Stretch-induced Ca²⁺ signalling in vascular smooth muscle cells depends on Ca²⁺ store segregation

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Aim
Calcium is a key second messenger that can be mobilized from both the extracellular medium and intracellular calcium stores. Pulmonary arterial smooth muscle cells (PASMCs) respond to stretch by a calcium increase, a mechanism enhanced during pulmonary hypertension (PH). We investigated the role of the spatial organization between plasma membrane stretch-activated channels (SACs) and intracellular calcium stores [sarcoplasmic reticulum (SR), mitochondria, and lysosomes] in response to stretch.

Methods and results
Studies were performed in freshly isolated PASMCs from both control and two different rat models of PH (chronically hypoxic and monocrotaline-treated rats). Co-immunolabellings revealed that the subcellular segregation between each subtype of SR ryanodine receptors (RyR1, RyR2, and RyR3), SERCA2 pumps (SERCA2a and SERCA2b), mitochondria, or lysosomes in freshly isolated PASMCs differs from control and PH PASMCs. In control PASMCs, stretching the membrane activates a Ca²⁺ influx through SACs. This influx is amplified by cell hyperpolarization, a calcium release by subplasmalemmal RyR1 and is then buffered by mitochondria. In two different PH rat models, the calcium response to stretch is enhanced due to hyper-reactivity of SACs and a greater calcium amplification by all RyR subtypes.

Conclusion
The spatial organization of RyR and calcium stores in PASMCs is important for cell signalling and plays a causal role in PH.

Keywords
Calcium signalling • Stretch-activated channel • Osmotic shock • Ryanodine receptor • Pulmonary hypertension

1. Introduction
The pulmonary circulation is involved in the control of the blood oxygenation by the lung. Characterized by a low pressure and high flow, the tone of this circulation is controlled by variations in the intracellular calcium concentration ([Ca²⁺]) within media pulmonary arterial smooth muscle cells (PASMCs).¹,² Whereas signalling pathways in response to hormonal stimuli such as endothelin-1 (ET-1) are relatively well described in intrapulmonary arteries (IPA),³ much less is known regarding calcium signalling pathways triggered by the mechanical forces developed by the blood flow.⁴ Yet, an increase in blood pressure is felt by SMC and induces their contraction, a phenomenon called myogenic tone.⁵ The latter is counteracted in IPA by phenomena such as vessel recruitment and distensibility in order to maintain a low value of pressure. However, under pathological conditions such as pulmonary hypertension (PH), the myogenic tone is increased and unmasked,⁶ maybe underlying different cellular signalling pathways.

While in IPA the myogenic tone mechanism was previously described in vessels,⁶ little is known about calcium signalling pathways induced by stretch at the cellular level. Plasma membrane stretch-activated channels (SACs) are able to transduce the mechanical signal of stretch into a contractile response since their activation allows a calcium influx from the extracellular medium to the cytosol.⁶–⁹ Although the calcium influx through SACs undoubtedly contributes to the stretch-induced [Ca²⁺]i increase and thus vessel contraction, we hypothesize that intracellular calcium stores in PASMCs could also be involved in this [Ca²⁺]i rise by a mechanism of calcium amplification. PASMCs display a specific intracellular organization of calcium stores and protein partners with segregated mitochondria, lysosomes, and proteins of the sarcoplasmic reticulum (SR). Calcium trafficking linked to SR stores is controlled by different proteins of the SR membrane: calcium release channels such as ryanodine receptors (RyR1, RyR2, and RyR3) and IP₃ receptors (IP₃R) as well as sarco/endoplasmic reticulum calcium ATPase pumps.
(SERCA2a and SERCA2b) that re-uptake calcium into the SR. This segregation supports specific signalling pathways as described for the ET-1 calcium response.

In this study, we investigate how the spatial organization of calcium stores is important for cell-signalling pathways in response to a mechanical stimulus, e.g. stretch. Furthermore, by using two classical animal models that mimic human PH, we examined whether this signalling pathway is altered during PH: (i) the chronically hypoxic (CH) rat that leads to PH with properties similar to those of human hypoxic PH and (ii) the monocrotaline-treated rat that leads to a severe PH.

