Published in "Pflügers Archiv - European Journal of Physiology
465(6): 829–838, 2013"
which should be cited to refer to this work.

Effect of aging on calcium signaling in C57Bl6J mouse cerebral arteries

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Abstract In cerebral arteries, alterations of vascular reactivity have been observed but not well molecularly characterized. Therefore, we have hypothesized that cerebrovascular reactivity could be modified by aging via a modification of Ca^{2+} signaling in smooth muscle cells. Ca^{2+} signals and gene expression implicated in contraction have been measured in posterior and middle cerebral arteries from young (2–3 months) and old (20–22 months) C57Bl6/J mice. Aging induced a decrease of KCl- and caffeine-induced contraction as well as a decrease of the amplitudes and an increase of the durations of KCl- and caffeine-induced Ca^{2+} signals. These results could be linked with the decrease of gene expression coding for Cav1.2, RyR2, SERCA2, PLB, STIM1, TRIC-B, and the increase of FKBP12.6 and TPCN1 gene expression. Finally, aging induced a modification of InsP3 subtype expression pattern responsible for a modification of the InsP3 affinity to activate Ca^{2+} signals. These results show that aging induces a decrease of contractility correlated with modifications of the expression of genes encoding Ca^{2+} signaling toolkit. Globally, the amplitude of Ca^{2+} signals was decreased, whereas their duration was increased by a defection of Ca^{2+} store refilling.

Keywords Aging · Cerebral artery · Ca^{2+}-induced Ca^{2+} release · Ca^{2+} channels

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Introduction

Aging has a significant effect on vascular functions, especially the decrease of compliance in large arteries and the decrease of reactivity in small arteries [37]. Aging induces direct structural and functional modifications of smooth muscle cells (SMC) that could affect contraction relaxation, cellular phenotype, and proliferation/regeneration. Previously well demonstrated, these cellular processes involve specific Ca^{2+} signals [2–4]. In addition, vascular SMC (VSMC) help to ensure the regulation of myogenic tone and blood pressure. The contraction/relaxation balance is regulated in part by Ca^{2+} signals and activation of Ca^{2+}-activated K^+ channels.

In VSMC, the Ca^{2+} signals are complex [3] as summarized in Fig. S1: depolarisation induces the activation of L-type currents mediated by voltage-gated Ca^{2+} channels (containing CaV1.2 subunits). The Ca^{2+} entry is then relayed and amplified by the release of Ca^{2+} stored in sarcoplasmic reticulum (SR) via the

Ca^{2+}-induced Ca^{2+} release mechanism (CICR) due to activation of ryanodine receptors (RyR). Hormones and neuromediators activate transduction pathways to produce second messengers responsible for the release of stored Ca^{2+} by three distinct mechanisms: (1) inositol 1,4,5-trisphophate (InsP3) via activation of InsP3 receptors (InsP3R); (2) cyclic ADP ribose (cADPR) via the decoupling between RyR2 and calstabin-2 (or FKBP12.6) known to stabilize RyR2 in closed conformation [20]; and (3) nicotinic acid adenine dinucleotide phosphate (NAADP) via activation of RyR and/or two pore channels encoded by TPCN genes [9]. The return to the basal Ca^{2+} concentration after an agonist- or depolarisation-activated Ca^{2+} response results from (1) Ca^{2+} extrusion by plasma membrane Ca^{2+}-ATPases (PMCA) and Ca^{2+} store refilling via sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA); (2) store-operated Ca^{2+} entry (SOCE) supported by the partnership between stromal interacting molecule (STIM) and Orai participated in the regeneration of the SR Ca^{2+} store [19, 31]; and (3) very recently, trimeric intracellular channels (TRIC) were described to contribute to blood pressure maintenance by the regulation of the store-dependent Ca^{2+} release [63], while SOCE-associated regulatory factor (SARAF) controls the store-operated Ca^{2+} entry to prevent excess Ca^{2+} refilling [45]. The simultaneous activity of these proteins (known altogether as the "Ca^{2+} signaling toolkit") participates to the intracellular signals necessary for cellular functions, particularly vascular contraction.

Aging is also characterized by the modulation of gene expression [46]. In the heart, the study of gene expression by DNA arrays showed that aging affected 10 % of expressed genes, indicating the complexity of aging effects on gene expression [29, 61]. These results provided pathways to describe and understand aging effects on physiology. For example, in pyramidal neurons, it has been demonstrated that aging induces changes in Ca^{2+} signaling [22, 56]. In aged animals, increases of L-type Ca^{2+} current and channel density were measured in aged hippocampal neurons, resulting in increased slow after hyperpolarization spikes [55, 56]. Consequently, a decrease of long-term potentiation was observed which could explain at least partly the aging-dependent cognitive impairments. Indeed, the use of nimoldipine, an inhibitor of L-type Ca^{2+} current, reduced the aging effect on slow after-hyperpolarization spikes to facilitate synaptic enhancement observed in the long-term potentiation [17, 43]. Finally, ryanodine, used to inhibit RyR and the amplification of Ca^{2+} entry via the L-type Ca^{2+} channels, had the same effect as observed with Nimoldipine [22]. Similar results were obtained in the heart: the age-associated decline of SERCA activity was related to the decrease of cardiac contraction induced by isoproterenol [25, 26]. Finally, during development phases, the Ca^{2+} signaling pathways were also modified. In sheep cerebral arteries, the expression of CaV1.2 was decreased (from fetus to adult) coupled to a modification of norepinephrine contraction [5]. However, in cerebral arteries, aging could also alter the Ca^{2+} signaling and could have functional consequences on vascular reactivity. This aspect of vascular aging has not yet investigated.

