The Role of Intracellular Ion Channels in Regulating Cytoplasmic Calcium in Pulmonary Arterial Smooth Muscle: Which Store and Where?

A. Mark Evans

Abstract The mobilisation of intracellular Ca^{2+} stores plays a pivotal role in the regulation of arterial smooth muscle function, paradoxically during both contraction and relaxation. Moreover, different spatiotemporal Ca^{2+} signalling patterns may trigger differential gene expression while mediating the same functional response. These facts alone serve to highlight the importance of the growing body of evidence in support of the view that different Ca^{2+} storing organelles may be selected by the discrete or co-ordinated actions of multiple Ca^{2+} mobilising messengers. In this respect, it is generally accepted that sarcoplasmic reticulum stores may be mobilised by the ubiquitous messenger inositol 1,4,5 trisphosphate. However, relatively little attention has been paid to the role of Ca^{2+} mobilising pyridine nucleotides in arterial smooth muscle, namely cyclic adenosine diphosphate-ribose and nicotinic acid adenine dinucleotide phosphate. This review will, therefore, focus on the role of these novel Ca^{2+} mobilising messengers in pulmonary arterial smooth muscle, with particular reference to hypoxic pulmonary vasoconstriction.

Keywords hypoxia • AMPK • NAADP • cADPR • ryanodine receptor • sarcoplasmic reticulum • lysosomes • artery • smooth muscle

1 Introduction

We know that agonist-specificity is determined, in part, by the release of Ca^{2+} from intracellular stores in a manner dependent on second messengers and their associated Ca^{2+} release channels. During pharmaco-mechanical coupling in smooth muscle, it has long been accepted that many G protein-coupled receptors induce the

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production of inositol 1,4,5-trisphosphate (IP_3), which leads to the activation of one or more of the known IP_3 receptor (IP_3R) sub-types on the sarcoplasmic reticulum (SR) and release of Ca^{2+} from this store.[1] However, there is a growing body of evidence to support a role for the Ca^{2+} mobilising pyridine nucleotides nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic adenosine diphosphate-ribose (cADPR) in the regulation of intracellular Ca^{2+} signalling in a number of cell types, including smooth muscle.[2–4] Furthermore, studies have raised the possibility that the spatiotemporal pattern of Ca^{2+} signals may also be determined via the selection of different intracellular Ca^{2+} stores in a manner dependent on the nature of the Ca^{2+}-mobilising messengers recruited by a given stimulus.[5–7]

Consideration of the role of Ca^{2+} signalling by pyridine nucleotides in arterial smooth muscle and of the Ca^{2+}-storing organelles that may be accessed by these messengers will therefore be central to advances in this field for some time to come. Our studies have revealed that in this respect the processes involved in pulmonary arterial smooth muscle are more complex than one might expect. In the context of pulmonary artery constriction and dilation, therefore, this chapter focuses on the role of pyridine nucleotide Ca^{2+}-mobilizing messengers, their receptors and the functional segregation of the Ca^{2+}-storing organelles they target.

2 Hypoxic Pulmonary Vasoconstriction

2.1 Regulation by Hypoxia of Calcium Mobilisation from Sarcoplasmic Reticulum Calcium Stores in Pulmonary Artery Smooth Muscle

In isolated pulmonary arteries, hypoxic pulmonary vasoconstriction (HPV) is biphasic when induced by switching from a normoxic to a hypoxic gas mixture [Fig. 4.1a(i)]. Thus, hypoxia induces an initial transient constriction (phase 1) and a slow tonic constriction (phase 2).[8,9] Both phases of constriction are superimposed on each other; that is, they are discrete events and are both initiated immediately on exposure to hypoxia. The initial transient constriction peaks within 5–10 min of the hypoxic challenge, whilst the underlying, tonic constriction peaks after 30–40 min. When the endothelium is removed, the gradual amplification of phase 2, which is driven by the release of an endothelium-derived vasoconstrictor, is not observed, and the phase 1 constriction now declines to a maintained plateau[8] [Fig. 4.1a(ii)]. Several investigations have suggested that phase 1 (first 5–10 min) of HPV is mediated, at least in part, by the release of Ca^{2+} from SR stores via ryanodine receptors (RyRs), but in general these studies did not demonstrate that this was an endothelium-independent process (see Chap. 12). The most significant study in this respect was that of Salvaterra and Goldman.[10] They determined that hypoxia triggered SR Ca^{2+} release in cultured pulmonary arterial smooth muscle cells, and that this led to consequent activation of a verapamil- and nifedipine-