2. Methods

An expanded Section 2 is available in Supplementary material online.

2.1 Animal models

Wistar male rats were bred in agreement with the Local Animal Care Ethics Committee (Comité d’Éthique Régional d’Aquitaine—referenced AP 2/11/2005; 200–350 g rats were randomly assigned into three groups: normoxic rats (Nx) were housed in ambient room air, CH rats were exposed 3 weeks in a hypobaric chamber (50 kPa) and monocrotaline rats (MCT) were intraperitoneally injected with 60 mg/kg of MCT. For experiments, rats were euthanized with i.p. injection of pentobarbital sodium (190 mg/kg).

2.2 PASMC isolation, electrophysiological recordings, and microspectrofluorimetric measurement of $[\text{Ca}^{2+}]$

PASMCs were obtained by enzymatic dissociation of media, as previously described. Voltage-clamp and current-clamp recordings were made with a standard patch-clamp technique, coupled to a microspectrofluorimetric indo-1 set-up. Cells were bathed in the 310 mOsmol solution. The $[\text{Ca}^{2+}]$-sensitive fluorescent probe indo-1 was used to record changes in $[\text{Ca}^{2+}]$, in cells. Mechanical stretch was performed by applying to the back end of the patch pipette a negative pressure of −40 mmHg by suction or by an osmotic shock with a hypo-osmotic 225 mOsmol solution (Supplementary material online, Table S1).

2.3 Fluorescence confocal imaging

In real-time fluorescence imaging, fluo-4/AM (2 μM), rhod-2/AM (1 μM), and fluo-5N/AM (10 μM) probes were used for measuring cytosolic ($[\text{Ca}^{2+}]$), mitochondrial ($[\text{Ca}^{2+}]_{\text{m}}$), and reticulum ($[\text{Ca}^{2+}]_{\text{SR}}$) calcium, respectively. An osmotic shock of 225 mOsmol was performed to induce a stretch. MitoTracker® Green FM (2 μM), LysoTracker® Red DND-90 (100 nM), and ER-Tracker™ Blue-White DPX (5 μM) were incubated in live cells for visualizing mitochondria, lysosomes, and SR, respectively. PASMCs were observed under a Nikon D-Eclipse C1 confocal scanning microscope.

2.4 Analysis of fluorescent labelling

For analysis, cells were subdivided into two defined areas that exclude the DAPI-labelled nucleus: the subplasmalemmal area ([S]) of 1.5 μm under the plasma membrane and the perinuclear area ([P]) within 1.5 μm of the DAPI nucleus, as previously described. When the nucleus was located close to the plasma membrane, this perinuclear/subplasmalemmal area was not analysed. For co-immunostaining, Pearson’s coefficient was used to determine the co-localization level with the Nis-Elements software. The co-localization was validated when the coefficient was > 0.80 in all tested cells (Supplementary material online, Figure S7).

2.5 Data and statistical analysis

Results are expressed as a mean ± SE of mean (SEM). Statistical analysis is performed on cells (the number n of tested responsive cells is indicated in each bar graph figure). N indicated the number of rats used for each experiment. The ANOVA test was performed to determine a statistical difference, differences with $P < 0.05$ or $P < 0.01$ were considered statistically significant. Student’s t-test was performed for Figure 2D and a two-way ANOVA for Figures 5 and 6D. All statistical analysis was performed with the Prism software (GraphPad).

3. Results

3.1 A calcium influx through SACs activates BKCa channels in PASMCs from Nx rats

In a cell-attached configuration, a stretch was induced by a negative pressure of −40 mmHg applied via the patch-clamp pipette to the plasma membrane of freshly isolated PASMCs from Nx rats (Nx-PASMCs). At −80 mV, a stretch induced a small inward unitary current (Figure 1A and B) that was blocked by the specific SAC blocker GsMTx-4 (5 μM) (Figure 1B and C). Several SACs were present under the patch-clamp pipette and thus multiple inward currents were recorded in the same time. At +80 mV, together with the SAC current, a large unitary outward current was recorded (Figure 1A–D), inhibited by Iberiotoxin (IbTx, 1 μM), a specific inhibitor of large conductance calcium-activated potassium channels (BKCa channels) (Figure 1D and E). In the absence of extracellular calcium, a stretch only activated SAC currents but not BKCa currents (Supplementary material online, Figure S1) pointing out that BKCa channels were not directly activated by stretch but by the calcium influx though SACs.

