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TOPICAL REVIEW

Pan-junctional sarcoplasmic reticulum in vascular smooth muscle: nanospace Ca^{2+} transport for site- and function-specific Ca^{2+} signalling

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Abstract This review focuses on how smooth muscle sarcoplasmic reticulum (SR), the major releasable Ca^{2+} store in these cells, performs its many functions by communicating with the plasma membrane (PM) and other organelles across cytoplasmic nanospaces, defined by membrane–membrane junctions less than 50 nm across. In spite of accumulating evidence in favour of the view that cytoplasmic nanospaces are a prerequisite for effective control of diverse cellular functions, our current understanding of how smooth muscle cells accomplish site- and function-specific Ca^{2+} signalling remains in its infancy. We first present evidence in support of the view that effective Ca^{2+} signalling depends on the restricted diffusion of Ca^{2+} within cytoplasmic nanospaces. We then develop an evidence-based model of the smooth muscle SR – the 'pan-junctional SR' model – that incorporates a network of tubules and quilts that are capable of auto-regulating their Ca^{2+} content and determining junctional $[Ca^{2+}]_i$ through loading and unloading at membrane–membrane nanojunctions. Thereby, we provide a novel working hypothesis in order to inform future investigation into the control of a variety of cellular functions by local Ca^{2+} signals at junctional nanospaces, from contraction and energy metabolism to nuclear transcription. Based on the current literature, we discuss the molecular mechanisms whereby the SR mediates these multiple functions through the interaction of ion channels and pumps embedded in apposing membranes within inter-organellar junctions. We finally highlight the fact that although most current hypotheses are qualitatively supported by experimental data, solid quantitative simulations are seriously lacking. Considering that at

Mark Evans (left) began his investigations into the regulation of cell and organ function by ion channels and calcium with his PhD in 1990 at the University of Edinburgh, under the supervision of Prof. B. L. Ginsborg. Subsequently, he has focused on pulmonary arterial smooth muscle as a Wellcome Trust Research Fellow at the University of Oxford. He was appointed to a Lectureship at the University of St Andrews in 2001, and then to the Chair of Cellular Pharmacology at the University of Edinburgh in 2009. **Nicola Fameli** (centre) obtained his PhD in 2000 from the Department of Physics and Astronomy of the University of British Columbia (UBC) in Vancouver, Canada, in experimental condensed matter physics. In the Dept of Anaesthesiology, Pharmacology and Therapeutics at UBC, under the supervision of Prof. Cornelis van Breemen, he embarked on quantitative physiology, including the development of realistic stochastic simulations of cation transport in cytoplasmic nanospaces during activation of vascular smooth muscle cells. **Cornelis (Casey) van Breemen** (right) obtained his DVM from the University of Toronto. He became interested in Ca^{2+} signalling and obtained his PhD at the University of Alberta under the supervision of Prof. E. E. Daniel. He is currently Professor Emeritus in the Dept of Anesthesiology, Pharmacology and Therapeutics at UBC, and Senior Emeritus at the Child and Family Research Institute also in Vancouver, researching the effects of Marfan Syndrome and ageing on vascular Ca^{2+} signalling at the Child and Family Research Institute.

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physiological concentrations the number of calcium ions in a typical junctional nanospace between the PM and SR is of the order of 1, ion concentration variability plays a major role as the currency of information transfer and stochastic quantitative modelling will be required to both test and develop working hypotheses.

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Abbreviations BK_{Ca}, Ca^{2+}-activated large conductance potassium (channel); CICR, calcium-induced calcium release; Cl_{Ca}, Ca^{2+}-activated chloride channel; HCX, H^+/Ca^{2+} exchanger; IP_3R, inositol 1,4,5-trisphosphate receptor; lysosome–SR (junction); MCU, mitochondrial uniporter; NAADP, nicotinic acid adenine dinucleotide phosphate; NCX, Na^+/Ca^{2+} exchanger; NFAT, nuclear factor of activated T-cells; NLCX, mitochondrial Na^+/Ca^{2+} exchanger; NKA, Na^+/K^+ ATPase; PM, plasma membrane; PMCA, plasma membrane Ca^{2+} ATPase; ROC, receptor-operated channel; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; STIM, stromal interaction molecule; STOC, spontaneous transient outward current; TRPM4, transient receptor potential melastatin-4; TPC, two pore segment channel; TRPC, transient receptor potential canonical; VGCC, voltage-gated Ca^{2+} channel.

