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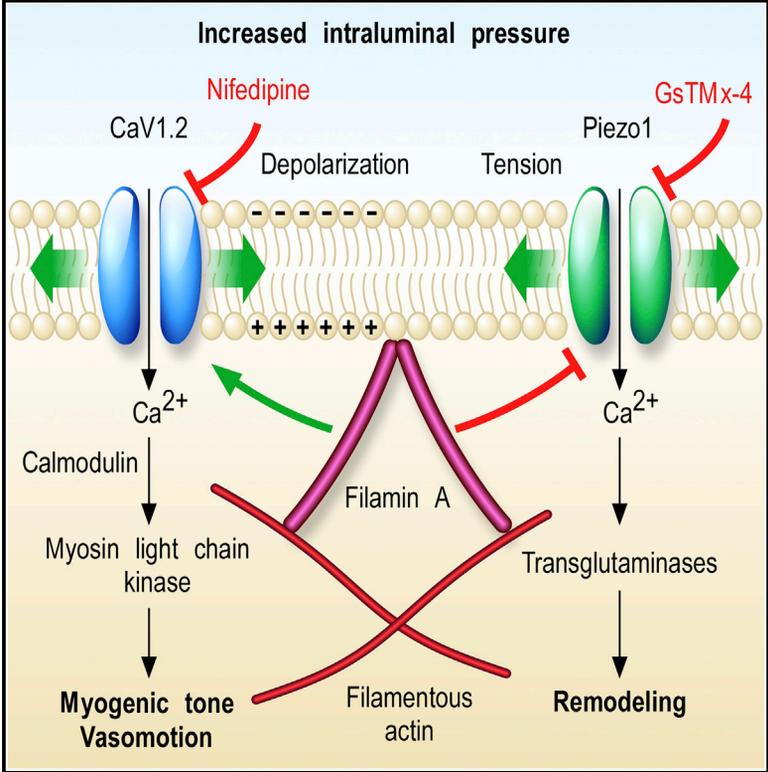
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Arterial Myogenic Activation through Smooth Muscle Filamin A

Graphical Abstract



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In Brief

Loss-of-function mutations in the cytoskeletal element filamin A are associated with severe arterial abnormalities. However, the specific role for FlnA in arterial myocytes remains poorly understood. Retailleau et al. show that smooth muscle FlnA is required for the arterial myogenic tone, by stimulating the opening of CaV1.2 at elevated pressure.

Highlights

- Deletion of smFlnA at the adult stage has a hypotensive effect
- smFlnA is critically required for the arterial myogenic tone
- Loss of smFlnA impairs pressure-dependent calcium influx
- Stretch activates the voltage-gated CaV1.2 channel in a FlnA-dependent manner

Arterial Myogenic Activation through Smooth Muscle Filamin A

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SUMMARY

Mutations in the filamin A (FlnA) gene are frequently associated with severe arterial abnormalities, although the physiological role for this cytoskeletal element remains poorly understood in vascular cells. We used a conditional mouse model to selectively delete FlnA in smooth muscle (sm) cells at the adult stage, thus avoiding the developmental effects of the knockout. Basal blood pressure was significantly reduced in conscious smFlnA knockout mice. Remarkably, pressure-dependent tone of the resistance caudal artery was lost, whereas reactivity to vasoconstrictors was preserved. Impairment of the myogenic behavior was correlated with a lack of calcium influx in arterial myocytes upon an increase in intraluminal pressure. Notably, the stretch activation of CaV1.2 was blunted in the absence of smFlnA. In conclusion, FlnA is a critical upstream element of the signaling cascade underlying the myogenic tone. These findings allow a better understanding of the molecular basis of arterial autoregulation and associated disease states.

INTRODUCTION

Filamin A (FlnA) is a cytoskeletal protein forming elongated V-shaped dimers through association of its C-terminal domain (Nakamura et al., 2011; Razinia et al., 2012). FlnA molecules bind to actin through the N-terminal domain and thereby orthogonally cross-link actin filaments. Moreover, FlnA acts as a versatile scaffold for numerous protein partners (Nakamura et al., 2011; Razinia et al., 2012). Notably, FlnA links the actin cytoskeleton to the plasma membrane by interacting with several trans-

membrane proteins, including membrane receptors, adhesion molecules, and ion channels (Ehrlicher et al., 2011; Nakamura et al., 2011; Razinia et al., 2012).

Null mutations in the X-linked *FlnA* gene are associated with a remarkably large variety of symptoms (Reinstein et al., 2013; Robertson, 2005). Hemizygotosity leads in the majority of the cases to embryonic lethality in males. In heterozygous females carrying null FlnA mutations, periventricular nodular heterotopia (PH) results from abnormal neuronal migration from the periventricular region to the cortical area, causing epileptic seizures (Robertson, 2005). Moreover, PH patients have a propensity for aortic dilatation, aneurysms, abnormalities of the microcirculation, premature stroke, and patent ductus arteriosus (Reinstein et al., 2013; Robertson, 2005; Zhou et al., 2007). In addition, FlnA missense mutations are linked to myxomatous valvular dystrophy and to otopalatodigital spectrum disorders (Robertson, 2005).

In line with the clinical observations, constitutive inactivation of FlnA in mice causes embryonic lethality associated with blood vessel leakage, further suggesting an important role for this cytoskeletal protein in the vasculature, at least during early development (Feng et al., 2006; Hart et al., 2006). Although the role for FlnA in neuronal migration has been extensively studied, its specific function in other cell types including arterial myocytes remains elusive.

