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Review

Imaging sympathetic neurogenic Ca^{2+} signaling in blood vessels

Withrow Gil Wier[a,b,*,1], Joseph R.H. Mauban[a]

[a] Department of Physiology, University of Maryland Baltimore, Baltimore, MD 21201, USA
[b] Department of Physiology, University of Arizona, College of Medicine, Tucson, AZ 85724, USA

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ABSTRACT

We review the information that has been provided by optical imaging experiments directed at understanding the role and effects of sympathetic nerve activity (SNA) in the functioning of blood vessels. Earlier studies utilized electric field stimulation of nerve terminals (EFS) in isolated arteries and vascular tissues (*ex vivo*) to elicit SNA, but more recently, imaging studies have been conducted *in vivo*, enabling the study of SNA in truly physiological conditions.

Ex vivo: In vascular smooth muscle cells (VSMC) of isolated arteries, the three sympathetic neurotransmitters, norepinephrine (NE), ATP and neuropeptide Y (NPY), elicit or modulate distinct patterns of Ca^{2+} signaling, as revealed by confocal imaging of exogenous fluorescent Ca^{2+} indicators. Purinergic junctional Ca^{2+} transients (jCaTs) arise from Ca^{2+} influx during excitatory junction potentials (eJPs), and are associated with the initial neurogenic contraction. Adrenergic Ca^{2+} waves and oscillations cause contraction while SNA-induced endothelial Ca^{2+} 'pulsars' cause relaxation.

In vivo: optical biosensor mice, which express genetically encoded Ca^{2+} indicators (GECI's) specifically in smooth muscle, combined with non-invasive imaging techniques has enabled imaging SNA-induced Ca^{2+} signaling and arterial diameter *in vivo*. SNA induces Ca^{2+} oscillations in intact arteries. $[Ca^{2+}]$ of arterial smooth muscle cells increased in hypertension, in association with increased SNA.

High resolution imaging has revealed local sympathetic, neurogenic Ca^{2+} signaling within smooth muscle and endothelial cells of the vasculature. The ongoing development of *in vivo* imaging together with an expanding availability of different biosensor animals promises to enable the further assessment of SNA and its effects in the vasculature of living animals.

1. Introduction

Chronically elevated SNA ('sympathetic hyperactivity') is thought to be involved in numerous disease states (Fisher et al., 2009, Malpas, 2010), including obesity, diabetes, metabolic syndrome, systemic hypertension (Blaustein et al., 2012), and heart failure (Parati and Esler, 2012). In one of its most well-known physiological roles, the sympathetic nervous system (SNS) participates integrally in control of arterial blood pressure through its regulation of vascular function (arteries and veins), cardiac function, baroreceptor function, renal and adrenal function, and cellular metabolism. Imaging the effects of sympathetic nerve activity (SNA) in arteries can provide an additional technique to the classical ones of microneurography or measurement of transmitter overflow and others (Guild et al., 2010). For example, in arteries containing appropriate optical Ca^{2+} indicators, the release of a single 'quanta' of ATP at sympathetic neuromuscular junctions can be seen to generate a unique Ca^{2+} transient in a smooth muscle cell, termed a junctional Ca^{2+} transient, or 'JCat' (Lamont & Wier, 2002). Similarly, neural release of a single 'quanta' of ATP on *vas deferens* can be detected optically as a 'neuroeffector Calcium transient' (NCT). The utility of optical recording of NCTs in locating neurotransmitter sites and measuring release probability at those sites has been described (Young et al., 2007).

Here we focus, first, on studies in which sympathetic, neurogenic Ca^{2+} signaling has been imaged in arteries or vascular tissues removed from the animal (*ex vivo*) and, second, on the more recent imaging of sympathetic neurogenic signaling in arteries of living animals (*in vivo*), either anesthetized or conscious. *In vivo* experimentation (Wier, 2014) is highly advantageous for examining the putative roles of SNA in pathological conditions such as hypertension.

Ex vivo imaging studies have utilized isolated vascular tissues loaded with the now classical exogenous organic Ca^{2+} indicators, such as fluo-4. The primary advantages of *ex vivo* studies are that many experimental manipulations are feasible and the highest quality images can be

* Corresponding author.
E-mail addresses: wgwier@email.arizona.edu (W.G. Wier), JMauban@som.umaryland.edu (J.R.H. Mauban).
[1] Present address: Department of Physiology, University of Arizona, College of Medicine, 1501 N. Campbell, Rm. 4103, PO Box 245051, Tucson, AZ 85724, USA.

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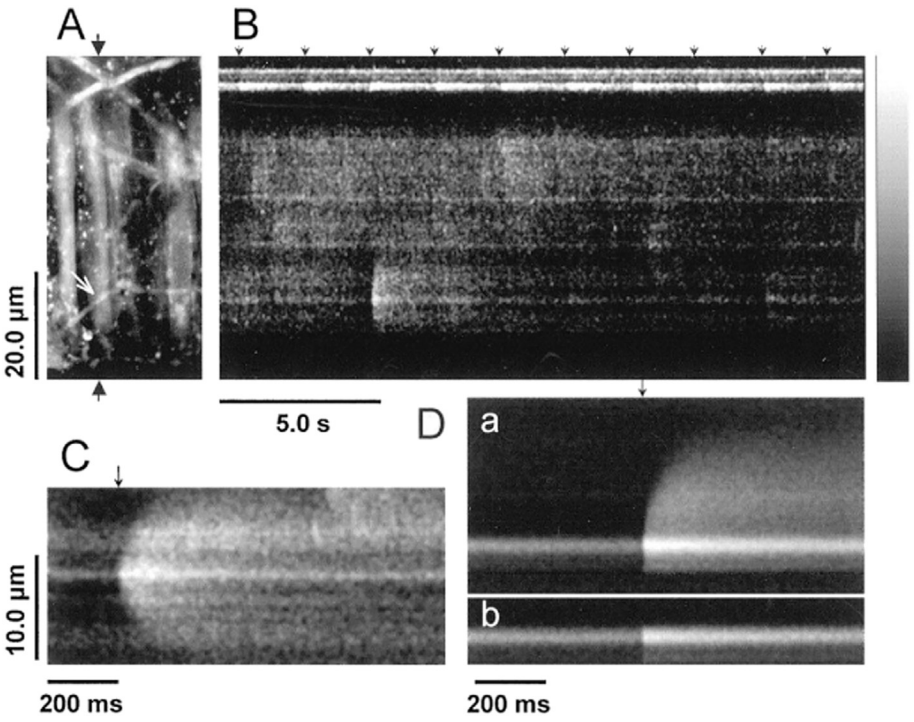

