which should be cited to refer to this work.

Up-regulation of ryanodine receptor expression increases the calcium-induced calcium release and spontaneous calcium signals in cerebral arteries from hindlimb unloaded rats

Jean-Luc Morel · Fabrice Dabertrand · Yves Porte · Anne Prevot · Nathalie Macrez

Abstract Microgravity induces a redistribution of blood volume. Consequently, astronauts' body pressure is modified so that the upright blood pressure gradient is abolished, thereby inducing a modification in cerebral blood pressure. This effect is mimicked in the hindlimb unloaded rat model. After a duration of 8 days of unloading, Ca^{2+} signals activated by depolarization and inositol-1,4,5-trisphosphate intracellular release were increased in cerebral arteries. In the presence of ryanodine and thapsigargin, the depolarization-induced Ca^{2+} signals remained increased in hindlimb suspended animals, indicating that Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release mechanism were both increased. Spontaneous Ca^{2+} waves and localized Ca^{2+} events were also investigated. Increases in both amplitude and frequency of spontaneous Ca^{2+} waves were measured in hindlimb suspension conditions. After pharmacological segregation of Ca^{2+} sparks and Ca^{2+} sparklets, their kinetic parameters were characterized. Hindlimb suspension induced an increase in the frequencies of both Ca^{2+} localized events, suggesting an increase of excitability. Labeling with bodipy compounds suggested that voltage-dependent Ca^{2+} channels and ryanodine receptor expressions were increased. Finally, the expression of the ryanodine receptor subtype 1 (RyR1) was increased in hindlimb unloading conditions. Taken together, these results suggest that RyR1 expression and voltage-dependent Ca^{2+} channels activity are the focal points of the regulation of Ca^{2+} signals activated by vasoconstriction in rat cerebral arteries with an increase of the voltage-dependent Ca^{2+} influx.

Keywords Calcium-induced calcium release · Calcium spark · Spontaneous calcium signal · Hindlimb suspension · Microgravity · Cerebral artery · Ryanodine receptor

Abbreviations

CICR	Ca^{2+}-induced Ca^{2+} release mechanism
HU	Hindlimb unloaded
CTL	Control conditions (i.e., not hindlimb unloaded animals)
InsP3R	Inositol 1,4,5-trisphosphate receptor
RyR	Ryanodine receptor
RyR1	Ryanodine receptor subtype 1
VSMC	Vascular smooth muscle cells
μG	Microgravity
NO	Nitric oxide

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Introduction

After only 1 week in space, blood volume is redistributed by microgravity (μG). The blood pressure gradient from 70 mmHg in the head to 200 mmHg in the legs and feet is replaced by a constant pressure near 100 mmHg in the whole body [18]. This modification could induce cardiac and vascular adaptations to regulate blood/tissues exchanges. Thus, an increase in blood pressure in vessels above the heart appears to be a consequence of μG, requiring the adaptation of the vascular system [52]. The vascular adaptation to μG likely involves a response of factors known to regulate blood pressure, including: (1) control of plasma volume [37]; (2) modulation of baro- and cardiopulmonary reflexes inducing the decrease of cardiac function [19]; and finally (3) adaptation of intrinsic properties of vascular endothelial and smooth muscle cells (VSMC). We have focused our study on this last factor and used the hindlimb unloaded (HU) rat model as it simulates the biological effects of μG; especially, modulations of vascular reactivity were described in this model [7, 28, 39, 53].

Vascular reactivity and tone are regulated by complex Ca^{2+} signals. Vasoconstriction is regulated by propagated Ca^{2+} signals in VSMC that are triggered by the Ca^{2+} influx or the activation of inositol 1,4,5-trisphosphate receptors (InsP3R) [24]. Both triggering events are amplified by the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism that is responsible for the release of Ca^{2+} from the sarcoplasmic reticulum through activation of Ryanodine receptors (RyR) [6, 21, 30]. Vasorelaxation is partially due to the activation of Ca^{2+}-activated K + currents regulated by localized Ca^{2+} events named Ca^{2+} sparks, encoded by ryanodine receptor subtype 1 (RyR1) and 2 (RyR2) in VSMC [8, 22, 35, 43]. Membrane depolarization, which is responsible for the Ca^{2+} entry, is principally due to the voltage-dependent L-type Ca^{2+} channels (VDCC), which opening can also activate localized Ca^{2+} events named Ca^{2+} sparklets [34, 44], that can in turn trigger both Ca^{2+} sparks and Ca^{2+} waves [2] and can modify transcription [36]. Theoretically, the variations of the membrane potential and the intracellular Ca^{2+} store content could spontaneously activate Ca^{2+} sparklets and Ca^{2+} sparks but also Ca^{2+} waves to assume the vascular tone and the spontaneous vascular contraction [1, 33].

A regulation of Ca^{2+} signals by μG and HU involving RyR function and expression has been previously shown but not in cerebral arteries [12, 29]. A decrease in K + currents and an increase in L-type currents have been proposed to explain respectively the increase in both myogenic tone and in part contraction [16, 51]. Therefore, the present study was designed to investigate the modulations of localized and propagated, as well as spontaneous and activated Ca^{2+} signals. Our results indicate that, in cerebral arteries, HU induced an increase in RyR1 expression and VDCC function that potentiated all spontaneous Ca^{2+} signals. HU also reshaped the CICR mechanism involved in vascular contraction likely to adapt the vascular reactivity to the increase in blood pressure solicitation.

Methods

Rats

The present investigations conform to the European Community and French guiding principles for the care and use of animals. Authorization to perform animal experiments (C33 10 029) was obtained from the Ministry of food and agriculture, direction of animal protection (France) and the protocol was validated by the Ethics committee (n°5012-0138-A). Wistar male rats (120–150 g 20 animals in control [CTL]) conditions; 20 animals in HU conditions, and finally nine animals for the experiment about the recovery of HU) were euthanized by cervical dislocation. Brains were immediately withdrawn and placed in PBS. In the following 10 min, middle cerebral arteries (MCA) were dissected and placed in M199 culture medium to be used for Ca^{2+} measurements and in appropriate buffer for RNA extractions.

Hindlimb unloading

Rats were HU by tail attachment during 8 days with a 30–40° angle in accordance with recommendations published by the National Aeronautic and Space Agency (NASA) [31]. The suspension duration was chosen because of significant modifications of soleus wet weight [40] and vascular adaptation [28] without any modification of body weight.

RT-PCR

Total RNA was extracted using the RNA preparation kit from Epicentre (Madison, WI, USA) following the instructions of the supplier and experiments were performed as described previously [17]. Sets of sense and antisense primer pairs specific for RyR1, RyR2 and RyR3 were used as previously described [8], and the expression of each RyR subtype was quantified by relative quantitative PCR (qPCR) performed with CFX96 thermal cycler and software (Bio-Rad). The normalization of RyR expression was realized with rat samples after verification of the stability of reference gene expression with GeNorm algorithms; actin and subunit A of the succinate dehydrogenase (SDHA) complex were chosen as references.