Recently, we demonstrated that FlnA regulates Piezo1 stretch-activated ion channels (SACs) in arterial myocytes (Retailleau et al., 2015). Absence of smooth muscle FlnA (smFlnA) greatly enhances the opening of SACs in response to pressure stimulation, indicating that this cytoskeletal element exerts a potent mechanoprotection over Piezo1. smPiezo1 deletion does not alter the arterial myogenic response, although it influences the hypertension-dependent structural remodeling of resistance arteries in a major way (Retailleau et al., 2015).

In the present report, using a conditional smooth-muscle-specific FlnA knockout mouse model (Retailleau et al., 2015), we

investigated the physiological role of this important cytoskeletal element in small arteries at the adult stage. We show that smFlnA is critically required for the myogenic response of small arteries but is dispensable for reactivity to vasoconstrictors.

RESULTS

Smooth-Muscle-Specific and Conditional FlnA Knockout Mouse Model

Given that FlnA constitutive inactivation is embryonic lethal (Feng et al., 2006; Hart et al., 2006), we used a tamoxifen-inducible conditional smMHC Cre* system to specifically delete FlnA in smooth muscle cells (smFlnA^{0/-}) of adult male mice, which are hemizygotic for FlnA (FlnA^{0/+}) (Figure 1). Two consecutive injections of tamoxifen (1 mg) resulted in a robust knockout of FlnA, as determined by qPCR in de-endothelized caudal cutaneous resistance arteries, at 2 weeks post-TAM injection and without compensation by FlnB or FlnC (Figure 1A). These findings were confirmed at the protein level by western blotting using a monoclonal antibody directed against the N-terminal domain of FlnA (Figure 1B). The band migrating at about 190 kDa represents a cleaved form of FlnA, presumably by calpain (Gorlin et al., 1990). The turnover of smFlnA was remarkably slow and a period of 12 weeks after TAM induction was required to achieve a complete deletion of the protein (Figure 1C). FlnA, as well as α , β , and γ smooth muscle actin isoforms visualized by confocal microscopy in isolated myocytes from the caudal artery were co-localized at the cell periphery (Figure 1D). In myocytes from smFlnA^{0/-} mice, FlnA expression was absent without an obvious change in the distribution of actin isoforms (Figure 1E), as previously reported for the constitutive FlnA knockout mice (Feng et al., 2006; Hart et al., 2006).

These data indicate that the smMHC Cre* system allows a robust inactivation of FlnA in arterial myocytes, without lethality or change in mouse body weight and size, at least 12 weeks post-TAM induction (Figures S1A and S1B). Thus, this conditional mouse model allows the study of FlnA in the adult circulation, without any influence of the developmental effects associated with its knockout. Next, we examined the effect of smFlnA deletion on blood pressure regulation in awake mice followed by telemetry measurements.

Deletion of smFlnA Lowers Systemic Blood Pressure

Arterial blood pressure was monitored in conscious mice using telemetry (Figures S1C–S1F). Notably, basal blood pressure was significantly reduced in the knockout animals, with a dominant effect on systolic blood pressure, both during day and night times (Figures S1D and S1F). Consequently, pulse pressure (the difference between systolic and diastolic pressure) was lowered by about 10 mm Hg in the absence of smFlnA. Upon infusion of angiotensin II (Ang II), a strong hypertensive effect was observed with a normalization of both diastolic and systolic pressure (Figures S1C–S1F).

Thus, smFlnA deletion lowers basal arterial pressure, but without affecting reactivity to Ang II. Next, we investigated whether this hypotensive effect might be linked to a change in the basal tone of the resistance arteries.

smFlnA Is Critically Required for the Caudal Artery Myogenic Tone

Raising intraluminal pressure resulted in a maintained vasoconstriction of the isolated caudal artery, a model of cutaneous resistance artery (Figures 2A–2C, S1G, and S1H). Prominent vasomotion was also visible on top of the myogenic tone (Figures 2D–2F, S1G, and S1H). Both the myogenic response and vasomotion were observed in de-endothelized caudal arteries (n = 3), required extracellular calcium, and were potently inhibited by nifedipine (n = 3), confirming a central role for the L-type voltage-dependent calcium channel CaV1.2 in the myogenic response (Figures S1G and S1H) (Moosmang et al., 2003). Of note, the AT1R antagonist losartan (10 μ M) did not significantly affect myogenic tone or vasomotion of the caudal artery (n = 6; data not shown).

smFlnA deletion dramatically reduced the amplitude of the caudal artery myogenic response, and the effect was graded in time after TAM induction (Figures 2A–2C). At 2 weeks post-TAM, no effect of the knockout was visible (Figure 2A). At 6 weeks, the myogenic response was only reduced above 100 mm Hg, while at 12 weeks post-TAM, the myogenic response was impaired over the whole pressure range (Figures 2B and 2C). In addition, the amplitude of vasomotion was reduced in the absence of smFlnA, with a significant effect seen at both 6 and 12 weeks post-TAM (Figures 2E and 2F). The myogenic tone in the absence of smFlnA was not rescued by the addition of either 200 nM iberiotoxin or 100 μ M L-NAME, thus ruling out the involvement of BK channels or nitric oxide in this phenotype (n = 3; data not shown). In the absence of extracellular calcium and in the presence of sodium nitropruside (SNP) and papaverine, the active myogenic response was fully suppressed and passive inner diameter was determined at increasing intraluminal pressure values (Figures S1I–S1K). A significant inward remodeling of the caudal artery was observed, but only at 12 weeks post-TAM induction (Figure S1K). Of note, myogenic tone and vasomotion were already significantly blunted at 6 weeks post-TAM induction, while passive diameter was not yet modified (Figures 2B, 2E, and S1J).

Thus, smooth muscle FlnA deletion strongly impairs the myogenic behavior of the caudal artery. Next, we investigated whether smFlnA deletion might similarly affect reactivity to various vasoactive agents.

