for 30 s at 1 frame/s. Hypertension was produced with combined high-salt diet [6% NaCl (wt/wt)] and infusion of ANG II for 9 days, via an implanted osmotic minipump. MAP (Fig. 5) was measured, via implanted aortic telemetric blood pressure transducers in each animal over 24 h periods, and during the period in which imaging took place. In conscious animals, MAP was 95.1 ± 1.5 mmHg prior to ANG II/salt, was 121.3 ± 6.1 mmHg during ANG II/salt, and recovered to 92.2 ± 4.8 mmHg (mean $\pm$ SE). During isoflurane anesthesia, MAP was significantly reduced at all times, being 73.8 ± 2.0 mmHg prior to ANG II/salt, 83.4 ± 4.2 mmHg during ANG II/salt, and recovering to 72.1 ± 4.0 mmHg. $[Ca^{2+}]$ in the basal state was 246 ± 42 nM ($n = 5$ animals), increased to 599 nM ($n = 2$ animals) on day 2 of ANG II/salt, declined to 331 ± 42 nM during ANG II/salt ($n = 5$ animals, days 3–6), and finally recovered to values almost identical to basal, 265 ± 47 nM ($n = 5$ animals).

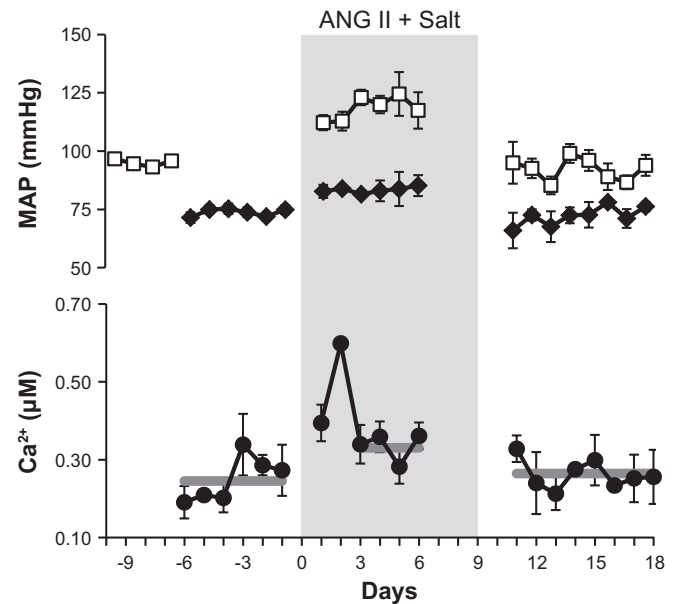


Fig. 5. MAP and arteriolar smooth muscle $[Ca^{2+}]$ increase in arterioles of exMLCK mice during chronic infusion with ANG II and a high-salt diet. MAP (top) in conscious state (open symbols) and in anesthetized state (closed symbols), during which the measurements of $[Ca^{2+}]$ (bottom) were obtained ($n = 5$ animals). For the $[Ca^{2+}]$ measurements, each point represents the mean $\pm$ SE of either 3 or 2 mice, since mice were imaged in 2 groups, on alternating days. Missing points are days in which imaging or MAP measurements were not performed. Gray lines indicate the mean values over the corresponding time periods. MAP was increased above basal levels during ANG II/salt (shaded box) when animals were conscious (26 mmHg), and by 11 mmHg above basal when anesthetized. During the last 4 measurements of the ANG II/salt period, mean $[Ca^{2+}]$ was increased by 85 nM. Removal of ANG II/salt reduced both MAP and arteriolar $[Ca^{2+}]$.

DISCUSSION

In Vivo Measurements and Utility for Hypertension Studies

The clear advantage of making $[Ca^{2+}]$ measurements in vivo is that the arterioles are in a state closer to physiological than can be reproduced in experiments outside the animal or even in surgically exposed tissues. Nevertheless, the method still requires anesthetization, which can have several consequences for cardiovascular function, including reduction of sympathetic nerve activity (22) and increased activity of the renin-angiotensin system (25). In the present experiments, anesthesia clearly reduced MAP, both under control conditions and in the hypertension produced by ANG II/salt (Fig. 5). In anesthetized mice, MAP was elevated during ANG II/salt above basal values by only 11 mmHg, whereas in the conscious state it was elevated by 26 mmHg. In the anesthetized mice, the elevated MAP was accompanied by an increase in $[Ca^{2+}]$ of 85 nM. In future studies, the ability of being able to image ear arterioles or other vascular beds, such as the cerebral vasculature (6), in the unanesthetized animal would provide a more improved model highly relevant to hypertension, stroke, and diabetes. Finally, the present experiments were limited to mouse ear vasculature, and the relevance of this vascular bed to the vascular changes that occur in hypertension is not yet known. However, ear arterioles are highly innervated by the sympathetic nervous system and are exposed to any circulating substances that might be involved in hypertension. To the best

of our knowledge, our new methodology is the first to permit repeated measurements of vascular smooth muscle Ca^{2+} before, during, and after the onset of experimental hypertension in the same group of animals.