Cytosolic Ca^{2+} measurements on cerebral arteries

Pial arborescence from MCA was micro-dissected and placed on glass slides coated with Cell-Tak™ to improve their adherence (BD biosciences, Le Pont de Claix, France), and incubated in M199 medium with Ca^{2+} indicators for 10 min at 37 °C

and 20 min at room temperature. Fluo8L-AM (K_d=1, 860 nmol/l), Fluo8-AM (K_d=390 nmol/l) and Fluo8H-AM (K_d=232 nmol/l) were used to record mediator-activated Ca^{2+} responses, spontaneous Ca^{2+} oscillations and localized Ca^{2+} events (Ca^{2+} sparks and Ca^{2+} sparklets), respectively. After this time, the glass slides were mounted in an experimental chamber and perfused with a physiological solution. Agonist-evoked Ca^{2+} responses were recorded indifferently with the MRC1024ES (Bio-Rad) and SP5 (Leica) confocal microscopes. Fluorescence was analyzed in each cell individually and only cells with clearly identified edges were used. All experiments were carried out at 26 ±1 °C. Spontaneous Ca^{2+} activity was recorded with the confocal TCS SP5 system (Leica-Microsystems, Nanterre, France); preparations were scanned at the speed acquisition of one image per 140 ms in an image series mode.

Flash photolysis

Cerebral arteries were loaded with the permeant form of InsP3 (cag-iso-2-145-InsP3, 5 μmol/l in M199) during 30 min at 37 °C before the Fluo8-AM loading. Photolysis produced by the xenon flash lamp (1 ms pulse) has been described previously [28]. The flash intensity was adjusted from 25 V to 250 V in these experiments to release InsP3.

Fluorescent binding using bodipy labeled pharmacological agents

Cerebral arteries used in Ca^{2+} measurements were perfused during 15 min with 100 nmol/l of ST-bodipy(−)-DHP and imaged with the TCS SP5 confocal system using the 535-nm excitation wavelength. The emitted light was collected between 580 and 620 nm. The labeling was measured in the 20 consecutive optical sections framing the Ca^{2+} measurement focal plane with a pitch of 1 μm. The fluorescence of each section was measured for each cell and compiled to compare the labeling of L-type voltage-gated Ca^{2+} channels in both control and HU conditions. Non-specific fixation of the ST-bodipy(−)-DHP was determined by simultaneous incubation of oxodipine 10 μmol/l and ST-bodipy(−)-DHP 100 nmol/l. A similar protocol was applied to evaluate the binding of FL-bodipy-thapsigargin (1 μmol/l) and FL-X- bodipy-ryanodine (100 nmol/l). The arteries used for this experiment were not loaded with the Ca^{2+} probe because of the overlap of the excitation wavelength (488 nm) and the window of emitted light retrieval (495–545 nm). The non-specific fixation of FL-bodipy-thapsigargin and FL-X- bodipy-ryanodine were respectively determined by simultaneous incubation of unlabelled thapsigargin (10 μmol/l) and FL-bodipy-thapsigargin, as well as unlabelled ryanodine (10 μmol/l) and FL-X-bodipy-ryanodine. All parameters of the confocal microscope setup were kept constant for all experiments.

Western blot analysis

The protocols used were previously described [9]. Pooled MCA were homogenized in 50 μl of PBS 10 % SDS solution. The supernatant was collected and equal amounts of proteins (10, 20 and 40 μg) from control and HU rats were heated at 95 °C for 5 min in Laemmli buffer, separated by electrophoresis in a 4–12 % gradient SDS-polyacrylamide gel (GeBaGel), and electrically transferred to a polyvinylidene difluoride (PVDF) membrane (70 min, 100 V, 4 °C). Non-specific binding was blocked by incubating the membrane in phosphate buffer–Tween 20 (0.1 %) containing 5 % nonfat dry milk for 1 h, and blots were incubated (overnight, 4 °C) with anti-RyR1, anti-RyR2 or anti-RyR3 (1:1,000). The primary antibody was detected with a horseradish peroxydase-coupled secondary antibody (1:3,000). The signal was detected using an enhanced chemoluminescence kit (GE Healthcare, Saclay, France). After revelation, the membrane was striped by incubation in 0.7 mmol/l β-mercaptoethanol and 0.4 % SDS solution (30 min, 55 °C), and revealed with anti-actin (1:1,000) antibody. All images were quantified using the KDS1D 2.0 software.

Data analysis

Localized Ca^{2+} events kinetic parameters were analyzed with spike2 software with a script written by Blaise Yvert (Institut des Neurosciences Cognitives et Intégratives d'Aquitaine, Bordeaux, France). Statistical analyses were produced with Graphpad prism software. Significance between two different conditions was tested using Student's *t*-test and ANOVA. *P* values <0.05 were considered significant and respectively completed with Tukey and Bonferroni post-hoc tests when needed. To compare the Ca^{2+} signals and ST-bodipy(−)-DHP labeling, the experiments were paired: arteries from one control and one HU animal were used the same day, loaded with the same fluorescent probe solution following exactly the same protocol (incubation duration, used solutions, etc.).

Chemicals and drugs

Fluo-8-AM, Fluo8L-AM, Fluo8H-AM and PBS were from Interchim (Montluçon, France). Caffeine was from Merck (Darmstadt, Germany). FL-bodipy-thapsigargin, ST-bodipy(−)-DHP, FL-X-bodipy-ryanodine, Medium M199, streptomycin and penicillin were from Invitrogen (Cergy Pontoise, France). Anti-RyR1-3 antibodies were from Millipore (Saint-Quentin en Yvelines, France). Cag-iso-2-145-InsP3 [D-2,3-*O*-isopropylidene-6-*O*-(2-nitro-4,5-dimethoxy)benzyl-myo-inositol 1,4,5-trisphosphate-Hexakis (propionoxymethyl) ester] was synthesized by SiChem (Bremen, Germany). Oxodipine was a gift from Dr. Galiano (IQB, Madrid, Spain). All other chemicals were from Sigma (Lyon, France).

Results

HU induces an increase in VDCC-dependent Ca^{2+} responses in MCA by increasing DHP receptor activity

To isolate the Ca^{2+} signal evoked by VDCC activation from the sarcoplasmic Ca^{2+} release, experiments were performed in the presence of 1 μmol/l thapsigargin, a SERCA inhibitor which neutralizes Ca^{2+} store refilling. Caffeine was classically used to activate RyR by decreasing the threshold of Ca^{2+} activation under the basal intracellular Ca^{2+} concentration. The absence of caffeine (10 mmol/l)-induced Ca^{2+} response was used to assess the inhibition of the sarcoplasmic Ca^{2+} store (Fig. 1a). The Ca^{2+} responses evoked by thapsigargin (Fig. 1a) were identical in both CTL and HU conditions (2.75 ±0.15, n =42 vs. 2.70±0.13, n =47, respectively). This result suggests that the Ca^{2+} store refilling was not different between CTL vs. HU. We also verified the expression level of SERCA by FL-bodipy-thapsigargin binding. The expression of SERCA was not modified by HU conditions (mean fluorescence of specific binding in CTL conditions: 21±2.2 vs. 18± 2.1 AU/μm^3 in HU conditions; n =37 cells in four different experiments).

In order to activate the depolarization of cerebral artery that further stimulates maximally L-type voltage dependent Ca^{2+} channels, the application of KCl (140 mmol/l) was performed to depolarize VSMC. In the presence of thapsigargin, KCl induced a larger Ca^{2+} response in cerebral arteries from HU animals compared to CTL animals, suggesting an up-regulation of the function or expression of L-type Ca^{2+} channels in HU conditions.