If the decline in vascular reactivity due to aging has been repeatedly measured, all molecular mechanisms affected by aging are not well documented. Therefore, we have investigated the effects of aging on Ca^{2+} signaling, gene expression of Ca^{2+} signaling toolkit, and contraction. Our results suggest that in cerebral arteries, the decrease of contraction due to aging was due to the modification of amplitude and kinetic parameters of Ca^{2+} signals. These modifications could be explained by the modification of gene expression of the Ca^{2+} signaling toolkit observed in old mice.

Methods

Animals

The care and use of animals complied with European Community and French guiding principles. The principal investigator is authorized by French authorities to perform animal experiments (no. C33-01-029). The average weights were 24±2 g (n=33) for young mice (2–3 months old) and 35±5 g (n=36) for old mice (20–22 months old). All C57Bl6/J were from Ets Janvier (Saint Berthevin, France). Mice were killed by cervical dissociation, brains were extracted of the skull, and the posterior (PCA) and middle (MCA) cerebral arteries were dissected.

Old animals (20–22 months) came into the laboratory at the age of 12 months. Approximately 20 % died before reaching the age of 22 months, whilst the remaining 80 % were in "good health," i.e., without visible characteristics of abating (no locomotor disorders, no difficulty with food or water intakes, and no lack of social interactions).

Quantitative real-time polymerase chain reaction after transcription

MasterPure® kit (Epicentre, Madison, WI) was used to isolate total RNA from cerebral arteries. RNA integrity and purity were verified by using RNA HighSens Analysis Kit (Experion, Bio-Rad, Marne-la-Coquette, France), and the concentration of RNA was measured with spectrophotometry (NanoDrop Technologies, Wilmington, DE). Reverse transcription (RT) reaction was carried out using the Sensiscript kit (Qiagen, Courtaboeuf, France). Obtained cDNA were amplified using the primers designed with primer3 software, and these were listed in the Supplementary Methods. The annealing temperature was determined with the gradient function of the CFX96 thermocycler (Bio-Rad), and the absence of primer dimerization was verified. The quantitative real-time polymerase chain reaction (qPCR) experiments were performed with the SYBR green PCR Master Mix (Bio-Rad) in the CFX96. The specificity of the amplification products was confirmed

by melting curve analysis. All samples were analyzed in duplicates. PCR efficiency was calculated from the slope of the standard curve. Gene expression levels were calculated using the 2−ddCt method normalized by reference genes. The optimal number and choice of reference genes have been experimentally determined by the GeNorm method [59] indicating the most stable reference genes in our experimental conditions (YWHAZ, RPL13A, and HPRT1). In the present experiments, we have used PCA and MCA from 15 young and old mice, and for the statistical analysis, we have restrained the samples to 12 young and old mice after the measurement of RNA quality and after GeNorm analysis.

Cytosolic Ca^{2+} measurements

After dissection, PCA and MCA were placed on glass slides coated with CellTak™ (BD-Biosciences, Le-Pont-de-Claix, France) and kept in M199 culture medium at 37 °C under an atmosphere supplemented with 5 % CO_2 before being used in experiments. The PCA were loaded with 2.10^{-6}mol/L Fluo8-AM Ca^{2+} probe for 20 min at 37 °C. Slides were then mounted in the experimental chamber, perfused with physiological solution [14], and placed on the confocal TCS SP5 system equipped with resonant scanner (Leica Microsystems, Nanterre, France), and images were acquired at 7 or 3 Hz in an image series mode. In experiments using cADPR and NAADP, arteries were permeabilized by using the previously described protocol [20]. Briefly, the physiological solution was replaced by a solution containing (in moles per liter) 1.4 10^{-1} KCl, 2 10^{-2} Hepes, 5 10^{-4} MgCl2, 10^{-4} ATP, and 10 μg/mL saponin. Experiments were performed 5 min after permeabilization, and, in order to limit the fluo8 leak, the Ca^{2+} response was recorded in the first 20 min after permeabilization. Fluorescence was analyzed with LAS-AF software (Leica Microsystems), and generated time course data were analyzed with a software developed by Michel Goillandeau (on request).