Simultaneously with patch clamp, $[\text{Ca}^{2+}]$, variations in cells were estimated with the indo-1/AM calcium probe. A membrane stretch induced a $[\text{Ca}^{2+}]$, increase which was concomitant with SAC and BKCa activities (Figure 1A, bottom). This $[\text{Ca}^{2+}]$, increase was reduced in the presence of IbTx (Figure 1F) and abolished in the absence of extracellular calcium (Supplementary material online, Figure S1). Altogether, these data suggest that a stretch induces a calcium influx through SACs which, in turn, directly activates BKCa channels since a depletion of internal calcium stores with cyclopiazonic acid (CPA, 10 μM), a SERCA inhibitor, did not alter BKCa activity observed after a stretch (Figure 1D and E).

3.2 Alteration of SACs and BKCa channel activities in PASMCs from PH rats

When the same stretch was applied in CH or MCT-PASMCs, the SAC current was enhanced in comparison with that observed in Nx-PASMCs (Figure 1B and C). In contrast, BKCa current was reduced in CH-PASMCs but unchanged in MCT-PASMCs (Figure 1D and E). The $[\text{Ca}^{2+}]$, increase observed after a stretch was greater in PASMCs from PH rats than in Nx ones (Figure 1F). This may suggest that PASMCs from PH rats present a hyper-reactivity to stretch. Same results were observed by measuring whole-cell currents after an osmotic shock of 225 mOsmol (Supplementary material online, Figure S2), a common used protocol used for stretch cells.

The activation of BKCa channels by an osmotic shock hyperpolarized the plasma membrane potential of Nx-PASMCs. This cellular hyperpolarization was reduced in CH-PASMCs in accordance with the reduced activity of BKCa channels previously observed (Figure 1E), but unchanged in MCT-PASMCs (Supplementary material online, Figure S3).
Figure 1 A calcium influx through SAC activates BKCa channels in Nx and PH PASMCs. Stretch is applied by a depression of −40 mmHg in a patch-clamp pipette in a cell-attached configuration. (A) Top: Voltage-clamp protocol. Middle: In a Nx-PASMC, in a cell-attached configuration, a stretch induces inward currents (through SAC at −80 mV) followed by outward currents (through BKCa channels at +80 mV). Bottom: simultaneously, the stretch induces a 
$[Ca^{2+}]_{i}$ increase. (B and D) Example of unitary inward (B) and outward currents (D) before and after a stretch in Nx, CH, and MCT-PASMCs, in the absence or presence of GsMTx-4 (5 μM), IbTx (1 μM), or CPA (10 μM). (C and E) Summary of the open probability of unitary inward (C, N = 3–5) and outward currents (E, N = 3–5), after a stretch. (F) Amplitude of the calcium peak induced by a stretch is enhanced in CH or MCT-PASMCs when compared with Nx ones (N = 3–5). *Significant difference for $P < 0.05$ with the Nx condition.
Osmotic shocks increase $[\text{Ca}^{2+}]_i$ in a subplasmalemmal area which is buffered by subplasmalemmal mitochondria. (A) Mitochondria, lysosomes, and SR are stained in green, red, and blue, respectively. In Nx-PASMCs, mitochondria are present in a subplasmalemmal area, whereas lysosomes are mostly present around the nucleus. In CH or MCT-PASMCs, mitochondria and lysosomes are distributed in the whole-cell area. N indicates nucleus locations. Scale bar: 10 µm. (B) Panel series of images of fluo-4/AM ($[\text{Ca}^{2+}]_i$ in green) and rhod-2/AM (mitochondrial calcium ($[\text{Ca}^{2+}]_m$) in red) recorded in an isolated CH-PASMC after an osmotic shock of 225 mOsmol at the confocal microscope. N indicates the nucleus, [S] the subplasmalemmal calcium area, and [P] the perinuclear calcium area. (C) Curve representing the $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ as the ratio $F/F_0$ during time. (D) Summary of the amplitude of the calcium peak observed in Nx ($N = 4–8$), CH ($N = 5$), and MCT ($N = 5$) rat PASMC subplasmalemmal (S) and perinuclear (P) areas. Osmotic shocks in Nx and CH PASMCs or an acute application of CPA (10 µM, 20 s) induce a greater $[\text{Ca}^{2+}]_i$ in the subplasmalemmal area than in the perinuclear one. An acute application of ET-1 (100 nM, 20 s), TG (1 µM, 20 s), or osmotic shocks in MCT-PASMCs induce a calcium rise simultaneously and in equal concentration in the whole cell (both S and P areas). *Significant difference for $P < 0.05$ between the subplasmalemmal and perinuclear areas. NS, non-significant.
3.3 SR calcium stores are involved in the stretch-induced calcium response