Fig. 1. JCaTs occur at sympathetic nerve varicosities. A, Confocal image of perivascular nerves and smooth muscle cells (SMCs) in a pressurized rat mesenteric small artery. The white arrow in (A) points to a sympathetic nerve varicosity that makes junctional contact with a SMC (whose long axis is vertical). To construct the line-scan image in (B), a single line, extending between the two black arrows in (A) was scanned for 20 s, during electric field stimulation (EFS) of the perivascular nerves. The chosen line crosses prominent nerve fibers at the top of the image, bisects the aforementioned smooth muscle cell longitudinally, and crosses a nerve fiber varicosity (white arrow). In the line-scan image, nerve fiber Ca^{2+} transients (accompanying nerve fiber action potentials) in the large fibers at the top (of A and B) are seen as periodic (0.5 s − 1) increases in fluorescence occurring at the time of the stimulus pulses (black arrowheads). (Nerve fibers contained more fluorescence than SMC and are evident in line-scan images as horizontal 'streaks'. A putative JCaT is seen in the SMC at 4.8 s in the line-scan image. C, jCaTs often arise at streaks in line-scan images. D, Region where a JCat occurred was scanned repeatedly. In (a), a JCat occurred. In (b), no JCat occurred, but a fluorescence transient occurred in the streak. We identify such transients as nerve fiber Ca^{2+} transients. These images demonstrate that jCaTs can arise precisely in the region of a nerve fiber varicosity.
Figure reproduced with permission from Lamont and Wier (2002).

obtained. The disadvantages are that SNA does not exist in these tissues, and it must be mimicked by EFS or bath application of sympathetic neurotransmitters. It is however, difficult, if not impossible, to reproduce experimentally the physiological pattern of SNA (*viz.* that ongoing in the living animal) in isolated tissues. By 'pattern', we mean the spatio-temporal occurrence of action potentials arriving at neuroeffector junctions in the wall of an artery. Yet, the signaling cascades triggered in the post-junctional cells, and thus the final cellular effects, are highly influenced by that pattern. In sympathetic varicosities in the walls of arteries, the amount and timing of the release of NE, ATP and NPY are dependent on the duration and frequency of the nerve action potentials (Todorov et al., 1999, Bradley et al., 2003). The reasons for this remain obscure, but a plausible hypothesis has been advanced and supported by Stjarne (2001). Briefly, he hypothesized that NE and ATP are stored in both 'big' or 'small' vesicles within sympathetic varicosities and that mechanisms exist to enable the selective secretion of 'big' quanta at low frequencies and 'small' quanta at high frequencies of action potential arrival in the nerve terminal. Inhibitory presynaptic autoreceptors (*e.g.* α_2-adrenoceptor and others) are also postulated to be involved in the different frequency dependence of ATP and NE release (Todorov et al., 1999). The optical studies of SNA induced Ca^{2+} signaling reviewed here provided new additional evidence for the differential dependence of ATP and NE release on action potential frequency.

The studies reviewed here also show that each of these neurotransmitters is associated with a distinct set of post-junctional signaling events and effects, ranging from immediate contraction to long-term trophic effects. Nevertheless, electric field stimulation (EFS) of nerve terminals in artery walls, or bath application of sympathetic neurotransmitters is fundamentally incapable of reproducing either the physiological or a pathophysiological spatio-temporal pattern of action potentials. *In vivo* imaging has the potential to overcome the shortcoming of the *ex vivo* studies.

In vivo imaging studies. *In vivo* imaging is now being recognized as having the ability to provide unique information under truly relevant physiological conditions (Wier, 2014, Lindquist and Niesner, 2015). The availability of genetically engineered Ca^{2+} biosensor mice (Isotani et al., 2004), combined with telemetric monitoring of physiological function and non-invasive, high resolution optical imaging (*viz.* two-photon fluorescence imaging) has enabled new *in vivo* experiments (Wier et al., 2008, Raina et al., 2009, Wier, 2014, Fairfax et al., 2014). In such studies, tonic SNA to arterioles has been associated with vasomotion and a particular kind of Ca^{2+} signaling. A point that will be emphasized here is that the use of such techniques permits *longitudinal* studies of vascular function (Mauban et al., 2014, Fairfax et al., 2014). Briefly, the use of non-invasive, non-perturbing experimental techniques in optical biosensor mice means that an individual animal can be studied over time, including the induction, development and reversal of an experimental condition or disease model. An example is the changes in global $[Ca^{2+}]$ during salt-dependent hypertension (Fairfax et al., 2014). With respect to SNA, the use of conscious animals (through techniques to be reviewed) is particularly important, because SNA is modified by anesthesia. Along similar lines, imaging neuronal activity in brains of conscious mice is now being developed (Shih et al., 2012, Guo et al., 2014, Svoboda, 2016) in order to study neuronal basis of behavior, which exists only in the conscious animal.