UV photolysis

Arteries were loaded with the membrane permeant derivative of caged InsP3: D-23-*O*-isopropylidene-6-O-(2nitro-4,5-demethoxy)benzyl-myo-Inositol 1,4,5-trisphosphate-Hexakis(propionoxymethyl)Ester (caged-145-InsP3; 2.10^{-6}mol/L) during 30 min before Fluo8-AM loading. Photolysis was produced by UV flash (10^{-3}s) from DIPSI (Chatillon, France).

Artery contraction

PCA were cannulised at both ends in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, VT) to measure the artery diameter continuously and were bathed in PSS (pH 7.4, Po_2 160 mm Hg, Pco_2 37 mm Hg). Pressure and flow were controlled. Contraction was expressed as the ratio between the passive diameter and the maximal artery diameter during agonist or pharmacological agent application [1].

Fluorescent pharmacology

Cerebral arteries were perfused during 20 min with 1.10^{-7} mol/L of ST-bodipy(-)-dihydropyridine (DHP) and imaged with the confocal TCS SP5 system using the 535-nm excitation wavelength during Ca^{2+} measurements. The emitted light was collected between 580 and 620 nm. The fluorescence of each cell was measured and reported to evaluate the binding of DHP on L-type voltage-gated Ca^{2+} channels (VDCC). Nonspecific fixation of the ST-bodipy(-)-DHP was determined by simultaneous incubation with oxodipine 1.10^{-6}mol/L. All parameters of confocal microscope setup were kept constant for all experiments.

Statistical analysis

Statistical analysis was performed with Graphpad prism software (Graphpad software Inc, La Jolla, CA). Data are expressed as means ± SE; *n* represents the number of tested cells or arteries. Significance between two different age groups (2–3 versus 20–22-month-old mice) or between control and treated arteries (in the absence versus presence of a pharmacological agent) was tested by means of Student's *t* test or one way ANOVA completed with a Tukey post hoc test. *P* values <0.05 were considered as significant and indicated by star symbols in the figures.

Pharmacological agents

Caffeine and ryanodine were purchased from Merck-Millipore (Nottingham, UK), caged-145-InsP3 was from SiChem (Bremen, Germany), ST-bodipy(-)-DHP, and M199 medium were from Life Technologies (Saint-Aubin, France); NAADP was from BioLog (Bremen, Germany) and Fluo8-AM, thapsigargin, and secondary antibodies were from Interchim (Montluçon, France). All other chemical compounds were from Sigma-Aldrich (Saint-Louis, MO).

Results

Aging effects on RyR-dependent Ca^{2+} signals

The RyRs were directly activated by caffeine and cADPR and engaged in CICR to amplify Ca^{2+} entry. The comparison of Ca^{2+} responses induced by caffeine application (0.01 mol/L) indicated that the amplitude of Ca^{2+} signals was reduced in old mice in PCA (Fig. 1a; 5.3±0.5 ratio units in young mice; n= 218 cells versus 2.9±0.6; n=237 cells in old mice; p=0.0001). Likewise, the Ca^{2+} signal, evoked by depolarization due to the

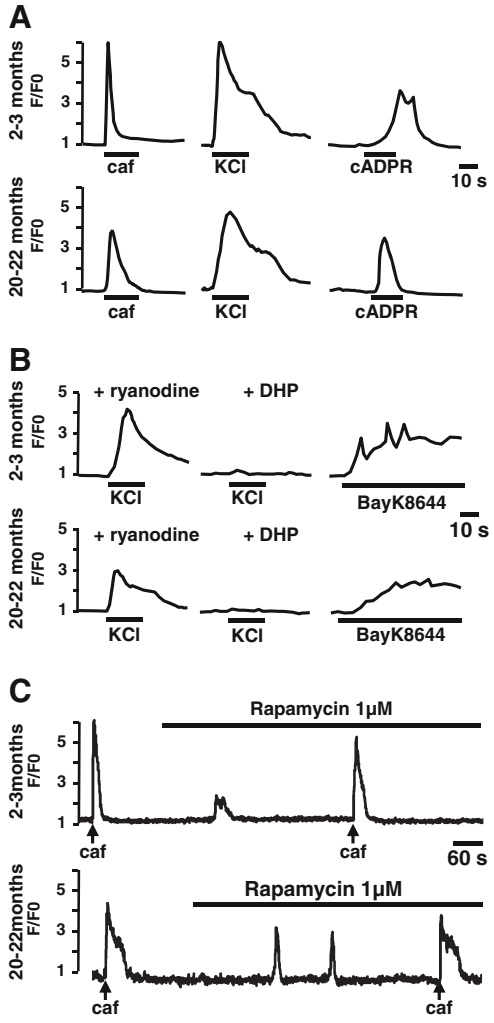

Fig. 1 Ca^{2+} signals in PCA from young and old mice. **a** Typical Ca^{2+} responses evoked by caffeine (*caf*), *KCl*, and cyclic ADP ribose (*cADPR*) in control condition. **b** Typical Ca^{2+} responses evoked by KCl in presence of ryanodine and DHP and typical BayK8644-evoked Ca^{2+} responses. **c** Typical Ca^{2+} responses evoked by caffeine in control condition and in presence of rapamycin