Reactivity of the Caudal Artery to Vasoconstrictors Is Preserved in the Absence of Smooth Muscle FlnA

We used isometric myography to test whether vasoconstriction in response to agonists might be altered in the absence of smFlnA (Figure S2). Reactivity to vasoconstrictors was mostly preserved upon smFlnA deletion, despite a mild decrease in the contractile response to KCl (80 mM) (Figures S2A–S2C). In addition, endothelium-dependent vasorelaxation in response to acetylcholine was unaltered by smFlnA inactivation (Figure S2D).

These findings demonstrate that smFlnA deletion predominantly impairs the myogenic tone of the caudal artery, without affecting reactivity to receptor agonists. Cross-linking of muscle actin filaments by low concentrations of FlnA reduces the concentration of myosin required for contraction of actin (Stendahl

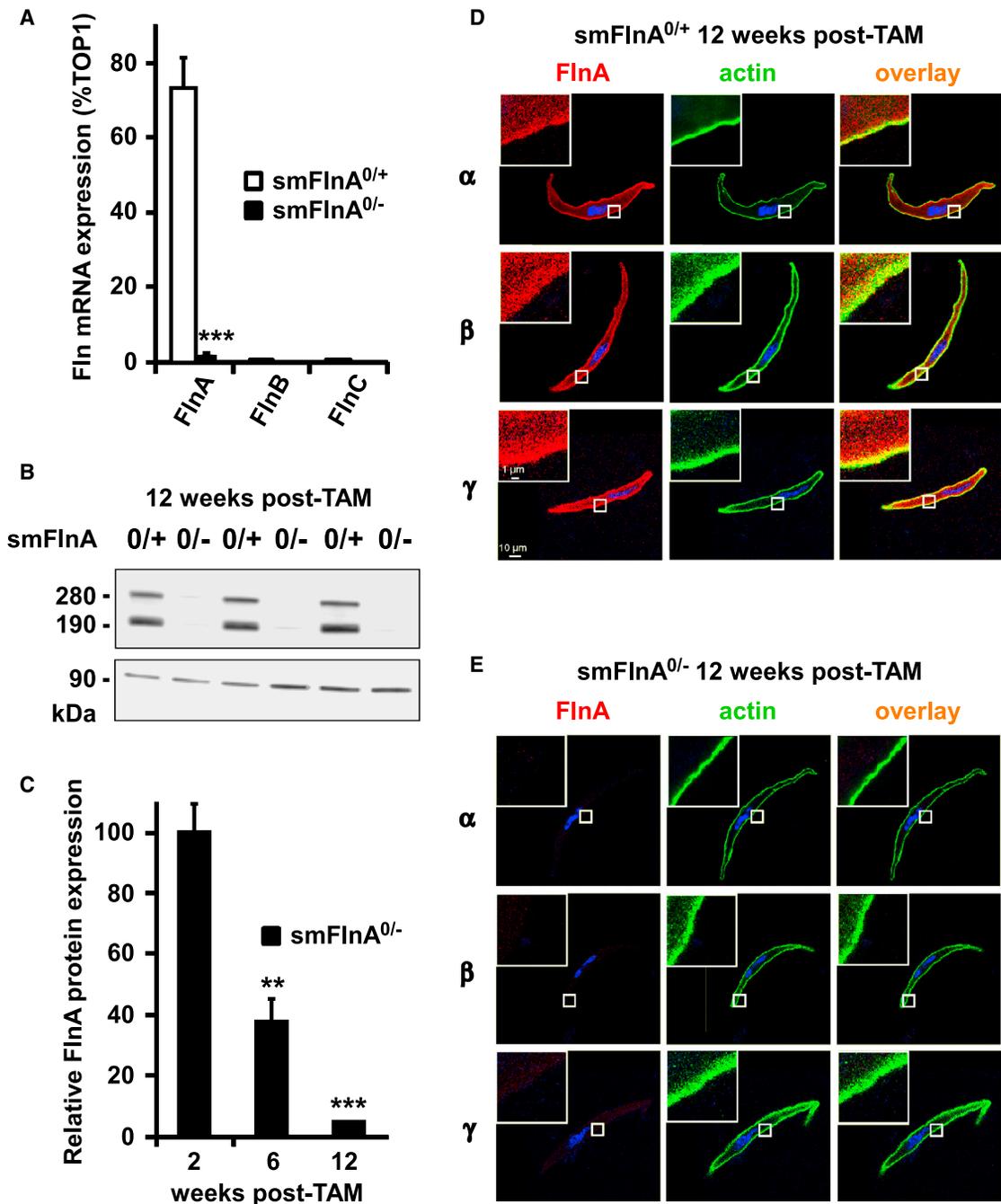


Figure 1. Smooth-Muscle-Cell-Specific and Conditional Knockout of FlnA

(A) qPCR data demonstrating the loss of FlnA mRNA in de-endothelized caudal artery segments from smFlnA^{0/+} (white bars) or smFlnA^{0/-} (black bars) mice at 2 weeks post-TAM induction. FlnB and FlnC expression is low in de-endothelized caudal arteries of either smFlnA^{0/+} or smFlnA^{0/-} mice.

(B) Western blot demonstrating a dramatic decrease in FlnA protein expression (top bands) in de-endothelized caudal arteries from smFlnA^{0/-}, as compared to smFlnA^{0/+} mice at 12 weeks post-TAM induction. The lower band shows calnexin expression, as a loading control.

(C) FlnA protein expression in de-endothelized caudal arteries, as function of time after TAM induction.

(D) Co-localization of FlnA with actin isoforms (α , β , and γ) in enzymatically dispersed myocytes from smFlnA^{0/+} caudal arteries. The inset shows a magnification of the boxed region.

