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# **DISTINCT EFFECTS OF VOLTAGE-AND STORE-DEPENDENT CALCIUM INFLUX ON STRETCH-INDUCED DIFFERENTIATION AND GROWTH IN VASCULAR SMOOTH MUSCLE\***

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Running head: Calcium entry and gene expression in vascular smooth muscle

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Stretch of the vascular wall stimulates smooth muscle hypertrophy by activating the MAPK and Rho/Rho kinase (ROK) pathways. We investigated the role of calcium in this response. Stretch-stimulated expression of contractile and cytoskeletal proteins in mouse portal vein was inhibited at mRNA and protein levels by blockade of voltage-dependent  $\text{Ca}^{2+}$  entry (VDCE). In contrast, blockade of store-operated  $\text{Ca}^{2+}$  entry (SOCE) did not affect smooth muscle marker expression but decreased global protein synthesis. Activation of VDCE caused membrane translocation of RhoA followed by phosphorylation of its downstream effectors LIMK-2 and cofilin-2. Stretch-activated cofilin-2 phosphorylation depended on VDCE but not on SOCE. VDCE was associated with increased mRNA expression of myocardin, myocyte enhancer factor (MEF) -2A and -2D and smooth muscle marker genes, all of which depended on ROK activity. SOCE increased ERK1/2 phosphorylation and c-Fos expression but had no effect on phosphorylation of LIMK-2 and cofilin-2 or on myocardin and MEF2 expression. Knock-down of MEF2A or -2D eliminated the VDCE-induced activation of myocardin expression and increased basal c-Jun and c-Fos mRNA levels. These results indicate that MEF2 mediates VDCE-dependent stimulation of myocardin

expression via the Rho/ROK pathway. In addition, SOCE activates the expression of immediate-early genes, known to be regulated by MEF2 via  $\text{Ca}^{2+}$ -dependent phosphorylation of histone deacetylases, but this mode of  $\text{Ca}^{2+}$  entry does not affect the Rho/ROK pathway. Compartmentation of  $\text{Ca}^{2+}$  entry pathways appears as one mechanism whereby extracellular and membrane signals influence smooth muscle phenotype regulation, with MEF2 as a focal point.

Smooth muscle growth in response to stretch is critical for the adaptation of blood vessels to altered transmural pressure (remodeling), and an increase in vessel wall thickness and contractile force is typically seen in hypertensive subjects. Contraction is triggered by an increase in intracellular  $\text{Ca}^{2+}$  level ( $[\text{Ca}^{2+}]_i$ ) and depends on the expression of contractile and cytoskeletal proteins characteristic of the differentiated smooth muscle phenotype (1). Both stretch and vasoactive substances influence  $[\text{Ca}^{2+}]_i$ , and several intracellular signal cascades regulating protein synthesis contain  $\text{Ca}^{2+}$ -dependent steps. Therefore, examination of the role of  $\text{Ca}^{2+}$  handling in stretch-dependent protein synthesis may shed light on the molecular mechanisms involved in vascular remodeling.

The tissue environment is crucial for the phenotype regulation of smooth muscle cells, and cell-cell and cell-matrix interactions also mediate transmission of mechanical signals. Due to the complex interrelationships of wall tension,

diameter, pressure and contractility, it is difficult to separate the influences of the different mechanical parameters in pressurized vessels, and a simpler geometry is an advantage. The portal vein of small laboratory animals is a vessel with intrinsic myogenic tone and mainly longitudinal musculature that rapidly hypertrophies under the influence of increased transmural pressure *in vivo* (2,3). Organ culture under longitudinal stretch *ex vivo* reproduces the effects of portal hypertension on protein synthesis, showing a prominent stretch-dependent effect on synthesis of e.g. smooth muscle  $\alpha$ -actin, actin-binding proteins such as SM22 $\alpha$  and calponin, and the intermediate filament protein desmin. All of these belong to the group of smooth muscle-specific proteins, the expression of which is regulated by serum response factor (SRF) binding to several CArG boxes in their promoter regions in association with the cofactors myocardin and myocardin-related transcription factors (MRTF; a.k.a. MAL / MKL1) (4). Stretch thus promotes the contractile phenotype and is a relevant stimulus to apply for investigation of mechanisms regulating growth and differentiation in vascular smooth muscle.

Several pathways interact in the response to stretch of the portal vein in organ culture (5). Focal adhesion kinase (FAK) phosphorylation in response to stretch is biphasic, with an acute (<15 min) component correlating with MAPK activation and a long term (>24 h) component correlating with Rho activation, downstream cofilin phosphorylation and actin polymerization. An increase in the cellular F/G-actin ratio has been shown to regulate the accumulation of MRTFs to the nucleus, which results in the transcription of SRF-regulated smooth muscle-specific genes and ultimately an increased contractile differentiation of the smooth muscle (6). Stretch thus stimulates both protein synthesis

generally via MAPK activation and synthesis of smooth muscle proteins specifically via a Rho-dependent pathway.

Increased  $[Ca^{2+}]_i$  in smooth muscle cells may arise in response to membrane depolarization, activating voltage-dependent calcium entry (VDCE), or to activation of voltage-independent channels (reviewed by (7)). Much evidence suggests that mammalian homologues of transient receptor potential (TRP) proteins may form voltage-independent channels, as homo- or heteromultimers, with varying tissue distribution and functional properties. In particular, activation of store-operated calcium entry (SOCE) by depletion of intracellular  $Ca^{2+}$  stores has been shown to be associated with proliferation of vascular smooth muscle cells (8). This mode of  $Ca^{2+}$  entry may therefore participate in the regulation of cell proliferation and growth, e.g. in association with atherosclerotic plaques and in neointima formation following angioplasty, growth processes that typically involve smooth muscle cells in proliferative rather than contractile phenotype. In fact, SOCE has been shown to be increased in proliferating vs. quiescent cells, and also increases in vascular tissue in the course of organ culture or in vascular injury, conditions that are associated with down-regulation of L-type channels and increased expression of TRPC1 protein, strongly implicated as a component of store-operated calcium channels (9-12). Hence any selective effects of SOCE vs. VDCE on proliferation and differentiation may suggest new therapeutic approaches to vascular disease by targeting  $Ca^{2+}$  entry mechanisms.

Studies in vascular tissue and smooth muscle cells have shown that increased  $[Ca^{2+}]_i$  causes phosphorylation of the transcription factor cAMP-responsive element (CRE) -binding protein (CREB) and transcription of *c-fos* (13,14). Transcriptional profiling indicated that VDCE and SOCE activate distinct but partially overlapping sets of CRE-containing genes (15). Calcium influx via nifedipine-sensitive channels has been shown

to activate smooth muscle-specific gene transcription in aortic smooth muscle cells, associated with Rho kinase (ROK)-dependent regulation of myocardin expression (16).

The MADS-box transcription factor Myocyte enhancer factor 2 (MEF2) has been shown to play a central role in the regulation of differentiation in cardiac and skeletal muscle (17). MEF2 has also been shown to be a transcriptional regulator of myocardin expression (18). Furthermore, MEF2 associates with histone deacetylase (HDAC) 4 to form a repressor complex controlling *c-jun* expression in vascular smooth muscle cells (19), suggesting that MEF2 may regulate phenotype differentiation in smooth muscle. Here we have investigated the role of calcium influx via L-type and store-operated channels in stretch-dependent growth and differentiation in vascular smooth muscle at the cell and tissue levels. The results reveal a novel role of the Rho/ROK pathway in regulating MEF2 expression.

## EXPERIMENTAL PROCEDURES

*Preparation of portal veins and organ culture-* Female NMRI mice (25-30 g) were euthanized by cervical dislocation or CO<sub>2</sub> as approved by the regional Animal Ethics Committee, Lund and Malmö. The portal vein was removed and dissected free of fat and connective tissue under sterile conditions. Organ culture with or without applied stretch for 24-72 h was performed as previously described (20). Protein synthesis was studied using autoradiography on polyacrylamide gels following [<sup>35</sup>S]methionine incorporation as described (20). Verapamil (1 μM), 2-aminoethoxydiphenylborate (2-APB, 30 μM), thapsigargin (1 μM), levcromakalim (10 μM), Bay K8644 (1 μM), Y-27632 (10 μM) or KN93 (30 μM) were added to the medium as indicated while vehicle (DMSO, <0.1 %),

where necessary, was added to the control medium.

*Force measurements-* Force measurements were conducted as described previously (5). Portal veins were attached to AME 801 force transducers (AE 801, SensoNor A/S, Horten, Norway) and equilibrated for 1 h in a HEPES-buffered Krebs solution (composition in mM : NaCl 135.5, KCl 5.9, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, glucose 11.6, HEPES 11.6, pH 7.4) at 37°C under a preload of 3 mN. To establish a reference force, vessels were contracted 2-3 times for 5 min with high-KCl solution (HK; 60 mM NaCl isotonicly exchanged for KCl), with 15 min recovery between contractions. Contractions were then repeated with increasing concentrations of either verapamil or 2-APB added.

To elicit SOCE-induced contractions, portal veins were incubated in Ca<sup>2+</sup>-free solution with 2 mM EGTA for 10 min, then exposed to caffeine (20 mM for 5 min) followed by thapsigargin (10 μM) in Ca<sup>2+</sup>-free solution with 1 μM EGTA for 20 min. Contractions were then elicited by repeated exposures to normal solution (2.5 mM Ca<sup>2+</sup>) followed by recovery in Ca<sup>2+</sup>-free medium. Increasing concentrations of 2-APB were added 10 min before and during each contraction, with 1 μM verapamil continuously present. Veins not exposed to 2-APB were mounted in parallel as time/vehicle controls. In one set of experiments, veins were dismantled following reference contractions in HK and then cultured for 3 days. The veins were then mounted again and SOCE-induced contractions studied as described above.