application of 0.14 mol/L KCl solution, was also decreased by aging in control conditions (Fig. 1a; 6.7±0.8 ratio units in young mice; n=178 cells versus 4.6±0.4; n=187 cells in old mice; p<0.0001) but also in the presence of 1.10^{-5}mol/L ryanodine to inhibit RyR and CICR (Fig. 1b; 3.6±0.3 ratio units in young mice; n=218 cells versus 2.0±0.4; n=237 cells in old mice; p<0.0001). The application of ST-bodipy(-)-DHP (inhibitor of L-type Ca^{2+} current, 1.10^{-6}mol/L) inhibited the KCl-induced Ca^{2+} response (Fig. 1b), whereas the application of BayK8644 (used to activate L-type Ca^{2+} current, 1.10^{-8} mol/L), evoked a smaller Ca^{2+} signal in arteries from old mice (Fig. 2b; 2.1±0.3 ratio units in young mice; n=79 cells versus 1.6±0.2; n=66 cells in old mice; p=0.004).

The interaction of RyR2 and calstabin-2 (FKBP12.6) was implicated in cADPR-induced Ca^{2+} response. In permeabilized PCA from old mice, the Ca^{2+} response evoked by cADPR (1.10^{-6}mol/L) was decreased in terms of area under the curve (1.90±0.21 ratio units/s, n=23 cells from young mice versus 1.10±0.18 ratio units/s, n=50 cells from old mice; p=0.02; Fig. 1a). The application of 1.10^{-6}mol/L rapamycin was used as a decoupling agent against FKBP12.6/RyR2 interaction. It was able to activate a Ca^{2+} signal but also affect the caffeine-induced Ca^{2+} response [20]. Rapamycin-evoked Ca^{2+} signals were increased in arteries from old mice (Fig. 1c); 6 min after rapamycin application, the amplitudes of the caffeine-induced Ca^{2+} response were decreased (Fig. 1c). These results suggested that aging reinforced the calstabin-2 regulation of Ca^{2+} signals.

Aging effects on InsP3-evoked Ca^{2+} responses

After loading of arteries with caged-145-InsP3 (2.10^{-6}mol/L) as described previously [14], we have performed its photolysis at several flash intensities to determine the apparent InsP3-affinity. Flash intensity is proportional to the quantity of released InsP3. In PCA, the apparent InsP3 affinity was decreased in old mice, but the maximal Ca^{2+} responses were identical in both ages (Fig. 2).

Aging effect on Ca^{2+} store refilling

To investigate the Ca^{2+} store refilling, we have performed different protocols using successive caffeine applications or inhibition of SERCA by thapsigargin. In Fig. 3a, a second caffeine application followed the first one immediately after the return to the basal level (60–90 s); the amplitude of the second response was decreased in the arteries from young as well as in old mice, but the effect was more pronounced in aged mice (−35 versus −47 % in young and old mice, respectively). In Fig. 3b, the delay between both caffeine applications was

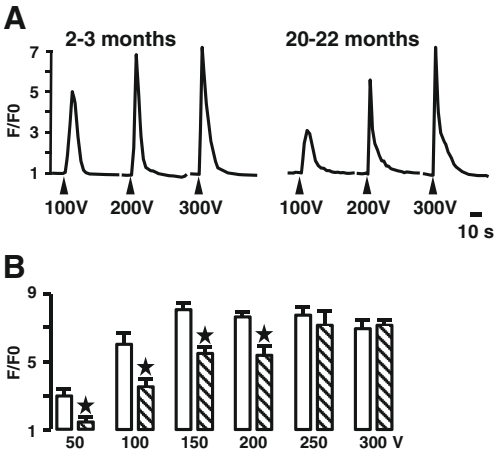

Fig. 2 InsP3-evoked Ca^{2+} release in PCA from young and old mice. **a** Typical Ca^{2+} responses evoked by photolysis of InsP3. **b** Mean of maximal amplitude of InsP3-activated Ca^{2+} responses in young (*open bars*) and old (*hatched bars*) mice. Sixty-five to 95 cells were tested for each condition. Data are expressed as mean ± SEM; *star* indicates p<0.05

