Ablation of SM22α decreases contractility and actin contents of mouse vascular smooth muscle

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Abstract The actin-binding protein SM22α marks contractile differentiation in smooth muscle, but its function is unknown. We tested its role in arterial contractility and stretch-sensitive vascular protein synthesis. Active stress in depolarised mesenteric resistance arteries and portal veins was reduced by 40% in SM22α$^{-/-}$ mice. Passive and active arterial circumference–force relationships were shifted leftwards, whereas α_1-adrenergic responses were increased. Actin contents were 10–25% lower in vessels from SM22α$^{-/-}$ mice, but protein composition was otherwise similar. Synthesis of SM22α, calponin and α-actin, but not β-actin, was sensitive to stretch. Ablation of SM22α did not affect stretch sensitivity of any of these proteins. Thus, SM22α plays a role in contractility, possibly by affecting actin filament organisation.

Key words: Actin; Calponin; Stretch; Differentiation; Resistance artery; Portal vein

1. Introduction

The actin-binding protein SM22 was isolated from chicken gizzard [1] and shown to be a globular 22 kDa basic protein with at least three isoforms (α, β, γ). The most abundant α isoform is specifically expressed in contractile mammalian smooth muscle cells together with other differentiation marker proteins, including α-actin, calponin and smooth muscle myosin [2]. The function of SM22α remains obscure, although its actin-binding properties have been characterised [3], and it has been shown to be identical to the protein transgelin, originally described to cause bundling of actin filaments [4,5].

SM22α expression is controlled by serum response factor (SRF), binding to CArG elements in its promoter [6], and is negatively regulated by globular actin [7]. Thus the organisation of the actin cytoskeleton is expected to influence SM22α expression and consistent with this, agents that depolymerise actin decrease SM22α synthesis [8]. The mechanical properties of smooth muscle correlate with SM22α expression, as short-term organ culture of rat portal vein in the absence of distension causes lower contractility and rate of actin and SM22α synthesis compared with culture under a mechanical load [8,9]. This may involve altered structure of cytoskeletal proteins, including actin filaments. The role of SM22α in this context is unknown, but it may be hypothesised to affect the structure and function of the actin filament. The actin cytoskeleton may play a dual role by sensing external forces influencing gene expression, on the one hand, and by generating and transducing contractile force, on the other. If so, it could serve both as an effector and as a target of stretch-sensitive gene expression.

The generation of mice lacking SM22α allows analysis of the physiological function of this protein [10]. These animals have an essentially normal phenotype, with normal development of the cardiovascular system and normal blood pressure. However, actin filaments in smooth muscle were more evenly distributed in the cytoplasm compared with control animals. The lack of overt phenotype may imply either that SM22α is of little functional importance or that its function is taken over by another protein, such as the closely related protein calponin. In addition, even if ablation of SM22α affects contractility, compensatory arterial remodelling could ensure essentially normal homeostasis.

To unravel the functional role of SM22α in contractility and mechanical sensing, we have studied contractile responses and determined stretch-sensitive expression of actin isoforms and calponin in isolated blood vessels from SM22α-deficient mice.

2. Materials and methods

2.1. Generation of SM22α$^{-/-}$ mice

SM22α$^{+/-}$LacZ mice were outbred with C57BL/6 and CD1 mice to generate SM22α$^{-/-}$LacZ and wild-type (WT) controls [10]. From these, genotyped homozygous-null (SM22$^{-/-}$LacZ) and WT breeder pairs were assigned to generate first and second generation offspring. Genotypes were confirmed by Western blotting of SM22α in aorta [8]. Animals of both genotypes were used at an age of 12 ± 1 weeks ($n = 15$ each).

2.2. Preparation of portal vein, aorta and mesenteric resistance vessels

Animals were killed by cervical dislocation as approved by the animal ethics committee, Lund and Malmö, and the intestine and mesentery, aorta, and portal vein removed. Preparations were mounted for force recording, fixed for histology, or extracted for protein analysis. In some experiments, endothelium was removed by running a hair through the lumen.

2.3. Force recording

Arterial segments were mounted in a myograph (610M, Danish Myo Technology, Aarhus, Denmark) using 40 μm stainless steel

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wire and were left unloaded or stretched to a circumference of 600 μm at 37°C. Protocols started after equilibration for 30 min in HEPES-buffered Krebs (composition in mM: NaCl 135.5, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11.6, HEPES 11.6, pH 7.4). High-K^+ solution (60 mM in Fig. 1, 140 mM in Fig. 2 and portal vein) was obtained by isomolar exchange of NaCl for KCl. Force was divided by two and normalised to vessel length. Portal veins were mounted as described [9] and stretched to 4 mN. Contractions were normalised to cross-sectional area, determined from the length and wet weight.

