

Shortening velocity and myosin heavy- and light-chain isoform mRNA in rabbit arterial smooth muscle cells

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Received 5 July 2001; accepted in final form 31 December 2001

Sherwood, Jennifer J., and Thomas J. Eddinger. Shortening velocity and myosin heavy- and light-chain isoform mRNA in rabbit arterial smooth muscle cells. *Am J Physiol Cell Physiol* 282: C1093–C1102, 2002. First published January 2, 2002; 10.1152/ajpcell.00307.2001.—In smooth muscle cells (SMCs) isolated from rabbit carotid, femoral, and saphenous arteries, relative myosin isoform mRNA levels were measured in RT-PCR to test for correlations between myosin isoform expression and unloaded shortening velocity. Unloaded shortening velocity and percent smooth muscle myosin heavy chain 2 (SM2) and myosin light chain 17b (MLC_{17b}) mRNA levels were not significantly different in single SMCs isolated from the luminal and adluminal regions of the carotid media. Saphenous artery SMCs shortened significantly faster ($P < 0.05$) than femoral SMCs and had more SM2 mRNA ($P < 0.05$) than carotid SMCs and less MLC_{17b} mRNA ($P < 0.001$) and higher tissue levels of SMB mRNA ($P < 0.05$) than carotid and femoral SMCs. No correlations were found between percent SM2 and percent MLC_{17b} mRNA levels and unloaded shortening velocity in SMCs from these arteries. We have previously shown that myosin heavy chain (MHC) SM1/SM2 and SMA/SMB and MLC_{17a}/MLC_{17b} isoform mRNA levels correlate with protein expression for these isoforms in rabbit smooth muscle tissues. Thus we interpret these results to suggest that 1) SMC myosin isoform expression and unloaded shortening velocity do not vary with distance from the lumen of the carotid artery but do vary in arteries located longitudinally within the arterial tree, 2) MHC SM1/SM2 and/or MLC_{17a}/MLC_{17b} isoform expression does not correlate with unloaded shortening velocity, and 3) intracellular expression of the MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms is not coregulated.

SM1; SM2; MLC_{17a}; MLC_{17b}; SMA; SMB; unloaded shortening velocity; arterial smooth muscle

MUSCLE CONTRACTION requires the interaction of myosin with actin. Muscle myosin is a member of a large family of motor proteins with a similar molecular structure, composed of two myosin heavy chains (MHCs) and two pairs of myosin light chains (MLCs). Smooth muscle (SM) expresses several different MHC and MLC isoforms. The alternate association of these SM MHC and MLC isoforms results in different myosin molecules that may have distinct contractile or structural roles. In striated muscle, myosin isoforms have been assigned unique contractile roles, but the

physiological significance has not yet been clearly defined for the SM myosin isoforms.

SM MHC isoforms result from the alternative splicing of a single gene. Alternative splicing near the 3' end of the MHC pre-mRNA results in expression of either one of two proteins, SM1 (204 kDa) or SM2 (200 kDa), differing only at their carboxy termini (1, 10). In studies performed in chemically permeabilized tissue strips, SM1 expression has been correlated with an increase in unloaded shortening velocity (21), an increase in calcium sensitivity, and also a decrease in shortening velocity (3). However, studies on purified proteins (23a), expressed proteins (33), and single cells (28) suggest that expression of the SM1 and SM2 isoforms results in similar in vitro ATPase activities (23a, 33) and similar maximal unloaded shortening velocities (28). Thus results in the literature are contradictory and the contractile properties of the SM1/SM2 MHC isoforms remain undefined.

Splicing at the 5' alternative splice site of the MHC gene results in two isoforms differing in the insertion (SMB) or omission (SMA) of seven amino acids near the putative ATP binding site (23a, 37). Expression of SMB is associated with a higher actin-activated Mg-ATPase activity, faster movement of actin filaments in in vitro motility assays (23a, 33), and faster maximum shortening velocity (6, 11).