Multiple smooth muscle activating mechanisms

Excitation–contraction coupling in all types of muscle (skeletal, cardiac and smooth) is largely controlled by interactions between voltage-gated Ca^{2+} channels (VGCCs) in the plasma membrane (PM) or its invaginations (T-tubules or caveolae) and Ca^{2+} release channels in the sarcoplasmic reticulum (SR). However, in smooth muscle cellular membrane interactions appear much more varied and crosstalk between the SR and other organelles, such as lysosomes and mitochondria also appears to contribute significantly to shaping the Ca^{2+} signal. In addition, smooth muscles display far greater heterogeneity and plasticity than skeletal and cardiac muscles. This should not be surprising considering that smooth muscle supplies the physical force for all homeostatic and reproductive functions in the body from functional hyperaemia in the brain for providing food for thought, to the delivery of babies to preserve our species. Not only does the function of smooth muscle vary from organ to organ, but it can also change over time in the same organ. Notable examples of such plasticity are uterine smooth muscle, which changes from quiescent to highly reactive during consecutive stages of pregnancy and birth, and the changes seen in arterial smooth muscle phenotype, from contractile to proliferative and migratory, during the cycle of injury and wound healing. Since these multiple functions are all controlled by Ca^{2+} signalling the overarching question is: how can fluctuations in the concentration of one ion, Ca^{2+}, exert such selective and multifaceted control? The generally accepted answer to this question is that both spatial and temporal details of the Ca^{2+} transients code for selective modulation of molecular targets and thereby transduce their multiple physiological effects. The underlying mechanisms are complex in nature, requiring strategic spatial positioning of cellular Ca^{2+} transporters and targets, each of which may be characterized by different kinetics and affinities for Ca^{2+} (Clark *et al.* 2010). Detailed discussion of the various channels and transporters that regulate SR-mediated Ca^{2+} signalling in smooth muscle has recently been provided by others (Wray & Burdyga, 2010). In this short review, therefore, we focus on how junctions of the main Ca^{2+} regulatory organelle, the sarcoplasmic reticulum (SR), provide cytoplasmic nanospaces, in which highly localized Ca^{2+} signals can be generated to select for different smooth muscle functions, from contraction and relaxation to gene expression and cell division. The main emphasis will be on vascular smooth muscle, while other types are briefly mentioned for comparison.

We propose that a ‘pan-junctional SR’ forms many different types of nanojunction with the PM, mitochondria, lysosomes and possibly other organelles, each performing separate, but coordinated functions. Figure 1 illustrates that smooth muscle SR may have eleven and possibly more types of nanojunction, hence the title ‘pan-junctional SR’. The most abundant PM–SR junctions selectively regulate luminal calcium concentration ($[Ca^{2+}]_{SR}$), hyperpolarization and relaxation, depolarization and vasomotion; the mitochondria–SR junctions regulate mitochondrial energy metabolism, apoptosis and SR Ca^{2+} loading, while the lysosomal–SR junction is essential for nicotinic acid adenine dinucleotide phosphate (NAADP)-initiated calcium-induced calcium release (CICR) from the SR, which may in turn modulate, for example, autophagy and cholesterol metabolism.

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Nanojunctions

The first nanojunction described in terms of its functional importance was the neuromuscular junction, and there can be no doubt as to how important this was to our understanding of neurotransmitter release and function (Del Castillo & Katz, 1956). However, until recently little attention has been given to the presence, function and plasticity of nanojunctions between intracellular membranes. Perhaps the one exception is in skeletal and cardiac muscles, where the importance to excitation–contraction coupling of the junctional complexes formed between the T-tubules of the sarcolemma and terminal cisternae of the SR is well recognized. The essence of a nanojunction, as suggested from observations in several cell types (Rosenbluth, 1962; Franzini-Armstrong, 1964; Ramesh *et al.* 1998), is that portions of two biological membranes, typically a few 100 nm in extension and each containing ion transporters and sensors, are separated by a highly structured cytoplasmic space 10–30 nm wide. Both the ultra-structure and electrostatic properties of the nanojunction, together with the composition of transport molecules embedded in their limiting membranes, ensure that cytoplasmic cation concentrations, $[Ca^{2+}]$ in particular, are locally determined. Cations may thus target sites of different affinities and modulate function appropriately.

Pan-junctional SR in vascular smooth muscle

In the early seventies the first convincing electron micrographs of smooth muscle SR revealed that in a number of peripheral areas of the long thin cells only a narrow gap of approximately 20 nm separated the SR from the PM (Gabella, 1971; Devine *et al.* 1972). A few years later careful $^{45}Ca^{2+}$ measurements, employing La^{3+} quenching of extracellular Ca^{2+}, demonstrated the existence of peripheral cytoplasmic domains between the PM and