2.4. Protocols

Circumference–tension relationships were determined in denuded second-order mesenteric arteries. Weight-matched animals (27 ± 2 and 28 ± 5 g for WT and SM22α$^{-/-}$, respectively, $n = 4$) were coded until analysis was completed. In pilot experiments a circumference of ~600 μm (calculated from wire diameter and separation) was found to be optimal (L_0) for contraction. Following equilibration (circumference ≈ 180 μm), vessels were contracted for 7 min and relaxed for 5 min in Ca^{2+}-free Krebs. Passive force was then determined (~0 mN in cycle one) and cycles repeated with increasing circumferences, ending at 800 μm. L_0 and active force at L_0 were determined from Gaussian fits to circumference–force data. Responses to the α_1-selective agonist cirazoline were assessed in mesenteric arteries stretched to L_0 and first contracted twice with high K^+. Cirazoline was added cumulatively (0.01–3 μM, 10 min each). Force responses were averaged over the incubation period.

2.5. Histology, calculation of wall thickness and cross-sectional area

Following relaxation in Ca^{2+}-free Krebs, arterial segments adjacent to those used in circumference–tension experiments were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) (30 min). Arteries were then washed in PBS containing 10% sucrose and methylene blue (2 × 10 min) and embedded in Tissue-Tek (Sakura). After freezing, 10 μm cross-sections were cut in a cryostat and stained with haematoxylin–eosin. Media thickness, obtained from four averaged measurements in predefined positions, and internal circumference were determined using a computerised image analysis system (Leica Q500MC).

2.6. Organ culture

Portal veins were cultured hanging undistended or with a 0.3 g gold weight attached [8]. After 2 days, veins were transferred to low-methionine (2 mg/l) medium and incubated for a further 24 h with [^{35}S]methionine. Veins were frozen and stored at −80°C for protein analysis.

2.7. Gel electrophoresis

Tissues (pulverised in liquid N_2) were extracted with urea- or sodium dodecyl sulfate (SDS)-containing buffer (for 2-D or 1-D electrophoresis). Protein concentration was determined using a Bio-Rad assay. For urea-extracted samples protein contents were determined by comparison on a 1-D gel with a standard sample. 1-D and 2-D [8] electrophoresis was done on 12% SDS–polyacrylamide gels. In the first dimension of 2-D gel separation 11 cm IPG strips with pH range 6–11, 4–7 or non-linear pH range 3–10 were used. 2-D gels were silver-stained, dried and autoradiographed. Gels and autoradiographs were scanned and analysed using gel analysis software (PD-Quest or Quantity-One, Bio-Rad).

2.8. Statistics

Mean values ± S.E.M. are shown. Statistical significance is indicated by $^*P < 0.05$, $^{**}P < 0.01$, using Student's t-test for unpaired data.