*Preparation of primary cultured vascular smooth muscle cells from portal vein-* After careful removal of the adventitia and the endothelium, portal veins were cut with fine scissors into small patches which were then transferred to 1 ml DMEM containing 1.4 mg/ml collagenase type II (GIBCO) and 100 units/ml penicillin and streptomycin. The patches were enzymatically digested for 3 h at 37°C in a humidified incubator with a mix of 5% CO<sub>2</sub> and 95% O<sub>2</sub> and then spun down at 1000 rpm for 3 min. The supernatant was discarded and 1 ml fresh complete

DMEM containing 10% FBS was added onto the pellet; the resulting suspension was transferred to a new 35 mm culture dish or 6-well plate. Fresh complete DMEM was added to make the final volume of culture medium 2 ml and the dishes were incubated for 4-5 days. The cells emerging from the patches (defined as passage 1) were then trypsinized and passaged according to standard procedures. All experiments were done on cells at passage 2.

*siRNA transfections*- Primary cultured vascular smooth muscle cells (VSMCs) were transfected with siRNA oligos and HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocols. To optimize siRNA transfection VSMCs were transfected with a series of siRNA concentrations ranging from 2 nM to 40 nM and with scrambled RNA, respectively. Cells were transfected for 3 h before the addition of fresh complete DMEM and incubated for an additional 48-72 h before treatment or harvest. Sequences of the siRNAs used in this study are as follows: MEF2A: CAC ATT CTG CTG AAT TAT TTA, MEF2D: CCG CCA GGT GAC CTT CAC CAA. Sequence of Allstars Negative Control siRNA (scrambled siRNA) is proprietary of Qiagen.

*Calcium imaging*- VSMCs of the second passage were maintained in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin, grown to approximately 70% confluence on glass bottom culture dishes (Mat Tek Corporation, U.S.A.) and serum starved in DMEM for 24 h. Cells were then incubated in Hank's balanced saline solution (HBSS) containing 3  $\mu$ M Fluo-4 AM (Molecular Probes) for 40 min at room temperature in the dark, and then the loading solution was replaced with fresh HBSS and the cells incubated for 30 min more to allow de-esterification of the dye in the cytosol. Fluo-4 fluorescence was excited at 488 nm and fluorescence changes in 520 nm light emission

from each cell contour were monitored using a Zeiss Pascal LSM 5 confocal microscope. All cells in one field were pooled to get the average fluorescence before and after the indicated treatments. Baseline fluorescence was averaged over 1-2 min before addition of agonist and peak fluorescence following addition was then evaluated to calculate the fold change relative to the basal level.

*Protein analysis*- Protein synthesis was studied using autoradiography on polyacrylamide gels loaded with equal amounts of protein in each lane following [<sup>35</sup>S]methionine incorporation during organ culture as described (20). Western blot analysis was carried out as described (5). Antibodies used included total/phosphor- ERK1/2 (Cell Signaling, 1:1000), total and phospho-Cofilin-2 (Cell Signaling, 1:500), total and phospho-LIMK-2 (Cell Signaling, 1:500). Either GAPDH (Millipore, 1:200) or beta-actin (Sigma, 1:5000) was chosen as internal reference for loading control.

*Immunohistochemistry*- After treatments as indicated, VSMCs were fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 for 10 min. For immunostaining, cells were incubated with anti-calponin (Sigma, 1:10000) or anti-smooth muscle  $\alpha$ -actin (Sigma, 1:10000) or anti-Rho (clone 55, Millipore, 1:5) monoclonal antibody for 2 h, followed by incubation with Cy2- or Cy5-conjugated anti-mouse secondary antibody for 1 h in the dark and subsequent nucleus staining with Sytox green. All specimens were imaged by a Zeiss Pascal LSM 5 confocal microscope system.

*Quantitative RT-PCR*- Total RNA was extracted from intact portal veins or portal vein cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and quantitative RT-PCR was carried out using QuantiFast SYBR Green RT-PCR kit (Qiagen) on a real-time thermal cycler (Light Cycler<sup>TM</sup>, Roche Diagnostics, Mannheim, Germany or StepOnePlus<sup>TM</sup>, Applied Biosystems). The following run protocol was used: reverse

transcription at 50°C for 10 min, and initial activation step at 95°C for 5 min, followed by 37 cycles of denaturation at 95°C for 10 sec and combined annealing/extension at 60°C. For normalization, expression of GAPDH was determined in parallel as an endogenous control. Primer sequences were as follows:

*GAPDH*- Sense 5' CCTGCCAAGTATG-ATGAC 3'; antisense 5'GGAGTTGCTGTT-GAAGTC 3',

*MEF2C*- Sense 5' CCCAATCTTCTG-CCACTG 3'; antisense 5' GGTGCGG-TATCCATTCC 3',

*MEF2D*- Sense 5' GCTATGGGTCATCT-GTTC 3'; antisense 5' ACTTGGAATGCT-GAACTG 3',

*c-fos*- Sense 5' ACTTCGACCATGATGT-TC 3'; antisense 5' ACTAGAGACGGACAG-ATC 3',

*Myocardin*- Sense 5' GCCACTGTGCGT-CCTCCTACC 3'; antisense 5' TCGGAACT-TCCTTCTAATCAGCAAAGAG 3'.

QuantiTect™ primer assays for *SM22α*, *calponin-1*, *desmin*, *MEF2A*, *MEF2B* and *c-jun* were purchased from Qiagen.

**Statistics-** Values are presented as mean ± S.E.M. unless otherwise stated. Student's t-test was used for statistical evaluation. For multiple comparisons one-way ANOVA and Bonferroni post test were used.  $p < 0.05$  was considered statistically significant.

## RESULTS

*Effects of voltage- and store-operated  $Ca^{2+}$  influx on contraction and intracellular  $Ca^{2+}$  in portal vein.* To establish conditions allowing dissection of effects caused by VDCE and SOCE, respectively, portal veins were exposed to the L-type channel blocker verapamil and to 2-APB, which is known to inhibit SOCE but not to affect VDCE, while it also has other actions including inhibition of inositol 1,4,5-

trisphosphate release (21). In the context of the present study 2-APB was used to inhibit SOCE without affecting VDCE, but it cannot be ruled out that other mechanisms related to intracellular  $Ca^{2+}$  stores may have been affected as well.

Exposure of the portal vein to a depolarizing high- $K^+$  solution (HK) containing 60 mM KCl results in a peak contraction followed by a plateau. To evaluate contraction, mean force was therefore calculated over a 5-min interval following exposure to HK. Effects of increasing concentrations of verapamil on HK-induced contractions are shown in Fig. 1A. Verapamil in a concentration of 1  $\mu$ M inhibits HK contractions by about 80 % and this concentration was used in experiments on intact portal veins. In Fluo-4 loaded primary portal vein cells 1  $\mu$ M verapamil was found to cause a >80% inhibition of depolarization-evoked  $Ca^{2+}$  increase, whereas 5  $\mu$ M verapamil totally inhibited the response (data not shown).

SOCE-induced contractions were elicited by readdition of  $Ca^{2+}$  (2.5 mM) to portal veins following incubation with thapsigargin and caffeine to deplete intracellular  $Ca^{2+}$  stores (see Experimental Procedures). This resulted in a contraction of about 30 % of the HK-induced contraction in the same vessel (Fig. 1B). The contraction was repeatable, since thapsigargin is an irreversible inhibitor of the sarcoplasmic reticulum Ca-ATPase, and was inhibited by 2-APB in a concentration-dependent manner. 2-APB in concentrations of 30 and 100  $\mu$ M completely inhibited the contraction in freshly prepared portal veins. Since SOCE is known to increase during organ culture (10), the effect of 2-APB was tested before and after 3 days of culture, revealing a slightly larger SOCE contraction relative to the HK response and a lower sensitivity to 2-APB, with about 40 % and 23 % of the SOCE contraction still present at 30 and 100  $\mu$ M 2-APB, respectively.

2-APB in concentrations of 30-100  $\mu$ M inhibited HK-induced contractions by about 30 % (Fig. 1C). This is in accordance with previous



observations and may be explained by a component of SOCE in the HK response, whereas 2-APB does not affect activation of voltage-dependent  $\text{Ca}^{2+}$  channels (22).

To verify the effect of 2-APB on SOCE, Fluo-4 loaded primary cultured rat portal vein cells were exposed to the same protocol of thapsigargin treatment and  $\text{Ca}^{2+}$  readdition as used on intact vessels. In the continued presence of verapamil (1  $\mu\text{M}$ ), readdition of  $\text{Ca}^{2+}$  (1.75 mM) following store depletion by thapsigargin elicited a sustained increase in intracellular  $\text{Ca}^{2+}$  concentration, which was completely inhibited by 2-APB at a concentration of 30  $\mu\text{M}$  (Fig. 1D).

Collected data showed that activation of VDCE induced a fold change of Fluo-4 fluorescence of  $4.1 \pm 1.0$  compared to the baseline level (58 cells from 5 expts,  $p < 0.01$ ) and that activation of SOCE caused a fold change of  $3.2 \pm 0.5$  (36 cells from 5 expts,  $p < 0.01$ ).

*Role of  $\text{Ca}^{2+}$  entry pathways for stretch-induced protein synthesis.* Stretch of the portal vein increases protein synthesis as determined either by [ $^3\text{H}$ ]-leucine incorporation (23) or by autoradiography following [ $^{35}\text{S}$ ]-methionine incorporation (5). The latter technique allows determination of radioactivity in specific protein bands and in the whole sample by integration over the entire lane following a single PAGE separation and was used here.