(E) FlnA is absent in myocytes from smFlnA^{0/-} caudal arteries induced by TAM for 12 weeks. The inset shows a magnification of the boxed region. Nuclei are shown in blue, FlnA is shown in red (left panels), and actin is shown in green (middle panels) and the merged images (right panels).

Data are shown as means \pm SEM.

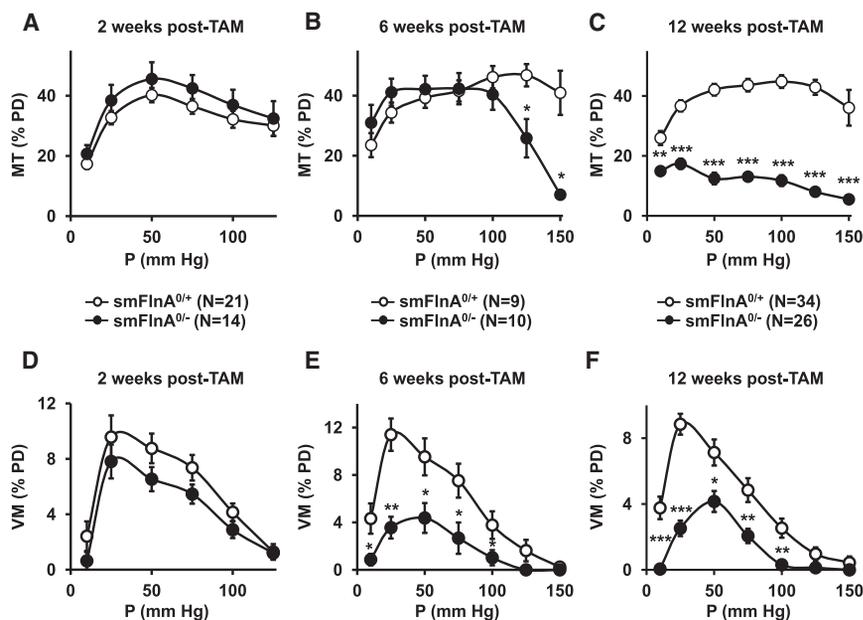


Figure 2. Myogenic Tone and Vasomotion of the Caudal Artery Are Dramatically Impaired in the Absence of smFlnA

(A) Myogenic tone (MT), expressed as a percentage of passive arterial diameter (PD) measured in isolated caudal artery segments of smFlnA^{0/+} (white circles) and smFlnA^{0/-} (black circles) mice as a function of intraluminal pressure at 2 weeks post-TAM induction.

(B) At 6 weeks post-TAM induction.

(C) At 12 weeks post-TAM induction.

(D) Vasomotion (VM), expressed as a percentage of passive arterial diameter measured in isolated caudal artery segments of smFlnA^{0/+} (white circles) and smFlnA^{0/-} (black circles) mice, as a function of intraluminal pressure at 2 weeks post-TAM induction.

(E) At 6 weeks post-TAM induction.

(F) At 12 weeks post-TAM induction. Two arterial segments per mouse were tested and the number of mice (N) is indicated.

Data are shown as means ± SEM.

and Stossel, 1980). Moreover, FlnA decreases the inhibitory action of caldesmon on actin-activated myosin ATPase and potentiates the reversal of this inhibition by calmodulin (Gusev et al., 1994). Thus, deletion of smFlnA is anticipated to impact on the general contractile properties of the arteries, as demonstrated by the modest reduction in the KCl-mediated vasoconstriction. However, the loss in myogenic tone was much more prominent and rather indicates a specific defect in smooth muscle mechanotransduction upon smFlnA deletion. Next, we investigated whether the renal circulation, another vascular bed characterized by a potent autoregulation, might be similarly altered upon smFlnA deletion (Carlström et al., 2015).

Basal Resistance of the Renal Circulation Is Dependent on smFlnA

In isolated kidneys, stepwise increase in arterial perfusion flow rate induced a progressive active increase in perfusion pressure (Figures S3A and S3D). Removal of extracellular calcium and addition of SNP fully suppressed this active response (Figure S3D, in red). Notably, the passive response in the absence of extracellular calcium together with SNP was similar between both mouse lines (Figure S3D, in red). The difference between the perfusion pressure in the presence and in the absence of calcium added with SNP represents the active tone (Figures S3A and S3D). Remarkably, the active increase in resistance of the renal circulation in the higher flow range was significantly blunted in the smFlnA^{0/-} mice (Figures S3A and S3D, black dots). By contrast, Ang II- and PE-mediated vasoconstrictions as well as endothelium-dependent renal artery dilatation induced by ACh were not significantly affected by deletion of smFlnA (Figures S3E–S3G).

In line with these ex vivo findings, the myogenic tone of isolated third-order intra-renal arteries measured by isobaric arteriography in vitro was lost in the absence of smFlnA at 12 weeks post-TAM induction (Figure S3B). Again, reactivity to Ang II

(normalization to the KCl response) was preserved, despite a mild decrease in the KCl response, as previously observed for the caudal artery (Figures S3H and S3I). No change in the passive diameter of the renal arteries was observed in the absence of extracellular calcium together with SNP and papaverine (Figure S3J).

Decreased active resistance of the renal circulation in the absence of smFlnA is anticipated to lower glomerular transcapillary hydraulic pressure. Accordingly, we observed a potent protection against Ang-II-induced proteinuria in the smFlnA^{0/-} mice (Figure S3C), although the hypertensive effect of Ang II infusion was comparable between both mouse lines (Figures S1C–S1F).

Thus, smFlnA has a major influence on the basal active resistance of the renal circulation and thereby impacts on glomerular filtration. Next, we investigated whether pressure-dependent calcium signaling might be altered in the absence of smFlnA.