We further studied the involvement of intracellular calcium stores in stretch-induced \([\text{Ca}^{2+}]_i\) increase. First, we examined the subcellular segregation of each calcium stores using live fluorescent probes. In Nx-PASMCs, mitochondria (in green) were present in the subplasmalemmal area and lysosomes (in red) were mostly present around the nucleus (N), forming two distinct clusters, whereas the SR was distributed in the whole-cell area (in blue) (Figure 2A). In contrast, in PH-PASMCs, mitochondria and lysosomes are distributed both in subplasmalemmal and perinuclear areas (Figure 2A).

Secondly, real-time fluorescence imaging for \([\text{Ca}^{2+}]_i\) in the cytosol ([C(2+)]_j) and mitochondria ([Ca2+]_m) was measured by a confocal microscope using fluo-4/AM and rhod-2/AM (Supplementary material online, Figure S4), respectively. \([\text{Ca}^{2+}]_i\) increases were measured in two distinct areas: subplasmalemmal (S) and perinuclear (P) (Figure 2B). In Nx-PASMCs, a stretch induced by an osmotic shock (225 mOsmol solution) increased \([\text{Ca}^{2+}]_i\) of 58 ± 18% in the subplasmalemmal area, which was followed by a minor perinuclear calcium increase of 10 ± 6% (Figure 2D and Supplementary material online, Figure S4B). Simultaneously with the cytosolic calcium rise, the \([\text{Ca}^{2+}]_m\) was increased in subplasmalemmal mitochondria (Figure 2B and C). This indicates that mitochondria do not participate to the stretch-induced \([\text{Ca}^{2+}]_i\) rise but rather buffer it.

Thirdly, to investigate the implication of the SR calcium in the stretch-induced \([\text{Ca}^{2+}]_i\), increase, \([\text{Ca}^{2+}]_m\), variations were measured with the ratiometric calcium probe indo-1/AM. An osmotic shock induced a rapid calcium increase in Nx-PASMCs (Figure 3A). This \([\text{Ca}^{2+}]_m\), response was strongly reduced by GsMTx-4 (5 \(\mu\)M) and completely abolished in the absence of extracellular calcium (Figure 3B). Ryonadine (an inhibitor of all RyR subtypes, 100 \(\mu\)M) and dantrolene (RyR1 and RyR3 antagonist, 18 \(\mu\)M) (Figure 3A and B) reduced osmotic shock-induced \([\text{Ca}^{2+}]_m\), increase of 24%. In contrast, the calcium response to an osmotic shock remained unaffected in the presence of the IP3R antagonist xestospongin C (1 \(\mu\)M) or L-type voltage gated calcium channel (VGCC) inhibitor nicardipine (1 \(\mu\)M) (Figure 3B). Likewise, lysosomes are not implicated in this calcium response since blockade of lysosomal calcium channels with trans-Ned-19-19-20 (100 \(\mu\)M) did not modify the \([\text{Ca}^{2+}]_m\), response (Figure 3B). These data indicate that RyR and, more precisely, RyR1 or RyR2, are implicated in the stretch-induced \([\text{Ca}^{2+}]_i\), increase via a calcium-induced calcium release mechanism (CICR) initiated by the calcium influx through SACs.