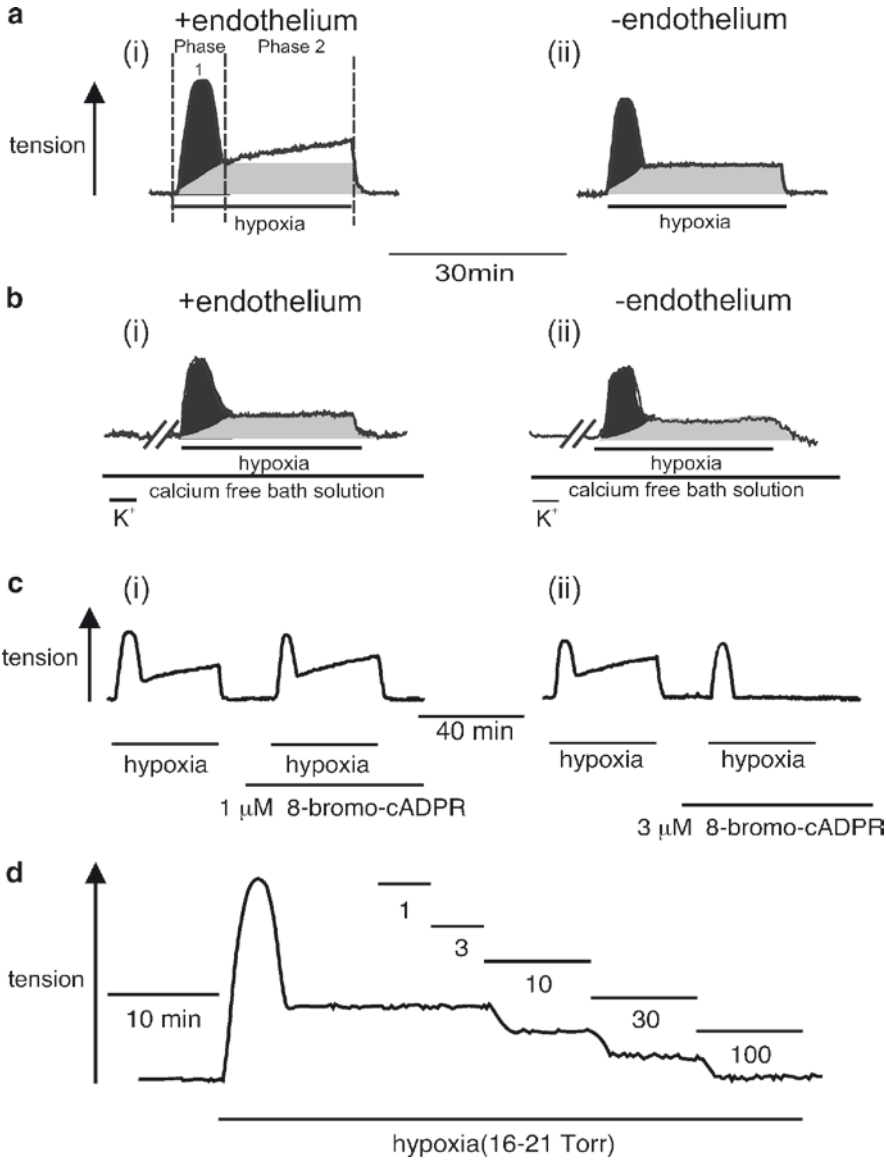

Fig. 4.1 Hypoxia triggers cADPR-dependent Ca^{2+} release from smooth muscle sarcoplasmic reticulum stores. **a**(i) Record indicating phase 1 and phase 2 of the response of an intact pulmonary artery ring to hypoxia, and the three identified components of HPV cADPR-independent SR Ca^{2+} release (*black*) cADPR-dependent SR Ca^{2+} release (*grey*) and endothelium-dependent (*white*). **a**(ii) Constriction by hypoxia of a pulmonary artery ring without endothelium. **b**(i) Constriction by hypoxia of an intact pulmonary artery ring in the absence of extra-cellular Ca^{2+}. **b**(ii) Constriction by hypoxia of a pulmonary artery ring without endothelium in the absence of extra-cellular Ca^{2+}. (**c**) Pre-incubating intact pulmonary arteries with 8-bromo-cylic ADP-ribose, a cADPR antagonist, at (i) 1 μ*M* has no effect but produces all-or-none block of phase 2 at (ii) 3 μ*M*. (**d**) Concentration-dependent reversal of maintained HPV by 8-bromo-cylic ADP-ribose in an artery without endothelium

insensitive transmembrane Ca^{2+} influx pathway that was not "modulated" by hypoxia. In short, hypoxia triggered SR Ca^{2+} release and store-operated Ca^{2+} entry in a manner that was mimicked by the SR Ca^{2+} ATPase (SERCA) antagonist thapsigargin and blocked by pre-incubation of cells with thapsigargin.

Some years later, we provided compelling evidence in support of a pivotal role for continued smooth muscle SR Ca^{2+} release via RyRs in the induction (phase 1 and 2) and maintenance (phase 2) of HPV in isolated pulmonary arteries both with and without endothelium.[8] Briefly, both phase 1 and phase 2 of HPV were shown to be abolished following block of SR Ca^{2+} release via RyRs with ryanodine and caffeine, whilst constriction in response to membrane depolarisation (80 m*M* K^+) and consequent voltage-gated Ca^{2+} influx remained unaffected (not shown). Furthermore, when the endothelium was absent, hypoxia triggered both the transient and maintained phases of constriction after removal of extra-cellular Ca^{2+}, despite the fact that constriction induced by depolarisation (by K^+) was abolished [Fig. 4.1b(ii)]. Thus, it would appear that smooth muscle Ca^{2+} release from ryanodine-sensitive SR stores underpins pulmonary artery smooth muscle constriction by hypoxia. It is notable, however, that maintained constriction of pulmonary artery rings was attenuated by up to 50% in Ca^{2+}-free medium,[11] consistent with the view that HPV is supported by consequent activation of store-depletion-activated Ca^{2+} entry.[12]

These findings suggested that in the intact artery the mobilisation by hypoxia of SR Ca^{2+} stores via RyRs was mediated by mechanisms intrinsic to pulmonary arterial smooth muscle cells. We therefore considered the possibility that cADPR, an endogenous regulator of RyRs,[13] may play a role in this process.

2.2 ADP-Ribosyl Cyclase and cADPR Hydrolase Activities Are Differentially Distributed in Pulmonary Versus Systemic Artery Smooth Muscle

Our initial findings were striking in that the enzyme activities for the synthesis and metabolism of cADPR were at least an order of magnitude higher in homogenates of pulmonary artery smooth muscle than in those of aortic or mesenteric artery smooth muscle.[14] Of further significance was the finding that the level of these enzyme activities was inversely related to pulmonary artery diameter. Thus, the differential distribution of these enzyme activities may offer, via amplification of the initial stimulus, the pulmonary selectivity required of a mediator of HPV and underpin, at least in part, the inverse relationship between the magnitude of constriction by hypoxia and pulmonary artery diameter.[15] This proposal was supported by direct measurement of cADPR content (estimated basal level ≥5 μ*M*) using a [^{32}P]cADPR binding assay. Hypoxia (16–21 Torr) increased cADPR levels twofold in second-order branches of the pulmonary arterial tree and tenfold in third-order branches.[14] Thus, like constriction by hypoxia and the distribution of the enzyme activities for cADPR synthesis, the

increase in cADPR content induced by hypoxia was inversely related to pulmonary artery diameter.

The mechanism by which hypoxia promotes cADPR accumulation in pulmonary artery smooth muscle remains to be confirmed. However, we have provided evidence to suggest that increased β-NADH beta-nicotinamide adenine dinucleotide formation under hypoxic conditions may facilitate cADPR formation from β-NAD^+ by augmenting adenosine diphosphate (ADP)-ribosyl cyclase or inhibiting cADPR hydrolase activities.[14] An alternative proposal is that hypoxia may initiate a paradoxical increase in reactive oxygen species (ROS) generation by mitochondria and thereby elicit SR Ca^{2+} release.[16] Most recently, however, we have obtained data consistent with the view that the metabolic sensor adenosine monophosphate (AMP)-activated protein kinase may couple the inhibition of mitochondrial oxidative phosphorylation by hypoxia to cADPR-dependent SR Ca^{2+} release in pulmonary arterial smooth muscle.[11] It is my view that the mobilisation by hypoxia of SR Ca^{2+} stores is mediated by the combinatorial effects of AMPK activation and β-NADH accumulation,[17] not only via the regulation of cADPR accumulation but also due to direct regulation by AMPK of, for example, RyRs, SERCA function and ion channels in the plasma membrane. Further investigations are, however, required to define the precise mechanisms involved.