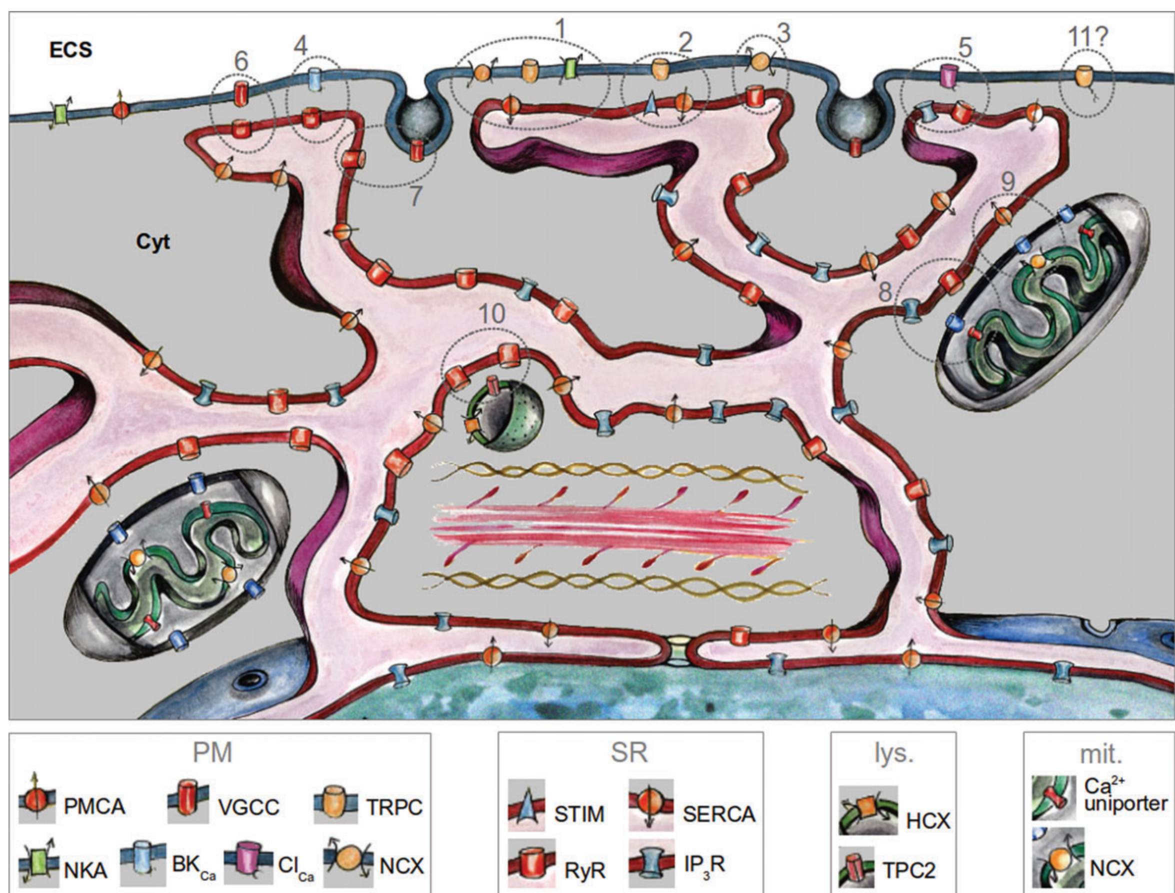

Figure 1
The above graphic illustration of the hypothetical pan-junctional SR features multiple nanojunctions between the SR, on the one hand, and the PM, mitochondria and lysosomes, on the other. Each nanojunction is identified with a number corresponding with its description in the text below. The ion transporter (codes shown below the picture) content of each junction is based on experimental evidence in the literature, which varies from solid to hypothetical. ECS: extra-cellular space; cyt: cytoplasm.

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peripheral SR, which were characterized by restricted diffusion (van Breemen, 1977). Once Ca^{2+} entered such a nanospace it was either pumped into the SR or diffused into the bulk myoplasm. The same study also showed that both the rates of Ca^{2+} entry through PM channels and the rate of SR Ca^{2+} uptake from the restricted peripheral cytoplasmic space determined the fraction of Ca^{2+} entry that was captured by the SR (see below for recent quantitative modelling). Since then progress has been slow and the concept that site- and function-specific Ca^{2+} signals may be supported by nanojunctions has received little attention. Perhaps we are now ready for a sea-change, given that the organelle-targeted fluorescent indicators developed in the past two decades (Rizzuto *et al.* 1993; Miyawaki *et al.* 1999) have opened the doors to elucidating the highly localized Ca^{2+} signalling events about the interface of junctional membrane pairs. We must 'mind the gap', however, as the dimensions of the nanojunctions are still well below the resolution of light microscopy and most supporting evidence remains indirect in nature. For this reason we will illustrate a stochastic modelling approach that helps formulate and test our hypotheses.

Our 'pan-junctional SR' hypothesis is encapsulated in Fig. 1, which illustrates a variety of nanojunctions, and the specific transporters that participate in shaping cation concentration transients within and between them. Below we describe their specific roles in regulating vascular smooth muscle function and the ionic mechanisms by which Ca^{2+} signalling within each nanospace may be modulated. Throughout the following discussion it is important to realize that the Ca^{2+} transients within the junctional nanospaces are segregated from those in the bulk myoplasm that determine contractile activity. Several factors participate in restricting $[Ca^{2+}]$ transients to nanojunctions:

(1) The geometry of the junctions, especially the distance between membranes, appears to control the retention of Ca^{2+} in the nanospace, as suggested by preliminary models (Fameli *et al.* 2007);
(2) The relatively low diffusivity of (free or buffered) cytosolic Ca^{2+} (Kushmerick & Podolsky, 1969; Allbritton *et al.* 1992), in combination with the above-mentioned restricted geometry, favours Ca^{2+} buffering by nanojunctions;
(3) The kinetics of Ca^{2+} sinks in the junctions, is another important element, tightly linked to the previous two factors; for example, if, as predicted, sarco/endoplasmic reticulum Ca^{2+} ATPase 2b (SERCA2b) is resident within PM–SR junctions of pulmonary arterial smooth muscle, its high affinity for Ca^{2+} (Verboomen *et al.* 1992) may provide a barrier to Ca^{2+} flux between the PM and the myofilaments and *vice versa* (Clark *et al.* 2010);
(4) There is ample evidence of electron opaque junction-spanning structures that are likely to provide physical obstacles to ion mobility in the junctions by increasing path tortuosity (Devine *et al.* 1972; Poburko *et al.* 2008); this is likely to be a more restrictive effect for diffusion than actual chemical buffering, since Ca^{2+} buffers likely contribute little at these spatial scales (Allbritton *et al.* 1992; Allbritton & Meyer, 1993).

However, it is important to note that even though the Ca^{2+} signalling domain for contraction may be of a larger scale, its distribution is far from homogeneous. Separate PM regions have been described for filament attachment and caveolae (Moore *et al.* 2004) and the density of myosin filaments appears to be less in the cell periphery than central myoplasm (Lee *et al.* 2002). In addition, it was shown that the functional Ca^{2+}-binding protein calmodulin is tethered to the myofilaments rather than free in solution (Wilson *et al.* 2002).