2. *Ex vivo* imaging studies

2.1. Optical indications of SNA in isolated vascular smooth muscle tissue

Studies on sympathetic neurogenic Ca^{2+} signaling in vascular smooth muscle utilized confocal imaging of Ca^{2+}-activated fluo-4 fluorescence in pressurized (70 mm Hg) rat mesenteric small arteries subjected to electrical field stimulation (EFS) (Lamont and Wier, 2002, Krishnamoorthy et al., 2014). The Ca^{2+} signaling underlying sympathetic neuroeffector Ca^{2+} transients (NCTs) in the mouse vas deferens has also been studied (Brain et al., 2002, Brain et al., 2003). A purinergic component of the vas deferens NCTs appears similar to the purinergic junctional Ca^{2+} transients (jCaTs) recorded from arterial smooth muscle, as discussed next.

2.1.1. ATP released from sympathetic varicosities activates P2X1 receptors to cause excitatory junction potentials (EJPs) and elementary Ca^{2+} signals (jCaTs).

To eliminate artery contraction and facilitate imaging, low

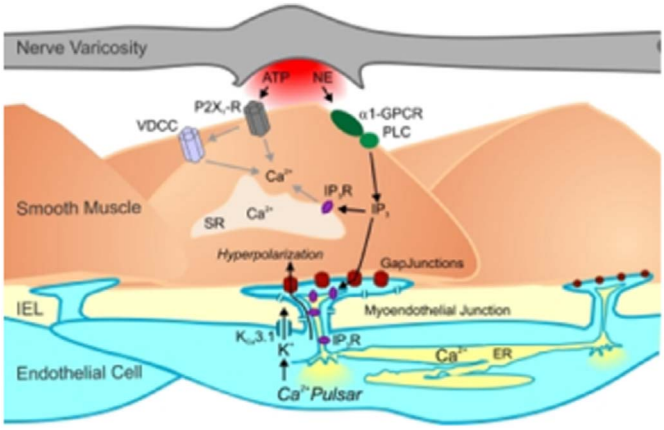

Fig. 2. Schematic illustration of sympathetic neuromuscular transmission, with negative feedback by the endothelium. ATP and NE released from sympathetic nerve varicosities activates P2X1 receptors and α_1-adrenoceptors, respectively. Ca^{2+} current through P2X1 channels depolarizes smooth muscle and activates voltage dependent Ca^{2+} channels (VDCC). The local, non-propagating change in [Ca^{2+}] has been termed a 'junctional Ca^{2+} transient' or 'JCat'. Activation of α_1-adrenoceptors (α_1-GPCR) results in production of IP_3 (inositol trisphosphate) *via* PLC (phospholipase C), which activates IP_3 receptors (IP_3R) and Ca^{2+} release from SR (sarcoplasmic reticulum). Endothelial cells (EC) project through holes in the internal elastic lamina (IEL) and connect to smooth muscle *via* myoendothelial gap junctions. IP_3 diffuses through myoendothelial gap junctions to activate IP_3R on EC ER (endoplasmic reticulum). The Ca^{2+} released from ER is called a 'pulsar', and activates EC potassium (K) channels ($K_{Ca}3.1$), which hyperpolarizes both EC and smooth muscle. The scheme explains how neutrally released ATP depolarizes smooth muscle, producing excitatory junction potentials (EJP) and activates voltage dependent Ca^{2+} entry that activates contraction. Neurally released NE activates contraction and other processes *via* Ca^{2+} released from SR. Negative feedback on SNA-induced artery contraction is provided by EC Ca^{2+} 'pulsars'. The negative feedback (hyperpolarization) may be involved in SNA-induced vasomotion, a characteristic of arteries receiving SNA *in vivo*.
Reproduced with permission from Nausch et al. (2012).

frequency (0.5 Hz to 0.67 Hz), low voltage EFS was used to excite the perivascular nerve fibers. This was referred to as 'sub-threshold' EFS, as it was sub-threshold for observable muscle contraction. A novel type of Ca^{2+} transient, arising near nerve fibers, was observed (Fig. 1). These were called 'junctional Ca^{2+} transients' or 'jCaTs', as they appeared to represent the post-junctional response to release of sympathetic neurotransmitter. Nerve fiber Ca^{2+} transients were also observed. The results showed that 1) nerve fibers are excited by each EFS pulse; 2) jCaTs occur nearly simultaneously with an EFS pulse, 3) jCaTs occur near nerve fibers, and 4) jCaTs are events of very low probability. JCaTs are larger in spatial spread and last longer than spontaneous Ca^{2+} sparks (Jaggar et al., 2000). JCaTs always occurred with brief latency to the EFS pulse. The spatial full-width-at-half-maximum (FWHM) for jCaTs was 4.8 μm, and the time taken to fall to half-amplitude, t1/2, (from the peak) is 145 ms. Unequivocal identification of the receptor(s) and ion channels that underlie jCaTs was accomplished through the use of the P2X1–receptor deficient mouse (Lamont et al., 2006). In the arteries of these animals, the non-degradable P2X receptor agonist, α, β-methylene ATP elicited no response, and jCaTs were completely absent during nerve stimulation, confirming the absolute requirement for P2X1receptors in the post-junctional responses to ATP.

In order to study selectively the Ca^{2+} signals and contractions generated by neurally released ATP, arteries were exposed to prazosin (1–10 μM) to block α_1-adrenergic receptors (Lamont et al., 2003). Purinergic receptor antagonists, such as suramin, abolish the small contractions that remain after prazosin (Gitterman and Evans, 2001). After prazosin treatment, approximately 74% of the initial transient contraction remained but only 5 % of the maintained contraction (Lamont et al., 2003), suggesting that the initial sympathetic neurogenic contraction is primarily purinergic. The changes in frequency and amplitude of jCaTs that might occur during prolonged SNA (EFS) were also characterized. The frequency of jCaTs declined markedly during the first 3 min of EFS, demonstrating a time-dependent decrease in the probability of neural release of ATP. In contrast to the frequency, the peak amplitude of the jCaTs changed little during 3 min of EFS. Propagating Ca^{2+} waves were not observed during EFS in the presence of prazosin. In summary, JCaTs occurred in sufficient numbers during the first 20s of EFS to produce a detectable elevation of average [Ca^{2+}] (fluorescence ratio), which paralleled the transient contraction that occurred during this time. On the other hand, jCaTs occurred at a very low frequency later in the EFS, when contractile force fell to very low levels (Lamont et al., 2003). These results indicate that the effects of neurally released ATP will be markedly dependent on the temporal pattern of SNA in the perivascular nerves. These results support the concept that sympathetic purinergic Ca^{2+} signaling underlies the rapid component of arterial blood pressure fluctuations (Golubinskaya et al., 1999).