To further investigate the increase in KCl-induced Ca^{2+} responses, we labeled the cerebral arteries with ST-bodipy(−)-DHP and evaluated the expression level of L-type voltage-gated channels, also known as DHP receptors. We firstly verified the ability of ST-bodipy(−)-DHP (100 nmol/l) to inhibit the residual Ca^{2+} response induced by KCl in the presence of thapsigargin (not shown), and secondly we measured the fluorescent binding of ST-bodipy(−)-DHP on VSMC previously used for Ca^{2+} measurement. As illustrated in Fig. 1b, the VSMC within the arterial wall were peripherally labeled with ST-bodipy(−)-DHP in CTL rats, while its binding was doubled in HU rats, suggesting that DHP receptors were effectively modified at the expression level.

The depolarization induced by the application of KCl (140 mmol/l) activated a Ca^{2+} influx that was increased by the CICR mechanism implicating RyR. In control conditions, the Ca^{2+} responses induced by KCl were higher in arteries from HU than CTL rats (Fig. 1c) and this difference persisted when arteries were incubated with 10 μmol/l ryanodine to inhibit RyR and CICR (P =0.027, Fig. 1c). The increase of Ca^{2+} influx via L-type dependent Ca^{2+} channels in HU rats was thus confirmed by this last result.

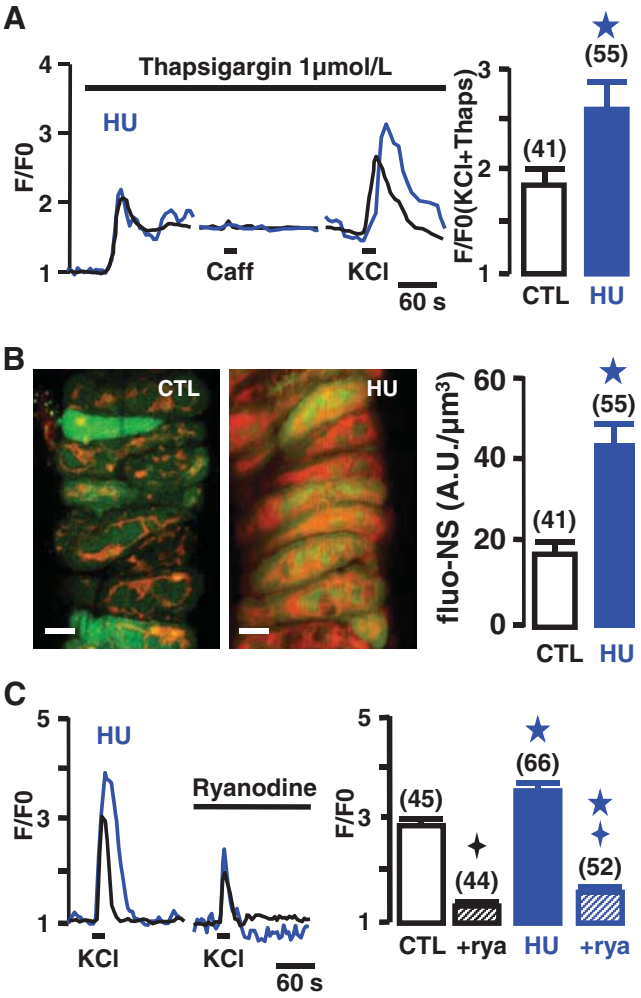

Fig. 1 Implication of the RS and RyR in KCl-evoked Ca^{2+} responses. **a** Typical Ca^{2+} responses induced by thapsigargin and KCl (140 mmol/l) in the presence of thapsigargin in cerebral arterial myocytes from CTL (*black*) and HU (*blue*) conditions in the *left panel* and compiled data of amplitude of KCl-induced Ca^{2+} responses in the presence of thapsigargin in the *right panel*. **b** Typical labeling of cerebral arteries with ST-bodipy(−)-DHP® from control (*CTL*) and HU rats (*HU*). Cells were loaded with fluo8-AM (in *green*) and labeled with 100 nmol/l ST-bodipy(−)-DHP (in *red*) to reveal DHP receptors in the left panel. Scale bar: 4 μm. Compiled data of specific fluorescence in the right panel. **c** Typical Ca^{2+} signals evoked by application of 140 mmol/l KCl in the left panel, with or without perfusion of 10 μmol/l ryanodine for 30 min and, in the right panel, compiled data showing the amplitude of Ca^{2+} signals with (*hatched bars*) or without (*open bars*) perfusion of ryanodine for 30 min. Arteries were loaded with Fluo8-AM (2 μmol/l). Data are means ± SEM with the number of tested cells indicated between parentheses. ★, P <0.05 between CTL and HU conditions; ✦,✦, P <0.05 between in the absence and in the presence of ryanodine in CTL and HU, respectively

HU induces also an increase in CICR

The increase in CICR can result from an increase in RyR expression, or function, in HU models. To test this hypothesis,

caffeine (10 mmol/l) was puff-applied on the cerebral arteries placed in a physiological solution without Ca^{2+} and loaded with the Ca^{2+} indicator Fluo8L-AM. In arteries from HU rats, the amplitude of the caffeine-induced Ca^{2+} response was significantly higher than in CTL (Fig. 2a,d). Caffeine-induced Ca^{2+} responses were totally inhibited in the presence of 10 μmol/l ryanodine for 20 min (not shown).

The CICR mechanism can also be activated by ionotropic P2X receptor activation in VSMC [26]. The application of 1 μmol/l αβMe-ATP, a selective activator of the P2X receptor was used to confirm the increase in CICR in HU models. In the presence of ryanodine (10 μmol/l), similar αβMe-ATP-induced Ca^{2+} responses were measured in cerebral arteries from HU and CTL conditions. However, in the absence of ryanodine, the amplitude of Ca^{2+} responses induced in cerebral arteries from HU were significantly higher than in CTL rats (Fig. 2b,e).

Finally, the CICR could also amplify the Ca^{2+} signals evoked by InsP3. To test this hypothesis we performed analysis of Ca^{2+} signals evoked by endothelin-1 (ET-1, known to activate the InsP3 pathway) and cag-iso-2-145-InsP3 (direct activator of InsP3R after photolysis). In the presence of ryanodine, the ET-1-induced Ca^{2+} responses were similar in CTL and HU conditions, whereas the Ca^{2+} signal was higher in HU than in CTL conditions in a normal physiological solution (Fig. 2c,f). Similarly, the Ca^{2+} response activated by cag-iso-2-145-InsP3 was doubled in arteries from HU animals for a 100 V intensity flash (Fig. 3).