2.3 The cADPR Antagonist 8-Bromo-cADPR Identifies cADPR-Independent and cADPR-Dependent Phases of Smooth Muscle SR Ca^{2+}Release by Hypoxia

The effects of a cADPR antagonist, 8-bromo-cADPR, on HPV in isolated pulmonary artery rings were quite different from the effects of ryanodine and caffeine. In arteries with and without endothelium, 8-bromo-cADPR had no effect on phase 1 of HPV. However, it abolished phase 2 in the presence of the endothelium and blocked the maintained constriction observed in arteries without endothelium[18] [Fig. 4.1c(ii)]. Thus, while cADPR-dependent SR Ca^{2+} release is required for the initiation and maintenance of phase 2 of acute HPV in isolated pulmonary artery rings, cADPR is not required to support the majority of SR Ca^{2+} release during the phase 1 constriction.

2.4 8-Bromo-cADPR Blocks Phase 2 of HPV in an All-or-None Manner

An unexpected and surprising observation of ours was that when arteries were pre-incubated with the cADPR antagonist 8-bromo-cADPR, phase 2 of HPV was blocked in an all-or-none manner.[18] Briefly, following pre-incubation of isolated pulmonary arteries with 1 μ*M* 8-bromo-cADPR, HPV remained unaltered [Fig. 4.1c(i)], but pre-incubation with 3 μ*M* 8-bromo-cADPR abolished the maintained

constriction observed during phase 2 [Fig. 4.1c(ii)]. This is entirely incompatible with the block by a competitive antagonist, such as 8-bromo-cADPR, of a simple process of "agonist"-receptor coupling.

The aforementioned finding was all the more curious given that, once initiated, the maintained phase of constriction, in pulmonary arteries without endothelium, was reversed by 8-bromo-cADPR in a concentration-dependent manner and with complete block only being attained at a concentration of 100 μ*M*,[18] nearly an order of magnitude higher than required for all-or-none block following pre-incubation with 8-bromo-cADPR (Fig. 4.1d). Unlike the all-or-none block observed following pre-incubation, this concentration-dependent reversal of maintained HPV is entirely consistent with the inhibition by a competitive antagonist of agonist-receptor coupling at a single population of receptors.

We concluded that these findings are reminiscent of the block by α-bungarotoxin of transmission at the neuromuscular junction, where greater than 45% of skeletal muscle nicotinic acetylcholine receptors must be blocked before neuromuscular transmission is compromised, and we proposed that a similar "margin of safety" may therefore be built into HPV. At the time, we suggested that the cADPR-dependent component of HPV may be initiated in an all-or-none manner, and that the all-or-none block of HPV by 8-bromo-cADPR could be due to it "blocking the activation by cADPR of a certain proportion of RyRs" or by the block of "cADPR-dependent Ca^{2+} mobilisation from a sub-population of RyRs" that are pivotal to the initiation of HPV.[2,18]

3 NAADP Induces Global Ca^{2+} Waves and Smooth Muscle Contraction in an All-or-None Manner

3.1 NAADP Triggers Ca^{2+}Bursts from Lysosome-Related Acidic Stores that Are Amplified by Ca^{2+}-induced Ca^{2+} release from the SR via RyRs

One possible explanation of the all-or-none block of HPV by 8-bromo-cADPR could be revealed by studies on NAADP, which is also synthesised by ADP-ribosyl cyclase, if NAADP or Ca^{2+} mobilisation from the store it accesses acts in concert with cADPR to mediate intracellular Ca^{2+} signalling by hypoxia in pulmonary arterial smooth muscle. This is clear from the fact that NAADP initiates global Ca^{2+} waves in an all-or-none manner and via a relatively complex two-pool system[19] comprised of the mobilisation of acidic, lysosome-related Ca^{2+} stores and subsequent Ca^{2+}-induced Ca^{2+} release (CICR) from the SR via RyRs.[5]

That NAADP may selectively elicit Ca^{2+} signals from lysosome-related Ca^{2+} stores in pulmonary arterial smooth muscle cells is supported by the fact that selective depletion of acidic Ca^{2+} stores by bafilomycin A1, which blocks the vacuolar H^+ ATPase (adenosine triphosphatase), abolishes NAADP-dependent Ca^{2+} signal-