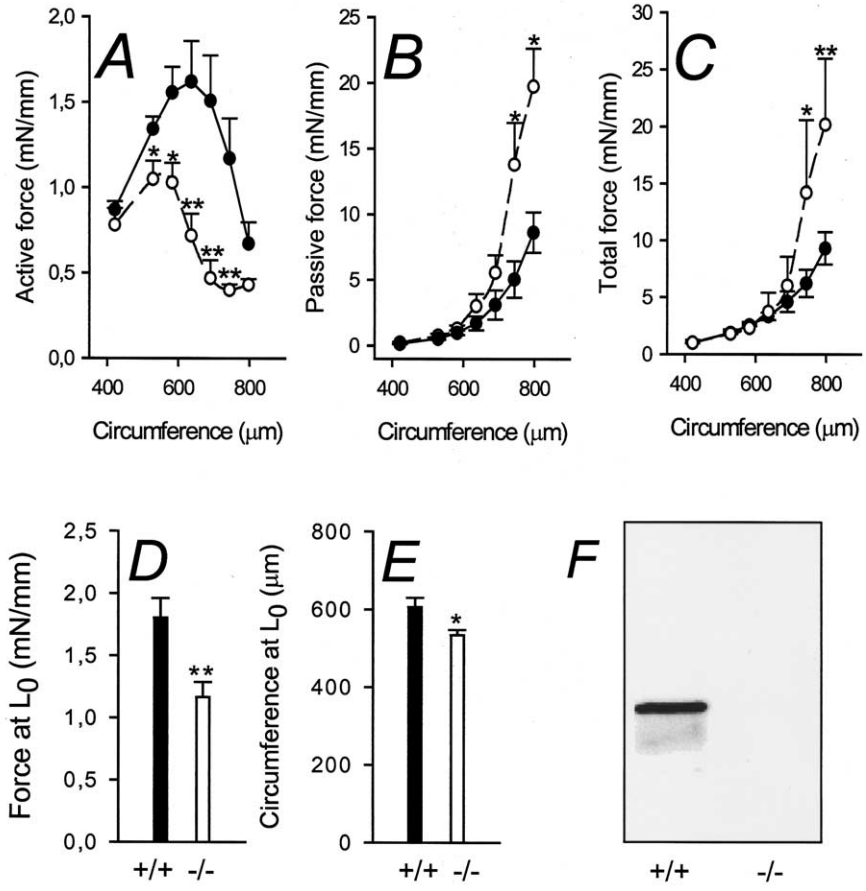

Fig. 1. Active (60 mM K^+, A), passive (B), and total force (C) of denuded mesenteric resistance arteries from SM22α$^{-/-}$ mice (open symbols) and controls (filled symbols) as a function of circumference. Two arteries were mounted per animal and four animals of each genotype were used. Maximal active force and optimal circumference (L_0) were reduced (D,E). Absence of SM22α was confirmed by Western blotting (F).

3. Results

3.1. Contractility of arteries and veins is lower in SM22α$^{-/-}$ than in WT mice

Circumference–tension relationships were determined in endothelium-denuded second-order mesenteric resistance arteries from control and SM22α$^{-/-}$ mice. In WT vessels, high-K$^+$-stimulated force at the optimal circumference (L_0) was about twice the passive force (Fig. 1A,B). In SM22α$^{-/-}$ L_0 was smaller and active force 40% lower than in WT (Fig. 1A,D,E). Passive force was greater in SM22α$^{-/-}$ mice at circumferences ~L_0 and above (Fig. 1B,E). Decreased contractility was also found in the SM22α$^{-/-}$ portal vein. Active stress in response to high K$^+$ was 8 ± 1 and 14 ± 1 mN/mm^2 ($n = 8$, $P < 0.05$) in portal veins from SM22α$^{-/-}$ and control mice, respectively.

3.2. Responses to α-adrenergic stimulation are greater in SM22α$^{-/-}$

Mesenteric resistance arteries from WT and SM22α$^{-/-}$ mice were stretched to L_0 and contracted, first with high K$^+$ and then with the α_1-selective agonist cirazoline. As in the series of experiments shown in Fig. 1, responses to depolarisation were smaller in SM22α$^{-/-}$ (Fig. 2A). The cirazoline responses in control vessels were only one sixth of the KCl response, but interestingly greater in SM22α$^{-/-}$ (Fig. 2B). The sensitivity to cirazoline was also greater in SM22α$^{-/-}$ (EC_{50} 118 ± 8 v. 220 ± 10 nM; $P < 0.05$).