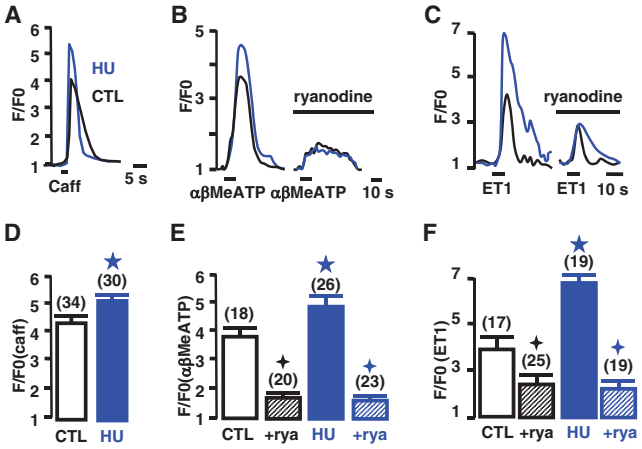

Fig. 2 Role of RyR in evoked Ca^{2+} responses in cerebral arteries from HU animals. **a**–**c** Typical Ca^{2+} signals evoked by application of 10 mmol/l caffeine, 1 μmol/l αβMe-ATP, and ET-1 (10 nmol/l), respectively, observed in smooth muscle cells within isolated cerebral arteries from CTL (*black*) and HU (*blue*) conditions; in control conditions and in the presence of ryanodine. **d**–**f** Compiled data showing the amplitude of Ca^{2+} signals evoked by caffeine, αβMe-ATP, and ET-1, respectively. Arteries were loaded with Fluo8L-AM (2 μmol/l). Data are means ± SEM with the number of tested cells indicated between parentheses. ★, $P<0.05$ between CTL and HU conditions; ✦, ✦, $P<0.05$ between in the absence and in the presence of 10 μmol/l ryanodine in CTL and HU, respectively. Arteries were loaded with Fluo8-AM (2 μmol/l)

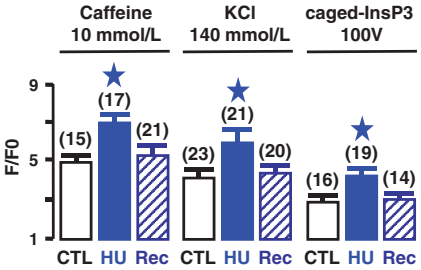

Fig. 3 Recovery of hindlimb suspension. Compiled data showing the maximal amplitude of Ca^{2+} responses evoked by caffeine, KCl and caged-iso-2-InsP3 in CTL (*open black bar*), HU (*open blue bar*) and 48 h recovery (Rec, *hatched bar*) rats. Data are means ± SE with the number of tested cells indicated in parentheses. Myocytes were loaded with Fluo8L-AM. ★$p<0.05$, between control and HU conditions (ANOVA). Experiments were repeated on three different sets of animals (one set containing one CTL, one HU and one Rec)

The effect of HU on RyR-dependent Ca^{2+} signaling is reversible

To test the recovery of vascular function, we used the same protocol as previously described [28]. Briefly, rats were detached and lived normally during 48 h before being euthanized. All studied Ca^{2+} signals were similar to those observed in CTL conditions after 48 h of recovery (Fig. 3). Taken together, these results suggest that the HU-induced modulation of RyR-dependent Ca^{2+} release in cerebral arteries was reversible after position recovery.

RyR1 expression is increased by HU

The increase in RyR function in Ca^{2+} signaling could be due to the increase of RyR expression. To evaluate this hypothesis, we measured the bodipy-FL-X-ryanodine binding (Fig. 4a). Thirty minutes after incubation of cerebral arteries in 1 μmol/l bodipy-FL-X-ryanodine, the specific fluorescence was weakly increased from 250±39 A.U./cell ($n=4$ arteries) in CTL conditions, to 287±41 AU/cell ($n=3$ arteries) in HU conditions, suggesting that RyR expression was potentially increased. To verify this hypothesis, a Western blot analysis of RyR subtypes expression was performed and revealed an increased RyR1/actin ratio in cerebral arteries from HU rats. However, HU had no effect on RyR2/actin and RyR3/actin ratios (Fig. 4b,c). Finally, to determine whether the increase in RyR1 expression was due to an increase in gene expression or a decrease in protein degradation, we investigated the expression level of mRNA encoding the RyR subtypes by RT-qPCR. In accordance with the previous results, only the expression of the RyR1 subtype was increased in HU conditions (Fig. 4d).

Because RyR could be implicated in spontaneous Ca^{2+} signaling, we have also analyzed the HU effects on localized and propagated Ca^{2+} signals.

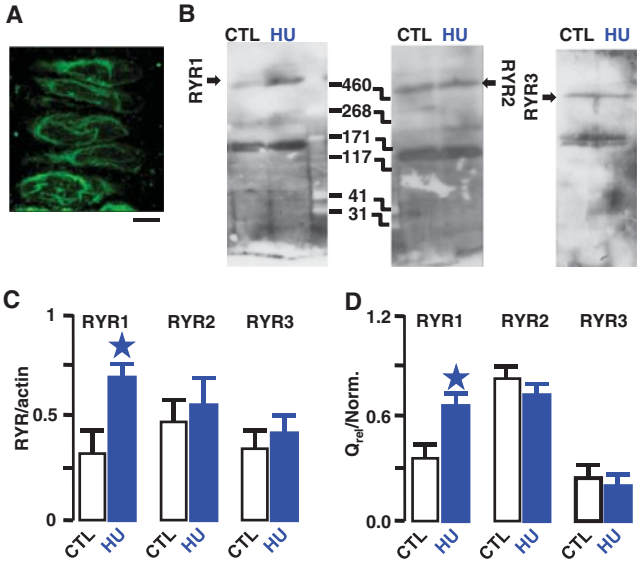

Fig. 4 Effect of hindlimb unloading on RyR expression in cerebral arteries. **a** Typical labeling obtained with ryanodine bodipy and observed in MCA collaterals from HU rats. **b** Typical bands obtained by Western blotting proteins (40 μg) from control and HU rats and realized with anti-RYR (1, 2 and 3) and anti-actin antibodies. **c** Intensity ratio of RyR1, RyR2 and RyR3 to actin Western blot bands in MCA from control (*black bars*) and HU rats (*blue bars*). Experiments were repeated five times, and two animals were used each time for each condition. **d** Expression of RyR in cerebral arteries revealed by RT-qPCR expressed as the normalized expression using SDHA and actin and reference genes. Experiments were repeated in duplicate on six different animals by condition. Data are means ± SE. ★, $P<0.05$ between CTL and HU conditions

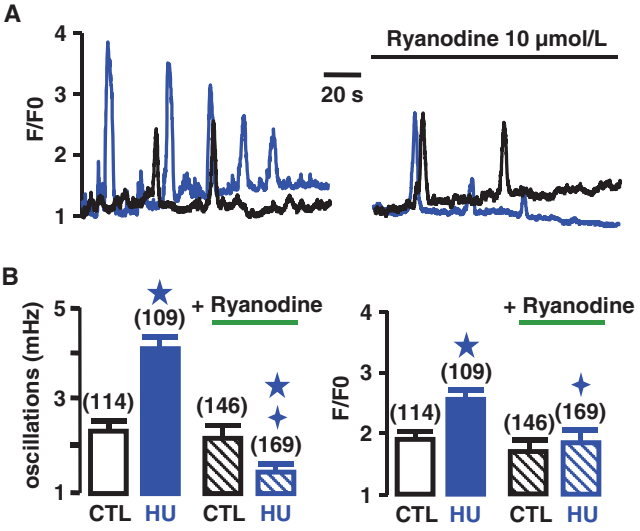

Fig. 5 Implication of RyR in spontaneous Ca^{2+} waves in cerebral arteries from HU and CTL rats. **a** Typical spontaneous Ca^{2+} oscillations measured in one VSMC of an isolated cerebral artery, in control conditions and in the presence of 10 μmol/l ryanodine for 20 min from control (*CTL*, *black*) and hindlimb unloaded (*HU*, *blue*) rats. **b** Frequency and mean amplitude of spontaneous Ca^{2+} oscillations measured in CTL (*black bars*) and in HU (*blue bars*) conditions. Parameters were calculated on 20 different preparations from ten CTL and ten HU rats. Data are means ± SEM with the number of tested cells indicated in parentheses. Arteries were loaded with Fluo8-AM (2 μmol/l). ★, $P<0.05$ between CTL and HU conditions; ✦, $P<0.05$ between in the absence and in the presence of 10 μmol/l ryanodine

Modulation of spontaneous Ca^{2+} waves by HU, role of RYR

Spontaneous Ca^{2+} waves, propagated into the VSMC, were detected in cerebral arteries taken from both CTL and HU rats (Fig. 5a). The percentage of cells presenting at least one spontaneous Ca^{2+} wave in a 5-min interval was 70 % in CTL rats and 80 % in HU rats (NS). The frequency of repeated spontaneous Ca^{2+} waves and the mean amplitude of these Ca^{2+} oscillations in MCA from HU rats were higher than in CTL rats ($P<0.0001$; Fig. 5b).