3.4 Calcium amplification by SR is enhanced in MCT but not in CH-PASMCs

In PH rats, the calcium response to an osmotic shock was higher in both CH and MCT rats, and even higher in MCT rats than in CH rats (Figure 3D–F). As for Nx-PASMCs, calcium responses in PH-PASMCs were reduced by GsMTx−4, ryanodine, and dantrolene but also by xestospongin C (Figure 3D–F). Furthermore, RyR and IP3R antagonists reduced the calcium response by 28% in CH-PASMCs but by 62% in MCT-PASMCs, whereas it was reduced by 24% in Nx-PASMCs (Figure 3). These data indicate that amplification by RyR and IP3R is more important in PASMCs from MCT rats, than from CH or Nx rats. In accordance with this greater calcium amplification by SR observed in MCT-PASMCs (Figure 3F), an osmotic shock induced a \([\text{Ca}^{2+}]_i\), increase both in subplasmalemmal and perinuclear areas (64 ± 16% and 52 ± 14%, respectively) (Figure 2D and Supplementary material online, Figure S4C). In contrast, in CH-PASMCs, the \([\text{Ca}^{2+}]_i\), increased by 117 ± 23% in the subplasmalemmal area was followed by a perinuclear increase by 23 ± 13% (Figure 2B–D). As observed for Nx rats, \([\text{Ca}^{2+}]_i\), increased after an osmotic shock in PH-PASMCs (Figure 2C and Supplementary material online, Figure S4C).

3.5 RyR and SERCA2 pumps are segregated in PASMCs from N and PH rats

To better understand the involvement of RyR subtypes in local calcium variations observed after an osmotic shock, we examined their subcellular segregation in freshly isolated PASMCs. Because SR calcium homoeostasis is also controlled by the activity of SERCA2 pumps, co-immunostaining for RyR subtypes (RyR1, RyR2, and RyR3) and SERCA pumps (SERCA2a and SERCA2b) was performed.

In Nx-PASMCs, the three RyR subtypes expressed were differentially distributed. RyR1 was the only subtype present in the subplasmalemmal area and did not co-localize with RyR2 (Figure 4A) and RyR3 (Figure 4B) that were present in the whole cell and in the perinuclear region, respectively. SERCA2b pumps were present in a subplasmalemmal region and co-localized with RyR1 (Figure 4C), whereas SERCA2a pumps were in the perinuclear region, and co-localized with RyR3 (Figure 4D). SERCA2a and SERCA2b formed two distinct clusters and they did not co-localize together (Figure 4E).

In CH-PASMCs, the labelling for RyR1 and RyR2 was the same as in Nx rats (Figure 4A). However, RyR3 had both a perinuclear and a subplasmalemmal labelling, even if, in this latter area RyR3 did not co-localize with RyR1, still forming two distinct clusters (Figure 4B). Besides, RyR1 still co-localized with SERCA2b in the subplasmalemmal level (Figure 4C) and RyR3 with SERCA2a both in perinuclear and subplasmalemmal areas (Figure 4D). In contrast, the subcellular expression of RyR and SERCA2 in MCT-PASMCs was completely different since RyR and SERCA2 were redistributed in the whole-cell SR without any defined pattern. That is, RyR1 co-localized with RyR2, RyR3, and SERCA2b in the cell and RyR3 co-localized with SERCA2a at both perinuclear and subplasmalemmal levels. Figure 4F schematically summarizes the distribution of RyR and SERCA2 in Nx, CH, and MCT rats and statistical analysis for localization and co-localization is illustrated in Supplementary material online, Figures S6 and S7.

3.6 RyR1 and SERCA2b are involved in the calcium response to an osmotic shock in Nx and CH rats

Immunolabellings in Nx-PASMCs have shown that RyR1 is associated with SERCA2b in the subplasmalemmal area, whereas RyR3 is associated with SERCA2a in the segregated perinuclear zone (Figure 4), forming two discrete stores. A previous study has shown that, in PASMCs, CPA preferentially inhibits one SR store refilled by the SERCA2b pump, whereas thapsigargin (TG), a distinct SR store, refilled by SERCA2a13 (Supplementary material online, Figure S8). Yet, acute application of CPA (10 \(\mu\)M) increased \([\text{Ca}^{2+}]_i\), mainly in a subplasmalemmal area, whereas TG (1 \(\mu\)M) elicited also a calcium rise in the perinuclear region (Figure 2D). Therefore, CPA is able to empty RyR1-containing SR calcium stores and TG to empty RyR3-containing SR calcium stores.