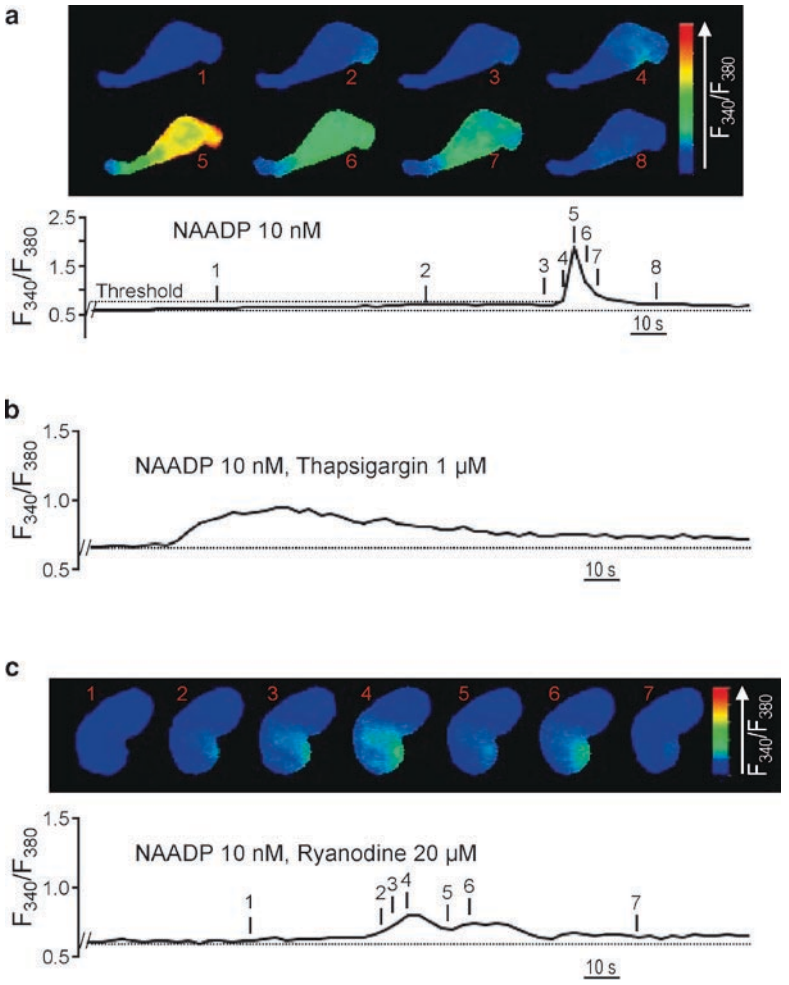

Fig. 4.2 NAADP triggers Ca^{2+} signals via lysosome-related stores in a manner that leads to subsequent Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs) in isolated pulmonary arterial smooth muscle cells. (**a**) *Upper panel* shows a series of pseudocolour images of the fura-2 fluorescence ratio (F_{340}/F_{380}) recorded in a pulmonary artery smooth muscle cell during intracellular dialysis of 10 n*M* NAADP. Note that a spatially localised 'Ca^{2+} burst' precedes the global Ca^{2+} wave (image 4). *Lower panel* shows the fura-2 fluorescence ratio against time. (**b**) Effect of intracellular dialysis of 10 n*M* NAADP after pre-incubation (20 min) of cells with 1 μ*M* thapsigargin. (**c**) *Upper panel* shows a series of pseudocolour images and the *lower panel* indicates F_{340}/F_{380} against time, obtained during the intracellular dialysis of 10 n*M* NAADP after pre-incubation (20 min) of cells with 20 μ*M* ryanodine. *Lower panel* shows the fura-2 fluorescence ratio against time. Note that in the absence of functional RyRs or SR stores replete in Ca^{2+}, spatially restricted Ca^{2+} bursts are triggered without initiation of a global Ca^{2+} wave

ling without effect on SR Ca^{2+} release via either RyRs or IP_3Rs.[19] Significantly, however, depletion of SR Ca^{2+} stores by inhibition of the sarcoendoplasmic reticulum Ca^{2+} pump (SERCA) with thapsigargin or block of RyRs with ryanodine (Fig. 4.2a-c) revealed spatially restricted bursts of Ca^{2+} release in response to NAADP that failed to propagate away from their point of initiation in the absence of either SR stores replete in Ca^{2+} or functional RyRs.[5,19] Thus, NAADP initiates highly localised "Ca^{2+} bursts" from lysosome-related stores that may either decline back to basal levels or precede and then trigger a global Ca^{2+} wave due to subsequent CICR from the SR via RyRs. NAADP-induced Ca^{2+} bursts must therefore breach a given threshold to elicit a global Ca^{2+} wave by CICR via RyRs on the SR, and this may be facilitated indirectly if Ca^{2+} bursts also serve to prime SR stores by increasing their Ca^{2+} load via SERCA-dependent uptake of a proportion of released Ca^{2+} into the SR (see section 5 see below). However, before this point can be discussed further we must consider the ways in which cADPR and Ca^{2+} may modulate RyR function.

3.2 Possible Role of cADPR in the Modulation of NAADP-Dependent Ca^{2+}Signalling

By sensitising RyRs to Ca^{2+}, cADPR may determine the threshold for CICR via RyRs in response to Ca^{2+} bursts elicited from lysosomes by NAADP, or any other stimulus, and the degree of amplification of the initial Ca^{2+} burst by CICR.[2] The set point for these processes will likely be determined by the basal cADPR concentration (≥ 5 μM) and the local Ca^{2+} concentration but will be open to modulation by stimulus-dependent changes in cADPR synthesis or degradation. Thus, it is quite possible that a competitive cADPR antagonist, such as 8-bromo-cADPR, could block in an all-or-none manner the amplification of NAADP-dependent Ca^{2+} bursts into global Ca^{2+} waves by raising the threshold for CICR via RyRs. Subsequent to the initiation of a global Ca^{2+} wave, however, increased cADPR accumulation alone in response to a given stimulus could also provide for the maintenance of regenerative Ca^{2+} waves and smooth muscle contraction by cADPR-dependent CICR via RyRs.[2] Such a paradigm would then allow for the concentration-dependent reversal of maintained constriction by 8-bromo-cADPR.

The process of CICR is therefore pivotal. This refers to the fact that RyRs may be activated by Ca^{2+} in its own right or to the fact that Ca^{2+} release via RyRs may be facilitated by released Ca^{2+} via positive feedback.[20–22] Thus, CICR offers cells the facility to amplify small, highly localised Ca^{2+} signals into global Ca^{2+} waves via the recruitment of neighbouring RyR complexes. This can be achieved in a highly regulated manner due to the limitations placed on Ca^{2+} diffusion (≤ 5 μm)[23] by the buffering capacity within the cytoplasm. CICR may therefore recruit, in concert, discrete clusters of RyRs to initiate highly localised, elementary Ca^{2+} release events such as Ca^{2+} sparks.[24,25] Alternatively, once a given threshold concentration is breached Ca^{2+} may induce a propagating global Ca^{2+} wave by the progressive

recruitment by CICR of RyR clusters distant from the site of initiation. As mentioned, cADPR, like Ca^{2+}, may act as an endogenous regulator of RyRs[13] and may also either activate RyRs directly or facilitate CICR via RyRs.[26,27] Thus, it is not surprising that Ca^{2+} also sensitises RyRs to activation by cADPR.[28] Therefore, when considering the regulation by cADPR of RyRs in a given cell type, the combinatorial effects of Ca^{2+} and cADPR are of fundamental importance,[4] not least with respect to the threshold for activation of RyRs by either agent. And, the threshold for CICR via RyRs may also be modulated by the luminal Ca^{2+} concentration of the SR,[29–33] which could in turn be primed by Ca^{2+} supplied by preceding lysosome-related Ca^{2+} release events.

The situation presented in vascular smooth muscle is more complex still given that RyR sub-types 1, 2 and 3 are highly co-expressed in these cell types,[34,35] not least because all three RyR sub-types can be expressed in a cADPR-sensitive form and each may exhibit different sensitivities to both Ca^{2+} and cADPR. Therefore, the RyR sub-type targeted by Ca^{2+} signals from lysosomes could affect markedly the characteristics of any subsequent amplification process, determine to a great extent the all-or-none initiation of global Ca^{2+} signals by NAADP in pulmonary arterial smooth muscle and, if a sub-population of RyRs on the SR were targeted, confer all-or-none block of this amplification step by pre-incubation with 8-bromo-cADPR (see below see section 4.1).

4 Lysosome-Sarcoplasmic Reticulum Junctions Form a Trigger Zone for Ca^{2+} Signalling by NAADP

4.1 Lysosomes Co-localise with a Sub-population of RyRs

Using LysoTracker Red as a fluorescent label for acidic organelles in acutely isolated pulmonary arterial smooth muscle cells, we demonstrated that a large proportion of lysosomes form tight clusters in a manner consistent with the spatially restricted nature of Ca^{2+} bursts triggered by NAADP. Importantly, lysosomal clusters were closely associated with a sub-population of RyRs labelled with Bodipy-Ryanodine [Fig. 4.3a(i)] and appear to be separated from these RyRs by a narrow junction or cleft that is beyond the resolution of deconvolution microsopy (<1 μm).[5] We proposed, therefore, that lysosomal clusters and RyRs may form a highly organised "trigger zone," or intracellular synapse, for Ca^{2+} signalling by NAADP in arterial smooth muscle. The presence of this trigger zone may explain, in part, why Ca^{2+} bursts by NAADP induce global Ca^{2+} signals in an all-or-none manner by further CICR from the SR via RyRs. This tight coupling of lysosomal Ca^{2+} stores to a sub-population of RyRs could also serve to provide the aforementioned "margin of safety" with respect to the initiation of HPV, should lysosome-related Ca^{2+} release play a role, and confer all-or-none block of HPV by 8-bromo-cADPR due to the consequent increase in the threshold for CICR.