Stretched and unstretched portal veins were incubated in organ culture in the presence or absence of verapamil (1  $\mu\text{M}$ ) and 2-APB (30  $\mu\text{M}$ ), as shown in Fig. 2A. Stretch of untreated control veins caused increased incorporation into a number of bands representing smooth muscle differentiation marker proteins, such as SM22, desmin and actin. Total protein synthesis was increased by stretch as evident from the summarized data in Fig. 2B. Blocking L-type calcium channels with

verapamil had no effect on overall protein synthesis in either stretched or unstretched veins, while inhibition of SOCE by 2-APB reduced total protein synthesis with maintained stretch sensitivity (Fig. 2B). Specific synthesis of stretch-sensitive smooth muscle proteins was differently affected by the inhibitors, as illustrated for SM22 and desmin (Fig. 2C,D). Verapamil decreased or abolished stretch sensitivity by inhibiting stretch-induced synthesis of these proteins, while 2-APB increased their synthesis relative to the total protein synthesis. However, the relative increase in synthesis (about 20 %) was matched by the decrease in total protein synthesis (Fig. 2B), so that synthesis of SM22 and desmin in absolute terms was essentially unaffected by 2-APB.

The effects of stretch and inhibitors on protein synthesis correlates with effects at the mRNA level as shown by quantitative RT-PCR for the smooth-muscle markers desmin, calponin-1 and SM22 $\alpha$ , normalized to GAPDH, as shown in Fig. 2E

To further investigate the specificity of protein synthesis in response to  $\text{Ca}^{2+}$  influx, we used the L-type calcium channel activator Bay K8644 (1  $\mu\text{M}$ ) to increase channel activity and the  $\text{K}^+$ -channel opener levcromakalim (10  $\mu\text{M}$ ) to cause hyperpolarization, which inhibits spontaneous activity (data not shown), and thereby inhibits activation of voltage-dependent channels. Bay K8644 increased smooth muscle-specific protein synthesis, whereas levcromakalim decreased synthesis and eliminated stretch sensitivity, as shown in Supplementary Fig S1.

Stretch-sensitive synthesis of smooth muscle-specific proteins is dependent on Rho activation and downstream cofilin-2 phosphorylation (20). This signaling pathway activates a net increase in actin polymerization and SRF-dependent transcription of smooth muscle-specific genes. In this study we have used cofilin-2 phosphorylation as an indicator of activity of the Rho pathway. Stretch of the portal vein for 24 h results in a 2-fold increase in phosphorylated relative to total cofilin-2 (Fig. 3A), which is consistent with previous

results (20). Since cofilin-2 is an SRF-regulated protein, the total amount of cofilin-2 also increases with stretch in long-term (72 h) culture (Supplementary Fig. S2), but this occurs later than the increase in phosphorylation. The effect of stretch on cofilin-2 phosphorylation is inhibited by verapamil. This closely resembles the effect seen on smooth muscle-specific protein synthesis (cf. Fig. 2).

*Effects of stretch and SOCE on ERK1/2 phosphorylation.* The basal level of ERK1/2 phosphorylation is increased considerably by dissection and handling of the tissue but reduced to low level by organ culture for 24 h (5). To allow basal phosphorylation to settle after dissection, effects of stretch on ERK1/2 phosphorylation were therefore investigated in portal veins cultured under unstretched conditions for 24 h, pretreated with verapamil, 2-APB or levcromakalim for 10 min, and then snap frozen after 5 min of stretch. None of the inhibitors affected the acute stretch-induced ERK1/2 phosphorylation relative to the effect seen in untreated vessels (Fig. 3B).

Even though ERK1/2 phosphorylation in response to acute stretch is unaffected by altered  $\text{Ca}^{2+}$  influx, SOCE is able to activate ERK1/2 as demonstrated by the experiment shown in Fig. 3C. Intracellular  $\text{Ca}^{2+}$  stores in stretched portal veins were depleted by 1  $\mu\text{M}$  thapsigargin, and the consequent SOCE caused a 100 % increase in ERK1/2 phosphorylation. This increase was totally blocked by 30  $\mu\text{M}$  2-APB but unaffected by verapamil.

In intact portal veins cofilin-2 is activated by stretch over a slower time scale (hours) than ERK1/2 (20). To evaluate the effect of SOCE on cofilin-2 phosphorylation, portal veins were incubated for 8 h in the presence and absence of 30  $\mu\text{M}$  2-APB following induction of SOCE by 1  $\mu\text{M}$  thapsigargin (Fig. 3D). The results show no effect of SOCE or its inhibition on

cofilin-2 phosphorylation, further indicating that SOCE does not influence activity of the Rho pathway and thereby the regulation of differentiation.

*Effects of VDCE on the Rho/ROK pathway, MEF2 isoform expression and smooth muscle differentiation in primary cultured VSMCs from mouse portal vein.* To identify pathways responsible for the specificity of  $\text{Ca}^{2+}$ -dependent gene expression, portal vein cells in primary culture were used. These cells express smooth muscle  $\alpha$ -actin and calponin in filamentous organization (Fig 4A), indicating smooth muscle differentiation. Recording of intracellular  $[\text{Ca}^{2+}]$  demonstrated that the cells express voltage- and store-operated channels, as evidenced by their responses to verapamil (data not shown) and 2-APB (Fig. 1D). In order to elucidate the role of  $\text{Ca}^{2+}$  entry pathways for the regulation of actin polymerization and smooth muscle differentiation, we determined the time course of RhoA activation and downstream signaling to cofilin-2. RhoA translocation from the cytosol to the membrane occurred as early as 30 seconds after high-KCl challenge (Fig. 4B), whereas phosphorylation of LIM kinase-2 peaked at 10 min (Fig. 4C) and that of cofilin-2 at 30 min (Fig 4D). Activation of RhoA and its downstream effectors is thus considerably faster in isolated cells than in intact portal veins (see above). In addition, the stimuli applied (KCl vs. stretch) may affect the time course differently.

Even though phosphorylation of LIM kinase and cofilin-2 are regulated by Rho activation following KCl stimulation, the possibility exists that  $\text{Ca}^{2+}$ -dependent effects may be mediated also by other pathways. One such possibility is direct phosphorylation of LIM kinase by CaMKIV, which has recently been shown in neuroblastoma cells (24). To test this, portal vein cells were incubated with the ROK inhibitor Y27632, the CaMK inhibitor KN93, or both, and activated by KCl. As expected, the depolarization-induced LIM kinase

phosphorylation was greatly inhibited by Y27632. KN93 had a small additional effect, while KN93 alone did not inhibit KCl-induced LIM kinase phosphorylation (Supplementary Fig. S3).

The dependence of MEF2 isoform expression on  $\text{Ca}^{2+}$  entry pathways was investigated using real time RT-PCR. Of the four isoforms, MEF2A and -2D expression was increased by membrane depolarization and inhibited by verapamil and Y27632, suggesting that the expression of these isoforms is regulated by  $\text{Ca}^{2+}$ -dependent activation of the Rho pathway in these cells (Fig. 5A,D). In contrast, the expression of MEF2B and C was nonresponsive to membrane depolarization (Fig. 5B,C). These effects on MEF2 isoforms were accompanied by parallel changes in myocardin mRNA levels (Fig 5E) and expression of the smooth muscle markers SM22 $\alpha$  (Fig. 5F), calponin-1 and calponin-2 (data not shown).

*SOCE activates c-Fos but not MEF2 or myocardin expression.* In order to elucidate the role(s) for SOCE in the regulation of VSMC proliferation or differentiation, we determined the time course of activation of the MAPK pathway following SOCE (Fig. 6). Incubation of VSMCs with 1  $\mu\text{M}$  thapsigargin for 10 min elicited a peak in ERK1/2 phosphorylation, which had entirely faded away at 30 min (Fig. 6A). In contrast, SOCE showed no significant effect on cofilin-2 phosphorylation (Fig. 6B). Exposure to 100 nM thapsigargin for 30 min led to a significant increase in c-Fos mRNA, which was entirely abolished in the presence of 30  $\mu\text{M}$  2-APB (Fig. 6C). However myocardin mRNA level was unaffected by thapsigargin treatment and thus not affected by SOCE (Fig. 6D). There was likewise no effect of thapsigargin on MEF2A and MEF2D mRNA expression (data not shown).

*Down-regulation of MEF2 eliminates calcium sensitivity of myocardin expression.* To further investigate the role of MEF2 in  $\text{Ca}^{2+}$ -dependent regulation we used siRNA to down-regulate the expression of the  $\text{Ca}^{2+}$ -sensitive isoforms MEF2A and -2D. Control experiments showed that 10 nM siRNA completely eliminated mRNA expression of these isoforms (Fig. 7A). As shown in Fig. 7B, down-regulation of both isoforms eliminated the KCl-induced increase in myocardin expression. The  $\text{Ca}^{2+}$ -sensitivity of SM22 expression was however not affected (Fig 7C). Basal c-Fos and c-Jun mRNA levels were significantly increased in MEF2A and -2D knocked-down cells, whereas their full expression upon stimulation of SOCE with thapsigargin remained unaffected. (Fig. 7D,E).

## DISCUSSION

As demonstrated in this study on differentiated vascular smooth muscle, SOCE stimulates immediate-early gene expression and overall protein synthesis but not synthesis of smooth muscle-specific proteins, which is instead driven by VDCE-dependent Rho/ROK activation, regulating myocardin expression and actin polymerization (16,20). The connection between VDCE and myocardin expression was found to involve MEF2.

MEF2 is well established as a regulator of differentiation in striated muscle, and given that myocardin has been shown to be a transcriptional target of MEF2 (18), it may also be expected to affect smooth muscle differentiation. Rho activation in C2C12 mouse myoblasts stimulates MEF2C expression and myogenic differentiation (25). Our results showing that expression of two isoforms of MEF2 is sensitive to the ROK inhibitor Y27632 suggest that MEF2 expression in vascular smooth muscle may also be under control of the Rho pathway. Moreover, knock-down of each of the  $\text{Ca}^{2+}$ -sensitive isoforms by siRNA is sufficient to eliminate the  $\text{Ca}^{2+}$  sensitivity of myocardin expression. The reason for this seeming

cooperativity between the isoforms needs to be further explored.