SR auto-regulation

For the SR to control and coordinate local cytoplasmic Ca^{2+} signals it must to some degree be able to regulate its luminal Ca^{2+} content independently from fluctuations in cytoplasmic $[Ca^{2+}]$. This is accomplished, in part, by PM–SR junctions, which may load the SR from the extracellular space to replenish it during activating waves of SR Ca^{2+} release (Lee *et al.* 2001) or extrude Ca^{2+} from the SR when it is resting, overloaded with Ca^{2+} (Nazer & van Breemen, 1998) or signalled to do so by vasodilators (Boittin *et al.* 2003).

SR loading (Fig. 1 : 1 and 2)

Consistent with smooth muscle heterogeneity and plasticity, refilling of the SR from the extracelluar space during stimulated Ca^{2+} release may be achieved by a variety of different mechanisms. We will focus our attention on one well-documented mechanism, which is illustrated in Fig. 1: 1. In this instance, the process is initiated by the opening of non-selective cation-permeable, receptor-operated channels (ROCs; e.g. transient receptor potential canonical channel TRPC6). These deliver Na^+ to the junctional nanospace in a manner coupled to Ca^{2+} entry mode Na^+/Ca^{2+} exchangers (NCX) in the PM, and thus supply Ca^{2+} to SERCA on the peripheral SR membrane (Lemos *et al.* 2007; Poburko *et al.* 2007). Both depolarization and local Na^+ accumulation favour reversal of NCX, and a transient rise in junctional $[Na^+]$ may be facilitated by the low-Na^+-affinity α_2 and α_3 isomers of the PM Na^+/K^+ ATPase (NKA) (Sahin-Erdemli *et al.* 1994). These isomers are typically localized near NCX and SERCA, probably in PM–SR junctions, and

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their lower Na^+ affinities (K_d ∼22–33 mM as reported in Zahler *et al.* 1997) would favour the generation of a higher junctional [Na^+] prior to Na^+ extrusion by the NKA (Juhaszova & Blaustein, 1997). The idea that NCX could be associated with regulation of SR Ca^{2+} content was first presented earlier (Reuter *et al.* 1973) although supporting data was not available at that time. The extensive present knowledge regarding the dynamics of the transport proteins, molecules and 3-D architecture of the nanojunctions finally permitted the formulation of quantitative hypotheses of this junctional Ca^{2+} transfer, from Ca^{2+}-entry-mode NCX to SERCA (Fameli *et al.* 2007). However, subsequent modelling outcomes suggested that greater structural complexity must limit Na^+ diffusion. This was adequately provided for by the inclusion of transjunctional protein complexes that had been originally identified in electron-micrographs (Gabella, 1971; Devine *et al.* 1972; Fameli *et al.* 2009), although we have yet to consider the possibility that the diffusion path could be shortened by observed tethering of NCX to TRPC channels (Rosker *et al.* 2004).

Smooth muscle SR reloading via SERCA may also be facilitated by Ca^{2+} influx through VGCCs (Takeda *et al.* 2011), ROCs (Albert *et al.* 2009; Shi *et al.* 2012), and the stromal interaction molecule (STIM)/Orai system (Fig. 1:2; Takahashi *et al.* 2007*a*,*b*; Berra-Romani *et al.* 2008), respectively, all of which have been shown to support homogeneous increases in SR calcium content. However, even when both VGCCs and ROCs are blocked the SR can be slowly refilled, presumably through an elusive Ca^{2+} leak in the PM (Deth & van Breemen, 1974). The extent to which these different SR-loading mechanisms may overlap in a single smooth muscle type or its proliferative and migratory phenotypes remains to be determined, but it seems likely that they will contribute to the heterogeneity in Ca^{2+} signalling mechanisms between different smooth muscles.

SR unloading (Fig. 1: 3)

During rest, when the SR is not actively releasing Ca^{2+}, NCX operates in the Ca^{2+} exit mode favouring unloading of the SR via a PM–SR junction, and functions together with Ca^{2+} extrusion via the plasma membrane Ca^{2+} ATPase (PMCA), located outside the PM–SR junctions, to maintain cellular Ca^{2+} homeostasis (Lee *et al.* 2002). Ca^{2+} exit mode NCX is clearly favoured by inactivation of ROCs and repolarization of the membrane. That aside, the mechanism proposed is that under these conditions Ca^{2+} released into PM–SR junctions from the peripheral SR would raise the local [Ca^{2+}] to stimulate Ca^{2+} extrusion (van Breemen *et al.* 1995; Nazer & van Breemen, 1998). Ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3Rs) are present at PM–SR junctions and may well serve this function, but at this time there is no concrete evidence for which release channel supplies Ca^{2+} for Ca^{2+} exit mode NCX in smooth muscle, although in endothelial cells it is clear that the RyR fulfils this function (Liang *et al.* 2004). Since the NCX is rheogenic, the hyperpolarization induced via RyRs linked to Ca^{2+}-activated large conductance potassium (BK_{Ca}) channels, described below, will promote SR Ca^{2+} unloading. For the sake of clarity, Fig. 1 shows separate nanojunctions for loading and unloading the SR (Fig. 1: 1, 2 and 3), but it is also possible that a single more complex PM–SR junction would allow NCX to oscillate between the Ca^{2+} exit and entry modes depending on the SR Ca^{2+} content, PM potential and the junctional Na^+ concentration.