SM also expresses several different MLC isoforms. Within a SM myosin molecule, one MLC₂₀ (20 kDa) and one MLC₁₇ (17 kDa) associate with each MHC. Two isoforms of MLC₂₀, expressed in SM, are produced by separate genes and differ by 11 amino acid substitutions within their sequences (29). MLC₂₀ phosphorylation is a key event in the regulation of SM contraction, but unique functional roles for the MLC₂₀ isoforms are unknown. SM expresses two isoforms of MLC₁₇: MLC_{17a} and MLC_{17b} (19, 20). The MLC₁₇ isoforms are produced by the alternative splicing of a pre-mRNA molecule (25, 30) and differ in the substitution of five of the last nine amino acids at their carboxy termini (18). Studies performed in whole tissues suggest an inverse relationship between MLC_{17b} expression and Mg-ATPase activity (20) and maximal shortening velocity (18, 26). This is in contrast to re-

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sults obtained in studies performed on purified proteins and isolated SM cells (SMCs), suggesting that there is no correlation between the MLC₁₇ isoform expression and mechanical function (8, 23a, 33). Thus the contractile properties associated with the MLC₁₇ isoforms remain to be resolved.

SM expression patterns are suggestive of unique functional roles for the SM myosin isoforms. Expression of the MHC and MLC isoforms is regulated in muscle hypertrophy (5, 21), in a tissue-specific (6, 10, 17, 20, 23a) and developmental manner (24, 37). In addition, several SM pathologies (e.g., asthma and arteriosclerosis) are characterized by changes in SM mechanical function along with changes in myosin isoform expression patterns (31). Although there are many examples of the simultaneous changes in myosin isoform expression and mechanical function, unique contractile properties have not yet been assigned to the SM myosin isoforms.

Despite studies indicating intercellular heterogeneity in SM myosin isoform expression within the arterial media (8, 9, 22, 27, 37, and unpublished data), there is a lack of information describing myosin isoform expression patterns within the media. In 1997, Eddinger and Meer (9) proposed the following hypotheses to describe the myosin isoform intercellular heterogeneity within the arterial media: 1) expression of the individual myosin isoforms may be confined to randomly distributed "clusters" of SMCs within the arterial media, 2) myosin isoform expression may vary with distance from the lumen of the artery, or 3) myosin isoform expression may be random and heterogeneous between the individual SMCs of the arterial media. Results from functional studies suggest differential expression of the myosin isoforms in the luminal and adluminal regions of the arterial media. In bovine carotid arteries, Garland and Keatinge (14) found differences in the acetylcholine-mediated constrictor responses of the adluminal and luminal arterial muscle layers. Additionally, SMCs within the inner tunica media of the artery have been shown to proliferate in experimental models of arteriosclerosis, but SMCs within the deeper layers of the tunica media only rarely proliferate (16, 31, 35). The mechanism for these differences remains unresolved. Several reports also suggest that myosin isoform expression is heterogeneous in arteries along the length of the arterial tree (6, 32). Physiological differences have been reported between the larger conduit arteries and the smaller muscular arteries (6, 31, 32). This study was designed to test the hypothesis that the SM myosin isoforms are differentially expressed in SMCs isolated from the luminal and adluminal regions of the carotid media and in SMCs isolated from arteries located longitudinally within the arterial tree.

Because of the intercellular heterogeneity in myosin isoform expression in SMCs within the arterial media (8, 9, 27), resolving correlations between myosin isoform expression and cell mechanical properties requires both parameters to be studied in the same single SMC. In this study, both unloaded shortening velocity and mRNA levels of the MHC SM1/SM2 and MLC_{17a/}

MLC_{17b} isoforms were measured in the same single SMC isolated from discrete locations within the artery. These methods allow correlations to be made between cell location, myosin isoform expression, and mechanical properties.