MEF2s are implicated in growth regulation via the MAPK pathway and interaction with class II histone deacetylases (HDACs) (17). In A10 vascular smooth muscle cells, MEF2 and HDAC4 have been shown to form a repressor complex controlling *c-jun* expression (19). The repression is enhanced by protein kinase A, whereas PDGF derepresses *c-jun* expression via  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases and novel protein kinase C $\delta$ , causing the translocation HDAC4 out of the nucleus. Therefore,  $\text{Ca}^{2+}$  may exert a dual effect via MEF2, by stimulating expression of myocardin, on the one hand, and of immediate-early genes, on the other. The present results suggest that these two mechanisms are selectively sensitive to the mode of  $\text{Ca}^{2+}$  entry, in that VDCE but not SOCE is able to activate myocardin expression, while SOCE stimulates the expression of immediate-early genes.

The cardiac isoform of myocardin stimulates MEF2 expression, thereby establishing a feed-forward loop that may help to stabilize the contractile phenotype in the terminally differentiated cardiac muscle (26). A similar mechanism does not appear to exist in smooth muscle, which lacks the cardiac myocardin isoform. This may be one basis for the reversible phenotype regulation in response to environmental influences, which is typical of adult smooth muscle cells. The present results suggest that VDCE is a positive modulator of myocardin expression via MEF2 expression. In addition, stretch of the smooth muscle cells promotes the contractile phenotype by enhancing actin polymerization, which impinges on the expression of smooth muscle markers by nuclear translocation of MRTFs (6,20), acting in synergy with increased myocardin expression. The finding that MEF2 knock-down eliminated the  $\text{Ca}^{2+}$  sensitivity of myocardin but not SM22 mRNA

levels may be related to the regulation of SRF-dependent smooth muscle gene expression via actin polymerization and nuclear translocation of MRTF. Knock-down of myocardin reduces the sensitivity of smooth muscle gene expression to VDCE (16). However, in the case of MEF2 knock-down as used here myocardin expression may not be sufficiently reduced to prevent VDCE-dependent smooth muscle gene expression, since myocardin and MRTF synergistically activate transcription of these genes (4).

MEF2A and -2D expression was found to be stimulated by KCl, and this effect was inhibited by verapamil and Y27632 in the portal vein cells. This suggests the novel concept that the expression of these MEF2 isoforms is under control of the Rho/ROK pathway in smooth muscle, although the details of this regulation remain to be investigated. In contrast no significant effect of VDCE on MEF2B and 2C expression was found. MEF2C is necessary for proper development of the vascular system (27), whereas MEF2A-deficient mice are viable at birth but develop severe cardiac malfunction soon thereafter (28). Neointima formation after balloon injury is associated with increased MEF2A, -2B and -2D, but not MEF2C expression (29). Finally, association of MEF2A with HDAC4 represses *c-jun* expression in vascular smooth muscle cells (19). Our data support a role for MEF2 as a negative regulator of growth/proliferation, as evidenced by increased basal c-Jun and c-Fos mRNA levels in MEF2A and -2D knocked-down cells.

The role of VDCE in regulating protein synthesis was demonstrated by inhibiting L-type channels using verapamil, by activating them using the dihydropyridine agonist Bay K8644 and by hyperpolarizing the cell membrane using the ATP-dependent  $\text{K}^+$  channel opener levcromakalim. Increased VDCE in the presence of Bay K8644 caused opposite effects to inhibition by verapamil by enhancing synthesis of smooth muscle marker proteins. This was associated with enhanced cofilin-2 phosphorylation, strengthening the

association between VDCE, the Rho/ROK pathway, actin polymerization, and expression of SRF-regulated smooth muscle genes. Levcromakalim, by its hyperpolarizing action, inhibits spontaneous activity and thus reduces the open probability of voltage-dependent  $\text{Ca}^{2+}$  channels. This was associated with decreased cofilin-2 phosphorylation and decreased synthesis of smooth muscle markers. Particularly, it is notable that the effect of stretch on smooth muscle marker expression was reduced by levcromakalim. This suggests that the enhanced  $\text{Ca}^{2+}$  influx associated with voltage-dependent channel activity during stretch (30), rather than stretch *per se*, has a role in regulating gene expression.

Depolarization of vascular smooth muscle by KCl has been shown to cause Rho activation, sensitive to removal of  $\text{Ca}^{2+}$  or inhibition of L-type channels (31). The effects of stretch in the portal vein are consistent with this finding, but the role of store-operated influx is less clear. Activation of SOCE was found to cause ERK1/2 phosphorylation that could be inhibited by 2-APB but not by verapamil. Furthermore, total protein synthesis in organ culture was inhibited by 2-APB but not by verapamil. This suggests that some level of SOCE is present under unstimulated conditions, which affects total protein synthesis but not stretch-dependent expression of smooth muscle markers. The ERK1/2 response to stretch of the portal vein can be inhibited by blockade of Ang II or ET-1 receptors and is sensitive to cholesterol depletion, suggesting involvement of autocrine mechanisms and lipid rafts (23,32). Vascular smooth muscle hypertrophy is stimulated by Ang II via a mechanism involving Ca/calmodulin dependent kinase (CaMK) II activation and phosphorylation of HDAC4 (33). A  $\text{Ca}^{2+}$ - and PYK2-dependent mechanism for Rho activation by Ang II has been identified (34). Taken together these results suggest that

Ang II may be involved in both growth and differentiation via  $\text{Ca}^{2+}$ -dependent mechanisms involving activation of CaMK II and RhoA, possibly via different routes of  $\text{Ca}^{2+}$  entry.

Pathways for growth and proliferation generally involve phosphorylation of the  $\text{Ca}^{2+}$ /cAMP response element (CRE)-binding protein (CREB), which regulates a large number of genes involved in growth, including immediate-early genes such as *c-fos* (35). The mode of  $\text{Ca}^{2+}$  influx has been shown to affect the transcription of CRE-containing genes in vascular smooth muscle cells, although the sets of genes regulated by voltage- and store-operated influx in vascular smooth muscle cells are partially overlapping (15). It is likely that the state of cell differentiation affects the selectivity between the modes of  $\text{Ca}^{2+}$  influx, since culture and vascular injury cause decrease of L-type channels and VDCE but increase of TRPC1 channels and SOCE (10,11).

ERK1/2 phosphorylation induced by 5 min of stretch was found to be unaffected by verapamil, 2-APB or levcromakalim. The mechanisms behind this rapid component seem to differ from those regulating basal ERK1/2 phosphorylation, (5), which may involve  $\text{Ca}^{2+}$  influx. In primary cultured portal vein cells ERK1/2 phosphorylation was found to peak at 10 min following initiation of SOCE, followed by an increase in c-Fos mRNA at 30 min. These effects were entirely abolished by 30  $\mu\text{M}$  2-APB.

In intact portal veins ERK1/2 phosphorylation was shown at 30 min following activation of SOCE. This was inhibited by 2-APB but unaffected by verapamil. There was however no effect of SOCE on cofilin-2 phosphorylation or myocardin mRNA, indicating a quite clear separation of the pathways. This applies both to primary cells and intact portal veins.

One concern might be that the levels of intracellular  $\text{Ca}^{2+}$  attained by SOCE are insufficient to activate steps in the Rho pathway. Inhibition of CaMK has been reported to inhibit KCl- but not ionomycin-induced RhoA activation in vascular

smooth muscle cells (31) and also to inhibit ionomycin-induced LIM kinase activity in neuroblastoma cells (24). Depending on the mode of  $\text{Ca}^{2+}$  elevation, CaMK activity may therefore affect the Rho pathway and downstream ROK activity and cofilin phosphorylation. However, CaMK inhibition did not affect depolarization-induced smooth muscle myosin heavy chain or  $\alpha$ -actin transcription in rat aortic cells (16). We have previously shown that stretch-induced cofilin phosphorylation in intact portal vein is dependent on ROK activity (20), and in the present work we found that CaMK inhibition had negligible effects on LIM kinase phosphorylation in portal vein cells. We conclude that the Rho/ROK pathway is the dominant mechanism for activation of LIM kinase and the downstream cofilin by membrane depolarization.

In the present study activation of SOCE by thapsigargin in primary cells resulted in a  $\text{Ca}^{2+}$  elevation amounting to about 75 % of that elicited by KCl. In intact rat tail and basilar arteries we have previously found that SOCE causes  $\text{Ca}^{2+}$  levels of about 60 % of KCl-induced levels in fresh arteries, rising to 90 – 175 % after organ culture (10). No comparable data exist for the intact portal vein, but it is notable that the sensitivity of SOCE-induced contractions to 2-APB was decreased following organ culture, suggesting that SOCE may have increased as in the arteries. These data suggest that SOCE-induced elevations in intracellular  $\text{Ca}^{2+}$  are substantial and that any sensitivity of the Rho pathway to SOCE-induced  $\text{Ca}^{2+}$  elevation has to be markedly lower relative to VDCE-induced effects. These results argue for an intracellular

compartmentation of  $\text{Ca}^{2+}$ -dependent pathways that requires further investigation.

As a result of the regulation of global protein synthesis by SOCE, the rate of smooth muscle-specific relative to total protein synthesis is actually increased when SOCE is inhibited by 2-APB. A possible explanation for this effect is the competition existing between ternary complex factors, dependent on the MAPK pathway, and myocardin/MRTFs for regulation of smooth muscle gene expression (36).