STOCs, sparks and hyperpolarization (Fig. 1: 4)

It was originally proposed that α-adrenergic stimulation of the guinea pig taenia coli released Ca^{2+} from an internal store to activate BK_{Ca} channels and cause large transient hyperpolarization (Bülbring & Tomita, 1977, 1987). Since this hyperpolarization was associated with relaxation, they proposed that the Ca^{2+} release was localized to a plasmalemmal domain proximal to the target channels. In 1986, spontaneous transient outward currents (STOCs) in visceral smooth muscle were observed (Benham & Bolton, 1986), which resulted from the activation of 75–100 BK_{Ca} channels localized in less than 3% of the cell PM and that this occurred in response to local RyR-mediated SR Ca^{2+} release. The picture was completed with the demonstration of Ca^{2+} sparks in vascular smooth muscle and their role in determining basal tension and evoked vasodilatation of cerebral arteries (Nelson *et al.* 1995). In these vessels, sparks play a critical role in the regulation of myogenic tone, and thus autoregulation of cerebral blood flow, by providing variable feedback regulation on the opening of VGCCs (Ledoux *et al.* 2006). Analysis of the relationship between spark intensity and STOC size suggests a distance between RyR in the SR and BK_{Ca} in the PM comparable with the width of diadic junctions in cardiac muscle (Pérez *et al.* 1999). Moreover, there is little doubt that SERCA maintains a releasable pool of Ca^{2+} within the superficial SR, which is linked to relaxation (Boittin *et al.* 2002, 2003). Evidence suggests that the type of SERCA (SERCA2b) located in the superficial SR may differ (by kinetics and mechanisms of regulation) from the SERCA type(s) that recycle Ca^{2+} into the deep SR (Clark *et al.* 2010).

Ca^{2+}-induced Ca^{2+} release (Fig. 1: 6 and 7)

Following initial descriptions in skeletal muscle (Endo *et al.* 1970), CICR was first demonstrated in intestinal

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smooth muscle (Saida, 1982) and vascular smooth muscle (Saida & van Breemen, 1983) by showing that, in saponin-skinned fibres, a sudden increase in $[Ca^{2+}]_i$ released additional Ca^{2+} from a caffeine-sensitive store. Since the ultra-structural relationship between the VGCC and RyR varies from one smooth muscle to another (compare, for example, Moore *et al.* 2004 with Gordienko *et al.* 2008), it might be expected that the nature of CICR will vary as well. The observation of a structural linkage between dihydropyridine receptors and RyRs in the bladder detrusor muscle of the guinea pig led to the hypothesis of a tight coupling between the two channels similar to that seen in cardiac muscle (10–20 nm; Moore *et al.* 2004). In contrast, it was proposed that CICR in smooth muscle of the rabbit urinary bladder is generally loosely coupled, meaning that the distance between VGCC and RyR is greater than 100 nm (Kotlikoff, 2003; Ji *et al.* 2006). That different smooth muscles display a range of coupling between Ca^{2+} entry and RyR activation, from tight to none at all, seems all the more likely when one considers the fact that constriction induced by membrane depolarization in pulmonary arteries appears insensitive to block of RyRs (Dipp & Evans, 2001; Dipp *et al.* 2001). The significance of this may lie in the fact that different smooth muscle types may utilise CICR in different ways, and may not necessarily require coupling between VGCCs and RyRs or IP_3Rs.

Electrical synchronization and vasomotion (Fig. 1: 5)

All smooth muscles exhibit Ca^{2+} wave activity. In large blood vessels, be they conduit arteries or capacitative veins, agonist-mediated activation elicits asynchronous Ca^{2+} waves generated by CICR at IP_3-sensitized IP_3R (Iino *et al.* 1994; Ruehlmann *et al.* 2000; McCarron *et al.* 2010). As mentioned above, PM–SR junctions are required to maintain this type of Ca^{2+} oscillation by refilling the SR. When small resistance arteries are stimulated with agonists they initially also exhibit asynchronous Ca^{2+} waves, which quickly convert into synchronous non-wave-like Ca^{2+} oscillations in adjoining smooth muscle cells. The resulting synchronized contractions are the basis of vasomotion and are achieved by rapid spread of Ca^{2+}-activated chloride channel (Cl_{Ca})-induced depolarization (Boedtkjer *et al.* 2008). According to this view the original periodic IP_3R/Ca^{2+}-mediated Ca^{2+} release near the PM is responsible for the initiation of each $[Ca^{2+}]_i$ oscillation in mesenteric resistance arteries (Peng *et al.* 2001). By contrast, auto-regulation of cerebral arteries presents an alternative example of an SR Ca^{2+} release-activated PM channel, the cation-permeable channel transient receptor potential melastatin-4 (TRPM4) (Gonzales & Earley, 2012). TRPM4 is essential for pressure-induced depolarization and contraction in cerebral arteries, which also exhibit vasomotion. TRPM4 is activated by SR Ca^{2+} release via IP_3R; however, prolonged exposure to Ca^{2+} inactivates the channel due to activation of phospholipase C and decreases in levels of phosphatidylinositol 4,5-bisphosphate (PIP_2). This is kept in check by local endogenous buffering, which ensures that Ca^{2+} release events are short and localized. This led to the conclusion that TRPM4 channels on the PM are less than 50 nm from the SR membrane, but not physically coupled to IP_3R. In short, the all-important electrical synchronization and vasomotion processes also appear to revolve around nanojunctions between SR and PM, and may vary between different types of smooth muscles. The two examples provided above illustrate that different junctional compositions may regulate the same function of synchronized smooth muscle activity and that Fig. 1 is only meant to provide a limited array of plausible examples. On the other hand it is equally clear that certain combinations of channels, for example, BK_{Ca} plus Cl_{Ca}, within the same junction would obviate any useful function.