To investigate the contribution of RyR in these spontaneous Ca^{2+} oscillations, cerebral arteries were superfused with a 10-μmol/l ryanodine solution during 20 min (Fig. 5a). In CTL, inhibition of RyR-mediated Ca^{2+} release with ryanodine decreased the percentage of oscillating cells from 75 % to 40 % in a significant manner, without affecting frequency or mean amplitude of the spontaneous Ca^{2+} oscillations. Yet, in HU, the ryanodine perfusion significantly decreased mean amplitude and frequency, as well as the percentage of oscillating cells to the values measured in CTL conditions (Fig. 5b). Furthermore, after 40 min in the presence of ryanodine, the spontaneous Ca^{2+} oscillations were totally inhibited in cerebral arteries from both CTL and HU rats. After the total extinction of spontaneous Ca^{2+} oscillations, the application of 10 mmol/l caffeine was not able to activate a Ca^{2+} response, as observed in the absence of ryanodine in external solution in cerebral arteries from CTL as well as HU rats (not illustrated). Similarly, after 10 min in the presence of thapsigargin (1 μmol/l), Ca^{2+} oscillations and caffeine-induced Ca^{2+} responses were totally inhibited in both CTL and HU conditions. These results suggest that (1) the maintenance of spontaneous Ca^{2+} waves is dependent on the activation of RyR and therefore HU induced an increased RyR-dependent spontaneous Ca^{2+} release and (2) the Ca^{2+} stores are necessary to generate Ca^{2+} spontaneous oscillations.

Modulation by HU of spontaneous Ca^{2+} waves, role of VDCC

After 15–20 min in ryanodine, the persistence of spontaneous Ca^{2+} oscillations in CTL conditions indicated that they could also be encoded by a source of Ca^{2+} other than the reticulum. As previously described [51], the Ca^{2+} influx was also modulated by HU conditions, and could also participate to spontaneous Ca^{2+} oscillations. As expected, in the absence of Ca^{2+} in the physiological solution, spontaneous Ca^{2+} oscillations were progressively inhibited in cerebral arteries from both CTL and HU rats (not illustrated). After 10 min in exempted Ca^{2+} solution, the spontaneous Ca^{2+} oscillations totally disappeared and the application of caffeine was not able to induce a Ca^{2+} response, indicating that the reticulum Ca^{2+} store was depleted. This result suggests that the Ca^{2+} influx could be

implicated in spontaneous Ca^{2+} oscillations preferentially as a modulator rather than a trigger signal.

Increasing the external Ca^{2+} concentration from 2 to 5 mmol/l had no effect on the presence and the frequency of the spontaneous Ca^{2+} oscillations observed in CTL rats (0.018 ±0.005 Hz, n=45, in 2 mmol/l solution vs. 0.023±0.007 Hz, n=36, in 5 mmol/l solution), yet it significantly increased the frequency of spontaneous Ca^{2+} oscillations in arteries from HU animals (0.038±0.005 Hz, n=59, in 2 mmol/l $CaCl_2$ solution vs. 0.058±0.007 Hz, n=36, in 5 mmol/l $CaCl_2$ solution, P=0.019). This was observed in 90 % of the cells studied. These data thus suggest that the contribution of the Ca^{2+} influx in spontaneous Ca^{2+} oscillations appears more important in HU conditions than in CTL conditions.

Therefore, we investigated the role of VDCC influx in the spontaneous Ca^{2+} oscillations. In the presence of dihydropyridines (oxodipine as well as ST-bodipy(−)-DHP), a drastic inhibition of the frequency of spontaneous Ca^{2+} oscillations in HU conditions (Fig. 6a, P=0.011) was observed without significant modification of the percentage of oscillating cells and amplitude of oscillations (Fig. 6b).

Finally, the application of 10 nmol/l BAYK8644, an activator of L-type VDCC, increased the frequency of spontaneous Ca^{2+} oscillations in HU conditions (0.065±0.007 Hz, n=31, P=0.005) without modification in the amplitude of the Ca^{2+} oscillations (F/F_0=2.61±0.7 ratio units, n=31). All observed cells presented Ca^{2+} oscillations in the presence of BAYK8644. These results indicate the role of VDCC in the spontaneous Ca^{2+} oscillations induced by HU.

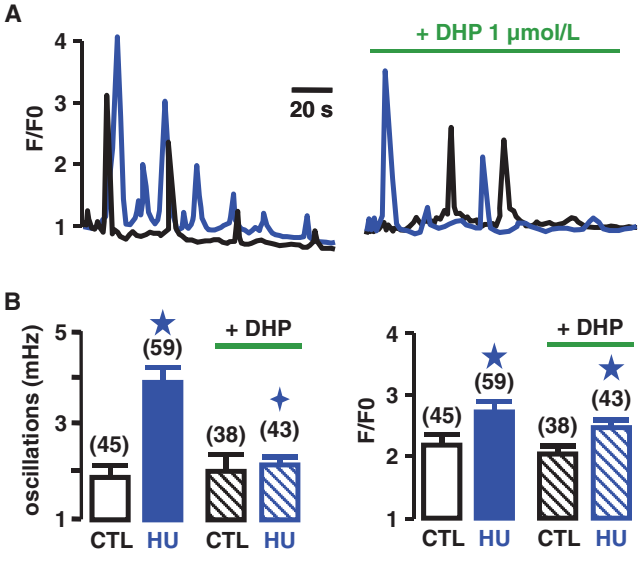

Fig. 6 Effect of DHP on spontaneous Ca^{2+} waves in cerebral arteries. **a** Typical spontaneous Ca^{2+} oscillations measured in one VSMC of an isolated cerebral artery from control (*CTL*, *black bars*) and hindlimb unloaded rats in the absence (*HU*, *blue*) and in control conditions and in the presence of 1 µmol/l oxodipine (*DHP*, *green*). **b** Frequency and mean amplitude of spontaneous Ca^{2+} oscillations measured in CTL (*black bars*) and in HU (*blue bars*) conditions with (*hatched bars*) and without (*open bars*) DHP. Data are means ± SEM with the number of tested cells indicated in *parentheses*. Arteries were loaded with Fluo8-AM (2 µmol/l). ★, P<0.05 between CTL and HU conditions; ✦, P<0.05 between in the absence and in the presence of 1 µmol/l oxodipine

Spontaneous localized Ca^{2+} events are modulated by HU

Ca^{2+} sparks and Ca^{2+} sparklets, resulting from the opening of a cluster of RYR and VDCC, respectively, are localized Ca^{2+} release events, as illustrated in Fig. 7a. Cerebral arteries from HU rats presented more myocytes with localized Ca^{2+} events than arteries from CTL rats (Fig. 7a). The actual number of localized Ca^{2+} events initiation sites per cell was not significantly modified by the HU; yet, the discharge frequency of localized Ca^{2+} events initiation sites was increased in HU (Fig. 7b). Parameters were calculated from 20 different preparations from ten CTL rats (47 localized Ca^{2+} events-displaying cells analyzed) and ten HU rats (103 localized Ca^{2+} events-displaying cells analyzed). These results indicate that localized Ca^{2+} activity was increased by HU.