2.1.2. *Sympathetic adrenergic (NE) Ca^{2+} signaling in isolated arteries*

Sympathetic neurogenic Ca^{2+} signals have also been observed during isometric contractions when high frequency EFS pulses were applied in trains, as opposed to the low frequency EFS used for studying purinergic transmission (Lamont et al., 2003). During the first 20s of high frequency EFS, force rose to a small peak, then declined, similar to that recorded previously and attributed to purinergic signaling. During this time, jCaTs were present at relatively high frequency. Propagating asynchronous Ca^{2+} waves, previously associated with bath-applied α-adrenoceptor agonists (Zang et al., 2001) were not initially present. During the next 2.5 min of EFS, force rose slowly, and asynchronous propagating Ca^{2+} waves appeared. The selective α1-adrenoceptor antagonist, prazosin, abolished both the slowly developing contraction and the Ca^{2+} waves, but reduced the initial transient contraction by only ~25%. These results suggested that sympathetic neurogenic contractions under these conditions consisted of an early, transient purinergic component and a later developing adrenergic component.

2.1.3. *NPY*

Neuropeptide Y (NPY) is co-released with ATP and NE at the sympathetic varicosities in the walls of arteries, and is known to modulate purinergic and adrenergic sympathetic functions (Pablo Huidobro-Toro and Veronica Donoso, 2004). Relatively little is known about any such modulatory effects on the post-junctional Ca^{2+} signals. Bath-applied 10 nM NPY increases the frequency of adrenergic asynchronous calcium waves induced by 2 μM PE and increased the PE-induced force by up to 3-fold (Wier et al., 2009).

2.2. *Optical indications of SNA effects in vascular endothelium*

Adrenergic contraction of arteries is enhanced by endothelial denudation, implying that endothelial function in arteries could be modulated by SNA to the artery, despite the absence of sympathetic nerve terminals (or α_1-adrenergic receptors) on endothelium High resolution studies have now revealed the mechanism of such an effect (Fig. 2, Nausch et al., 2012). Using an *en face* preparation of mouse mesenteric artery, Ca^{2+} signaling in endothelial cells was observed with confocal microscopy during EFS of sympathetic nerve terminals in the underlying arterial wall. $InsP_3$ (inositol trisphosphate), released into the cytosol of the smooth muscle cells after EFS diffused into endothelial cells through myo-endothelial gap junctions, to activate Ca^{2+} release through EC InsP3 receptors, which in turn activated EC Ca^{2+} activated K^+ channels, and thus exert a relaxant effect. These studies showed an indirect effect of SNA on endothelial function. As discussed further next, SNA *in vivo* is often associated with oscillatory arterial contraction (vasomotion). We had postulated earlier that the cellular basis of such vasomotion may involve diffusion of Ca^{2+} from smooth muscle into EC, activating K + channels (intermediate and small conductance KCa channels) (Mauban and Wier, 2004). The more recent

optical imaging studies (Nausch et al., 2012; Boerman et al., 2016) suggest a somewhat different mechanism, involving InsP3, rather than Ca^{2+}. Thus it seems likely that SNA induced artery vasomotion *in vivo*, involves this same heterocellular mechanism. Oscillatory vasomotion is an indicator of SNA *in vivo*.

3. *In vivo* imaging studies

The development of optical biosensor mice has been critical to the advancement of *in vivo* imaging studies of SNS control of arterial function (Zhang et al., 2010, Mauban et al., 2013, Zacharia et al., 2013, Mauban et al., 2014, Ji et al., 2004). These mice express genetically engineered Ca^{2+} sensor molecules specifically in smooth muscle. Mice have also been developed that express Ca^{2+} sensor specifically in endothelial cells, and have been used for investigating the SNA induced Ca^{2+} signaling in endothelium. The most successful strain of smooth muscle Ca^{2+} sensor mice, known as 'exMLCK' expresses a FRET (Förster Resonance Energy Transfer) – based sensor molecule that utilizes cyan and yellow fluorescent proteins that have been linked to the smooth muscle isoform of the Ca^{2+}/Calmodulin activated myosin light chain kinase (MLCK) (Isotani et al., 2004, Wier et al., 2008, Raina et al., 2009). Upon binding Ca^{2+}/Calmodulin, exMLCK undergoes a conformational change that decreases FRET between the cyan and yellow FPs, whilst also activating the myosin light chain kinase activity. exMLCK therefore serves as an indicator of the activation of MLCK, and as a Ca^{2+} indicator. Other types of genetically engineered biosensor mice useful for studying SNA on arteries include smooth muscle GCaMP mice (Ji et al., 2004) and cyclic GMP biosensor mice (Thunemann et al., 2014). The use of optical biosensor mice therefore obviates the need to 'load' tissues with a fluorescent dye and provides a phenotypically normal mouse in which artery Ca^{2+} signaling (or other) and artery diameter can be readily measured. There are advantages also of biosensor mice, since the problems of transient expression in cultured cells (Hirata and Kiyokawa, 2016), noted for FRET studies, under control of strong (e.g. CMV) promoters is avoided; expression in biosensor mice can be stable and non-perturbing.