Vasomotion and Ca^{2+} waves and oscillations are strongly influenced by mitochondria, not only because they require a regulated energy supply, but also due to complex Ca^{2+} exchange between the SR and mitochondria.

SR-mitochondrial Ca^{2+} exchange (Fig. 1: 8, 9)

Mitochondria–SR junctions regulate mitochondrial energy supply, apoptosis and SR Ca^{2+} loading. There is now a vast literature on this subject, which has seen exciting recent progress with the identification of the main Ca^{2+} transporters: the mitochondrial uniporter (MCU) and mitochondrial Na^+/Ca^{2+} exchanger (NLCX), and may be accessed via an excellent recent review (Pizzo *et al.* 2012). Although mitochondria are capable of slow Ca^{2+} uptake from the bulk cytoplasm, which is in steady state with extrusion mainly via NLCX and partly H^+/Ca^{2+} exchangers (HCX), rapid mitochondrial Ca^{2+} transients depend on close approximations of the SR release channels, IP_3R and possibly RyR and the MCU. This process has been elegantly visualized using Ca^{2+} indicators targeted to the outer mitochondrial membrane, which record hot spots of 20–30 μM Ca^{2+} during activation of IP_3R (Giacomello *et al.* 2010). Ca^{2+} accesses the MCU via the large voltage-dependent anion channels in the outer membrane, which may be rate limiting. In smooth muscle the large transient mitochondrial Ca^{2+} gain is subsequently extruded via the NLCX which fuels SERCA-mediated re-uptake into the SR of smooth muscle cells (Szado *et al.* 2003; Poburko *et al.* 2009). In addition, evidence suggests that during the refilling process described above, peripheral mitochondria

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'funnel' Ca^{2+} lost from the PM–SR junction back into the SR (Poburko *et al.* 2009). Evidence has also been presented for a more central population of mitochondria that are insensitive to activity of the PM NCX (Szado *et al.* 2003). Besides the direct involvement of MCU and NLCX in cellular Ca^{2+} movements, mitochondrial Ca^{2+} transport also regulates Ca^{2+} channels in the SR and PM. Recent work showed that strategically localized, immobile mitochondria remove Ca^{2+} from active clusters of IP_3R to prevent their inhibition, thus allowing propagation of CICR-mediated Ca^{2+} release waves (Olson *et al.* 2010). An interesting variation on such mitochondrial control over excitatory Ca^{2+} waves in vascular smooth muscle was suggested in an article showing that the return supply of Ca^{2+} delivered by NLCX towards the IP_3R cluster served as an activating pacemaker mechanism (Ishii *et al.* 2006). Both studies cited above provide a mechanistic explanation for the earlier observation that rotenone-induced mitochondrial depolarization drastically interfered with smooth muscle Ca^{2+} oscillations (Swärd *et al.* 2002). Mitochondrial modulation of VGCCs and store-operated channels in the PM has also been documented, but this has not as yet been shown in smooth muscle. The limited data presented above indicate that SR–mitochondrial nanojunctions are important in smooth muscle Ca^{2+} signalling; however, caution is warranted in relation to any results obtained with pharmacological agents, as they modify many other mitochondrial functions. Rapid advances are now anticipated due to genetic manipulation of MCU and NLCX (Pizzo *et al.* 2012).

The lysosome–SR junction (Fig. 1: 10)

In pulmonary arterial smooth muscle cells NAADP appears to activate a unique, but converging, Ca^{2+} signalling pathway via lysosome–SR (L–SR) junctions, and one which may mediate Ca^{2+} signalling by endothelin-1. NAADP releases Ca^{2+} from lysosome-related stores in a manner that requires two pore segment channel subtype 2 (TPC2; Calcraft *et al.* 2009; Agbani *et al.* 2011). Subsequently this lysosomal Ca^{2+} release is amplified, in an all-or-none manner (Boittin *et al.* 2002), by CICR from the SR via RyR3, which is resident on the SR membrane participating in L-SR junctions (Kinnear *et al.* 2004, 2008). Thereafter, RyR2 may be recruited to carry, by CICR, a propagating Ca^{2+} wave across the wider cell in order to facilitate contraction (Kinnear *et al.* 2004; Clark *et al.* 2010). This is supported by observations that SERCA2a, RyR2 and RyR3 are located on the deep SR, which may comprise a segregated contractile domain.

What could be the advantage of RyR3 in the lysosome–SR junction? With respect to CICR, RyR3 exhibits the highest EC_{50}, and would therefore provide for a greater 'margin of safety' with respect to the all-or-none initiation of CICR by Ca^{2+} bursts from lysosomal Ca^{2+} stores at the L–SR junction. Furthermore, RyR3 exhibits a higher gain in open probability (P_o). Thus, once the threshold for activation is breached RyR3 would offer greater amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores. On the other hand RyR2 having a more diffuse cellular distribution (Kinnear *et al.* 2004; Clark *et al.* 2010) and lower EC_{50} for CICR would ensure that once initiated a propagating Ca^{2+} wave would be less prone to failure.

Although such dynamic considerations point intriguingly toward specific mechanisms, they remain in the realm of conjecture due to the near-inaccessibility of the lysosome–SR junctions by current calcium imaging techniques. However, as we briefly consider next, precise hypotheses can further our understanding by means of quantitative simulations.