In summary, voltage- and store-operated  $\text{Ca}^{2+}$  influx differentially regulate gene expression and protein synthesis in vascular smooth muscle. A schematic incorporating some of the mechanisms discussed here is shown in Fig. 8. The increase in smooth muscle marker expression during maintained stretch of the vascular wall is mediated by Rho/ROK activation and actin polymerization, and VDCE contributes to this activation. Increased expression of MEF2 is a link in this chain of events as an activator of myocardin expression. Basal ERK1/2 activity and expression of immediate-early genes, as well as overall protein synthesis, are influenced by  $\text{Ca}^{2+}$  via SOCE. This may involve release of a MEF2-HDAC4 repressor complex, suggesting a role for MEF2 in pathways regulating both differentiation and growth/proliferation. The relationship between these two roles for MEF2 is unknown, but in principle the two mechanisms might act together under given conditions, as e.g. both growth and differentiation are promoted by stretch. Inhibition of SOCE shifts the pattern of protein synthesis towards that characteristic of the contractile phenotype, suggesting that phenotype regulation in vascular smooth muscle may be affected by selective targeting of  $\text{Ca}^{2+}$  influx over the plasma membrane.

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## FOOTNOTES

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The abbreviations used are:

Ang II Angiotensin II

2-APB 2-Aminoethoxydiphenyl borate

CamKII Calcium/calmodulin-dependent protein kinase II

CREB cAMP-responsive element-binding protein

EGTA Ethylene glycol- bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid

ERK1/2 Extracellular-signal regulated kinase 1 and 2

ET-1 Endothelin-1

FAK Focal adhesion kinase

HBSS Hank's balanced salt solution

HDAC Histone deacetylase

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HK High KCl

LIMK LIM kinase

MAPK Mitogen activated protein kinase

MEF2 Myocyte enhancer factor 2

MRTF Myocardin related transcription factor

PYK2 Proline-rich tyrosine kinase 2

ROK Rho-associated kinase

SOCE Store-operated Ca<sup>2+</sup> entry

SRF Serum response factor

TRP Transient receptor potential

VDCE Voltage-dependent Ca<sup>2+</sup> entry

VSMC Vascular smooth muscle cell

## FIGURE LEGENDS

**Figure 1.** *Inhibitory effects of verapamil and 2-APB on contractions in portal veins and on SOCE in primary VSMCs.* (A) Portal veins were contracted with high- $K^+$  medium (HK) containing 60 mM KCl, in the presence of increasing concentrations of verapamil (n=4). (B) Freshly dissected (triangles) or 3-day cultured (squares) portal veins were subjected to SOCE-induced contractions (see Experimental procedures) in the presence of increasing concentrations of 2-APB (n=4). (C) HK-induced contraction were elicited in the presence of increasing concentrations of 2-APB (triangles). Time controls with no 2-APB added (squares) were run in parallel (n=4). (D) Primary VSMCs loaded with Fluo-4AM were treated with 1  $\mu$ M thapsigargin (TG) in  $Ca^{2+}$ -free medium and then  $Ca^{2+}$  (1.75 mM) was added in the presence or absence of 30  $\mu$ M 2-APB as indicated. Verapamil (1  $\mu$ M) was present throughout the procedure. Representative fluorescence recordings (arbitrary units) are shown here.

**Figure 2.** *Differential effects of verapamil and 2-APB on stretch-induced protein synthesis.* (A) Mouse portal veins were cultured for 72 h under stretched or unstretched condition, in the presence or absence of either 1  $\mu$ M verapamil or 30  $\mu$ M 2-APB, as indicated, with [ $^{35}$ S] methionine present during the last 24 h. Proteins were separated by SDS-PAGE and bands were visualized by autoradiography. Bands representing desmin, actin and SM22 are labeled by dots. In each autoradiograph, (B) total protein synthesis, (C) SM22/total protein synthesis, and (D) desmin/total protein synthesis were evaluated and values normalized to the value of the stretched control vein in each experiment (n=4-5). (E) mRNA levels for desmin, calponin-1 and SM22 $\alpha$  were determined by quantitative RT-PCR (n=3).

\*\*\*p<0.001, \*\* p<0.01, \* p<0.05 vs. stretched control; n.s.: not significant.

**Figure 3.** *Differential blockade of  $Ca^{2+}$  influx pathways specifically affects cofilin-2 and ERK 1/2 phosphorylation.* (A) Mouse portal veins were cultured for 24 h under stretched or unstretched conditions in the presence or absence of 1  $\mu$ M verapamil. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-P-cofilin-2 and anti-cofilin-2 antibodies. Phosphorylated and total cofilin-2 were evaluated and normalized to stretched control in each experiment (n=4). Representative blots are shown beneath summarized data. (B) Portal veins were cultured under unstretched condition for 24 hours and then pretreated with 10  $\mu$ M levcromakalim, 30  $\mu$ M 2-APB or 1  $\mu$ M verapamil for 10 min before being stretched for 5 more min and then snap frozen. Phospho-ERK1/2 and Total ERK 1/2 were detected by western blotting using anti-phospho-ERK and anti-total ERK antibodies. Data were normalized to stretched control (n=4). (C) Portal veins were cultured under stretched conditions and incubated with either 1  $\mu$ M verapamil or 30  $\mu$ M 2-APB as indicated for 10 min before being exposed to 1  $\mu$ M thapsigargin for 30 min. Phosphorylated and total ERK were detected by western blotting (n=4). (D) Portal veins were cultured under stretched conditions and incubated with or without 30  $\mu$ M 2-APB for 10 min before being exposed to 1  $\mu$ M thapsigargin for 30 min. After extensive wash with culture medium, portal veins were then cultured for 8 more hours under stretched conditions in the continuous presence or absence of 30  $\mu$ M 2-APB. Phosphorylated and total cofilin-2 were detected by western blotting. Data are normalized to untreated control veins cultured in parallel (n=4). \*\*\* p<0.001, \*\*p<0.01, \*p<0.05; n.s.: not significant.

**Figure 4.** (A) Time course of RhoA activation and downstream signaling following activation by VDCE. VSMCs of the second passage were incubated with anti-SM  $\alpha$ -actin or anti-calponin monoclonal antibodies and visualized by confocal microscopy following labeling with Cy5 conjugated secondary antibody (red) and nucleus staining with Sytox green. Bar: 20  $\mu$ m. (B) VSMCs were fixed after exposure to high-KCl (60 mM) for the indicated times and immunostained for RhoA using a Cy2-conjugated secondary antibody (green). Translocation of RhoA shown by arrows. Bars: 20  $\mu$ m. (C, D). VSMCs were treated with high-KCl and verapamil for times as indicated. Proteins were separated by SDS-PAGE and immunoblotted for phosphorylated and total (C) LIM kinase or (D) cofilin-2. Representative blots are shown beneath the summarized data. Values were normalized to those in the control cells (n=4-5). \*\*p<0.01, \*p<0.05 vs. control.

**Figure 5.** Dependence of MEF2A, MEF 2D and Myocardin on VDCE and RhoA activation. VSMCs of the second passage were treated with 60 mM KCl in the presence or absence of either 5  $\mu$ M verapamil or 10  $\mu$ M Y27632 as indicated. Total RNA was extracted and subjected to real time RT-PCR. (A-F) mRNA expression of MEF2A, -2B, -2C, -2D, myocardin and SM22 was normalized to that of control relative to GAPDH (n=6). \*\*\*p<0.001, \*\*p<0.01, \*p<0.05 vs. control; n.s.: not significant.

**Figure 6.** SOCE activates ERK1/2 phosphorylation and c-Fos mRNA expression, but does not affect cofilin-2 phosphorylation and myocardin expression in VSMCs. (A) VSMCs of the second passage were treated with 100 nM thapsigargin for times as indicated. Proteins were separated by SDS-PAGE and immunoblotted for phosphorylated and total (A) ERK1/2 or (B) cofilin-2. Representative blots are shown beneath the summarized data. Values were normalized to control (n=4). After treatment of VSMCs with 100 nM thapsigargin for 30 min in the presence or absence of 30  $\mu$ M 2-APB, total RNA was extracted and real-time RT-PCR carried out to determine mRNA expression for (C) c-Fos and (D) myocardin. Values were normalized to the control relative to GAPDH (n=4-6). \*\*\*p<0.001, \*\*p<0.01, \*p<0.05 vs. control; n.s.: not significant.

**Figure 7.** Silencing of MEF2A and MEF2D decreases sensitivity of myocardin to VDCE and increases basal mRNA expression of c-Fos and c-Jun in VSMCs. (A) VSMCs of the second passage were treated with siRNA targeting MEF2A or MEF2D for 48 h and the knock-down effect was evaluated by real-time RT PCR. Wild type control, vehicle control and negative control were set up in parallel. MEF2A- or MEF2D- knocked-down VSMCs were then subjected to high KCl (60 mM) challenge for 24 h. Total RNA was extracted and real-time RT-PCR carried out to measure mRNA expression of (B) myocardin and (C) SM22 $\alpha$  (n=3). MEF2A- or MEF2D- knocked-down VSMCs were treated with 1  $\mu$ M thapsigargin for 30 min before total RNA extraction and subsequent real-time RT-PCR. Levels of mRNA for (D) c-Fos and (E) c-Jun were measured and normalized to control relative to GAPDH (n=3). \*\*\*p<0.001, \*\*p<0.01, \*p<0.05, Thapsigargin-treated vs. basal. ## p<0.01, #p<0.05, basal MEF2A or -2D knock-down vs. basal WT .

**Figure 8.** Simplified schematic showing suggested effects of SOCE and VDCE on growth and differentiation of vascular smooth muscle. For details see text. Ca<sup>2+</sup> inflow via the two pathways has specific and different effects on cell signaling, suggesting that the resulting intracellular Ca<sup>2+</sup> pools are functionally separate. MEF2 participates in both pathways but whether this occurs via different pools and/or isoforms needs to be established.