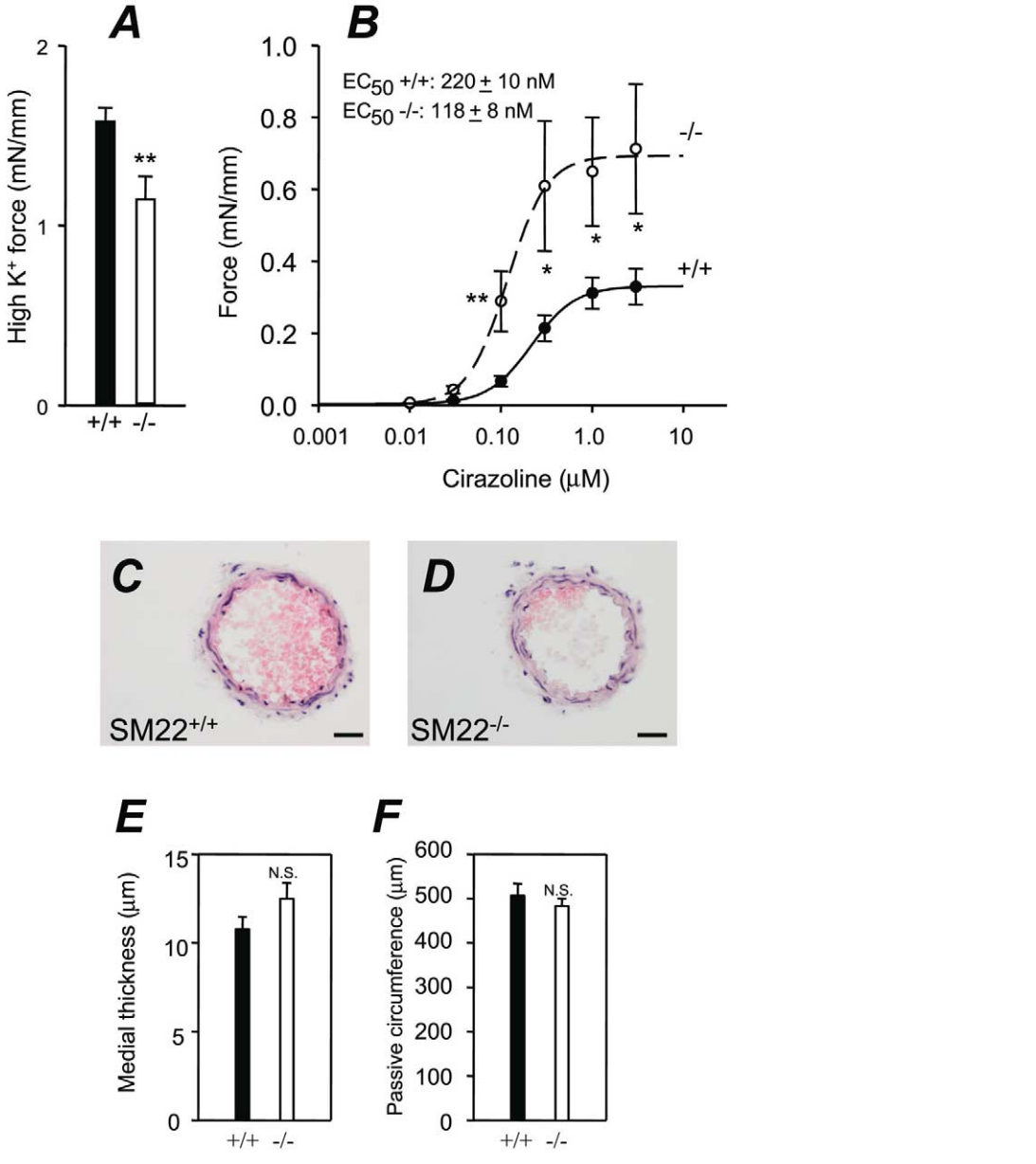

Fig. 2. Reduced response to depolarisation, increased potency and maximal response to α_1 stimulation (A,B, $n = 5$), but similar arterial morphometry (C–F) of SM22α$^{-/-}$ v. WT mesenteric resistance arteries. Haematoxylin–eosin-stained mesenteric resistance arteries from control (C) and SM22α$^{-/-}$ (D) mice. Scale bars: 25 μm. Summarised data (n: numbers as in Fig. 1) of media thickness (E) and internal circumference (F).

3.3. Media thickness is similar in SM22α$^{-/-}$ and WT mice

Arterial segments adjacent to those used in the mechanical recordings were sectioned (Fig. 2C,D) and examined morphometrically. Neither the thickness of the smooth muscle layer (media) nor the circumference differed between WT and SM22α$^{-/-}$ mice (Fig. 2E,F). Thus medial areas were also not different. It can be concluded that at any given circumference the media thickness will not differ between WT and SM22α$^{-/-}$, and thus a difference in active force (Fig. 1A,D) represents a similar difference in active stress.