3.1. Theory of in vivo FRET measurements in biosensor mice

Intravital FRET measurements are particularly challenging (Tao et al., 2015). The theory of the use of the exMLCK FRET ratio has been discussed extensively in our previous publications (Wier et al., 2008, Raina et al., 2009, Zhang et al., 2010, Wang et al., 2013). Briefly, the fractional occupancy of exMLCK by Ca^{2+}/Calmodulin (Y) can be calculated as $Y = (R - R_{min}) / (R_{max} - R_{min})$. The calcium concentration wherein $Y = 0.5$ represents the EC50. The fluorescence ratios R_{max} and R_{min}, respectively, represent 100 and 0% fractional occupancy of exMLCK by Ca^{2+}/Calmodulin. 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 EC50 (KA) of 0.892 μM (pCa, 6.05) and a Hill coefficient (*n*) of 1.4 (Wang et al., 2013). Methods for determination of intrinsic fluorescence, spectral overlap and other considerations for quantitative imaging of $[Ca^{2+}]$ using exMLCK FRET ratio measurements under these conditions were described previously (Wier et al., 2008, Zhang et al., 2010, Mauban et al., 2014).

3.2. In vivo preparations

The types of murine (mouse) arteries that have been utilized in optical *in vivo* imaging studies of SNS control of Ca^{2+} signaling of arteries are 1) femoral artery (FA), 2) cremaster muscle arterioles and small arteries, 3) subcutaneous arterioles of the ear, and 4) mesenteric arteries (Boerman et al., 2016). FA are conduit arteries, and thus contribute little to total peripheral vascular resistance (TPR), the key hemodynamic parameter regulated by the SNS. Rather, SNA in conduit arteries may control arterial stiffness. In any case, murine FA are highly innervated by the SNS, and tonic SNA is readily revealed as a strong vasodilation after autonomic ganglion blockade. The murine cremaster muscle is a classical intravital imaging preparation that provides experimental access to small arteries and arterioles (~20 μm diameter) of the type that do contribute a major component to TPR. Recently, endothelial cell Ca^{2+} pulsars were recorded in surgically exposed mesenteric small arteries of anesthetized Ca^{2+} biosensor mice (Boerman et al., 2016), as discussed above (Section 2.2).

Subcutaneous arteries and arterioles of the mouse ear are used for non-invasive studies, since these can be imaged through intact skin with two-photon fluorescence microscopy.

Key tools for detecting or measuring SNA in all these preparations are 1) total autonomic ganglionic blockade, using such classical drugs as hexamethonium, and 2) systemic or local application of sympathetic neurotransmitter receptor agonists and antagonists, such as phenylephrine (PE), prazosin and others.

3.3. Optical indications of SNA in surgically exposed arteries of anesthetized biosensor mice

Surgical exposure of arteries in anesthetized animals has numerous advantages for optical imaging of arteries, and enables many experimental manipulations, but has the significant disadvantage that anesthesia affects SNA and other physiological functions. Even those anesthetic mixtures that do not strongly affect arterial blood pressure, may affect cardiac and vascular functions in compensatory ways (*e.g.* decreasing heart rate but increasing vascular resistance). Isoflurane does produce a dose-dependent decrease in arterial BP, and a depression of SNA, even at the accepted anaesthetic concentration (1.5%) (Seagard et al., 1984).

Initial *in vivo* studies with anesthetized exMLCK mice were directed at validating the optical methods for quantification of arterial $[Ca^{2+}]$ and for developing methods to manipulate SNA, measure arterial blood pressure (BP) and other physiological parameters (Zhang et al., 2010, Wang et al., 2013). Using wide-field FRET microscopy, outer diameters of FAs in anaesthetized Ang II-infused mice (a model of salt-induced hypertension) were found to be significantly less than those of saline-infused (control) mice. Contractile activation and diameter of FAs were known to be under continuous control of SNA (Zacharia et al., 2013), a characteristic that could help reveal CNS-induced changes in SNA in hypertension. Finally, conduit arteries are of interest because they are importantly involved in the pathophysiology of hypertension, where increased conduit stiffness (possibly due to increased contractile activation) contributes to increased cardiac work (Sparks et al., 2011, Sudano et al., 2011). Approximately 90% of the vascular tone in FAs in anaesthetized biosensor mice is attributable to SNA (Zacharia et al., 2013, Mauban et al., 2013) and specifically to activation of α1A and α1D receptor subtypes (Zacharia et al., 2013). In Ang II–salt hypertension, an increased 'depressor' (decrease in mean arterial BP) response to systemic block of autonomic ganglionic transmission can be demonstrated (Kuroki et al., 2012), suggesting increased SNA-induced vascular tone. While these results indicated clearly that the additional FA tone in mice infused with Ang II for 2–3 weeks was associated with increased sympathetic α1-adrenergic receptor activation, other mechanisms that mediate BP increase during early phase (*i.e.* hours to days) Ang II infusion, which were not tested here, might also exist (Feng et al., 2010).

4. Summary of SNA-induced Ca^{2+} signaling in arteries, as observed *ex vivo*

Fig. 2 summarizes the concepts gained from the studies of SNA-induced Ca^{2+} signaling, as it can be observed in *ex vivo* experiments. It can be expected that this scheme might operate somewhat differently *in*

vivo because of the many factors that are present in the living animal that are not reproduced in *ex vivo* experiments. For example, the high-resolution recording of Ca^{2+} 'pulsars' induced by SNA to the smooth muscle cells, could not, for technical reasons, be done in the presence of the normal arterial blood pressure and blood flow. Yet, these factors influence the membrane potential of the smooth muscle cells and of the endothelial cells, and hence, the cytosolic [Ca^{2+}] and the activation and/or availability of many voltage-sensitive ion channels.

5. *In vivo* studies (conscious animals)

Recently, the first non-invasive measurements of intracellular smooth muscle [Ca^{2+}] in arterioles of conscious mice has been reported. Two major considerations motivated this work: (1) Ca^{2+} signaling in arteries under physiological conditions with normal arterial blood pressure and intrinsic regulation *via* circulating hormones and the autonomic nervous system had never before been observed. (2) Studies of Ca^{2+} signaling in experimental hypertension have been complicated by the effects of anesthesia to reduce arterial blood pressure probably by reducing sympathetic nerve activity (SNA) to arteries. As a consequence, genuinely representative *in vivo* measurements of intracellular [Ca^{2+}] did not exist. The methods required for these measurements will be discussed in some detail.