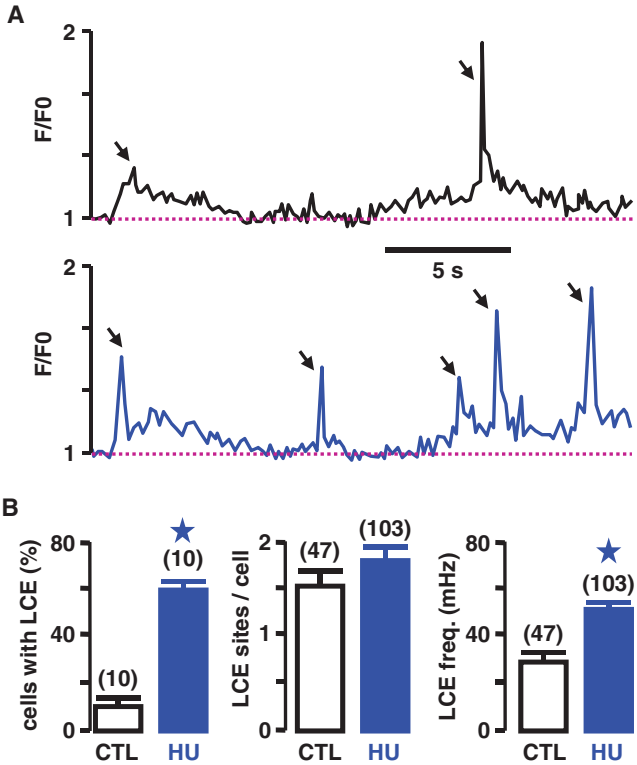

Fig. 7 Effect of HU on localized Ca^{2+} events in cerebral arteries. **a** Localized Ca^{2+} events measured in cerebral arteries from both CTL and HU rats. *Arrows* indicate the initiation sites of localized Ca^{2+} events. **b** Percentage of cells presenting localized Ca^{2+} events, number of localized Ca^{2+} events discharging regions per cell and frequency of reactivation of each site of localized Ca^{2+} events in CTL (*black bars*) and in HU (*blue bars*) conditions. Parameters were calculated on 20 different preparations from ten CTL and ten HU rats. Data are means ± SEM with the number of tested cells indicated in *parentheses*. Arteries were loaded with Fluo8H-AM (2 µmol/l). ★, P<0.05 between CTL and HU conditions

Ca^{2+} sparks can be discriminated from Ca^{2+} sparklets by their pharmacology and their amplitude and duration [34]. Only localized Ca^{2+} events with an amplitude superior to the median plus one standard deviation were considered and their duration, maximal amplitude, surface above threshold and upstroke velocity were qualified. Using an external solution depleted in Ca^{2+} (0 mmol/l) to inhibit Ca^{2+} influx, the persistent localized Ca^{2+} signals showed the characteristics of Ca^{2+} sparks (Fig. 8a, F/F_0=2.66±0.12 ratio units; duration=0.25±0.04 s; corrected area=0.19±0.02 ratio unit/s; upstroke velocity=13.02±0.07 ratio unit/s; n=110). Therefore, localized Ca^{2+} events characterized by a duration inferior to 0.3 s were considered as Ca^{2+} sparks when analyzing localized Ca^{2+} events in CTL and HU rats in a normal external medium solution (ME 2Ca^{2+}). As illustrated in Fig. 8a (right inset), in the presence of 1 µmol/l of oxodipine or ST-bodipy(−)-DHP, the persistent spontaneous localized Ca^{2+} signals presented characteristics of Ca^{2+} sparks (duration inferior to 0.3 s, F/F_0=2.62±0.17 ratio units, corrected area=0.17±0.02 ratio unit/s upstroke velocity=10.9±0.1 ratio unit/s; n=59 in HU conditions). In HU conditions, the use of dihydropyridines also induced the decrease of the Ca^{2+} sparks frequency to levels near those observed with the use of the 0 mmol/l Ca^{2+} solution (0.035±0.003 Hz, n=26; P=0.02).

The presence of 10 µmol/l ryanodine applied 30 min before Ca^{2+} recording drastically inhibited localized Ca^{2+} events in cerebral arteries from both CTL (n=4) and HU (n=5) rats. All Ca^{2+} events with duration inferior to 0.3 s were inhibited. The remaining Ca^{2+} events were characterized by a longer duration (1.55±0.21 s, with amplitude=1.78±0.03; corrected area=0.67±0.12 ratio unit/s; n=19). The amplitude and kinetic parameters were similar in both HU and CTL rats (Fig. 8b; n=19 and 12, respectively). Therefore, the localized spontaneous Ca^{2+} events recorded in the presence of ryanodine were probably Ca^{2+} sparklets.

Previous data indicated that the activation of VDCC can modulate the frequency of Ca^{2+} sparks. Therefore, we tested this hypothesis. In MCA from HU rats, the increase of Ca^{2+} concentration of the physiological solution from 0 to 5 mmol/l increased the localized Ca^{2+} events (Fig. 9a). By the kinetic identification of Ca^{2+} sparks ($\Delta F/F_0$>1.5 ratio units; upstroke velocity >10 ratio unit/s), it was possible to measure the increase of the Ca^{2+} sparks frequency (from 0.021±0.003 Hz, n=22, in 0 mmol/l Ca^{2+} solution to 0.060±0.006 Hz, n=83 in 5 mmol/l Ca^{2+} solution; P=0.0005). Moreover, the addition of 1 nmol/l BayK8644 increased the Ca^{2+} sparks frequency to a level similar to the one observed with the use of the 5 mmol/l Ca^{2+} solution (0.069±0.009 Hz, n=19; Fig. 9b). In contrast, in CTL conditions the Ca^{2+} sparks frequency was neither significantly increased by the increase in Ca^{2+} concentration of the physiological solution superfusing cerebral arteries, nor by application of BayK8644 (Fig. 9b). Taken together, these last data suggest that the increase of the frequency of localized Ca^{2+} events observed in HU conditions was due to an increase in both RyR1 expression and VDCC function. The increase in VDCC function or expression was able to increase the Ca^{2+} spark frequency via the increase in Ca^{2+} sparklet frequency.