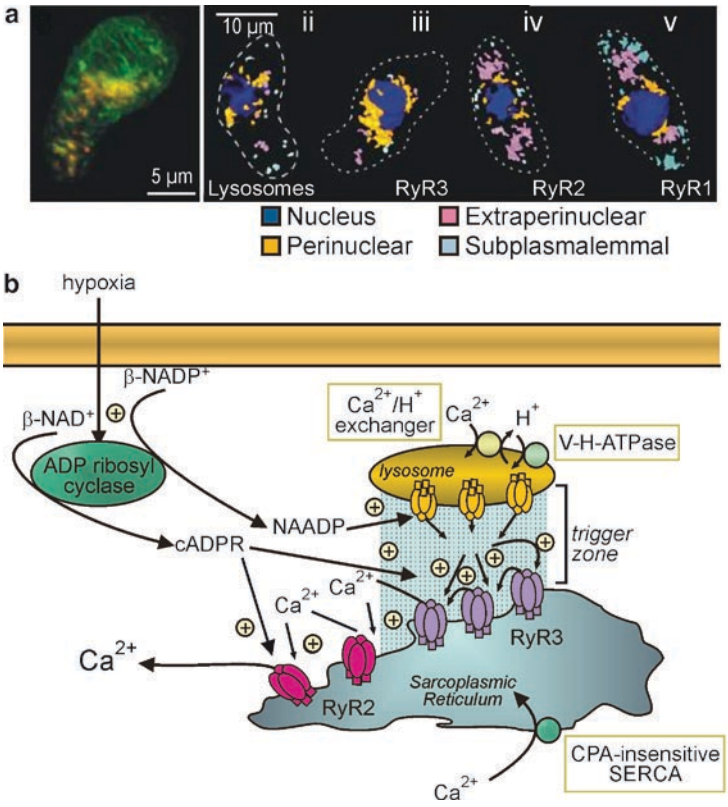

Fig. 4.3 Lysosomes preferentially co-localise with ryanodine receptor sub-type 3 to form a trigger zone for calcium signalling in response to NAADP. (**a**) 3D reconstruction of a deconvolved Z-stack of images from a pulmonary artery smooth muscle cell showing co-localisation (*yellow*) of lysosomes labelled with LysoTracker Red (*red*) and ryanodine receptors (RyRs) labelled with Bodipy-Ryanodine (*green*). (**b**) 3D reconstruction of deconvolved Z-stacks of images showing the distribution of individual volumes of lysosomal (αlgp120), RyR3, RyR2 and RyR1 labelling coloured to indicate their respective distribution in defined regions of the cell: the perinuclear volume, extra-perinuclear volume and sub-plasmalemmal volume. (**c**) Schematic diagram depicts the proposed trigger zone for Ca^{2+} signalling by NAADP and the possible amplification of NAADP-dependent Ca^{2+} signals by cADPR. *NAADP* nicotinic acid adenine dinucleotide phosphate; *cADPR* cyclic adenosine diphosphate-ribose; *SERCA* sarcoendoplasmic reticulum Ca^{2+} ATPase; *RyR* ryanodine receptor; *V-H-ATPase* vacuolar proton pump

4.2 Lysosomes Co-localise with RyR Sub-type 3 to Form a Trigger Zone for Ca^{2+}Signalling by NAADP in Pulmonary Arterial Smooth Muscle

Our most recent studies sought to determine whether lysosomes selectively couple to one of the three RyR sub-types expressed in arterial smooth muscle, namely, RyR1, RyR2, or RyR3.[36] The distribution of labelling for a given protein by density was determined for each of three defined regions of the cell relative to the nucleus (defined by DAPI 4'-6-Diamidino-2-phenylindole labelling), namely,

the perinuclear (within 1.5 μm of the nucleus), the sub-plasmalemmal (within 1.5 μm of the plasma membrane) and the extra-perinuclear region (the remaining volume of the cytoplasm). The density of labelling for the lysosome marker (αlgp120) was about twofold greater in the perinuclear than observed within the extra-perinuclear region and about fourfold greater than was observed in the sub-plasmalemmal region of cells, with dense clusters of labelling evident in the perinuclear region compared to a more diffuse distribution of labelling outside this region [Fig. 4.3a(ii)]. In common with the distribution of lysosomes, but to an even greater extent, the density of RyR3 labelling was concentrated within the perinuclear region of the cell, where it was about 4- and ~14-fold greater than that in the extra-perinuclear and sub-plasmalemmal regions, respectively. Furthermore, the density of RyR3 labelling within the perinuclear region was about twofold higher than that for either RyR1 or RyR2 [Fig. 4.3a(iii-v)].

Further insight was provided by analysis of the density of co-localisation between lysosomes and each RyR sub-type. Within the perinuclear region of the cell, RyR3 was found to co-localise with about 41% of the total volume of lysosome labelling, with the density of co-localisation about 4- and about 60-fold greater than that observed in the extra-perinuclear or sub-plasmalemmal regions, respectively. In marked contrast, labelling for RyR2 and RyR1 co-localised with only 13 and 14%, respectively, of the total volume of lysosome labelling within the perinuclear region, and their respective density of co-localisation was approximately twofold lower than that for RyR3. Furthermore, the mean volume of co-localisation between RyR3 and lysosomes was about twofold greater than that for either RyR1 or RyR2. We concluded, therefore, that lysosomal clusters preferentially co-localise with RyR3 in the perinuclear region of the cell to form a trigger zone for Ca^{2+} signalling by NAADP.