3.4. Vascular protein composition of WT and SM22α$^{-/-}$ mice

Due to the small amount of tissue in mesenteric resistance arteries, proteins separated on SDS gels were visualised by silver staining, which is more sensitive than Coomassie blue but less quantitative. Using gels loaded with different amounts of extract (Fig. 3A), protein bands were evaluated within their linear ranges, as shown by control experiments. Slopes were not identical for different proteins, but the staining should report any difference in individual proteins between samples. Comparisons between corresponding bands in WT and SM22α$^{-/-}$ (Fig. 3B) indicate generally similar composition, with the exceptions that the contents of actin and of a 24 kDa protein of unknown identity were reduced in SM22α$^{-/-}$ vessels (72 ± 2% and 59 ± 2% of WT, $n = 4$, $P < 0.05$). To corroborate these findings in a larger artery, gels of the aorta were stained with Coomassie blue (Fig. 3C). Actin contents in SM22α$^{-/-}$ were 87 ± 5% ($n = 5$, $P < 0.05$) of WT. In line with the findings in arteries, actin contents of the SM22α$^{-/-}$ portal vein were 91 ± 3% ($n = 10$, $P < 0.05$) of that in control vessels. Using 2-D gels it was found that the actin isoform composition did not differ between WT and SM22α$^{-/-}$ portal veins (Fig. 4A).

3.5. Stretch-dependent protein synthesis

SM22α is a highly stretch-regulated protein as revealed in organ culture of rat portal vein [8]. By its association with actin it may influence the cytoskeleton, which has been shown

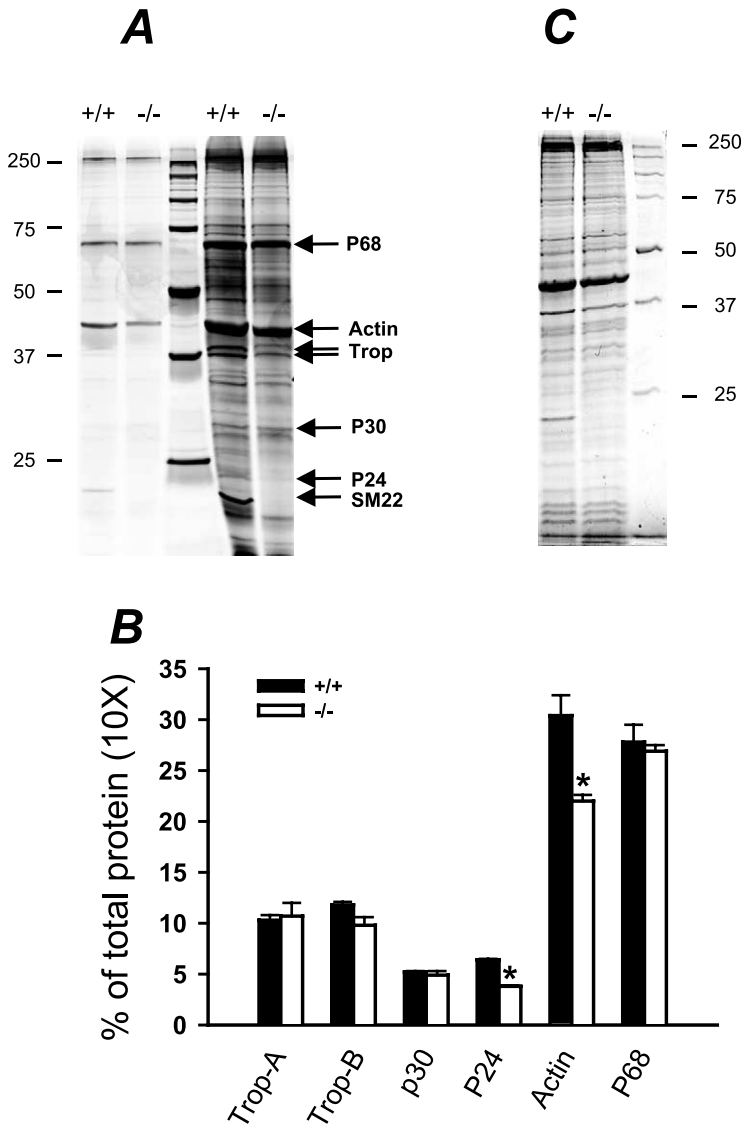

Fig. 3. Vascular protein composition in WT and SM22α$^{-/-}$ mice. Silver-stained gels loaded with two different amounts of extract of mesenteric artery (A). Comparison of selected protein bands in mesenteric arteries ($n = 4$), all evaluated in the linear range and normalised to total protein in the respective sample (B). Coomassie-stained gels of aorta (C).