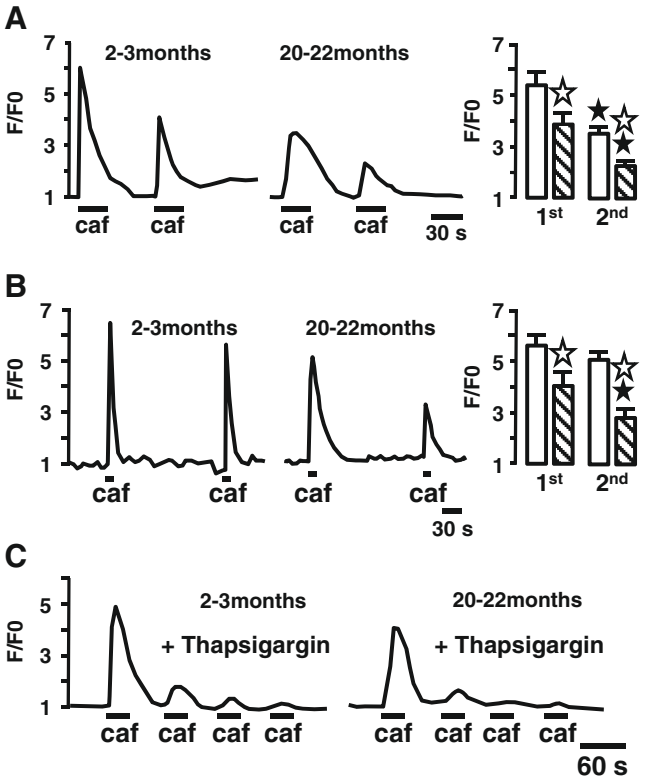

Fig. 3 Ca^{2+} store refilling in PCA from young and old mice. **a–b** On the *left*, two consecutive caffeine-evoked Ca^{2+} responses separated by 60 s (**a**) or 210 s; on the *right*, mean of maximal amplitude of evoked Ca^{2+} responses in young (*open bars*) and old (*hatched bars*) mice. Forty-six to 61 cells were tested for each condition. Data are expressed as mean ± SEM; *white star* indicate $p<0.05$ between young and old mice; *black star*, $p<0.05$ between the first and the second responses. **c** Typical Ca^{2+} responses evoked by successive caffeine application in presence of thapsigargin

increased to 180–210 s; the second caffeine-induced Ca^{2+} response was identical to the first one in young mice and decreased (−35 %) in arteries from old mice. These effects were significant (graph bars in Fig. 3a–b). Finally, successive caffeine applications were performed on arteries in the presence of thapsigargin 10^{-6}mol/L (Fig. 3c). In this last case, the disappearance of the signal was more rapid and more pronounced in old mice. In all cases, the rates of decay of the caffeine-induced signals were significantly slowed in old mice (from 1.02±0.04 ratio units (RU) s^{-1} (n=200) in young mice to 0.26±0.03 (n=91) RU s^{-1} in old mice). Taken together, these results indicate that the refilling of Ca^{2+} stores implicating SERCA and store-operated channels was decreased in old mice.

Aging effects on expression of gene encoding Ca^{2+} signaling

As revealed by using antisense strategies or overexpression of genes encoding channels and pumps implicated in Ca^{2+} signaling, the decrease or the increase of gene expression were translated in terms of Ca^{2+} signals (data not shown) [12, 13, 20]. For this reason, we have investigated the expression levels of proteins implicated in recorded Ca^{2+} signals in cerebral arteries from young and old mice.

In PCA (Fig. 4a), aging induced the decrease of expressions of CaV1.2, RyR2, and the increase of FKBP12.6, as revealed by RT-qPCR. We have evaluated expression levels of proteins by using a fluorescent pharmacological agent and immunostainings because mouse cerebral arteries were too small for western blot analysis. As illustrated in Fig. 4b, the decreases of CaV1.2 labeling with ST-bodipy(-)-DHP (p=0.004) and RyR2 immunostaining (p=0.04), and the increase of FKBP12.6 immunostaining (p=0.003) have been measured. These results could explain the modulation due to aging of KCl-, caffeine-, and cADPR-evoked Ca^{2+} signals.