Figure 5 shows that an osmotic shock-induced \([\text{Ca}^{2+}]_i\), increase was reduced by CPA but not by TG, indicating that the RyR subtype involved in the CICR is the RyR1 subtype.

In contrast to osmotic shock-induced calcium responses, ET-1 which increases \([\text{Ca}^{2+}]_i\), via a RyR3-dependent pathway induced a calcium...
Figure 3 Involvement of the SR in calcium responses to an osmotic shock in Nx, CH, and MCT rats. Original traces (A, C, and E) and summary bar graph responses [(B) (N = 4–15), (D) (N = 4–15), and (F) (N = 4–6)] of an osmotic shock-induced calcium response measured with indo-1 in Nx-PASMCs (A and B) CH-PASMCs (C and D) and MCT-PASMCs (E and F); with 2 mM extracellular calcium. For all rats, calcium responses are significantly smaller in the presence of GsMTx-4 (5 μM), ryanodine (RYA, 100 μM), dantrolene (DT, 10 μM) but not altered with trans-Ned-19 (Ned-19, 100 μM), nicardipine (1 μM). In Nx-PASMCs, the responses are completely abolished in the absence of extracellular calcium (+1 mM EGTA). In CH-PASMCs and MCT-PASMCs, calcium responses are also reduced by Xestospongin C (XestoC, 1 μM) and methyl-β-cyclodextrin (MβCD, 5 mM). *Significant difference for P < 0.05 with the condition without any inhibitor for Nx, CH, MCT-PASMCs. #Significant difference for P < 0.05 with the Nx condition, $with the CH condition. NS, non-significant.
Figure 4  Immunolabelling of RyR and SERCA pumps in Nx, CH, and MCT rat PASMCs. Images showing the distribution labelling of RyR1 (green), RyR2 (red), RyR3 (red), SERCA2a (green) and SERCA2b (red), and co-immunostainings between these proteins, as indicated [(A) RyR1 and RyR2; (B) RyR1 and RyR3; (C) RyR1 and SERCA2b; (D) SERCA2a and RyR3; (E) SERCA2a and SERCA2b] in PASMCs from Nx, CH, and MCT rats. Arrow heads indicate some staining points. Nucleuses are stained in blue by DAPI. Scale bar: 10 μm. In Nx PASMCs, RyR1 co-localize with SERCA2b in the subplasmalemmal area, whereas RyR3 co-localize with SERCA2a in the perinuclear area. In CH PASMCs, RyR1 co-localize with SERCA2b in the perinuclear area in distinct clusters than RyR3 and SERCA2a which are present both in perinuclear and subplasmalemmal levels. In MCT-PASMCs, RyR subtypes and SERCA2 pumps are expressed in both areas. (F) Schematic representation of the distribution of RyR subtypes and SERCA2 pumps summarizing immunostaining from (A) to (E).
increase which was only inhibited by TG and not by CPA (Supplementary material online, Figure S5).

In CH-PASMCs, although RyR1 and RyR3 were present in the sub-plasmalemmal area, they constituted two different clusters and each subtype was still associated to a specific SERCA pump, as in Nx rats. Thereby, CPA was still able to empty RyR1 SR calcium stores and TG, RyR3 ones. Osmotic shock-induced [Ca\(^{2+}\)], responses were only reduced by CPA but not by TG (Figure 6), showing that, in PASMCs from CH rats, RyR1 is, again, the RyR subtype involved in the CICR mechanism. In MCT-PASMCs, where the subcellular localization of RyR subtypes and SERCA pumps is diffused and where the CICR is greater, both TG and CPA were able to reduce by 62% calcium responses to an osmotic shock (Figure 5).