Pressure-Dependent Calcium Signaling Is Impaired in the Absence of smFlnA

Using ratiometric Fura-2 calcium imaging in isolated pressurized caudal arteries, we monitored a rapid and reversible rise in intracellular calcium occurring in a pressure-dependent manner (Figure 3A). When nifedipine was added to inhibit CaV1.2 calcium channels, this pressure-dependent calcium signal was greatly blunted (Figure 3B). Our recent work has demonstrated that the stretch-activated cationic channel Piezo1 is dispensable for the myogenic tone of both caudal and cerebral arteries (Re-tailleau et al., 2015). In line with these findings, smPiezo1 deletion did not alter pressure-dependent calcium signaling in the caudal artery (Figure 3B; gray circles). When extracellular calcium was omitted the increase in intracellular calcium was fully suppressed, further demonstrating that a calcium influx is at play (Figure 3B). Upon deletion of smFlnA, a rise in basal cytosolic

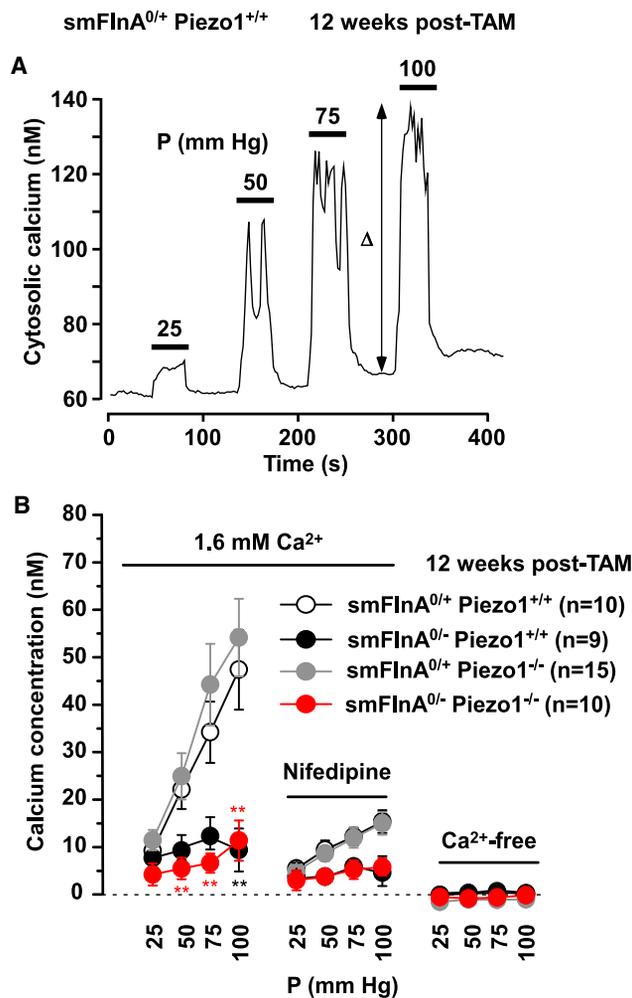


Figure 3. Pressure-Dependent Calcium Influx in Arterial Myocytes Critically Requires smFlnA

(A) Intracellular calcium was monitored using Fura-2 ratiometric fluorescence calcium imaging on a pressurized whole caudal artery (intraluminal stop flow). The artery (smFlnA^{0/+} Piezo1^{+/+} at 12 weeks post-TAM induction) was initially bathed in a control saline solution containing extracellular calcium (1.6 mM) and challenged with 80 mM KCl at an intra-luminal pressure of 75 mm Hg or with 1 μ M phenylephrine (see Figure S4). After a period of stabilization at 75 mm Hg, intraluminal pressure was gradually increased from 10 to 100 mm Hg. Between each pressure step, pressure was stepped back to 10 mm Hg. (B) Calcium transient (Δ ; as indicated in A) induced by the increase in pressure (the basal signal at 10 mm Hg has been subtracted for each pressure step) in the presence of extracellular calcium (1.6 mM). Subsequently, 3 μ M nifedipine was added to inhibit L-type calcium channels. Finally, extracellular calcium was omitted together with added 500 μ M EGTA, before calibration. Control mice (smFlnA^{0/+} Piezo1^{+/+}) are indicated by white circles. smFlnA was deleted alone (smFlnA^{0/-} Piezo1^{+/+}; black circles) or together with Piezo1 (smFlnA^{0/-} Piezo1^{-/-}; red circles). In addition, Piezo1 was deleted alone (smFlnA^{0/+} Piezo1^{-/-}; gray circles). The number of arteries (n) is indicated. Data are shown as means \pm SEM.

calcium was observed at 10 mm Hg (Figure S4A; black circles). Deletion of smPiezo1 together with FlnA prevented this basal increase in intracellular calcium (Figure S4A, red circles). Remarkably, the pressure-dependent calcium influx (Δ) was dramatically

reduced upon smFlnA deletion, with or without Piezo1 (Figures 3B and S4A; black and red circles). On the contrary, a large calcium transient could still be induced by cell depolarization (80 mM KCl at 75 mm Hg) or by PE addition (at 75 mm Hg) in the absence of smFlnA (Figure S4A). Moreover, whole-cell patch-clamp recordings indicated that both voltage-dependent calcium and potassium channels were unaltered upon smFlnA deletion (Figures S4B–S4G). Of note, the impairment of both myogenic tone and vasomotion of the caudal artery in the absence of smFlnA was independent of Piezo1 (Figures S4H and S4I).

These findings indicate that smooth muscle L-type calcium channels (CaV1.2) are functional in the absence of FlnA, although they fail to open in response to an increase in intraluminal pressure. In line with these results, next we investigated whether CaV1.2 might be directly mechanosensitive.