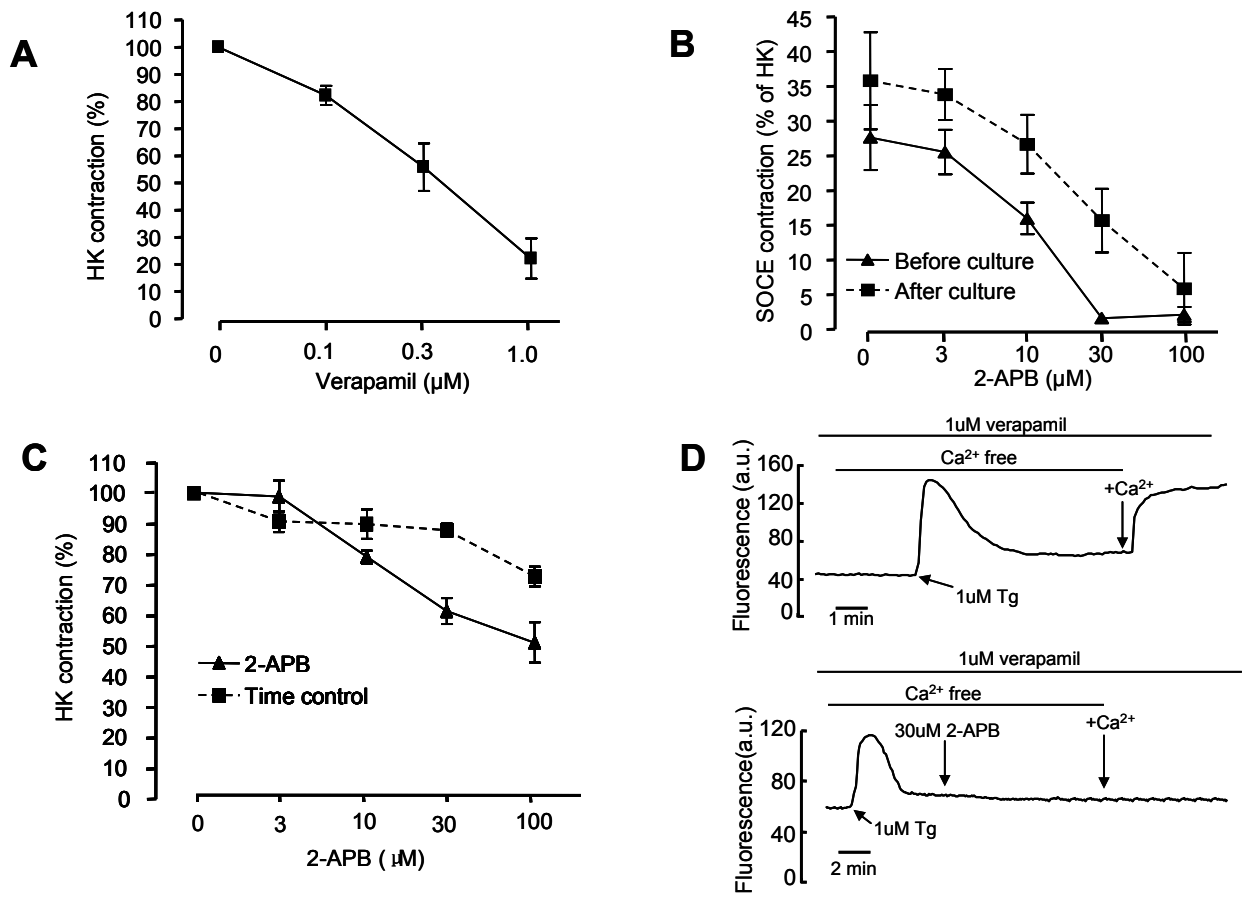


Figure 1

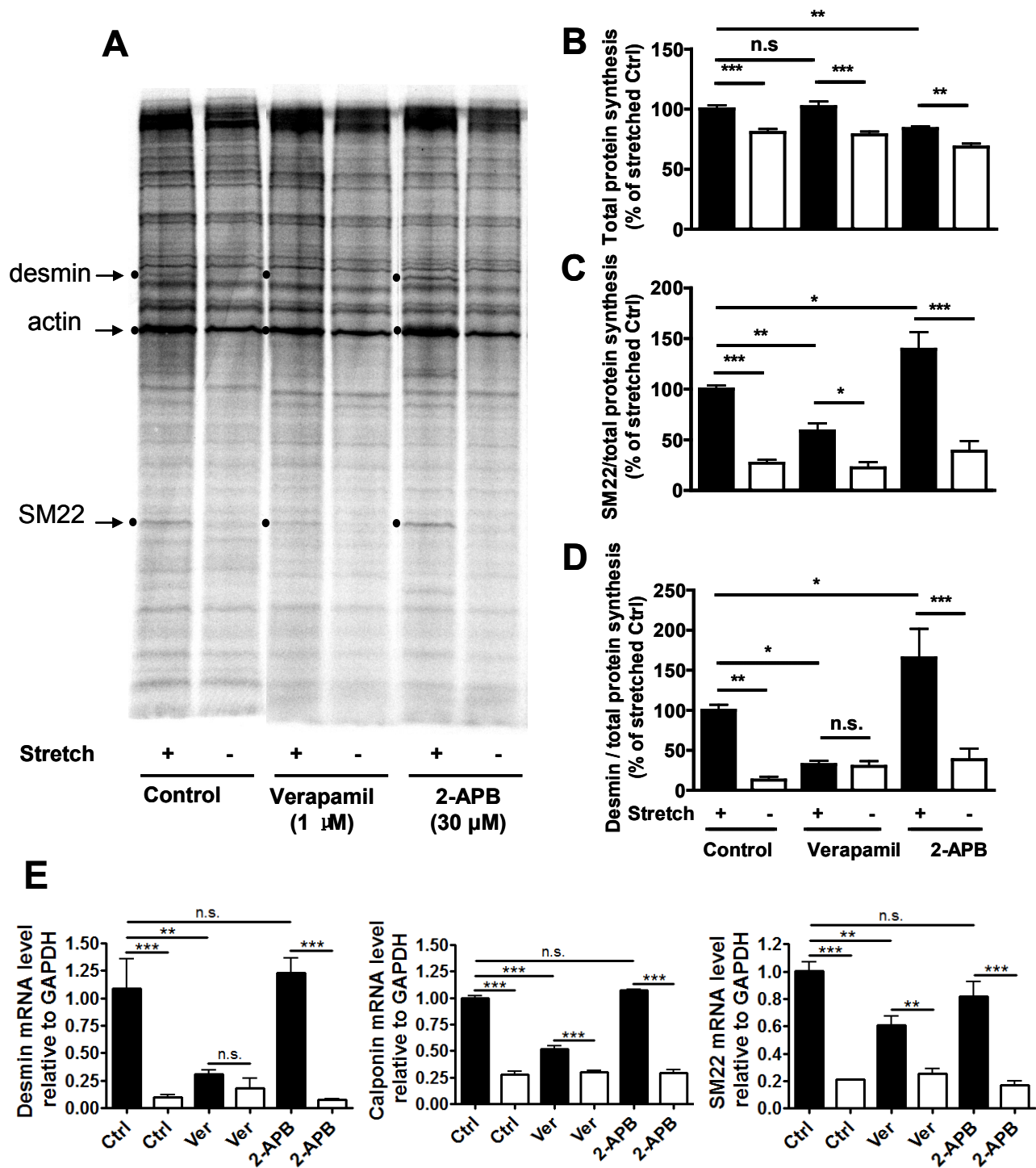


Figure 2

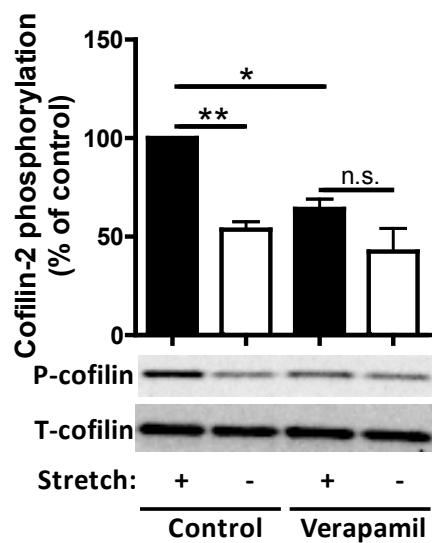
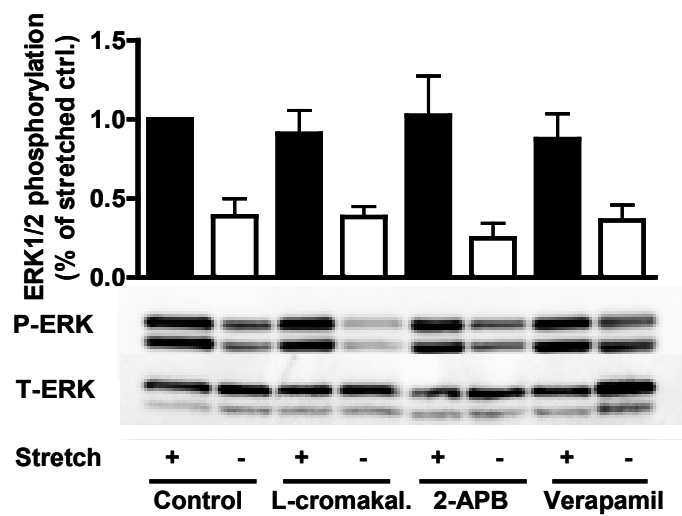
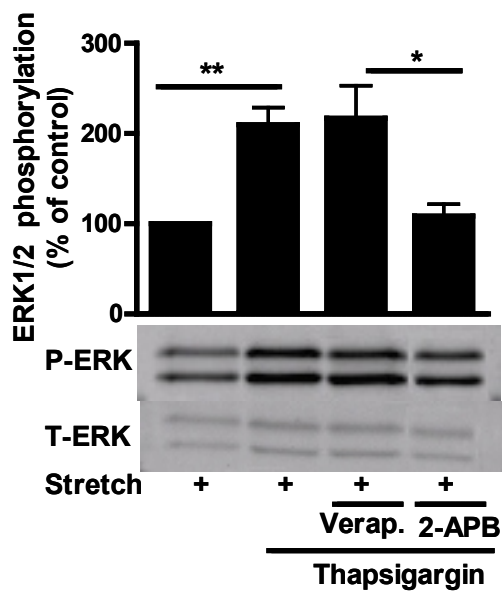
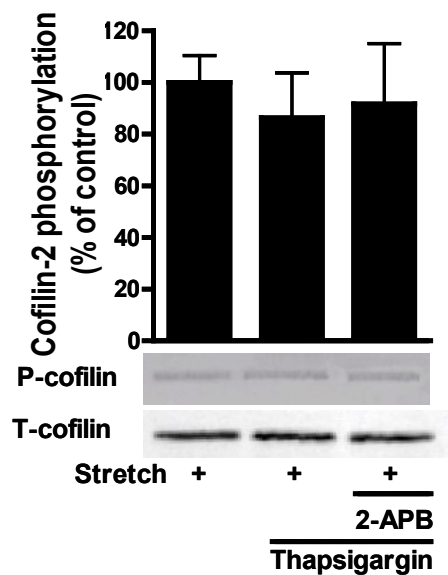
**A****B****C****D**