5.1. Methods for imaging in awake (conscious) optical biosensor mice

A methodological problem in *in vivo* experiments, whether the animal is conscious or not, is movement. Clearly the optical section obtained by high-resolution imaging will not be maintained during even small movements. In anesthetized animals, respiratory movements are a major problem as conscious animals attempt movement. These issues are typically addressed by some form of immobilization. For imaging subcutaneous arteries of the ear or cerebral arteries (through a glass window), head-fixation is required. This involves affixing a rigid structure to the skull (surgically) such that the head can be held immobile beneath the microscope objective. Further immobilization or restraint of the animal may also be required. In our recent experiments (Fairfax et al., 2014), the animals' heads were fixed in position by attaching the threaded bar to an anchored stage, their bodies were immobilized inside a custom-made conical tube, and their depilated ears were positioned on a flat horizontal silicone platform as previously detailed (Mauban et al., 2014). This arrangement immobilized the ear, without directly pinning it, and provided a convenient configuration for use of a dipping objective lens (20x, 1.0 NA). Telemetric blood pressure measurements distinguished the anesthetized and unanaesthetized/awake conditions since isoflurane dependably lowers MAP.

A Zeiss LSM 710 NLO microscope was utilized for two-photon microscopy, equipped with a femtosecond pulsed near infrared (IR) laser (Chameleon Vision, Coherent, Inc., Santa Clara, CA). The frame rate and pixel size of the bidirectional scanning varied for each experiment, but ranged from 2 to 5 Hz and 0.59 to 0.83 μm/pixel, respectively. The microscope was enclosed in a light-tight enclosure and room lights were turned off to eliminate background signals. The CFP moiety of exMLCK was excitation at 820 nm. Emission light received IR filtration before being separated into two channels (CFP and YFP). CFP and YFP emission was band pass filtered from 460 to 500 nm and 520 to 560 nm, respectively. Two binary GaAsP photodetectors within the Zeiss LSM BiG module detected fluorescence emission. The gains of the two detector channels were held constant for all experiments at levels optimizing exMLCK fluorescence. The configuration settings of the detectors were reproduced during calibration experiments, which determined the maximum and minimum FRET ratios obtainable from our microscope (Mauban et al., 2014).

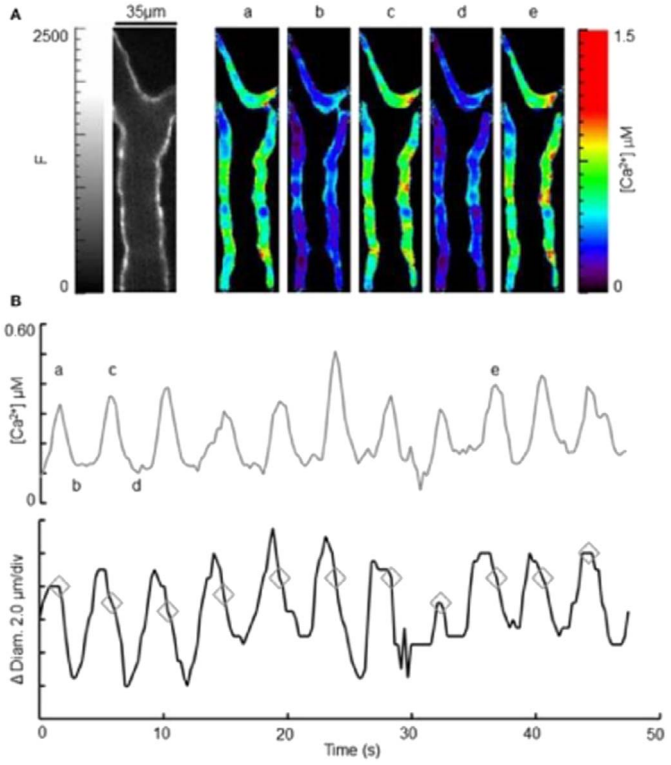

Fig. 3. Intracellular [Ca^{2+}] and diameter during oscillatory vasomotion in an ear arteriole of a conscious mouse. (A) Left image shows an ear arteriole using CFP fluorescence (0–2500). Scale bar is 35 μm and applies to all images. White triangles denote the 5 ROIs used to measure [Ca^{2+}]. Rainbow-colored images to the right demonstrate the range of calculated [Ca^{2+}] (0–1.5 μM) over the time-course of spontaneous oscillatory vasomotion, as indicated by the small letters above each image, relating to (B). (B) Average [Ca^{2+}] and change in wall position (change in diameter) from the 5 ROIs labeled in (A). Peak oscillations in [Ca^{2+}] are indicated by gray diamonds in the diameter trace, and correspond with the rapid decrease in diameter during vasomotion.
Figure reproduced with permission from Fairfax et al. (2014).

5.2. Arterial Ca^{2+} signaling and SNA in awake biosensor mice

Mice typically required 7–10 days to regain normal MAP following surgical implantation of telemetric blood pressure transducers and head restraints. MAP recordings began as the animal was engaging in normal behavior within its housing cage. Recordings continued into isoflurane induction while the animal was affixed to the restraint device and also during restoration of consciousness (upon removal of isoflurane) while restrained. Because two-photon fluorescence imaging was being used, the immediate environment of the animal was dark, a factor which may have influenced the MAP in the conscious restrained state. After awakening in the dark, MAP in the conscious restrained animal was not significantly different from that in the conscious, unrestrained animal. In contrast, 1.5% isoflurane lowered MAP consistently. Mice periodically attempted activity ~3–4 times per minute, and this was associated with transient increases in MAP, as occurs in the normal basal state.