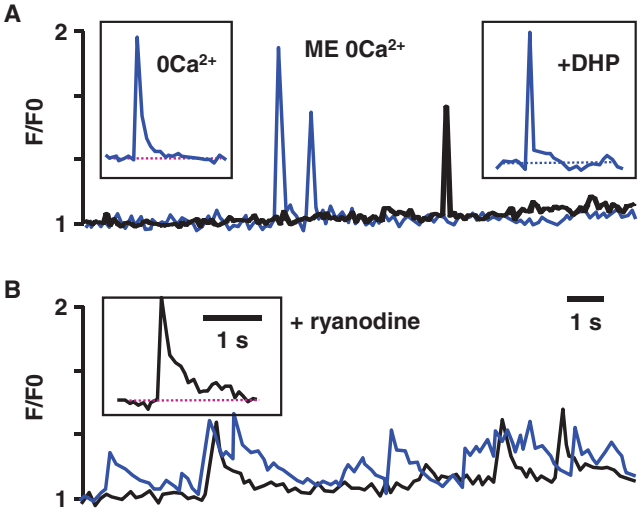

Fig. 8 Effect of ryanodine and external Ca^{2+} depletion on localized Ca^{2+} events in cerebral arteries. **a** Localized Ca^{2+} events (Ca^{2+} sparks) measured in cerebral arteries from both CTL (*black trace*) and HU (*blue trace*) rats in the presence of a Ca^{2+}-depleted external solution (Mac Ewen solution without Ca^{2+}; ME 0Ca^{2+}). **b** Localized Ca^{2+} events (Ca^{2+} sparklets) measured in cerebral arteries from both CTL (*black trace*) and HU (*blue trace*) rats in the presence of 10 µmol/l ryanodine. Arteries were loaded with Fluo8H-AM (2 µmol/l)

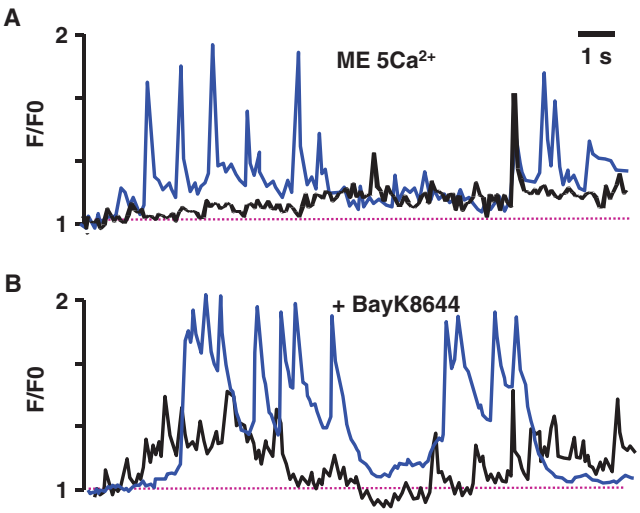

Fig. 9 Effect of BayK8644 and 5 mmol/l Ca^{2+} external solution on localized Ca^{2+} events. **a** Localized Ca^{2+} events measured in cerebral arteries from both CTL (*black trace*) and HU (*blue trace*) rats in external solution containing 5 mmol/l $CaCl_2$ (ME 5Ca^{2+}). **b** Localized Ca^{2+} events measured in cerebral arteries from both CTL (*black trace*) and HU (*blue trace*) rats in the presence of 1 nmol/l BayK8644. Arteries were loaded with Fluo8H-AM (2 µmol/l)

Discussion

An increase in vascular reactivity of cerebral arteries induced by HU conditions was previously suggested by the measurement of myogenic tone [16], contractions induced by KCl or agonists [53] and also NO-dependent relaxation [46]. Indeed, vascular reactivity could be modified by changes in cell interactions, contractile proteins or transduction pathways and expression level, but also by the functional state of proteins involved in the regulation of the contraction/relaxation equilibrium. The regulation of this balance is assumed in part by the regulation of (1) agonist-activated Ca^{2+} signals and (2) spontaneous Ca^{2+} signaling controlling the myogenic tone via Ca^{2+} sparks and spontaneous contraction via Ca^{2+} waves, respectively. Therefore, the aim of the present study was to test the effect of an 8-day HU on the Ca^{2+} signaling toolkit of the VSMC in MCA from HU rats. Our results strongly suggest that the level of RyR expression plays a crucial role in the adaptation of VSMC from animals exposed to gravity modulation [4, 12].

Effects of the HU-induced increase of RyR1 expression on Ca^{2+} signals

The increase in the expression of RyR1 correlated with the increase in spontaneous Ca^{2+} activity, thereby indicating that the RyR1 subtype has a regulatory function in cerebral arteries from HU rats. An increase in vascular reactivity of cerebral arteries was previously shown by the measurement of myogenic tone [16] and contractions induced by KCl or agonists [53]. The modification of RyR expression could be linked to a change in vasoconstriction, as observed during aging [17]. The increase in RyR1 expression and function in VSMC of HU rats fits with the different effects measured previously and provides a new insight complementary to the increase in L-type Ca^{2+} current density and membrane VDCC α_{1C} subunit expression described in cerebral VSMC after HU [51]. RyR1 appears to be a major target of vascular adaptation to HU and the regulation of its expression depends on the effects of HU on the intravascular blood loading [7, 12, 28]. If the cellular sensor of gravity is not yet described, the modulation of RyR1 expression was described before not only in VSMC but also in skeletal muscle [3].

By using ST-bodipy(−)-DHP, oxodipine or a Ca^{2+}-free external solution, it was possible to isolate localized Ca^{2+} events that have characteristics similar to the Ca^{2+} sparks defined in VSMC [22]. The increase in cells presenting spontaneous Ca^{2+} sparks, with higher frequency, in HU compared to CTL rats is consistent with the increase of RyR1 expression [8], the increase in Ca^{2+} influx and the published increase in the activity of Ca^{2+}-activated K^+ channels (BK_{Ca}) [49]. The increase of Ca^{2+} sparks frequency could explain the increase of vascular reactivity observed in rats exposed to HU conditions via two opposite effects. First, an elevation of intravascular pressure in rat cerebral arteries activated by the HU position induces an increase in Ca^{2+} sparks frequency, acting as a negative feedback mechanism through hyperpolarizing BK_{Ca} activity, and thus consequently increasing the relaxation ability [45]. This increase of Ca^{2+} sparks frequency can in turn result in an increase of the contractility of cerebral arteries. Moreover, Ca^{2+} sparks can also directly activate chloride currents, commonly named spontaneous transient inward currents (STICs), and can be one of the mechanisms explaining why isolated cerebral VSMC from HU rats had a more depolarized membrane potential, thus a higher excitability, than CTL rats [15].

The function of RyR1 in smooth muscle was previously studied in transient [8] or complete [14] inhibitions of its expression. In these studies, we have shown that RyR1 was expressed near the plasma membrane and was responsible for the CICR mechanism induced by Ca^{2+} influx activation and implicated in the upstroke velocity of Ca^{2+} responses involving the reticulum Ca^{2+} release. In the present study, we have measured an increase in RyR1 expression that was correlated with the increase in Ca^{2+} influx-induced Ca^{2+} release, indicating that the level of RyR1 expression can finely regulate the kinetic of Ca^{2+} release from the reticulum. The increase in RyR1 in HU animals could explain why the increase of CICR was induced by P2X1 activation (near 40 %). This result could be correlated with the spatial distribution and/or coupling of P2X1, VDCC and RyR subtypes in VSMC [10, 11, 23, 43].