4.3 Why Might RyR3 Be Targeted to Lysosome–SR Junctions?

Determining factors in this respect could be the relative sensitivity of each RyR sub-type to CICR, the maximum gain in response to Ca^{2+} and the relative sensitivity of each receptor sub-type to inactivation by Ca^{2+}.[37,38] The threshold for activation of RyR1, RyR2 and RyR3 is similar, with channel activation at cytoplasmic Ca^{2+} concentrations above 100 n*M*. However, estimates of the EC_{50} are different, with half-maximal activation at about 250 n*M* for RyR2 and about 400 n*M* for RyR3. The higher EC_{50} exhibited by RyR3 could be significant because this would provide for a higher "margin of safety" with respect to the all-or-none amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores by CICR via RyRs at the lysosome–SR junction; that is, the probability of false events being initiated would be lower for RyR3 than for RyR2. Another factor that may be of significance is that whilst the mean open times versus cytoplasmic Ca^{2+} concentration for RyR2 and RyR3 are comparable and increase approximately tenfold over their activation range, the mean open time for RyR1 is much lower and increases only twofold over its activation range. Furthermore, comparison of the Po versus cytoplasmic Ca^{2+} concentration curves

shows that RyR3 (0–1) exhibits a higher gain in Po than does RyR2 (0–0.9), whilst RyR1 (0–0.2) exhibits relatively little gain in Po with increasing cytoplasmic Ca^{2+} concentration. Thus, once the threshold for activation is breached, RyR3 would offer greater amplification of Ca^{2+} bursts from lysosomal Ca^{2+} stores than would RyR2, whilst amplification via RyR1 would be marginal. There is also marked variation in the relative sensitivity of each RyR sub-type to inactivation by Ca^{2+}. RyR3 exhibits the lowest sensitivity to inactivation by Ca^{2+} with an IC_{50} of 3 m*M* whilst that for RyR2 is 2 m*M*; in each case, channel activity may still be observed at concentrations above 10 m*M*. In marked contrast, RyR1 inactivation occurs within the micromolar range, and full inactivation is achieved by 1 m*M* Ca^{2+}; this may, in part, explain the low gain in Po for RyR1 in response to activation by Ca^{2+}. Its sensitivity to inactivation by Ca^{2+} would therefore render RyR1 unsuitable for a role in the amplification of Ca^{2+} bursts at lysosome-SR junctions because the local Ca^{2+} concentration may exceed the threshold for RyR1 inactivation. Thus, the functional properties of RyR3 make it best suited to a role in the amplification of Ca^{2+} bursts at lysosome–SR junctions.

4.4 How May Ca^{2+}Signals Propagate Away from Lysosome–SR Junctions to the Wider Cell If RyR3 Is Targeted to the Perinuclear Region of Cells?

Significantly, the density of RyR3 labelling declines markedly (between 4- and 14-fold by region) outside the perinuclear region of the cell.[36] It seems unlikely, therefore, that RyR3 functions to carry a propagating Ca^{2+} wave far beyond the point of initiation of CICR within the proposed trigger zone for Ca^{2+} signalling via lysosomes. Given this finding, it may be of significance that the density of labelling for RyR2 increases markedly in the extra-perinuclear region when compared to the perinuclear region and exhibits about a threefold greater density of labelling within this region than observed for either RyR3 or RyR1. This suggests that RyR2, but not RyR1, may function to receive Ca^{2+} from RyR3 at the interface of the lysosome–SR junction and thereby allow for further propagation of the Ca^{2+} signal via CICR. Such a role would be supported by the lower EC_{50} for CICR via RyR2, which would ensure that once initiated a propagating Ca^{2+} wave would be less prone to failure. Furthermore, relative to RyR1, its greater intrinsic gain and lower sensitivity to inactivation by Ca^{2+} would render RyR2 most suitable to a role in the wider propagation of a global Ca^{2+} wave.

If clusters of RyR3 do indeed sit within the lysosome–SR junction and an array of RyR2 carries propagating Ca^{2+} signals away from this and Ca^{2+} signalling via this junction triggers HPV, pre-incubation of pulmonary arteries with 8-bromo-cADPR could block HPV in an all-or-none manner by increasing the threshold for CICR via RyR3 or RyR2. Furthermore, once initiated, if regenerative, propagating Ca^{2+} waves via RyR2 are maintained by an increase in cADPR accumulation in the

absence of further Ca^{2+} release from lysosome-related stores, 8-bromo-cADPR could reverse associated pulmonary artery constriction in a concentration-dependent manner (Fig. 4.3c).

4.5 RyR1 Is may be the predominant RyR subtype in the Sub-plasmalemmal Region of the Cell

The even distribution of RyR1 across the three specified regions of pulmonary artery smooth muscle cells suggests that it may contribute in some way to the regulation of Ca^{2+} signalling within each region. However, RyR1 is predominantly targeted (three- to fivefold by density of labelling) to the sub-plasmalemmal region and may therefore play a prominent role in Ca^{2+} signalling between the SR and Ca^{2+}-sensitive ion channels in the plasma membrane.[36]

5 Discrete SR Compartments Underpin Ca^{2+}-Dependent Vasodilation and Vasoconstriction

5.1 Cyclopiazonic Acid and 8-Bromo-cADPR Reveal Two Functionally Segregated SR Ca^{2+}Stores

An unexpected observation during our studies on the role of cADPR in HPV was that the SERCA pump antagonist cyclopiazonic acid blocked the phase 1 constriction but had no effect on phase 2 (Fig. 4.4a-b),[18] even though Ca^{2+} release from ryanodine-sensitive SR stores in the smooth muscle underpins both phases of HPV.[8] This was precisely the reverse of the effect of 8-bromo-cADPR, which abolished phase 2 of HPV without effect on phase 1 (Fig. 4.4c, d).[18] At the time, we concluded that phase 1 might be mediated by the mobilisation of an SR compartment served by a cyclopiazonic acid-sensitive SERCA that may be inhibited by hypoxia due to a fall in adenosine triphosphate (ATP) supply, and that to allow for this and a second phase of maintained cADPR-dependent SR Ca^{2+} release, one would require the presence of a second, spatially segregated SR Ca^{2+} store that is served by a discrete, cyclopiazonic acid-insensitive SERCA pump.[2,18]

We began to square this circle when studying the effect on cytoplasmic Ca^{2+} concentration of intracellular dialysis of cADPR (from a patch pipette). High concentrations of cADPR (100 μM) induced a small but sustained (unlike NAADP or IP_3)[19] and global increase in intracellular Ca^{2+} concentration (unpublished data). However, relatively low concentrations (20 μM) only increased cytoplasmic Ca^{2+} concentration at the perimeter of the cell and elicited a concomitant membrane hyperpolarisation (not shown).[39] The hyperpolarisation was reversed by the highly selective BK_{Ca} channel antagonist iberiotoxin, by chelating intracellular Ca^{2+} with BAPTA, by selective block of RyRs with ryanodine and by pre-incubation with