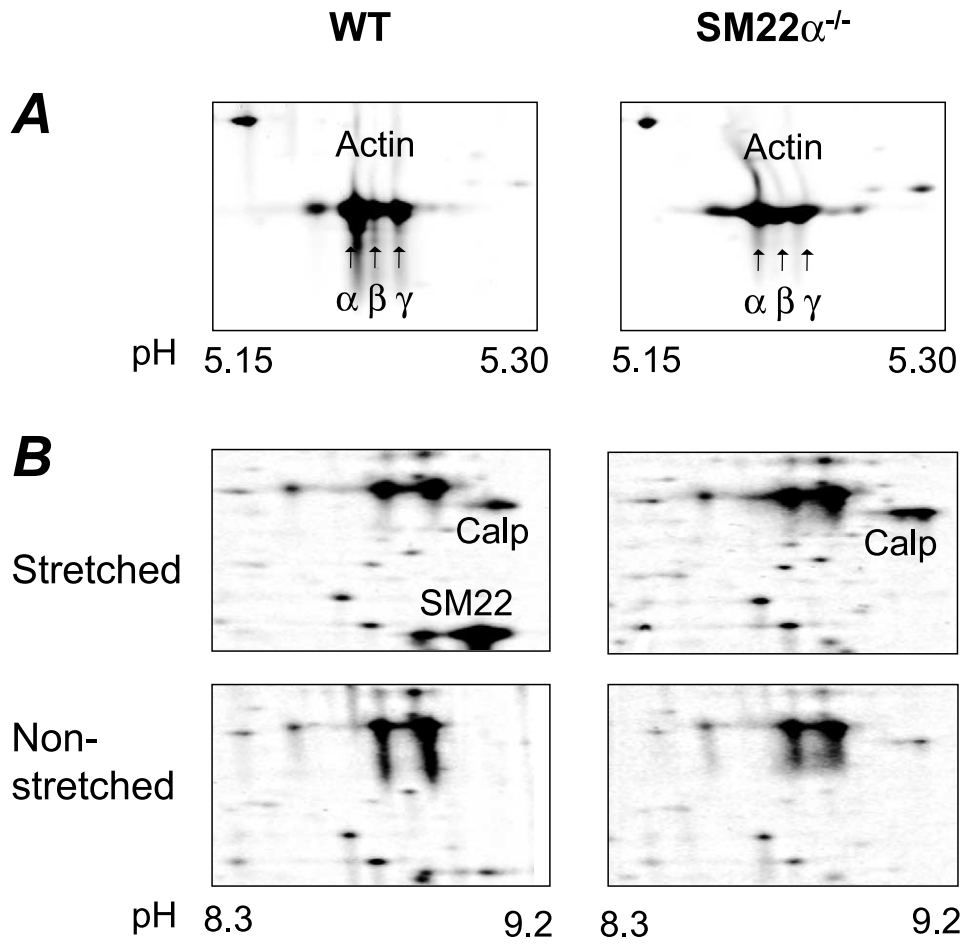

Fig. 4. Silver-stained 2-D gels showing actin isoform (α, β, γ) composition in WT and SM22α$^{-/-}$ portal veins (A). Autoradiographs showing stretch-sensitive [^{35}S]methionine incorporation (B). Locations of calponin and SM22α are indicated.

to be essential for stretch signalling [8,11]. We therefore analysed stretch-sensitive protein expression using [^{35}S]methionine and autoradiography of 2-D gels. Portal veins were cultured for 3 days undistended or under an isotonic preload (0.3 g), approximately causing distension to L_0. Autoradiographs from the basic region containing SM22α and calponin are shown in Fig. 4B. Stretched preparations generally showed higher rates of protein synthesis, although clear differences between individual spots were seen. The radiolabel incorporation into SM22α in stretched control veins was 28 ± 11 times that in unstretched veins ($n = 5$), while no incorporation was detected in SM22α$^{-/-}$. Calponin contents were not significantly altered in SM22α$^{-/-}$ portal veins (78 ± 13% of that in WT, $n = 6$, $P > 0.05$) and its rate of synthesis showed similar stretch sensitivity in WT and SM22α$^{-/-}$ (36 ± 10 v. 36 ± 14, $n = 5$ and 3). While synthesis of β-actin was not sensitive to stretch, α- and, to a lesser extent, γ-actin showed stretch sensitivity that did not differ between WT and SM22α$^{-/-}$. Radiolabel incorporation into α- relative to β-actin was 74 ± 7% and 78 ± 14% v. 13 ± 2% and 20 ± % in stretched v. unstretched portal veins of WT and SM22α$^{-/-}$ mice ($n = 3$–4). The corresponding values for γ- relative to β-actin were 79 ± 11% and 88 ± 4% v. 49 ± 7% and 60 ± 4%.