Figure 3

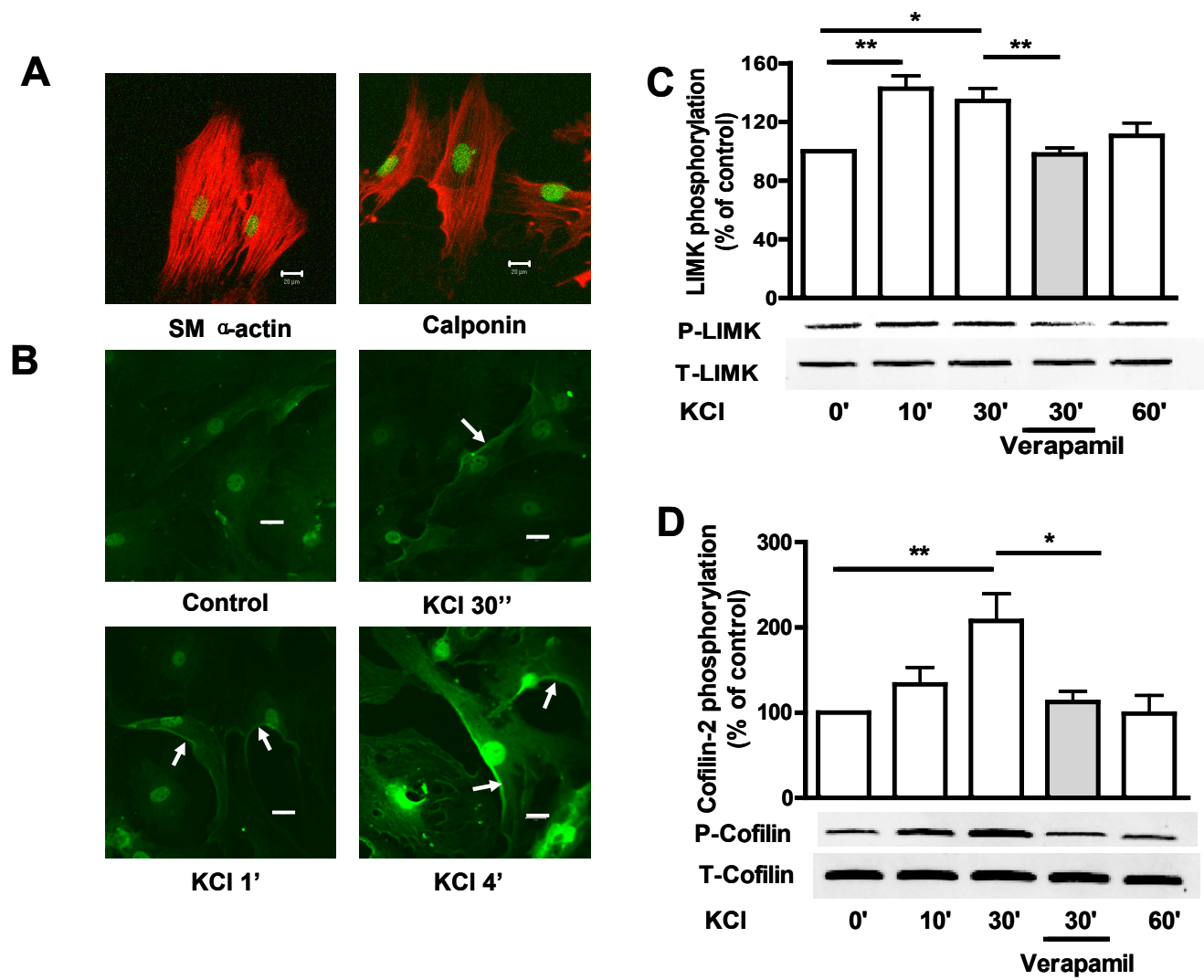


Figure 4

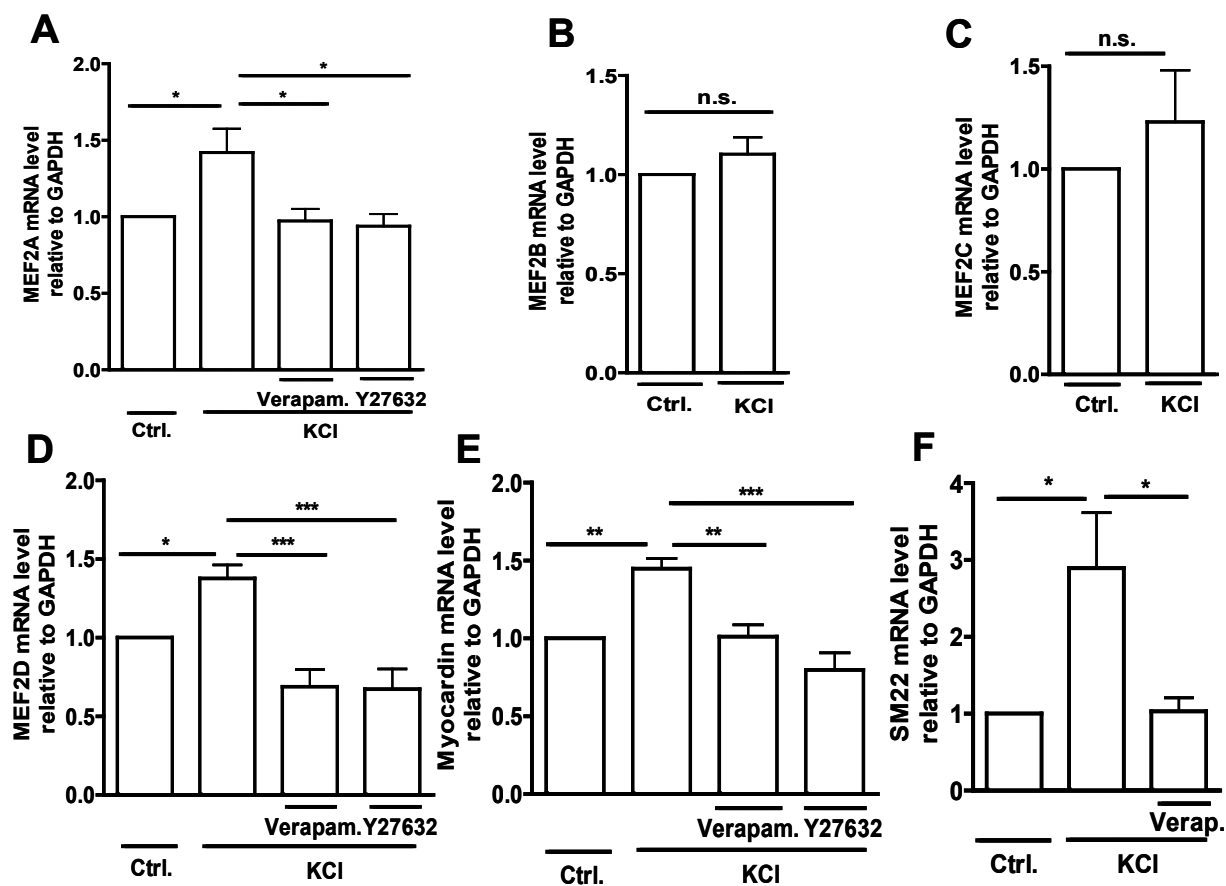


Figure 5