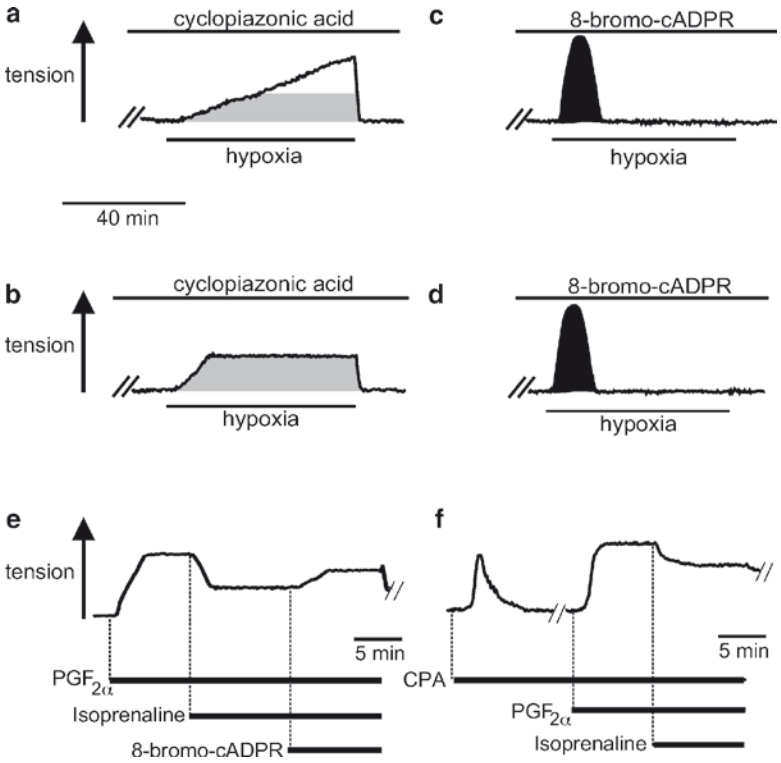

Fig. 4.4 Pharmacologically distinct smooth muscle sarcoplasmic reticulum Ca^{2+} stores underpin pulmonary artery dilation and constriction. Constriction by hypoxia (16–21 Torr) of a pulmonary artery ring (**a**) with and (**b**) without endothelium following pre-incubation (20 min) with cyclopiazonic acid (CPA) (10 μ*M*); (**c**) with and (**d**) without endothelium following pre-incubation (20 min) with 8-bromo-cADPR (300 μ*M*). Vasodilation by isoprenaline (100 n*M*) of a pulmonary artery ring, without endothelium, following pre-constriction with prostaglandin F2α (50 μ*M*) and the effect of (**e**) 8-bromo-cADPR (300 μ*M*) and (**f**) pre-incubation (20 min) with CPA (10 μ*M*)

cyclopiazonic acid. Most importantly, hyperpolarisation by cADPR was blocked by two different cADPR antagonists. Given that cADPR synthesis is up-regulated in a cAMP- (cyclic adenosine monophosphate-) and protein kinase A- (PKA)-dependent manner in cardiac muscle,[40] it seemed likely that cADPR could mediate hyperpolarisation by adenylyl cyclase-coupled receptors, such as β-adrenoceptors. Consistent with this proposal and previous studies on smooth muscle from a variety of tissues (for review, see Ref. [24]), we found that isoprenaline and cAMP induced hyperpolarisation in isolated pulmonary arterial smooth muscle cells and demonstrated that in each case hyperpolarisation exhibited a similar pharmacology to that induced by cADPR.[39] Strikingly, however, the selective PKA antagonist H89 blocked hyperpolarisation by both isoprenaline and cAMP, respectively, but was without effect on hyperpolarisation by cADPR. Thus, it would appear that cADPR is a downstream element in this signalling cascade. Further support for this proposal

was derived from studies of isolated pulmonary artery rings without endothelium. Vasodilation evoked in response to β-adrenoceptor activation by isoprenaline was inhibited (~50%) by blocking cADPR with the membrane-permeant antagonist 8-bromo-cADPR (Fig. 4.4e), RyRs with ryanodine and, consistent with the hyperpolarisation, by pre-incubation with cyclopiazonic acid (Fig. 4.4f). We concluded that cADPR-dependent Ca^{2+} signalling via RyRs on a cyclopiazonic acid-sensitive SR store was responsible, in part, for BK_{Ca}-dependent vasodilation by isoprenaline in isolated pulmonary arteries.[39]

Although these findings provided evidence of functionally segregated stores and allowed for further interpretation of our anomalous findings, they presented us with a paradox. That is, our data suggested that cADPR-dependent SR Ca^{2+} release via RyRs mediates both vasodilation and vasoconstriction of pulmonary arteries in a stimulus-dependent manner. We concluded that this could only be explained if (1) β-adrenoceptor signalling targets, via PKA-anchoring proteins, PKA-dependent cADPR synthesis to a particular RyR sub-type, possibly RyR1 (section 4.5 see above), in the "peripheral" SR that is in close apposition to BK_{Ca} channels in the plasma membrane (2) cADPR-dependent vasoconstriction results from the activation of discrete RyR sub-types localised in the "central" SR. Clearly, however, our data suggest that these discrete SR compartments would have to be served by different SERCA pumps. More precisely, an SR compartment in close apposition to the plasma membrane would be served by a SERCA pump that is sensitive to cyclopiazonic acid and, by contrast, a central SR compartment in close apposition to the contractile apparatus would be served by a SERCA pump that is relatively insensitive to cyclopiazonic acid.[2,39] This conclusion is supported by the fact that both SR Ca^{2+} release in response to hypoxia[10] and HPV (unpublished data) are abolished following SR store depletion by block of SERCA with thapsigargin.

In complete agreement with our proposal, previous studies on smooth muscle, the pulmonary vasculature included, have provided evidence of discrete SR compartments.[41–46] Most significantly, a number of these studies shared one common and convincing piece of evidence: The SERCA pump antagonist cyclopiazonic acid selectively depletes one of at least two functionally segregated SR compartments. We therefore sought to determine whether multiple SERCA were expressed in pulmonary arterial smooth muscle and, if so, their respective spatial distribution.

5.2 SERCA2a and SERCA2b Serve Discrete SR Compartments in Pulmonary Arterial Smooth Muscle

Western blots and immunocytochemistry carried out with sequence-specific antibodies raised against each SERCA isoform identified protein bands for SERCA2a and SERCA2b but not SERCA1 or SERCA3 (not shown). In agreement with previous studies on vascular smooth muscle,[47] therefore, it would appear that only SERCA2a and SERCA2b are functionally expressed in pulmonary arterial smooth muscle.