Oscillatory vasomotion and accompanying regular oscillations in [Ca^{2+}] were present in 71% of the arterioles observed in the conscious, restrained state (Fig. 3). Changes in [Ca^{2+}] were nearly uniform and synchronous between smooth muscle cells. Such synchronous Ca^{2+} oscillations were not reported previously in any of the arterioles examined in anesthetized biosensor mice (Mauban et al., 2014) and were less frequent, at 14%, during anesthetized measurements in this study. For the arteriole illustrated, the time-averaged diameter was about ~35 μm and the oscillatory vasomotion involved wall movements of ~5–6 μm and [Ca^{2+}] oscillations from ~0.1 to ~0.5 μM. Maximum [Ca^{2+}] occurred during the rapid decrease in diameter, as would be expected for a Ca^{2+} indicator that binds Ca^{2+} with similar affinity to

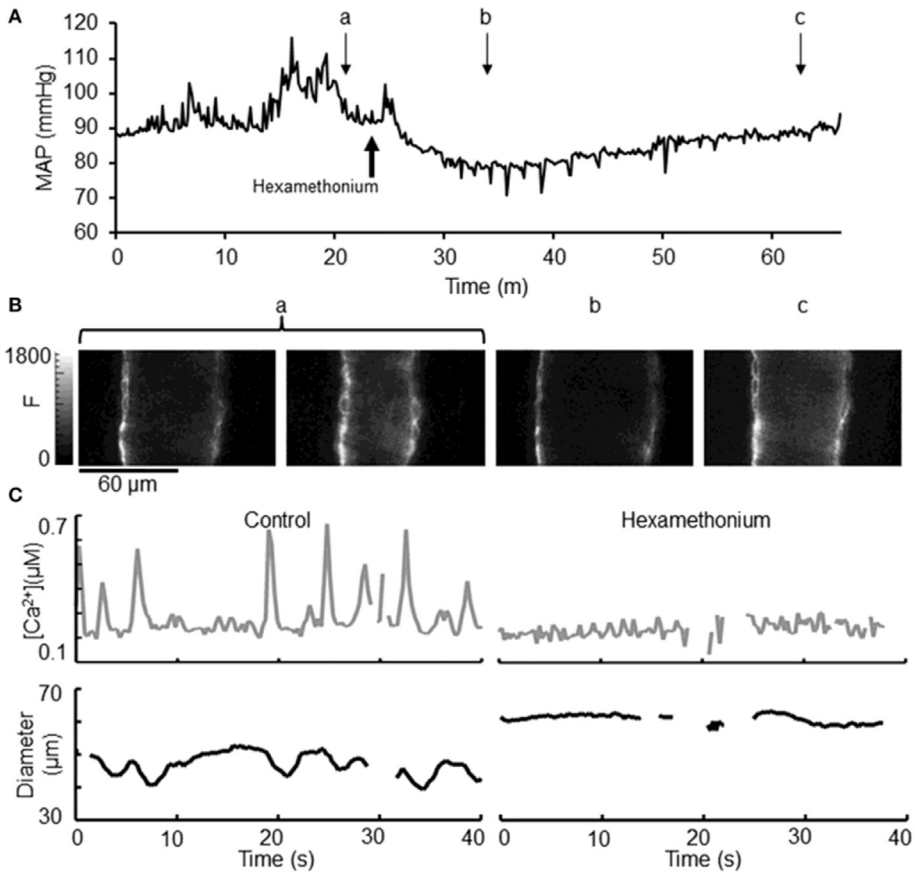

Fig. 4. Effect of hexamethonium on $[Ca^{2+}]$ oscillations, vasomotion, and diameter in exMLCK ear arterioles of conscious mouse. (A) Mean arterial pressure (MAP) before, during and following recovery of i.p. hexamethonium injection (30 µg/g BW). Brief attempts of activity by the mouse are indicated by transient changes in MAP. Small letters indicate the time point of the corresponding CFP images shown in (B). (B) Selected images demonstrate that arteriolar vasomotion (a) is eliminated by hexamethonium treatment (b). Recovery of tone (c) occurred ~ 30 min after hexamethonium injection. (C) Transient increases in $[Ca^{2+}]$ and corresponding decreases in diameter during irregular vasomotion during conscious, restrained conditions (left). Hexamethonium eliminated vasomotion, $[Ca^{2+}]$ transients, and caused vasodilation (right). Baseline $[Ca^{2+}]$ was not affected by hexamethonium.
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the endogenous MLCK and which activate cross-bridge cycling. In some instances, very rapid and brief Ca^{2+} transients were observed and these were accompanied by similarly rapid and brief vasoconstriction. In this case, the Ca^{2+} transient peaked before contraction was observed; contraction was evident in the subsequent frame.

A key observation was the effect of autonomic ganglionic blockade on MAP, arteriolar dimensions and Ca^{2+} signaling (Fig. 4). Intra-peritoneal (i.p.) injection of hexamethonium (30 µg/g BW) (which transiently blocks all SNA in anesthetized mice), reduced MAP within a few minutes of injection. The small artery in Fig. 4 was undergoing vasomotion, however during the minimum MAP following hexamethonium treatment, no vasomotion was present in this artery and the diameter was significantly increased. During recovery, MAP slowly increased and this artery regained some of its vasomotion behavior and initial tone. In a separate experimental animal, hexamethonium treatment was also seen to abolish the Ca^{2+} signaling associated with vasomotion.