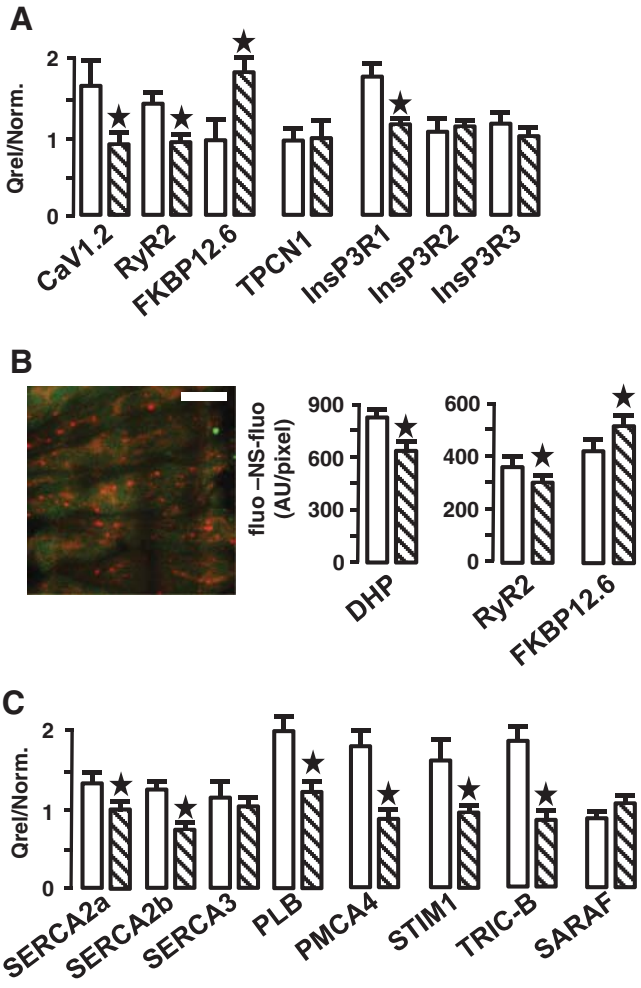

Fig. 4 Expression of channels and pumps. **a–c** Mean ± SEM of gene expression level quantified after RT-qPCR in PCA from young (*open bars*, n=12) and old (*hatched bars*, n=12) mice. *Star* indicates $p<0.05$. **b** *From left to right*: typical labeling with ST-bodipy(-)-DHP labeling (*red*) and fluo-8 (*green*) in PCA. Mean of fluorescence emitted by ST-bodipy(-)-DHP in PCA from young (*open bars*) and old (*hatched bars*) mice and means of fluorescence emitted by immunostaining of RyR2 and FKBP12.6 with specific antibodies. Data are expressed as mean ± SEM; *star*, $p<0.05$

Aging effects on expression of gene encoding Ca^{2+} store refilling

By RT-qPCR, the decreases of expression of SERCA2a, SERCA2b, PLB, STIM1, TRIC-B, and PMCA4 were measured in PCA (Fig. 4c) from old mice. The expression of SARAF, Orai1, PCMA1, STIM2, TRIC-A, TPCN1, and TPCN2 remained unchanged (Figs. 4a, c, and S2). The decreases of STIM1 and SERCA2 expressions were also verified by specific immunostaining ($p=0.02$, Fig. S2). These results suggested that the aging-induced modifications of Ca^{2+} store refilling could also be explained by a modulation of gene expression.

Aging and contraction of cerebral arteries

Finally, these modifications of Ca^{2+} signals could have physiological significance as measured in contraction experiments. All signals were measured on the arteries from mice just after euthanasia; thus, we thought that vascular contraction would be the best indicator of physiological functioning. For technical reasons, only PCA contraction was measured. The contractions induced by caffeine (0.01 mol/L) or KCl (0.14 mol/L) were both decreased (Fig. 5), whereas the phenylephrine (10^{-5} mol/L)-induced maximal contraction was unchanged in PCA from old mice, suggesting that aging was unable to modify maximal signals induced by InsP3 as observed in Ca^{2+} experiments.

Aging effects on MCA

As illustrated in the Supplementary Data, the studies of Ca^{2+} signaling and gene expression were also performed in MCA from young and old mice. The KCl-, caffeine-, and cADPR-induced Ca^{2+} responses as well as the Ca^{2+} refilling due to SERCA activation were affected by aging in MCA as observed in PCA (Fig. S3). In MCA, similar aging effects were observed on gene expressions encoding CaV1.2, RyR2, FKBP12.6, and proteins implicated in Ca^{2+} store refilling (Fig. S4).

However, a marked difference was found concerning the InsP3-dependent pathway. At low flash intensities, the InsP3-activated Ca^{2+} signal appeared similar in MCA from young and old mice, but at high flash intensity, the signal was significantly decreased in MCA from old mice (Fig. S5a–b). A bell-shaped concentration-Ca^{2+} response curve was observed for arteries from old mice (Fig. S5b). Obviously, we have controlled that the response obtained to 300-V intensity flash was not due to a deleterious effect of flash on cell components: the caffeine-evoked Ca^{2+} responses obtained after the flash delivery were not different to those obtained in cells that were not exposed to the UV flash. Furthermore, the effect of aging on InsP3R expression was different in MCA. If only a decrease of InsP3R1 was observed in PCA, in MCA, a decrease of InsP3R3 and an increase of InsP3R2 were measured (Fig. S5c). The modification of the InsP3R expression pattern could be linked to the modification of InsP3 affinity and Ca^{2+} regulation that we observed.