Stretch Activation of CaV1.2 Is Modulated by smFlnA

Exogenous CaV1.2 was transfected in M2 melanoma cells expressing FlnA, as well as native FlnB (Cunningham et al., 1992; Sverdllov et al., 2009) (Figures 4 and S4J–S4M). Mock-transfected M2 cells lack native voltage-gated calcium channels (Figures 4A, 4C, and S4J). Upon co-transfection of the α 1C, α 2 δ 1, β 1, and β 2 CaV1.2 subunits, we recorded depolarization-activated inward barium currents in the cell-attached patch configuration (Figure 4B). During a maintained membrane depolarization to -20 mV (near the predicted reversal potential of non-selective SACs), we applied pulses of pressure of increasing amplitude (Figures 4A and 4B). In EGFP-transfected cells, no channel activity was seen, while stretch-induced inward currents were consistently elicited in the CaV1.2 expressing cells ($n = 6$; Figure 4B). A reversible enhancement of α 1C alone by a steady pressure stimulation of -35 mm Hg was also observed at 0 mV, despite lower current amplitude in the absence of regulatory subunits (Figures 4C and 4D). Next, we analyzed in more detail the mechanism of CaV1.2 modulation by membrane stretch (Figures S4J–S4M). Mild steady pressure stimulation (-35 mm Hg), allowing the construction of I-V curves without patch disruption, reversibly shifted the activation curve by about 10 mV toward more negative membrane potentials (Figures S4K–S4M). This negative shift of activation resulted in about a 2-fold increase in current amplitude at negative membrane potentials (Figures S4K–S4M). By contrast, voltage-dependent inactivation was not altered by membrane stretch (Figure S4L, inset). These findings, along with previous observations demonstrating shear stress stimulation of α 1C (Lyford et al., 2002), indicate that CaV1.2 is sensitive to mechanical stress.

Next, we recorded native CaV1.2 channels in arterial myocytes using the same patch configuration (Figures 4E–4G). We used isolated caudal artery myocytes from smPiezo1^{-/-} mice, to avoid the contribution of non-selective SACs (Retailleau et al., 2015). Depolarization to 0 mV elicited small inward barium currents (black trace, Figure 4E). When the patch was mechanically stimulated by a steady pressure of -35 mm Hg, the amplitude of the inward current was reversibly enhanced (red and blue traces, Figure 4E). In the range of the resting membrane potential of arterial myocytes

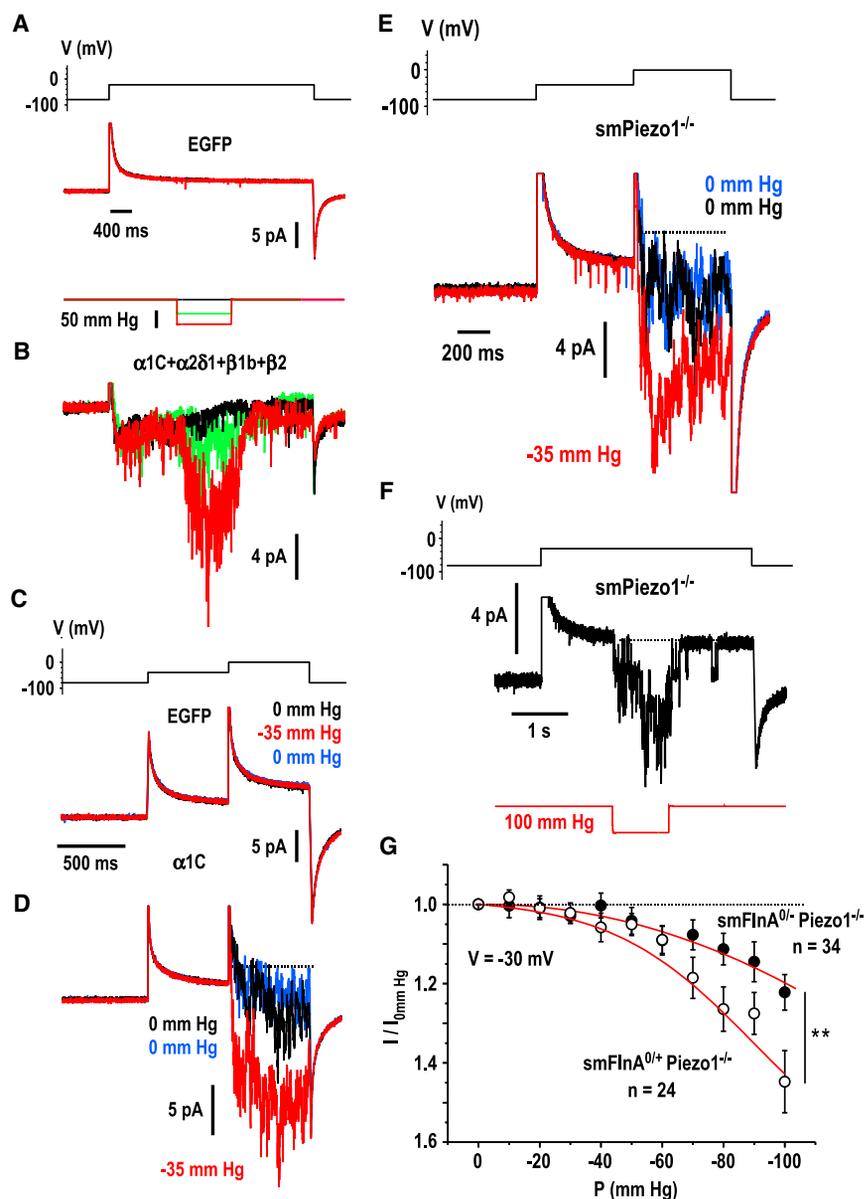


Figure 4. Upregulation of CaV1.2 by Membrane Stretch Is Influenced by FlnA

(A) EGFP-transfected M2 cell. The voltage protocol is shown on top, the current trace in the middle, and the pressure protocol (color coded) in the bottom.