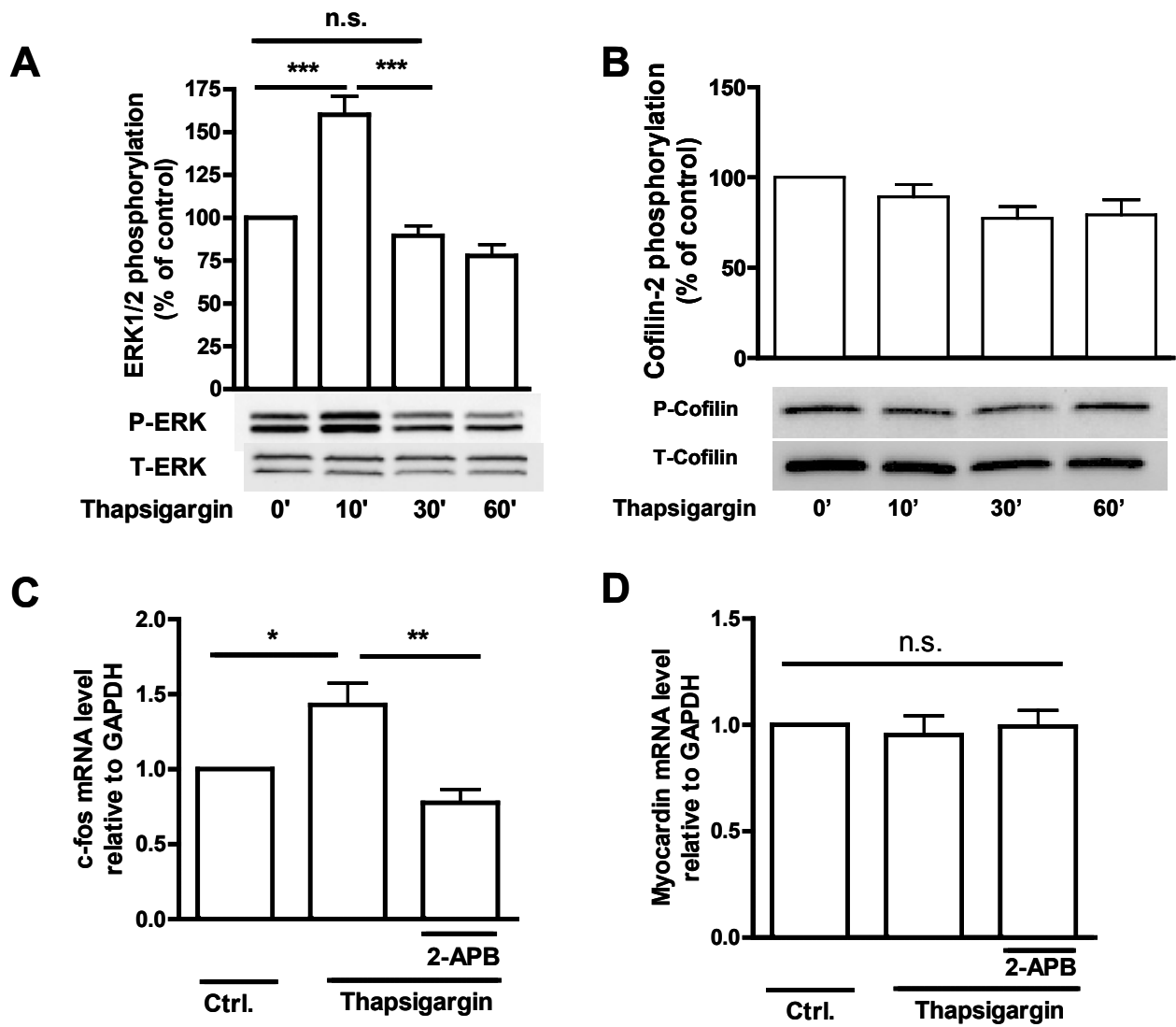


Figure 6

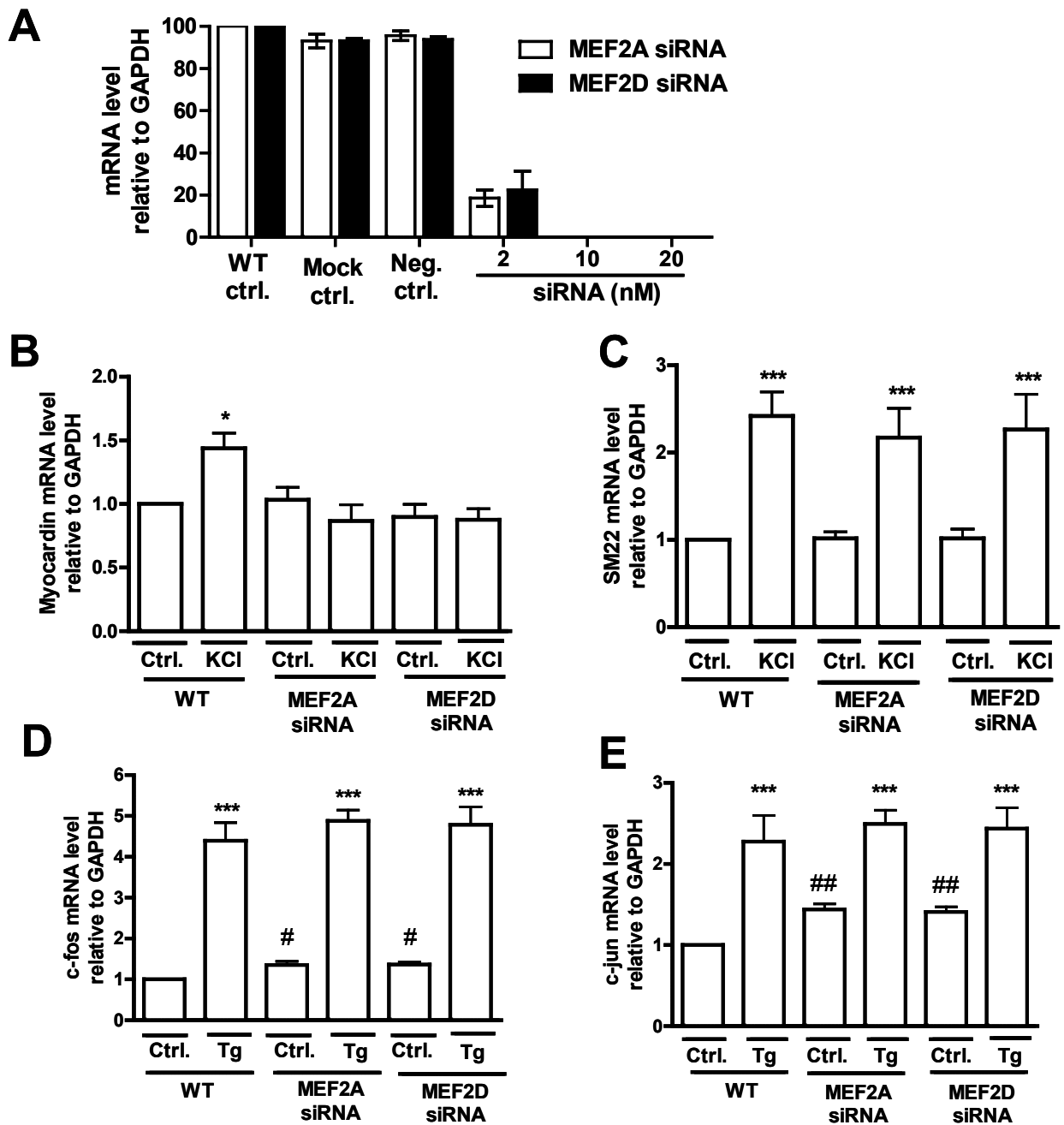


Figure 7

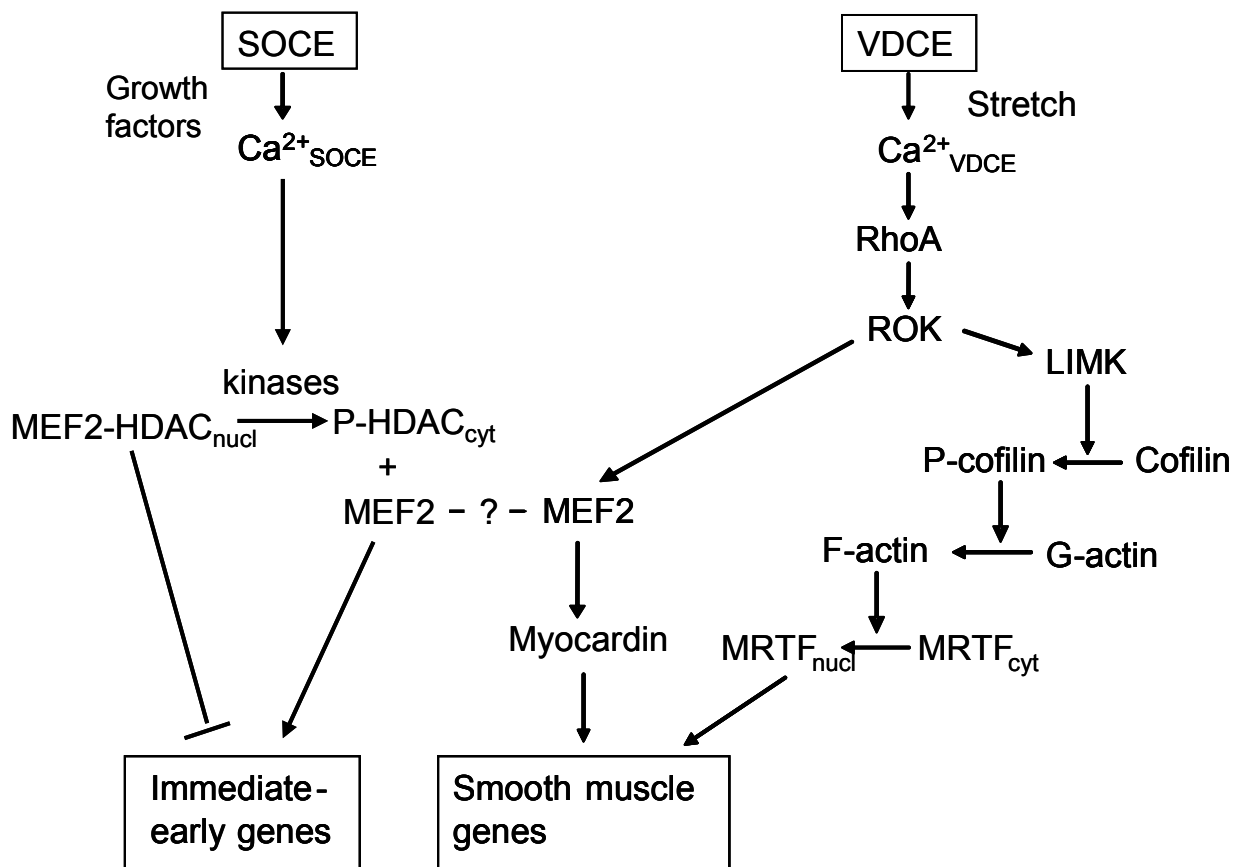


Figure 8