Finally, aging regulated the NAADP-evoked Ca^{2+} responses in MCA but not in PCA. The application of 1.10^{-7} mol/L of NAADP on permeabilized arteries induced similar Ca^{2+} signals in PCA from young and old mice (2.0±0.19 ratio units/s, $n=23$ cells from young mice versus 2.85±0.48 ratio units/s, $n=21$ cells from old mice; $p=0.17$), but, in MCA, the NAADP-evoked Ca^{2+} signals were significantly increased by aging (Fig. S6a–b). Recently, the TPCN channels were proposed as a target of NAADP. If the TPCN1 expression was not modulated by aging in PCA (Fig. 4a), in MCA, an increase of TPCN1 expression was revealed by RT-qPCR as well as by immunostaining (Fig. S6c–d). Thus, the increase of NAADP-induced Ca^{2+} response could be due to the increase of TPCN1.

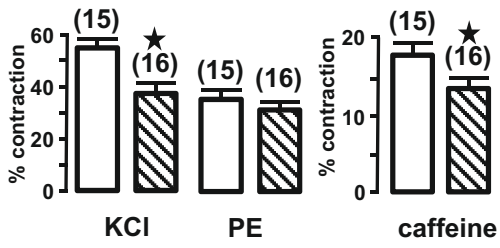

Fig. 5 Mean of KCl-, phenylephrine-, and caffeine-induced contraction. Number of tested PCA was indicated *in parenthesis* in young (*open bars*) and old (*hatched bars*) mice. Data are expressed as mean ± SEM; *star* indicates $p<0.05$

Discussion

Effects of aging on the Ca^{2+} signaling have been well described in skeletal muscle [44] and neurons [10, 56] yet not exhaustively in VSMC [23]. Therefore, we focused our study on the channels and pumps involved in Ca^{2+} signaling implicated in the regulation of vascular reactivity. Our major result is that the Ca^{2+} signaling is affected by aging. If aging can affect vascular contraction, it is possible that the other Ca^{2+}-dependent cellular mechanisms could be disrupted.

Aging affects vasoconstriction by decreasing the efficiency of second messengers to induce Ca^{2+} signals

In VSMC, hormones and neurotransmitters converge on calcium signals via different transduction pathways. The vasoconstrictions induced by alpha-adrenergic, angiotensin-II, and endothelin-1 stimulations implicate InsP3, CaV1.2, and NAADP [32, 41, 54], cADPR and CaV1.2 [18, 40], and InsP3 and NAADP [6, 33], respectively. In elderly humans, the vasoconstriction induced by alpha-adrenergic stimulation was decreased [52], but the mechanism was not described.

Assuming that the affinity of agonists and transduction pathways could be modified, ultimately the Ca^{2+} signal relies on second messenger activation.

The decrease of fluorescent DHP binding found in our study could reflect a decrease in the expression of L-type Ca^{2+} channels rather than a post-transductional change that would result more in a change in ligand affinity [28]. Thus, the decrease of depolarisation-induced Ca^{2+} signal and contraction could be attributed to the decrease of CaV1.2 expression. The calstabin-2/RyR2 interaction is regulated by cADPR to control CICR mechanism and Ca^{2+} oscillations induced by neurotransmitters [20]. As expected, the overexpression of calstabin-2 in association with the decrease of RyR2 affected the cADPR-evoked Ca^{2+} signal.

In the present study, aging causes a decrease of InsP3R1 in benefit of InsP3R3 in PCA and a slight increase of InsP3R2 expression in MCA. The InsP3R subtypes have different sensitivities to InsP3 and are modulated by Ca^{2+}. The InsP3R2 has the highest sensitivity to InsP3 and was regulated by Ca^{2+} in a bell-shaped concentration-dependent manner, whereas the InsP3R3 has the lowest InsP3 sensitivity and was very weakly modulated by Ca^{2+} in comparison with both other subtypes [36, 42, 53]. As we have previously shown in smooth muscle [21, 39], the increase of InsP3R3 proportion could explain the decrease of the InsP3 affinity to induce Ca^{2+} signals without change in the maximal Ca^{2+} response. On the other hand, in MCA, the increase of InsP3R2 proportion induces a higher InsP3 affinity and leads to inhibition by Ca^{2+} of InsP3 receptors [58], inducing a large modification of the maximal response as described in engineered cells expressing different combinations of InsP3R subtypes [53]. The apparent modification in the downstroke velocity could probably be due to the decrease of extrusion mechanisms. Finally, NAADP evoked Ca^{2+} signals by activation of RyR and TPCN channels [27, 67] directly or after interaction with a NAADP-binding cytosolic protein [60]. TPCN1 is required in NAADP-induced Ca^{2+} signal [7] and as observed in MCA, the increase of TPCN1 is sufficient to explain the increase of NAADP-induced Ca^{2+} response. Taken together, our results indicate that the Ca^{2+}-induced Ca^{2+} release by agonist depends on the effect of second messengers on their receptor channels to induce contraction.