(B) $\alpha 1C + \alpha 2\delta 1 + \beta 1b + \beta 2$ -transfected M2 cell. Same voltage and pressure protocols as (A).

(C) EGFP-transfected M2 cell. The voltage protocol is shown on top, the current trace is shown at the bottom. This patch has been recorded at 0 mm Hg (black trace), -35 mm Hg (red trace), and back to 0 mm Hg (blue trace).

(D) $\alpha 1C + \alpha 2\delta 1 + \beta 1b + \beta 2$ -transfected M2 cell. Same protocol as (C).

(E) Native L-type (CaV1.2) calcium channel currents in a caudal artery myocyte from a *smPiezo1*^{-/-} mouse recorded in the cell-attached patch configuration under membrane stretch. The voltage protocol is shown on top and the current traces are below. Currents have been recorded at 0 mm Hg (black trace), -35 mm Hg (red trace), and back to 0 mm Hg (blue trace). The extrapolated leak current at 0 mV is shown by a dotted line.

(F) Stretch activation of native L-type calcium channels at a membrane potential of -30 mV in a myocyte from a *Piezo1*^{-/-} mouse, recorded in the cell-attached patch configuration. The extrapolated leak current at -30 mV is shown by a dotted line. The voltage protocol is shown on top, the current trace is in the middle, and the pressure protocol is at the bottom.

(G) Pressure-effect curve of the endogenous inward currents recorded at a membrane potential of -30 mV for caudal artery myocytes from *smFlnA*^{0/+} *Piezo1*^{-/-} and *smFlnA*^{0/-} *Piezo1*^{-/-} mice. Numbers of patches are indicated in the legend.

Data are shown as means \pm SEM.

(between -40 and -30 mV), inward currents could be reversibly elicited upon pressure stimulation (Figures 4E-4G). Notably, the pressure-effect curve of CaV1.2 (in the *Piezo1*^{-/-} background) was shifted toward more negative pressure upon *smFlnA* deletion (Figure 4G).

Finally, we explored the eventual contribution of mechano-sensitive K⁺ channels in caudal artery myocytes. Previous findings suggested a possible role for stretch-inactivated K⁺ channels in the initiation of the mesenteric artery myogenic response (Schleifenbaum et al., 2014). In caudal artery smooth muscle cells from both *smFlnA*^{0/+} *Piezo1*^{-/-} (n = 37) and *smFlnA*^{0/-} *Piezo1*^{-/-} (n = 41) mice, we found no significant effect of stretch (-50 mm Hg) on potassium currents over the whole voltage range, when recorded in the cell-attached patch configuration (data not shown; E.H. unpublished data).

reactivity to vasoconstrictors is preserved. Notably, *smFlnA* is critically required for pressure-dependent calcium influx through CaV1.2 in arterial myocytes. Thus, these findings place *smFlnA* as an upstream element in the mechanotransduction signaling cascade responsible for the myogenic activation of arterial myocytes.

Our recent findings rule out a role for the cationic non-selective stretch-activated channels (SACs) in the myogenic response of both resistance and cerebral arteries (Retailleau et al., 2015). Indeed, *Piezo1* is fully dispensable for the myogenic response but is critically required for SAC activity in arterial myocytes (Retailleau et al., 2015). Similarly, the cationic channel TRPC6 is dispensable for the arterial myogenic tone (Schleifenbaum et al., 2014). Alternatively, recent results suggest that inhibition of smooth muscle voltage-dependent K⁺ channels (sensitive to

DISCUSSION

Our findings indicate that specific deletion of *smFlnA* in adult mice dramatically impairs the arterial myogenic tone of both the caudal and renal arteries, while

the KCNQ blocker XE991, but different from KCNQ3, 4, or 5) might contribute to mesenteric artery smooth muscle cell depolarization at elevated intraluminal pressure, resulting in the secondary opening of the voltage-dependent CaV1.2 channels (Moosmang et al., 2003; Schleifenbaum et al., 2014). A mechanical agonism of the Ang II type 1 receptor (AT1R), independently of Ang II, has been implicated in both the pressure-dependent inhibition of smooth muscle cell K⁺ channels and the arterial myogenic response of both mesenteric and renal arteries (Blodow et al., 2014; Mederos y Schnitzler et al., 2008; Schleifenbaum et al., 2014). Whether FlnA might be involved in the mechanical agonism of AT1Rs (although we found no effect of losartan on the myogenic tone of the caudal artery) and/or required for the downstream modulation of K⁺ channels is an intriguing possibility. However, in the present study, we found no evidence for stretch-inactivated K⁺ channels in caudal artery myocytes, suggesting that an alternative mechanism might be at play in this arterial bed.