Previously, this laboratory has demonstrated a significant positive correlation between mRNA and protein expression levels for the MHC SM1/SM2 (27, 28), SMA/SMB (11), and MLC_{17a/}MLC_{17b} (8) in numerous rabbit SM tissues. Levels of mRNA were shown to be an indicator of protein expression levels for these isoforms in rabbit SM tissues. Therefore, the results of this study are interpreted to suggest that myosin isoform expression and unloaded shortening velocity are similar in the luminal and adluminal SMCs of the carotid media but that they do vary along the length of the arterial tree. SMC MHC SM1/SM2 or MLC_{17a/}MLC_{17b} isoform expression ratios do not correlate with unloaded shortening velocity, and intracellular expression of the MHC SM1/SM2 and MLC_{17a/}MLC_{17b} isoforms are not coordinately regulated.

METHODS

Tissue preparation. Two-kilogram, ten-week-old female New Zealand White rabbits were euthanized by CO₂ asphyxiation as approved by the Marquette Animal Care and Use Committee ($n = 10$ rabbits). The arteries (carotid, femoral, and saphenous) were excised, cleaned of adhering tissue, and stored in a physiological salt solution (PSS; in mM: 140 NaCl, 4.7 KCl, 1.2 Na₂HPO₄, 1.2 MgSO₄, 1.6 CaCl₂, 5.6 glucose, 0.02 EDTA, and 2.0 MOPS, pH 7.2) at 4°C until processed for use. Mechanical studies were performed within 5 days post mortem. There was no difference in the unloaded shortening velocity measured in SMCs the first day post mortem (saphenous 0.020 ± 0.003 , $n = 11$) compared with SMCs measured 5 days post mortem (saphenous 0.018 ± 0.003 , $n = 13$).

Cell isolation. Intact arteries (intima, media, and adventitia) were incubated for 15 min in 0 Ca²⁺ PSS (in mM: 140 NaCl, 4.7 KCl, 1.2 Na₂HPO₄, 2.4 MgSO₄, 0.0 CaCl₂, 5.6 glucose, 0.02 EDTA, and 2.0 MOPS, pH 7.2 at 4°C) and balloon digested for 10 min in a 37°C enzyme solution: 20 U/ml papain and 2 mM dithiothreitol (DTT) in 0 Ca²⁺ PSS (7). After balloon digestion, cells were obtained from the luminal region of the carotid media by flushing 0 Ca²⁺ PSS ($10 \times 750 \mu\text{l}$) through the lumen of the vessel into a silicone-coated petri dish. Flushing provided the mechanical perturbation to liberate SMCs from the inner surface of the vessel wall. Cells from the adluminal side of the carotid media were obtained by everting the carotid artery before balloon digestion and flushing cells from the lumen of the arterial tube. Femoral and saphenous SMCs were balloon digested in a papain solution as described above. After digestion, these arteries were bisected longitudinally and tissue strips were teased from the luminal side of the artery, releasing cells into the bathing solution. After isolation, the dissociated cells were stored in 0 Ca²⁺ PSS on ice until used (<4 h).

Mechanical measurements. Unloaded shortening velocity was measured at room temperature (24°C) as described in Eddinger et al. (8). Isolated SMCs were transferred to a depression slide, flushed with relaxing solution (pCa 9.0; 14.5 mM creatine phosphate, 7 mM EGTA, 20 mM imidazole, 1 mM free Mg²⁺, 4 mM free MgATP, 5.42 mM MgCl₂, 79.16 mM KCl, 4.74 mM ATP, and 16.33 μM CaCl₂, pH 7.0), and permeabilized by a 7-min incubation in 250 hemolytic U/ml