3.7 Another calcium store is present in PASMCs from PH rats

When an osmotic shock is applied in the absence of extracellular calcium, no calcium response is observed in Nx-PASMCs (Figure 3), but in CH-PASMCs, a calcium increase is measured (Figure 6). This calcium increase is reduced by GsMTx-4, ryanodine, dantrolene, xestospongin C, CPA, and TG but not by nicardipine, a Ca\(^{2+}\) and Na\(^{+}\) free solution or trans-Ned-19 (Figure 6B). SR calcium is implicated in this response because an osmotic shock induced a depletion of SR calcium (measured with the fluo-5N probe) in the absence of extracellular calcium only on CH rats (Figure 6D). Finally, we observed that caveolae are essential in these calcium responses, because they were fully inhibited when cells were pre-treated with the caveolae disruptor MβCD (5 mM, Figure 6A and B). These data indicate that an additional mechanism is present in PH rats involving caveolae. This was further confirmed by the fact that an osmotic shock-induced [Ca\(^{2+}\)] response in the presence of extracellular calcium was also strongly reduced by MβCD in PH-PASMCs (Figure 3D), but not in Nx-PASMCs (Figure 3B).

4. Discussion

We report that the specific spatial organization of calcium stores is important for specific calcium responses, according to the stimulus. Our study also reveals that calcium responses to stretch are increased under pathological conditions such as PH and that this increase is associated with a different spatial distribution of RyR subtypes.

Stretch of PASMCs by either a patch-clamp pipette or an osmotic shock activates SACs, as we previously described. Stretch induces a calcium entry through SACs that directly activate I\(\text{bTX}\)-sensitive potassium channels (BK\(\text{Ca}\) channels). The potassium efflux through BK\(\text{Ca}\) channels leads to cell hyperpolarization and increases the electrochemical gradient for calcium ions. By this mean, when SACs are activated and PASMC stretched, the hyperpolarization amplifies the calcium entry through SACs and thus cell contraction. Stretch-induced hyperpolarization also explains why VGCC is not implicated in the stretch-induced calcium response, as revealed by the absence of the effect of VGCC antagonist nicardipine on stretch-induced [Ca\(^{2+}\)] increase.

In PASMCs, we show that RyR1 is implicated in calcium release from the SR by a CICR mechanism after a stretch, RyR1 associated with SERCA2b is only present in the sub-plasmalemmal SR, whereas RyR3 and SERCA2a are expressed within the depth of the cell, around the nucleus. A similar subcellular localization pattern of RyR1/2 and RyR3 has been described in airway smooth muscle cells, but it is not ubiquitous in smooth muscle cells as in cerebral arteries RyR2 is the most expressed isoform in the sub-plasmalemmal area. This segregation between RyR1 and RyR3 has previously been described in PASMCs, but we extend these findings by showing that RyR3 is actually present in a different cluster from RyR1 since they do not co-localize together. Thereby, this spatial segregation of RyRs accounts for specific signalling pathways. For example, ET-1 allows a calcium leak from perinuclear lysosomes which is amplified by perinuclear RyR3. We confirmed that the ET-1-induced calcium response is inhibited only when RyR3-associated calcium stores are emptied using TG. In contrast, our experiments show that the stretch amplification response by RyR1 is only inhibited by CPA; the exact opposite response to what is observed for ET-1. To confirm the involvement of RyR1 and SERCA2b, we also show that acute application of CPA or osmotic shock induces a calcium rise mostly in the sub-plasmalemmal region, where only RyR1 is expressed and where the contractile apparatus is located in freshly isolated PASMCs (Supplementary material online, Figure S4D). Besides, it is noteworthy that previous results in PASMCs...
Figure 6 [Ca\(^{2+}\)] of responses to osmotic shocks in PASMCs from PH rats in the absence of extracellular calcium. (A) [Ca\(^{2+}\)] responses measured by indo-1 recorded in a CH rat PASMC following an osmotic shock of 225 mOsmol in the absence of extracellular calcium +1 mM EGTA and with methyl-β-cyclodextrin (MβCD). (B and C) Summary of the amplitude of the calcium peak observed after an osmotic shock in PASMCs from CH (B: N = 3–10; C: N = 4–10). (D) Curve representing the [Ca\(^{2+}\)]\(_{SR}\) as the ratio F/F\(_0\) during time after an osmotic shock. [Ca\(^{2+}\)]\(_{SR}\) is measured by a confocal microscope with the fluo-5N probe. The [Ca\(^{2+}\)]\(_{SR}\) decrease after an osmotic shock in CH-PASMCs (N = 6, N = 3) but not in Nx-PASMCs (N = 6, N = 3).
indicating that RyR2 is involved in TRPV4 channel-dependent calcium responses.  