Increase of VDCC-dependent Ca^{2+} influx in HU

In the presence of ryanodine, the KCl-induced Ca^{2+} responses were still higher in HU conditions, confirming the increase in depolarization-induced Ca^{2+} influx [50, 51]. In our study, the increase in ST-bodipy(−)-DHP labeling further suggests that VDCC were expressed more and/or activated more in HU conditions, as previously observed in electrophysiology experiments [49, 51]. This could potentially be due to an increase in channel expression and/or an increase in channel activity. In fact, the DHP labeling could be increased by an increase in channel phosphorylation leading to membrane addressing as well as an increase in channel synthesis [27]. Of note that the use of the pharmacological detection of VDCC is more appropriate than the detection of CaV1.2 protein or RNA, since it has been shown recently that CaV1.2 subunits expression was not inevitably correlated with L-type current in VSMC [42].

The result of this up-regulation of VDCC was the increase in Ca^{2+} influx activated by depolarization (present study) and potentially the enhancement of angiotensin-II responses, as previously described [30] and recently suggested in the HU model [50]. Whatever triggered the response, the result is an

increase in calcium influx and mainly an increase in CICR. Moreover, a role of VDCC in spontaneous localized Ca^{2+} signaling was recently proposed. The most important technical difficulty in VSMC is to isolate and measure the localized Ca^{2+} signaling. Pharmacologically it was possible to individualize Ca^{2+} sparklets [34] by inhibiting Ca^{2+} sparks with high concentration of ryanodine. The use of ST-bodipy(−)-DHP and oxodipine both resulted in an inhibition of localized Ca^{2+} events having Ca^{2+} sparklets kinetic characteristics, thus suggesting that they were encoded by VDCC. Ca^{2+} sparklets have been firstly described by total internal reflection fluorescence (TIRF) microscopy designed to measure Ca^{2+} signals near the plasma membrane [34]. Here, by the labeling of cerebral arteries with ST-bodipy(−)-DHP, we have suggested that VDCC could be expressed in the plasma membrane included in a confocal section in the area where localized Ca^{2+} events were measurable. Moreover, both 5 mmol/l $CaCl_2$ external solution and BayK8644 increased the spontaneous localized Ca^{2+} events having kinetic parameters resembling those previously identified as Ca^{2+} sparklets in the presence of ryanodine. The presence of Ca^{2+} sparklets was more important in HU conditions than in CTL conditions, thus confirming an increase of VDCC activity in HU conditions.

SERCA and InsP3R activity appeared not intrinsically modified, contrary to CICR

In VSMC, an increase in intracellular calcium concentration $[Ca^{2+}]_i$ is determined mainly by Ca^{2+} influx through the plasma membrane but also by Ca^{2+} release from intracellular stores, as well as Ca^{2+} removal by Ca^{2+}-ATPases. The InsP3-dependent Ca^{2+} signals have long been regarded as the main Ca^{2+} signals in smooth muscles responsible for the contraction activated by neurotransmitters and hormones [47]. Therefore, the investigation of this signal has been performed. Our results suggest that 8 days of HU increased the InsP3-(cag-iso-2-InsP3)-induced Ca^{2+} responses as well as caffeine- and KCl-induced responses. However, in the presence of ryanodine, the ET1-induced Ca^{2+} responses were identical in both control and HU animals, suggesting that InsP3 pathways were not modified.

Reversibility of HU effects

Based on our own expertise, in the rat portal vein, the effect of HU on Ca^{2+} signaling is significant after 7 days and persists until the 14th day during HU. In the same model, the reversibility of the vascular deconditioning induced by HU appeared after stopping the suspension protocol for 48 and 72 h after a duration of 7 and 14 days, respectively [28]. In cerebral arteries, we show here that the duration of 8 days is sufficient to induce a modulation in Ca^{2+} signaling as observed at the level of BK_{Ca} expression [48]. Here, we have clarified that the reversibility of the HU effect was already effective after 2 days following the end of the suspension protocol. Of note that the reversibility of the HU effect may also be induced by countermeasure protocols applied during HU. Daily short-term standing for example was sufficient to reverse HU effects in mesenteric arteries, but it was inefficient in cerebral arteries [51].

Potential role of the increase of spontaneous Ca^{2+} oscillations

Our results indicate that the number of oscillating cells was not modified by μG simulation, whereas the frequency and the amplitude of the Ca^{2+} oscillations were increased. The spontaneous Ca^{2+} waves preceding contraction and the amplitude of these Ca^{2+} waves could encode the intensity of the contraction. Therefore, the spontaneous contractions of cerebral arteries would be increased, and further be involved in the increase in basal vascular tone [16]. Moreover, the frequency of Ca^{2+} oscillations could also regulate gene expression. In fact, an increase of frequency can activate specific effectors such as calmodulin kinase II [13], which is not only involved in contraction [32] but also in the modulation of gene expression [38] to induce proliferation and migration in VSMC [5, 20]. This second effect of the increase in spontaneous Ca^{2+} waves may also be implicated in the increase of expression of RYR1 and L-type Ca^{2+} channels to generate a positive feedback on Ca^{2+} signaling.

Role of RyR in HU and microgravity

In 1997, we suggested for the first time that HU could modify RyR expression in portal vein VSMC [28] and we have specified that RyR1 expression was decreased after μG exposure during spaceflight in animals as well as in cultured cells [12]. In the same time a decrease of RyR2 expression was found in mesenteric arteries and veins [4]. In these cases, the modification of the Ca^{2+} signal only depended on the decreases in RyR1/2 expressions (without modification in VDCC-induced signals) and this was correlated with a decrease of contraction and the suspected decrease of blood loading induced by HU or μG. In MCA, we have observed the opposite effect: an increase of RyR1 expression correlated with the suspected increase of blood loading. Very recently, it has been demonstrated that vasoconstriction was decreased in basilar arteries from mice exposed to μG during spaceflight [41]. However, in HU animals our results are all clearly in favor of an increase in vascular reactivity. A modulation of myogenic tone or an increase of vasorelaxation could result from the increase of Ca^{2+} sparks frequency, however the increase of Ca^{2+} responses activated by caffeine or purinergic stimulation strongly inclines towards an increase in mediator-activated vasoconstriction.

It therefore is necessary to continue investigations on models having undergone spaceflight (in terms of signaling), but also in the HU model (in terms of contractility).

Perspectives

Our main results (i.e., increase of VDCC and RyR1 activities) support the increase of cerebral vascular activity observed in HU used as a model of simulated μG. Nevertheless, it is necessary to study the functions activated by spontaneous Ca^{2+} oscillations in cerebral arteries, and especially the molecular targets implicated in the proliferation/apoptosis balance, such as transcription factors observed in endothelial cells [25], and also the Ca^{2+} sensitivity of myofilaments. Finally, this adaptation appeared to have settled rapidly in 1 week and could persist during 28 days [49, 51], yet the time course of the molecular adaptation as well as the effect of a longer exposure to HU and μG remain to be further evaluated.

Acknowledgments We particularly thank Dr. B. Yvert for spike2 analysis script to discriminate localized Ca^{2+} events; Laure Diaz, masteral student, and Nathalie Biendon for technical support and J. Mironneau for helpful discussion. JLM is the manager of the project. FD and JLM equally participated to the conception, design of the experiments, collection, analysis and interpretation of Ca^{2+} imaging. YP performed qPCR experiments. NM and AP participated to interpretation of data and writing, respectively. This work was supported by grants from the Centre National d'Etudes Spatiales (CNES), the Agence Nationale pour la Recherche (AdapHyG, n° ANR-09-BLAN-0148) and a postdoctoral fellowship from CNES to F. Dabertrand. Equipment (confocal microscopes, thermocycler) was financed by Conseil Régional d'Aquitaine.

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