Staphylococcus aureus α -toxin solution. Isolated cells were examined with phase-contrast imaging. SMCs were identified, based on morphology, as long and spindle-shaped with tapering ends and bright haloes (12). One end of the cell was attached to the micropipette (2- μ m tip) with gentle suction. To free the SMC from adhering to the slide, the micropipette was used to lift the SMC off the bottom of the depression slide. The cell was then maneuvered to a horizontal position and stimulated to contract by addition of an activating mixture [1 μ M histamine and 1 μ M phenylephrine in activating solution (pCa 6.0): 14.5 mM creatine phosphate, 7 mM EGTA, 20 mM imidazole, 1 mM free Mg^{2+} , 4 mM free MgATP, 5.31 mM $MgCl_2$, 68.55 mM KCl, 4.79 mM ATP, and 4.91 mM $CaCl_2$, pH 7.0]. Cell contractions were monitored by a charge-coupled device camera (XC-75; Sony, Park Ridge, NJ), stored on a videocassette recorder, and later digitized with NIH Image 5.0 software. Changes in cell length over time were plotted. To plots characterized by a smooth sigmoidal curve, a linear function was fit to the steepest portion of the curve. The slope of this line was used as the maximal unloaded shortening velocity of the SMC. After mechanical measurements were made, the contracted SMC was placed into an empty, sterile microcentrifuge tube for RT-PCR analysis.

Phosphorylation controls. We are unaware of methods to confirm phosphorylation levels in single SMCs. Therefore, to confirm the assumption that our activation protocol was stimulating maximal phosphorylation of MLC₂₀, permeabilized SMCs were incubated in a phosphatase inhibitor before stimulating contraction. Six α -toxin permeabilized cells were incubated in 1 μ M okadaic acid before activation. At this concentration, okadaic acid acts as a protein phosphatase type I inhibitor, inhibiting MLC phosphatase dephosphorylation of MLC₂₀ (2). Incubating SMCs in okadaic acid before activation did not change the mean normalized unloaded shortening velocities measured in these cells (see RESULTS). Further support that our activation protocol yields maximal phosphorylation of the MLC₂₀ was obtained by incubating 10 isolated, α -toxin-permeabilized SMCs in 4.65 mM adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) before activation (see RESULTS). MLC₂₀ is thiophosphorylated by incubation in ATP γ S and cannot be dephosphorylated (4). There was no difference in the mean normalized unloaded shortening velocity of α -toxin-permeabilized SMCs activated with and without prior incubation in ATP γ S. These results are consistent with previous work, suggesting that our activation protocol results in maximal phosphorylation of MLC₂₀ in isolated SMCs (8, 11, 28).

RNA extraction and reverse transcription. Rnazol B (Tel-test, Friendswood, TX) was used to extract total RNA from arterial tissues. Briefly, tissue was homogenized in Rnazol B, and the RNA was purified with a series of phenol-chloroform and alcohol washes. RNA (1.0–1.5 μ g) was added to the reverse transcription (RT) reaction mix [5 \times RT buffer, 0.2 μ g/ μ l random hexamers, 2 U/ μ l RNasin, 0.1 μ g/ μ l acetylated BSA, 500 μ M 2-deoxynucleotide-5'-triphosphate (dNTP), 1.23 mM DTT, and 10 U/ μ l Moloney murine leukemia virus-RT] to a final volume of 10 μ l and incubated at 37°C for 2 h. For single SMC RT, cells were lysed with a freeze-thaw cycle, and the 10- μ l RT mixture was added to the tube containing the isolated SMC.

PCR. Two oligonucleotide primers (Operon, Alameda, CA) were synthesized for each of the SM MHC (SM1/SM2) (27), MHC (SMA/SMB) (11), and MLC_{17a}/MLC_{17b} (8) isoforms. Each primer pair for the myosin isoforms flanks the unique exon difference between the isoforms and an intron in the genomic DNA so that only PCR products amplified from the cDNA are of the correct size.

For single SMC analysis, the product of the 10- μ l RT reaction mix was divided into two 5- μ l aliquots, and duplicate PCRs were performed (one reaction with the MHC SM1/SM2 and the other reaction with the MLC₁₇ primers). For tissue analysis, 2 μ l of the RT reaction products were amplified in a PCR with primers specific for the MHC SMA/SMB isoforms. The PCR mix [1 \times *Taq* polymerase buffer (Promega, Madison, WI); 3 mM $MgCl_2$ (Promega); 0.2 mM of each dNTP (Promega); 2 μ M 5' primer; 2 μ M 3' primer; and 1 unit *Taq* polymerase (Promega)] was added to the products of the RT reaction (total PCR volume: 50 μ l), overlaid with 100 μ l of mineral oil, placed in a thermocycler, and cycled 60 times (35 times for tissue analysis) through the temperature profiles [3 min 94°C hot start; 1.5 min 94°C denaturation, 60°C annealing (2 min 55°C for tissue PCRs and 3 min 72°C for primer extension)]. In single SMC PCR, a second addition of *Taq* was made after the first 30 cycles.