Finally, the subcellular organization of other calcium stores than SR (mitochondria and lysosomes) also contributes to the specificity of signalling pathways. In freshly isolated PASMCs, we show that mitochondria are mostly located in a subplasmalemmal area, whereas lysosomes are present in the perinuclear zone. Mitochondria do not contribute to the calcium increase induced by a stretch but rather reduce the calcium spread by buffering the cytosolic calcium, probably to maintain the calcium homoeostasis. Besides, lysosomes do not participate to the stretch-induced [Ca$^{2+}$]i rise but rather to the calcium signalling pathway for ET-1, as previously described.  

These results were obtained in freshly isolated cells whose shape is round, compared with the more elongated shape observed in intact vessels. This change in the morphology of cells is linked to the dissociation process. 

With respect to pathophysiological conditions such as PH, PASMCs, from both CH and MCT rats exhibit higher calcium responses to stretch, leading to a greater contraction of IPA, as previously shown. This increased calcium response is due to a combination of three factors. (i) SACs from PH rats are hyper-responsive to a membrane stretch and allow a higher calcium influx. (ii) The CICR amplification by the SR is enhanced. In MCT-PASMCs, all RyR subtypes and SERCA2 pumps are redistributed in the whole-cell area and participate to a greater amplification by 62% instead of 24% in Nx-PASMCs. Previous studies show that MCT rats develop a more severe PH than CH rats and this may result from this stronger reorganization of SR calcium proteins. Furthermore, there is also a calcium amplification by IP$_3$Rs. A likely hypothesis is that calcium itself, because of its larger concentration, could activate IP$_3$Rs which are calcium sensitive. (iii) In CH-PASMCs, the amplification mechanism is different due to a specific coupling between SACs and RyR. The calcium increase in the subplasmalemmal level in CH-PASMCs is able to only activate RyR1 but not RyR3 (since only CPA, and not TG, is able to reduce calcium responses; Figure 5). A hypothesis could be that SACs and RyR1 are strongly coupled in CH rats. Caveolae are good candidates to participate to this coupling by forming a complex microenvironment adequate for bringing closer membrane SACs and SR RyR1. We show in CH-PASMCs, that the caveolae disrupter MβCD strongly reduces stretch-induced calcium responses (but not in Nx-PASMCs), possibly by separating SACs and RyR1. If SACs are physically coupled to RyR1, a change in SAC conformation will subsequently activate RyR1 leading to calcium leak from the subplasmalemmal SR, without the contribution of extracellular calcium, as we shown (Figure 6). Depletion of a SR calcium store (measured with the fluo-5N probe) during stretch without extracellular calcium confirm that SACs could be coupled to SR by a mechanism different of CICR. Similar calcium responses to an osmotic shock in the absence of extracellular calcium were previously observed in CH-PASMCs, but we further show that these responses are completely abolished with the caveolae disrupter MβCD. Furthermore, in MCT rats IPA and in human PH, caveolin-1 expression is augmented in SMC, suggesting that caveolae are important in the pathogenesis of PH and can participate to increase the myogenic tone.  

Altogether, these findings suggest that, for each stimulus, PASMCs can mobilize a segregated calcium store independently and produce a biological response precisely adapted to the stimulation received by the pulmonary artery (summarized in Figure 7). Indeed, we have shown by co-immunostaining and functionally experiments that (i) in the...
subplasmalemmal area, RyR1 is associated with SERCA2b and mitochondria to respond to a mechanical stretch stimulus, whereas (ii) in the perinuclear region, RyR3 is associated with SERCA2a and lysosomes which are recruited by ET-1. Moreover, in PH-PASMCs, the equilibrium between the stimulus and the biological response is shifted to a greater calcium increase and in fine, to a larger IPA contraction mainly due to a hyper-reactivity of SAC, a reorganization of all calcium stores and caveole.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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