Aging attenuates amplification of Ca^{2+} signals by CICR

Another crucial step in the Ca^{2+} signals is the amplification due to the CICR. It is well accepted that RyR2 has a predominant function in the mechanism of CICR after depolarization of VSMC [11, 13]. Our results confirm and precise those obtained on mesenteric arteries from 30-month-old C57Bl6/j mice, describing a decrease of caffeine-induced Ca^{2+} signaling [16]. Moreover, the decrease of RyR expression could also explain the alteration of myogenic tone observed in cerebral arteries from old mice [24]. Indeed, RyR are responsible for Ca^{2+} sparks and Ca^{2+}-activated K^+ channels, yet they were also implicated in the regulation of myogenic tone [15].

These results also suggest that the level of RyR2 expression could be a regulator of the VSMC reactivity in aged subjects as well as in physiopathological conditions such as in dystrophic mdx mice [38]. Moreover, we have demonstrated, by RT-qPCR as well as by immunostaining, an increase of calstabin-2 gene expression that could increase the inhibition of RyR2. In cardiomyocytes, the overexpression of calstabin-2 was responsible for a decrease of RyR-dependent Ca^{2+} leak [48]. In fact, calstabin-2 stabilizes RyR2 in a resting state via molecular interactions, which results in inhibiting the release of SR-stored Ca^{2+} [30].

Aging reduces the velocity of Ca^{2+} store refilling

Our results have shown that Ca^{2+} store refilling was decelerated with aging. Both ATPase pumps (SERCA and PMCA) are implicated in the return to basal level of the Ca^{2+} signals. It has been demonstrated in aging neurons [35] and in CHO cells [8] that a change of 20–25 % in their expression level can significantly modify Ca^{2+} signals. Our consistent results thus suggest that the decrease of the expression of PMCA4 and SERCA2 could be responsible for the aging effect on the return to basal Ca^{2+} level.

The refilling of the Ca^{2+} store also depends on store-operated Ca^{2+} entry (SOCE) that cannot be easily measured in native VSMC. As illustrated in Fig. S1, the mechanism of SOCE includes a stoichiometric interaction of Orai or TRPC in the plasma membrane with STIM in the SR [50]. The decrease of STIM expression observed in our study suggests that SOCE. Recently, two new regulators of store refilling have been identified and located in the sarcoplasmic membrane: SARAF [45] and the TRIC-A and -B channels [62, 63, 65]. The decrease of SARAF expression induced the increase of resting $[Ca^{2+}]i$ associated with the decrease of the reticulum Ca^{2+} loading [45], whereas the TRIC-B knockout induced a reticulum Ca^{2+} overloading and decrease of resting $[Ca^{2+}]i$ [62]. These data therefore suggested that SARAF and TRIC-B have antagonist functions. Likewise, our data suggest that TRIC-B and SARAF expressions were modulated in opposite way by aging to neutralize a putative effect on resting $[Ca^{2+}]i$. However, we did not measure a significant modification in resting $[Ca^{2+}]i$ by using a combination of Ca^{2+} probes (not shown).

Conclusion and perspectives

Our results strongly suggest an adaptation to time as our study has examined the effects of aging in otherwise healthy animals having access to a balanced diet. The adaptation of the Ca^{2+} signaling toolkit induces a modulation of vascular reactivity (decrease of CICR) but could also affect lysosomal/endosomal

functions and cell regeneration implicated in aging-dependent vascular physiology and pathologies. Aging can affect directly or indirectly many molecular assemblies implicated in vascular reactivity such as Ca^{2+} signaling (this study), the contractile apparatus [49], probably its Ca^{2+} sensitivity via the Rho/Rho-kinase pathway, decrease of voltage- and Ca^{2+}-dependent potassium channels [34] (Georgeon-Chartier, Morel, personal preliminary data), and basal NO level in endothelial cells [51]. Our results could help understand the effects of aging on cardiovascular pathological risks. As we observed in mice, a decrease of expression of CaV1.2 in atherosclerotic carotids was described in elderly men [57]. The overexpression of calstabin-2 was also implicated in cell hypertrophy and apoptosis [66]. Finally, the increase of TPCN1 could increase the arteriosclerosis risk through the implication of NAADP in autophagy [47] and inflammation [64], two components of arteriosclerosis.

Therefore, our results could indicate that the regulation of gene expression of proteins implicated in Ca^{2+} signaling is an adaptation to aging as well as a risk factor for vascular diseases linked to old age.

Acknowledgments CGC has performed RT-qPCR experiments, fluorescent pharmacology, and Ca^{2+} measurements; CM has performed contraction experiments, supervised experiments, and interpretation of results; and JLM and AP wrote the manuscript. We thank A. Joutel for a helpful discussion; D. Berracochea who gave us old mice to initiate the project; A. Donadieu and N. Biendon for technical assistance; and M. Goillandeau for software development (IMN software facilities). The study was supported by grants from the Centre National des Etudes Spatiales, from CNRS (longevity and aging specific action), and Agence Nationale pour la Recherche (AdapHyG no. ANR-09-BLAN-0148) and Region Aquitaine (CGC doctoral fellowship and confocal microscope).

Conflict of interest There is no conflict of interest with funding providers.

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