Gel electrophoresis and densitometry. The PCR products were resolved on an 8% SDS-PAGE gel using the methods of Giulian et al. (15). Gels were stained in ethidium bromide solution (1 μ g/ml), and PCR products were visualized under an ultraviolet light. Band density was quantified by image analysis of gel photographs using Ambis 2000 software (Ambis, San Diego, CA).

Controls for validity. Only one set of primers was used in each PCR to amplify the MHC SM1/SM2, SMA/SMB, or MLC_{17a}/MLC_{17b} isoforms. Therefore, the intensities of the gel bands depend solely on the concentration of cDNA reverse transcribed from the mRNA present for each isoform within the sample (13). Previous work in this laboratory has shown RT-PCR to be an accurate and precise method of measuring the relative mRNA levels of SM1/SM2 (27), SMA/SMB (11), and MLC_{17a}/MLC_{17b} (8) in single SMCs. We have also shown that relative levels of SM1/SM2, SMA/SMB, and MLC_{17a}/MLC_{17b} mRNA correlate well with the relative amounts of protein for each of these isoforms (8, 11, 27). Controls were performed to determine the validity of these methods (27). Duplicate RT-PCRs of the same RNA samples were shown to yield similar PCR product ratios. Densitometric data of gels loaded with a 16-fold concentration range of the PCR products showed that sample loading is in the linear range for quantitation.

Statistical analysis. Throughout the text, the mRNA levels of the MHC SM1/SM2 are listed as percent SM2 [(SM2/SM1 + SM2) \times 100] and the levels of the MLC_{17a}/MLC_{17b} isoforms as percent MLC_{17b} [(MLC_{17b}/MLC_{17a} + MLC_{17b}) \times 100]. Sample means for the mRNA expression of the MHC and MLC isoforms and unloaded shortening velocity were tested for significance by a one-way ANOVA ($P < 0.5$) and a post hoc Tukey test. In data failing the normality test, significance was determined by a one-way ANOVA on ranks ($P < 0.05$) and a post hoc Dunn's test. All reported means are given with SE. Data from the carotid, femoral, and saphenous arteries was not pooled for analysis; instead, linear regressions (least-squares method) were fit to plots of cellular myosin isoform mRNA ratios and unloaded shortening velocity for each artery separately. Significance of the correlation coefficients was tested with a *t*-test with two degrees of freedom. Unloaded shortening velocities are reported normalized to initial cell length to control for differences in contractile units in series.

RESULTS

Single SMCs were isolated from different regions of rabbit arterial system to compare and correlate cellular and mechanical properties. Cellular characteristics

Table 1. Characteristics of smooth muscle cells isolated from the luminal and adluminal sides of the carotid media

Carotid Medial SMC Origin	Initial Length, μm	Final Length, μm	% Cell Shortening	Shortening Velocity, $L/s \times 1,000$	% SM2 mRNA	% MLC _{17b} mRNA
Adluminal region	205.8 \pm 7.4	71.0 \pm 3.1*	64.6 \pm 2.1	14 \pm 2	24.6 \pm 3.4	31.9 \pm 1.7
Luminal region	189.1 \pm 5.5	62.2 \pm 2.1	66.7 \pm 1.0	18 \pm 2	17.1 \pm 2.3	28.1 \pm 2.6

Values are means \pm SE; $n = 21$ –25 (adluminal) and 21–32 (luminal). *Difference from carotid luminal smooth muscle cells (SMCs) is significant ($P < 0.05$).