GCaMP2sm Biosensor Mice

GCaMP2 appears to be slightly advantageous over exMLCK for observing dynamic Ca^{2+} signaling in arterial smooth muscle cells in vivo, probably because of its somewhat larger dynamic range (Fig. 1), relative greater brightness, and the fact that tissue penetration of the laser excitation light is better at 920 nm than at 820 nm (as used for exMLCK). These factors also make GCaMP2 somewhat more advantageous than exMLCK for detailed observation of artery structure and diameter in vivo. However, at the basal state $[Ca^{2+}]$ of ~ 200 nM (as measured with exMLCK), GCaMP2 would be expected to be $\sim 50\%$ saturated, and, while still able to respond to small changes in $[Ca^{2+}]$, to do so with a relatively reduced dynamic range and sensitivity. The major disadvantage of GCaMP2, however, is that it is a single-wavelength, intensity-based GECI. GCaMP2 fluorescence will depend on protein expression level, and fluorescence transients may be more subject to motion artifacts. It therefore lacks the advantages of ratio-metric biosensors, primarily that its signal cannot be converted to $[Ca^{2+}]$.

exMLCK Biosensor Mice

For quantification of $[Ca^{2+}]$ the FRET biosensor exMLCK is appropriate. The fact that it is an integrated biosensor molecule, with CFP and YFP on the same molecule (MLCK), is highly advantageous, conferring a fixed stoichiometric ratio of CFP to YFP of 1:1, and thus facilitating quantification of the FRET ratio. Ratio-metric measurements are required for quantification of $[Ca^{2+}]$ in the presence of variable levels of exMLCK expression. Thus exMLCK appears to be an ideal indicator of both myosin regulatory light chain phosphorylation and $[Ca^{2+}]$. exMLCK appears to be excluded from smooth muscle cell nuclear regions and, in A7r5 cells, is bound to actin stress filaments (13). Although exMLCK successfully phosphorylates myosin light chains in transfected cells (13), it is not yet known whether exMLCK expressed in smooth muscle cells of mice contributes to actual contractile activation. Prior studies have shown that exMLCK FRET ratio tracked both $[Ca^{2+}]$ (measured with indo-1 or fura-2) and myosin regulatory light chain phosphorylation (5, 27). Furthermore, after neural stimulation of bladder strips, the latencies for the onset of $[Ca^{2+}]$ changes and exMLCK FRET ratio were not significantly different. Nevertheless, compared with an “ideal” cytoplasmic Ca^{2+} indicator, the K_D is relatively high, and therefore, small changes in $[Ca^{2+}]$ can be difficult to detect, without relatively prolonged signal integration. We show here that measurement for 8 s provides adequate signal-to-noise ratio to distinguish small differences in $[Ca^{2+}]$, and that Ca^{2+} transients in individual cells can be measured with a temporal resolution of 0.5 s, just adequate to resolve Ca^{2+} waves and oscillations. The goal of obtaining adequate signal-to-noise ratios prevents faster rates of acquisition, even with highly sensitive detectors present in the microscope system. A key point is that measurements of R_{min} and R_{max} made ex vivo are applicable to the in vivo

measurements, since two-photon excitation provides selective excitation of CFP in both settings. The highly focused two-photon excitation, combined with use of image masks for analysis, means that tissue intrinsic fluorescence, both from smooth muscle and from other tissues, does not complicate the measurements.

Summary

In summary, the use of optical biosensor mice and two-photon microscopy does enable noninvasive, longitudinal measurements of $[Ca^{2+}]$, vessel diameter, and vascular reactivity in arterioles of the mouse ear. Arterial blood pressure can be measured simultaneously with imaging, facilitating longitudinal studies of hypertension. GCaMP2 will be most useful for measurements of Ca^{2+} dynamics and artery structure. exMLCK will be most useful for detecting small, steady-state changes in cytoplasmic $[Ca^{2+}]$, although it is also capable of measuring Ca^{2+} dynamically. An increase in $[Ca^{2+}]$ during ANG II/salt hypertension can be measured by this technique, although it may be blunted by the anesthesia (as is the increase in MAP).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.R.H.M., S.T.F., M.A.R., J.Z., and W.G.W. conception and design of research; J.R.H.M., S.T.F., and W.G.W. performed experiments; J.R.H.M., S.T.F., and W.G.W. analyzed data; J.R.H.M., S.T.F., M.A.R., J.Z., and W.G.W. interpreted results of experiments; J.R.H.M., S.T.F., and W.G.W. prepared figures; J.R.H.M., S.T.F., and W.G.W. drafted manuscript; J.R.H.M., S.T.F., M.A.R., J.Z., and W.G.W. edited and revised manuscript; J.R.H.M., S.T.F., M.A.R., J.Z., and W.G.W. approved final version of manuscript.

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