We observed a stretch sensitivity of CaV1.2 (α 1C), in agreement with a previous report demonstrating shear stress stimulation of a human intestinal α 1C isoform (Lyford et al., 2002). The stimulation of Cav1.2 opening by membrane stretch in the voltage range of the resting membrane potential of arterial myocytes occurs independently of Piezo1 but is significantly influenced by smFlnA. Without FlnA, the pressure-effect curve of CaV1.2 was shifted toward more negative pressure, resulting in a blunted calcium influx in response to an increased intraluminal pressure. How can smFlnA influence CaV1.2 opening in response to pressure? Channel density and voltage-dependent gating in the absence of mechanical stimulation were not altered by smFlnA deletion (Figures S4B–S4G). Moreover, intrinsic CaV1.2 mechanosensitivity, although shifted toward higher pressure values, was still operating in the absence of smFlnA (Figure 4G). Thus, one possible defect in the absence of smFlnA might be altered force transmission to the CaV1.2 complex. Interestingly, the actin binding protein FlnA also interacts through its C-terminal domain with a variety of transmembrane proteins and thereby physically links the actin cytoskeleton to the plasma membrane (Nakamura et al., 2011; Razinia et al., 2012; Zhou et al., 2007). An important membrane partner of FlnA is caveolin-1 (Echarri and Del Pozo, 2015; Muriel et al., 2011; Stahlhut and van Deurs, 2000; Sverdlov et al., 2009). FlnA anchors caveolae to actin stress fibers by interacting with caveolin 1 (Echarri and Del Pozo, 2015). Caveolae are cholesterol-rich membrane invaginations highly abundant in mechanically stressed cells (Echarri and Del Pozo, 2015), including arterial smooth muscle cells of the caudal artery (data not shown). Notably, cholesterol sequestration or caveolin-1/cavin-1 deletion impairs pressure-dependent calcium influx and the arterial myogenic response, thus mimicking the effect of smFlnA deletion (Adebiyi et al., 2007; Balijepalli et al., 2006; Drab et al., 2001; Dubroca et al., 2007; Swärd et al., 2014). Interestingly, previous findings have demonstrated an interaction between caveolin-1 and the C-terminal domain of α 1C, with a localization of CaV1.2 in caveolae of mesenteric artery smooth muscle cells (Suzuki et al., 2013). Altogether, these findings could be consistent with a possible role for FlnA in force transmission to the pool of CaV1.2 channels located within cav-

eoalae. Without smFlnA, caveolae would buffer membrane tension resulting in a blunted modulation of CaV1.2 by force (Sinha et al., 2011). The definitive demonstration of this attractive hypothesis will need to await the availability of highly resolutive and quantitative molecular tools allowing the measurement of membrane tension specifically within caveolae of arterial myocytes.

In conclusion, the cytoskeleton element smFlnA plays a key role in the arterial myogenic tone. Our data indicate that smFlnA is critically required for the pressure-dependent calcium influx through CaV1.2 initiating the myogenic response and vasomotion (Moosmang et al., 2003; Schleifenbaum et al., 2014). By contrast, smFlnA negatively regulates the opening of Piezo1 that influences arterial remodeling through activation of transglutaminases (Retailleau et al., 2015). Altogether, our findings demonstrate that the actin cytoskeleton network acts in concert with calcium channels at the plasma membrane of arterial smooth muscle cells to sense changes in intraluminal pressure. Not only do these results provide information about the molecular basis of the arterial myogenic tone, they also suggest a more general role for voltage-gated calcium channels in cellular mechanotransduction. The function for CaV1.2, and possibly for other CaV isoforms, as force-gated ion channels will need to be further investigated in the context of mechanosensory transduction, mechanical nociception, and baroreceptor reflex control of blood pressure. In summary, our study indicates that CaV1.2 acts in parallel with Piezo1 in arterial myocytes as mechanosensors responsible for pressure-dependent autoregulation (the present report) and hypertension-dependent remodeling (Retailleau et al., 2015), respectively. Finally, the present results will also contribute to a better understanding of the vascular phenotype of PH patients.

EXPERIMENTAL PROCEDURES

Mice

Experiments were carried out in accordance with the guidelines of the Institutional Ethical Committee for Experimental Animals and conform to the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996). The study was approved by the local Committee for ethical and safety issues (CIEPAL-Azur). The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community standards on the care and use of laboratory animals.

TAM-inducible smMHCCre⁺ male mice (inducible Cre⁺ is inserted in the Y chromosome [Wirth et al., 2008]) were crossed with female FlnA^{lox/lox} mice to achieve a smooth-muscle-specific deletion of FlnA at the adult stage (smMHCCre⁺ FlnA^{0/-}), thus avoiding developmental effects of the knockout (Feng et al., 2006). Of note, male mice are hemizygotes for FlnA as the gene is on the X chromosome (FlnA^{0/+}). Mice were backcrossed at least 12 times onto a C57BL/6 background. Twelve-week-old adult male mice were injected twice intra-peritoneally with TAM (50 mg/kg/day dissolved in peanut oil, EtOH 10%). Mice were studied within 12 weeks after TAM injection. In the control group, we included FlnA^{0/lox} and smMHCCre⁺ mice injected with TAM, and, since no significant difference was observed between both genotypes, data were merged (smFlnA^{0/+}). smPiezo1^{-/-} mouse lines have been previously described (Retailleau et al., 2015).

Statistics

Significance of the differences was tested with a permutation test (R Development Core Team: <http://www.r-project.org/>) (n < 30) or two samples t test (n > 30). One star indicates p < 0.05, two stars p < 0.01, and three stars

$p < 0.001$. Data represent mean \pm SEM. N indicates the number of mice studied. n indicates the number of cells or arteries.

A detailed materials and methods section is available in the [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.02.019>.

AUTHOR CONTRIBUTIONS

K.R. performed the arteriography/myography experiments, analyzed the histological data, and generated the figures. M.A. performed the staining experiments and was in charge of breeding the FlnA mouse lines. S.D. performed the Ang II hypertension experiments. R.P. did some of the patch-clamp recordings. V.B. performed the telemetry arterial pressure measurements and analysis of the data. M.J. isolated smooth muscle cells from caudal arteries. J.B. performed the isolated perfused kidney experiments. D.H. contributed to the writing of the manuscript. S.O. provided the smMHC^{Cre} line. F.N. provided antibodies and discussed the results. Y.F. provided the FlnA lox/lox mice. A.P. was involved in the molecular biology experiments, design of the experiments, and writing of the manuscript. F.D. performed the calcium imaging experiments and contributed to data analysis. E.H. performed some of the electrophysiological experiments, designed experiments, and wrote the manuscript.

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