4. Discussion

As shown here, SM22α ablation reduces vascular contraction in response to depolarisation. Since blood pressure is normal in SM22α$^{-/-}$ mice [10], reduced wall tension should be associated with a narrowed lumen (inward remodelling) according to Laplace's law. In fact, passive and active circumference–tension relationships in mesenteric resistance arteries from SM22α$^{-/-}$ mice were shifted to the left. Inward remodelling is observed in small subcutaneous vessels from human hypertensive subjects [12], where the adaptation normalises wall stress [13]. In the present case, the adaptation would instead accommodate decreased force generation. No difference in media thickness was noted, indicating that the decreased active force is associated with decreased active stress, the reason for which likely involves the force-generating apparatus. The observed remodelling seems to be an adaptation to chronically altered contractility, but since all animals were of the same age, we cannot determine from the present results if remodelling progresses with age.

In contrast to the decreased force response to membrane depolarisation, sensitivity and maximal force response to α-adrenergic stimulation were increased in SM22α$^{-/-}$. This suggests increased activity of adrenergic receptor mechanisms, involving e.g. receptor density, intracellular Ca^{2+} release or sensitivity of the contractile system to Ca^{2+}. Possibly a decreased myogenic tone in SM22α$^{-/-}$, giving lower force at a given membrane potential, is compensated physiologically by an increased response to adrenergic stimulation. Even though it cannot be excluded that the greater adrenergic response in

SM22α$^{-/-}$ represents a primary effect of the gene ablation, this does not imply a greater force-generating capacity of the contractile system, as maximal α_1-stimulated force was only one sixth (WT) to one half (SM22α$^{-/-}$) of the respective KCl response. We conclude that SM22α ablation decreases active stress in response to depolarisation, which is compensated by remodelling and increased adrenergic responsiveness.

The lower response to depolarisation in SM22α$^{-/-}$ was paralleled by reduced actin contents, which was observed in all vessels studied. It is thus likely to be a general phenomenon in the vasculature. The decreased contents of actin and an unidentified 24 kDa protein were the major alterations in protein composition noted, whereas there were no changes in several other proteins. We do not exclude that more extensive analysis would reveal additional differences. Decreased actin expression might be a factor behind the decreased contractility. Since a change in the aggregation pattern of actin filaments in intestinal and vascular smooth muscle has been found in SM22α$^{-/-}$ [10], a possible explanation for the decreased contractility is altered structure/function of the thin filament system. Such an alteration, if present, does however not appear to affect the function of the actin cytoskeleton in mechanosensitive gene expression, as the synthesis rates of α-actin and calponin showed similar stretch sensitivity in portal veins from WT and SM22α$^{-/-}$ mice. Actin isoforms were similarly distributed in SM22α$^{-/-}$ and WT and differed similarly with respect to stretch sensitivity. Whereas β-actin forms a ubiquitous cytoskeleton, α-actin is associated with contractile filaments [14]. We have previously reported that actin synthesis is stretch-sensitive in the portal vein [8]. The present results show that this is confined to the SRF-regulated smooth muscle α- and γ-actin, while β-actin is stretch-insensitive. The smaller stretch sensitivity of γ-actin may reflect that this spot is a mixture of smooth muscle and non-muscle proteins [14]. The results suggest that SM22α is not needed for either contractile or cytoskeletal actin synthesis, but that this molecule regulates more subtle aspects of the filament organisation.

SM22α belongs to the type 3 family of calponin homology domain proteins and is closely related to calponin [15]. It is interesting that our findings recapitulate some properties reported in *h*1-calponin$^{-/-}$ mice. First, aorta and vas deferens from calponin$^{-/-}$ mice generated ~40% less force compared to WT muscle in response to depolarisation [16,17]. Second, actin expression was reduced by 25–50%, and actin filament abundance was also decreased [18]. The effects seen in the present study are specific for SM22α since calponin contents were not significantly altered. The closely related proteins calponin and SM22α thus both affect contractility, although the structural basis needs to be clarified in both cases. Notably, both proteins affect actin filament stability, but the mechanisms may differ [15]. In conclusion, this study demonstrates decreased vascular contractility and actin contents in SM22α$^{-/-}$ mice. This may be related to a role of SM22α in promoting actin filament stability.

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