Astonishingly clear differences in the spatial organisation of SERCA2a and SERCA2b, respectively, were evident even on visual inspection of deconvolved Z sections and three-dimensional (3D) reconstructions of all cells labelled for SERCA2a and SERCA2b. This fact was confirmed by determining the distribution by density of labelling for each SERCA isoform within the sub-plasmalemmal (within 1 μm of the plasma membrane), the perinuclear (within 1.5 μm of the nucleus) and the extra-perinuclear (remainder) volumes.[48] The vast majority of SERCA2b labelling, about 70%, lay within the sub-plasmalemmal region, with only about 8% and about 20% of labelling present in the extra-perinuclear and perinuclear regions, respectively (Fig. 4.5a). In marked contrast, SERCA2a labelling was almost entirely (~90%) restricted to the perinuclear region of pulmonary arterial smooth muscle cells (Fig. 4.5a). These data suggest, therefore, that native SERCA2b may be sensitive to cyclopiazonic acid and supply an SR compartment that sits proximal to the plasma membrane and underpins Ca^{2+}-dependent vasodilation via adenylyl cyclase-coupled receptors, while SERCA2a may supply a central SR compartment and represent a cyclopiazonic acid-insensitive, thapsigargin-sensitive SERCA that underpins pulmonary artery constriction by hypoxia (Fig. 4.5b).

5.3 Possible Role of cADPR-Independent and cADPR-Dependent Phases of SR Ca^{2+}Release by Hypoxia

Given that vasodilation in response to activation of adenylyl cyclase-coupled receptors and phase 1 of HPV are inhibited by cyclopiazonic acid, they likely utilise a common SR store. It is possible, therefore, that SR Ca^{2+} release by hypoxia serves two purposes. Hypoxia may primarily trigger constriction by cADPR-dependent Ca^{2+} release from a central SR compartment that is in close apposition to the contractile apparatus and served by a cyclopiazonic acid-insensitive SERCA pump (SERCA2a). A secondary action of hypoxia may be to deplete a peripheral SR compartment by inhibition of a cyclopiazonic acid-sensitive SERCA pump (SERCA2b) in close apposition to the plasma membrane and that normally mediates vasodilation by releasing Ca^{2+} proximal to the plasma membrane to trigger membrane hyperpolarisation and thereby facilitate Ca^{2+} sequestration via plasma membrane Ca^{2+} ATPases and the Na^{+}/Ca^{2+} exchanger. This would explain why pulmonary vasodilation by β-adrenoceptor activation is abolished by hypoxia[49] and why maintained HPV is enhanced by cyclopiazonic acid[50] but abolished by thapsigargin (unpublished data).

6 Summary

In pulmonary arterial smooth muscle, considerations on the direct regulation of Ca^{2+} release from a single SR compartment are insufficient to explain current experimental observations of intracellular Ca^{2+} signalling. With respect to vasocon-

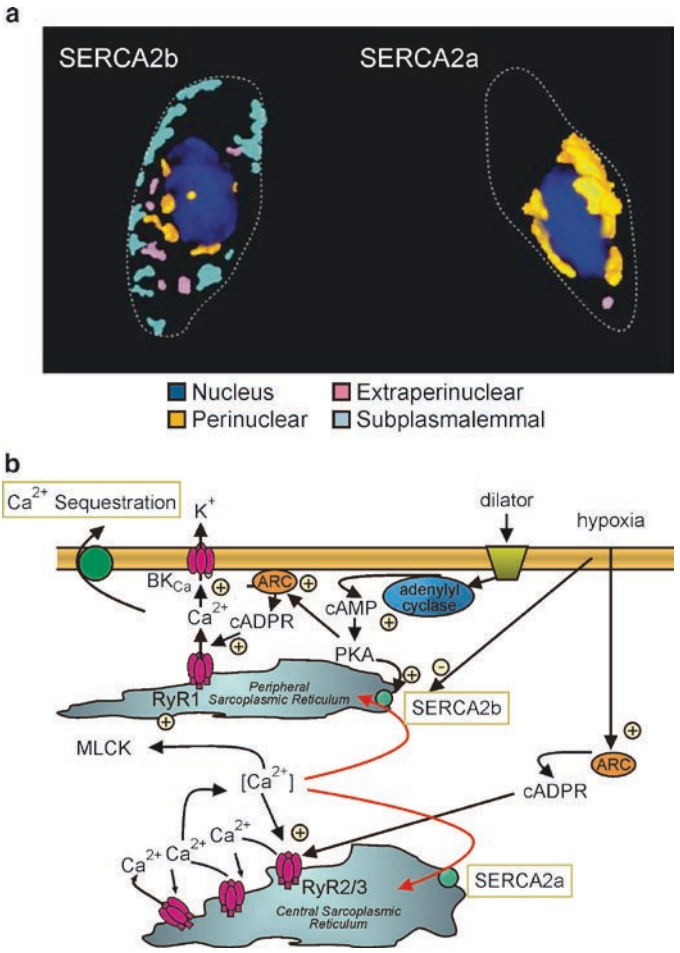

Fig. 4.5 SERCA2a and SERCA2b are differentially distributed within isolated pulmonary arterial smooth muscle cells and may serve functionally segregated SR Ca^{2+} stores. (**a**) 3D reconstruction of deconvolved Z-stacks of images showing the distribution of individual volumes of SERCA2b and SERCA2a labelling coloured to indicate distribution by defined regions of the cell: the perinuclear volume, extra-perinuclear volume and sub-plasmalemmal volume. (**b**) Schematic representation of the proposed spatial and functional compartmentalisation of the sarcoplasmic reticulum in a pulmonary arterial smooth muscle cell: *SR* Ca^{2+} *ATPase* SERCA; *ARC* ADP-ribosyl cyclase; *cADPR* cyclic adenosine diphosphate-ribose; *RyR* ryanodine receptor; BK_{Ca} Ca^{2+}-activated potassium channel; *cAMP* cyclic adenosine monophosphate; *PKA* protein kinase A

striction, it is now clear that Ca^{2+} signals may be initiated via a trigger zone, or intracellular synapse, between lysosome-related Ca^{2+} stores and the SR. Thus, mobilisation of lysosome-related stores by NAADP or other stimuli may be amplified by subsequent CICR via RyR3 and RyR2 on a central SR compartment that is served by SERCA2a and that feeds the contractile apparatus. Both cADPR and IP_3 may modulate Ca^{2+} release from this SR store independently or may coordinate

Ca^{2+} signals in concert with each other or NAADP. A second SR compartment may, however, lie in close apposition to the plasma membrane, is likely served by SERCA2b and supports cADPR-dependent Ca^{2+} release via a discrete RyR subtype (possibly RyR1) to recruit plasmalemmal BK_{Ca} channels to elicit smooth muscle cell hyperpolarisation and pulmonary artery dilation. In each case, however, the view proposed is most likely an oversimplification of the Ca^{2+} signalling apparatus available to the cell, and this point is emphasised by the fact that lysosome-related Ca^{2+} stores are mobile. In short, the complex nature and versatility of cellular Ca^{2+} signalling mechanisms should not be underestimated.

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