The major advantage of making $[Ca^{2+}]$ measurements in a conscious animal should be that arterioles are in a state closer to physiological than can be achieved in any other condition. Our expectation is that cardiovascular control systems, autonomic nerve activity, endothelial, hormonal, and myogenic mechanisms should be operating similarly to the animal's normal basal state under the conditions of our imaging experiments. The primary indication of this is MAP at normal basal levels, rather than being altered. MAP in the conscious restrained mice, set up for two-photon imaging of ear arterioles, was not different from that when the animal was moving freely before the experiment began. Furthermore, MAP fluctuates during imaging, and such fluctuations in MAP are associated with activity. Such fluctuations in MAP in the freely moving animal are associated with activity, and in the conscious restrained state, with attempted activity (as evidenced by slight movements that were not fully prevented). With respect to MAP, these results are somewhat different than those reported earlier (Gross and Luft, 2003) where restraint of mice did result in significant elevation of MAP. In those experiments, mice were not restrained in the dark, and were under a different system of restraint. Whatever the reason for the differences, the key result for cardiovascular experiments of the type reported here is that both MAP and HR were not altered by stress during the system of conscious restraint that we used. Therefore, studies of experimental hypertension or investigations of mechanisms of normal vascular control may not be complicated by stress-induced or anesthesia-induced alterations in SNA or hormonal control systems. Blood flow and arterial pressure are maintained so that endothelial flow-sensitive mechanisms and pressure sensitive mechanisms (myogenic tone) should be operating normally.

Arterioles undergoing oscillatory vasomotion (Aalkjaer et al., 2011) (Figs. 3 and 4) were found much more frequently in conscious mice than in anesthetized mice (in agreement with an earlier study (Drew et al., 2011)), and such vasomotion was strongly reduced or abolished by inhibition of SNA by hexamethonium. With respect to Ca^{2+} dynamics, diameter changes and frequency, the vasomotion appeared similar to that elicited by exposure of isolated arteries to adrenergic receptor agonists, such as phenylephrine (Mauban et al., 2001). Thus, it seems likely that the vasomotion we observed *in vivo* was due to the action of the sympathetic neurotransmitter, norepinephrine (NE), acting on α_1-adrenergic receptors (α1-AR) (Zacharia et al., 2013). The cellular mechanisms of SNA induced vasomotion remain unclear, but likely involve 1) depolarization and adrenergic elevation of Ca^{2+} in smooth muscle, 2) diffusion of smooth muscle Ca^{2+} into EC, 3) activation of EC KCa channels *via* 'pulsars', 4) subsequent hyperpolarization of smooth muscle and EC. The fact that volatile anesthetics such as Isoflurane induce membrane hyperpolarization and vasodilation (Yamazaki et al., 1998), likely explains at least part of the mechanism for reduced vasomotion relative to the conscious state. Interestingly, NE induces vasomotion more readily in mesenteric small arteries from spontaneously hypertensive rats (SHR) than in normotensive rats (Chen et al., 2010). Although the physiological significance of such

vasomotion remains unclear, it is thought likely to enhance tissue dialysis (Aalkjaer et al., 2011).

Purinergic JCaTs are too fast and small to be detectable with exMLCK in ear arterioles, as are Ca^{2+} sparks. As discussed above, asynchronous propagating Ca^{2+} waves are readily produced by EFS, simulating SNA, in isolated arteries. However, asynchronous propagating Ca^{2+} waves were not seen in any blood vessel in the present study. Such Ca^{2+} signals were also not detected in our previous studies, either in cremaster muscle arterioles (Mauban et al., 2013) or femoral arteries (Wang et al., 2013, Zacharia et al., 2013) that were surgically exposed, or in intact ear arterioles (Mauban et al., 2014). Thus, it appears that the Ca^{2+} waves observed *in vitro* may remain as elementary signals and fail to emerge as a phenomenon in a fully intact physiological system.

6. Summary and conclusions

High resolution imaging of Ca^{2+} signaling in isolated blood vessels (*ex vivo*) has revealed distinct post-junctional cellular mechanisms activated by the sympathetic neurotransmitters, ATP and NE. NPY is co-released, but appears to modulate the purinergic and adrenergic signaling mechanisms. In isolated arteries, high resolution imaging can reveal 1) the Ca^{2+} transients in single sympathetic varicosities (*e.g.* Fig. 1), 2) the local, elementary, post-junctional smooth muscle Ca^{2+} transient resulting from the released ATP (JCat or NCT), 3) the post-junctional smooth muscle Ca^{2+} waves or oscillations generated by the released NE, and 4) local Ca^{2+} signals in endothelial cells. In arteries, SNA thus provides a system by which rapid, tonic, or oscillatory contraction can be generated, and in which the arterial endothelium can exert negative feedback to influence contraction.

Clearly, it is important to determine to what extent these concepts apply to the actions of SNA and control of artery contraction *in vivo*. The availability of Ca^{2+} biosensor mice and non- invasive imaging (two-photon) has made possible the observation of Ca^{2+} signaling in a variety of situations, including in the smooth muscle cells of blood vessels in the ears of conscious mice. Arterioles of the mouse ear were found to be under tonic control by the SNS, and the SNA was associated with oscillatory vasomotion and Ca^{2+} oscillations. The vasomotion, and perhaps low frequency blood pressure oscillations, that exist in living animals may thus be a consequence of the observed SNA induced Ca^{2+} signaling arterial smooth muscle and endothelium (Fig. 2).

Finally, the use of awake optical biosensor mice may be particularly important in hypertension research. Although not yet accomplished, it should be possible to observe optical signals over the course of the lifetime of an individual animal, without the confounding effects of anesthesia, not just over a period of two weeks, as presently achieved. A point to be reiterated is that FRET based biosensors are quantitative. That is, they provide a signal that can be related to an absolute quantity of interest, such as Ca^{2+} concentration. This facilitates greatly the comparison of measurements made at different times in one animal or between animals. Finally, a recent study utilizing anesthetized optical Ca^{2+} biosensor mice and surgically exposed mesenteric arteries, showed that endothelial cell Ca^{2+} pulsars were reduced 85% in old (24–26 months) mice, compared to young (3–6 months) mice. Thus, it seems likely that the ability of SNA to promote vasodilation by evoking endothelial cell Ca^{2+} signaling would be markedly reduced by age. We may speculate that this is a component of the increased risk of hypertension with aging.

Conflict of interests

The authors state no conflict of interests.

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