3-D quantitative modelling: an invaluable tool to study transport processes in nanojunctions All documented instances of the nanojunctions outlined in Fig. 1 require an experimental resolution of <5 nm to visualize the important Ca^{2+} (and Na^+, and possibly other species) transients generated therein. This cannot be satisfactorily accomplished by currently available instrumentation. Nevertheless, by quantitative 3-D modelling we can study nanojunction-based hypotheses on mechanisms of Ca^{2+} signalling, develop them further and thereby generate new questions that may drive further experimentation. This cycle of renewal leads naturally to the development of yet more accurate quantitative models by incorporating all new biophysical features as they become available from experimental study. A very small population of ions is predicted to be present in the volumes of cytoplasm that reside within a single junction, and increases of one single Ca^{2+} ion may increase the local concentration from nanomolar to micromolar. A stochastic approach is thus required to model these signalling events. The quantitative stochastic models we develop are typically informed by confocal and electron microscopy, including immunofluorescence and immunogold labelling, transporter densities and kinetics.

Initial simplified models focused on Ca^{2+} flux across the PM–SR junction. While they only accounted for the stochastic element of diffusion, encouraging results were obtained. However, they highlighted the need to incorporate in the models more realistic intracellular architecture reconstructions, transporter kinetics and density, and reaction implementations. That aside, it appeared that the functional integrity of PM–SR junctions relies heavily on the close apposition of the two membranes, since in the models a separation of less than 50 nm adequately provided for compartmentalized Ca^{2+} signalling, which was lost when the separation of PM and junctional SR was greater than approximately 50 nm (Fameli *et al.* 2007).

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Employing a more sophisticated set of modelling tools, we have explored the L–SR nanojunction described in the previous section (Fameli, 2011).

Figure 2 illustrates some of the steps in the process of building a 3-D stochastic Monte Carlo model of Ca^{2+} transport in L–SR nanojunctions and some preliminary results. From several electron micrographs like the one in Fig. 2*A*, we recreated a 3-D representation of the relevant junctional geometry including one lysosome (grey object in Fig. 2*B*), partially wrapped by a portion of the SR (blue object in Fig. 2*B*), using open source '3-D content creation' software (blender.org). We then simulated the junctional $[Ca^{2+}]$ ($[Ca^{2+}]_{NJ}$) transient formed by the release of lysosomal Ca^{2+} (small white dots in Fig. 2*B*) via TPC2 (red objects in Fig. 2*B*) as stimulated by NAADP, in the presence of SERCA pumps on the nanojunctional SR (yellow objects in Fig. 2*B*) and mobile Ca^{2+} buffers (larger green dots in Fig. 2*B*) in the junctional and neighbouring cytoplasm. To study the influence of the junctional geometry on the transient, we repeated the simulations with different L–SR junctional widths. A plot of the $[Ca^{2+}]_{NJ}$ transient peak values *vs.* the junction width (Fig. 2*C*) suggests that Ca^{2+} accumulation in the lysosome–SR junction is extremely sensitive to applied changes in junctional dimensions and in particular on junctional width. Based on the information that RyR3 requires about 8–10 μM Ca^{2+} to release about 60–90% of SR Ca^{2+} (green band in Fig. 2*C*; Takeshima *et al.* 1995), our simulation results so far suggest that sufficiently high $[Ca^{2+}]_{NJ}$ transients to activate the L–SR-resident RyR3s can occur in L–SR junctions if these are less than about 50 nm wide. In other words, it is evident from this model that 'merely' altering the nanojunctional geometry causes significant disruption of a key element in the NAADP-dependent Ca^{2+} signalling pathway that leads to RyR-based CICR and eventual vascular smooth muscle contraction. Preliminary outcomes support the view that, as found for the PM–SR junctions, appropriate regulation and control of Ca^{2+} signalling is compromised when junctional membranes of the lysosome and SR are separated by distances greater than 50 nm. This also implies that 'functional uncoupling' of lysosome–SR junctions could be readily achieved and may contribute to plasticity of function.

It is clear, therefore, that quantitative simulations are essential for testing hypotheses related to transport processes occurring in specific nanojunctions. However, we also contemplate the need for a similar approach to the integration of activities within the entire population of nanojunctions, including the mechanisms of regenerative waves of SR Ca^{2+} release. As discussed above, the SR performs a multitude of functions at different sites within the smooth muscle cell even though its entire lumen, including that of the nuclear envelope, appears contiguous (McCarron & Olson, 2008). This paradox has been kept alive for more than 50 years as a running controversy related to the pros and cons of smooth muscle SR compartmentalization, eloquently summarized in the recent review (Wray & Burdyga, 2010). We envision future development of a dynamic model based on the SR as a continuous quilted network of membranes containing clusters of Ca^{2+} release channels as well as SERCA pumps, which are probably also inhomogeneously

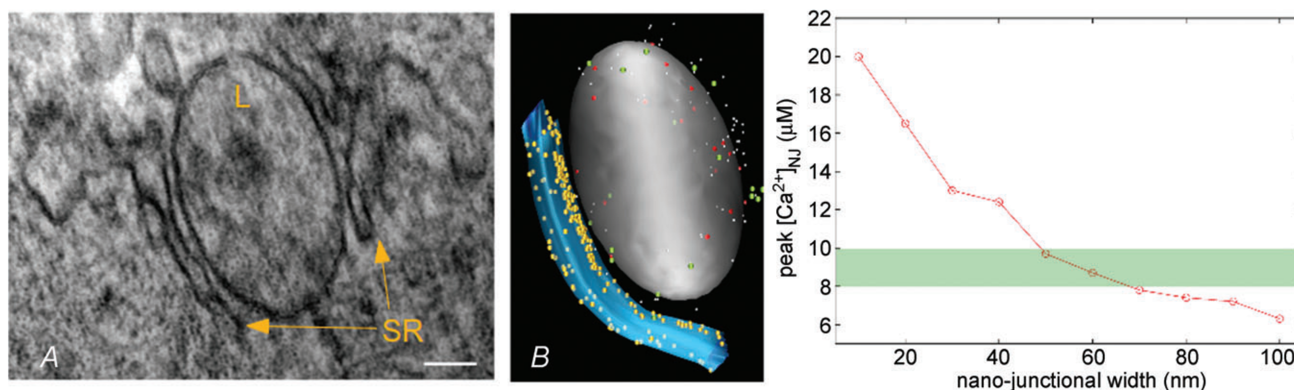

Figure 2
A, electron micrograph of a partially SR-surrounded lysosome (L). Tissue: rat pulmonary artery smooth muscle. Scale bar: 100 nm. *B*, 3-D software reproduction of a lysosome (grey)–SR (blue) nanojunction inspired by a series of observations from micrographs as in *A*; red transporters represent TPC2 Ca^{2+} release channels, yellow transporters stand for SERCA pumps; white molecules are Ca^{2+} and larger green ones are mobile buffers. *C*, simulation output as peak $[Ca^{2+}]_{NJ}$ *vs.* width of junction. Initial $[Ca^{2+}]_{lys}$ was set to 500 μM as reported in mast cells (Lloyd-Evans *et al.* 2008). Ca^{2+} Brownian motion trajectories are reproduced by means of accurate and extensively validated random walk algorithms implemented in the stochastic particle-simulator MCell (mcell.org) (Stiles *et al.* 1996; Stiles & Bartol, 2001; Kerr *et al.* 2008), which also allows the placement of relevant transporters and their appropriate reaction kinetics on the recreated object geometries. The green bar indicates the approximate threshold value for CICR at RyR3.

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distributed over the entire surface. For example, SERCA may be concentrated in PM–SR junctions functioning in refilling during excitatory Ca^{2+} waves. If, as can be clearly observed in both electron microscopy and confocal imaging, the lumen width varies considerably, then there would be regions within the continuous lumen that impede rapid diffusion between adjacent SR domains. The clustering of release channels (SR Ca^{2+} sinks) and SERCA (luminal Ca^{2+} sources) combined with SR sections offering resistance to diffusion would be ideal to create dynamic apparent Ca^{2+} compartments without the need to postulate the existence of membranous barriers between them. Such a system would be ideally suited for the propagation of Ca^{2+} waves as well as for replenishment of SR luminal Ca^{2+} for maintenance of the Ca^{2+} oscillations.

Does appropriate control of transcription require nanojunctions of the SR (Fig. 1: 11)?

The link between Ca^{2+} signalling and long-term changes in terms of remodelling (proliferation and migration) and cell death is not yet elucidated. In this respect, nuclear factor of activated T-cells (NFAT) translocation to the nucleus is significant. We know that released Ca^{2+} activates calcineurin, which is bound to the scaffolding protein AKAP79 on the PM. The Ca^{2+}–calcineurin complex dephosphorylates NFAT and thus induces translocation from the cytoplasm to the nucleus (Nilsson *et al.* 2008). Yet this apparently simple process is controlled by a complex, but poorly understood Ca^{2+} signalling mechanism. This is clear from the fact that Ca^{2+} entry through TRPC1, Ca^{2+} entry through STIM/Orai complexes, Ca^{2+} release through IP_3Rs and mitochondrial Ca^{2+} release have all been shown to be required for NFAT translocation, which also appears to require the presence of both PM–SR and SR–mitochondria nanojunctions. This is perhaps not surprising given that protection of transcription-activating sites from physiological Ca^{2+} transients activating myofilaments is clearly essential in order to prevent inappropriate vascular remodelling in response to normal cell and tissue function. Consistent with this idea, Ca^{2+} uptake by SERCA2 is required not only to preserve smooth muscle Ca^{2+} waves, but to afford protection against NFAT signalling (Bobe *et al.* 2011). However, while the case for different roles for SERCA, TRPC1 and STIM/Orai complexes in calcineurin/NFAT activation is evident, future studies must address the fact that this site on the PM is segregated from the nuclear membrane by SR, mitochondria and, not least, by the contractile domain itself. In this respect it is interesting that recent experiments have shown that smooth muscle Ca^{2+} waves evoked by endothelin-1 are deflected by the nuclear envelope (Esfandiarei *et al.* 2013), consistent with previous proposals that the nuclear membrane provides a buffer barrier (al-Mohanna *et al.* 1994; Wamhoff *et al.* 2002) that may determine in some respect Ca^{2+} signalling to the nucleus. It seems likely, therefore, that additional nuclear nanospaces may be conferred by the pan-junctional SR.

Conclusion

We hypothesize that nanojunctions between the SR, on the one hand, and PM, mitochondria, lysosomes and other organelles, on the other, are the basis for segregating localized calcium signals for the independent regulation of contraction, relaxation, energy metabolism, apoptosis, proliferation and migration. Variations in the prevalence, ultra-structure and molecular makeup of the nanojunctions could explain both smooth muscle heterogeneity and plasticity. Therefore, elucidating the mechanisms of ion transport within nanospaces is essential to our further understanding of calcium signalling not just in smooth muscle, but all cell types. A combination of high-resolution dynamic imaging of localized ion concentrations, ultrastructural 3-D reconstruction and (stochastic) quantitative modelling could lead to specific testable hypotheses, with the caveat that the signalling architecture of each cell type be considered unique and studied separately.

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