of single SMCs isolated from the inner media (luminal) and outer media (adluminal) side of the carotid artery are listed in Table 1. Before contraction, carotid luminal (189.1 \pm 5.5 μm) and adluminal SMCs (205.8 \pm 7.4 μm) were similar in length. Isolated carotid SMCs were stimulated to contract with a phenylephrine/histamine/calcium solution. Carotid luminal and adluminal SMCs contracted to a similar percentage of their initial length, but carotid luminal SMCs (62.2 \pm 2.1 μm) reached a shorter final length ($P < 0.05$) than carotid adluminal SMCs (71.0 \pm 3.1 μm). Maximal unloaded shortening velocities were not significantly different in SMCs isolated from the adluminal [0.014 \pm 0.002 lengths (L/s)] and luminal (0.018 \pm 0.002 L/s) regions of the carotid media (Fig. 1).

Cellular characteristics of single SMCs isolated from arteries along the length of the arterial tree are listed in Table 2. Isolated femoral SMCs (223.13 \pm 7.1 μm) were significantly longer ($P < 0.05$) than carotid (195.9 \pm 4.6 μm) but not saphenous (202.9 \pm 8.4 μm) SMCs. After phenylephrine/histamine/calcium stimulated contraction, all arterial SMCs shortened significantly to lengths ranging from ~ 26 to 122 μm . After contraction, femoral SMCs (85.4 \pm 3.4 μm) were significantly longer ($P < 0.001$) than both carotid (65.8 \pm 1.8 μm) and saphenous (68.1 \pm 4.4 μm) SMCs. Cell shortening was more variable in femoral (shortening ranging from 40 to 90% of initial cell length) and saphenous SMCs (30–90% of initial cell length) than in carotid SMCs (ranging from 40 to 80% of initial

length). Femoral SMCs shortened to 61.0 \pm 1.8% of initial cell length, which was significantly less shortening ($P < 0.05$) than saphenous SMCs (66.1 \pm 2.3% of initial cell length) but not carotid SMCs (65.8 \pm 1.0% of initial cell length). Figure 2 is a histogram of the unloaded shortening velocities measured in 48 carotid, 33 femoral, and 35 saphenous SMCs. Saphenous SMCs (0.024 \pm 0.003 L/s) shortened significantly faster ($P < 0.05$) than femoral SMCs (0.014 \pm 0.002 L/s) but not than carotid SMCs (0.016 \pm 0.002 L/s).

Differences in MLC₂₀ phosphorylation levels result in differences in unloaded shortening velocity. To ensure that the differences we detected in single SMC unloaded shortening velocity were not due to differences in MLC₂₀ phosphorylation levels, we performed experiments where we incubated α -toxin-permeabilized SMCs in okadaic acid [inhibits MLC₂₀ phosphatase activity (2)] or ATP γ S [irreversibly phosphorylates MLC₂₀ (4)] before agonist activation. Mean unloaded shortening velocity measured in SMCs after these experimental manipulations were not significantly different from the mean unloaded shortening velocity measured in SMCs without these experimental treatments. Unloaded shortening velocity was 0.014 \pm 0.002 L/s ($n = 30$) in untreated femoral and 0.01 \pm 0.002 L/s ($n = 6$) in okadaic acid treated femoral SMCs. Unloaded shortening velocity was 0.016 \pm 0.001 L/s ($n = 48$) in untreated carotid and 0.023 \pm 0.006 L/s ($n = 10$) in ATP γ S-treated carotid SMCs. These results are similar to previous reports that our activation protocol stimulates maximal phosphorylation in isolated arterial SMCs a (8, 11, 28).

To make correlations between mechanical properties and myosin isoform expression, RT-PCR on the RNA of contracted SMCs isolated from different regions of rabbit arteries was performed using primers for the MLC_{17a}/MLC_{17b} and MHC SM1/SM2 isoforms. Previous work in this laboratory has shown that mRNA levels correlate with the protein expression for these isoforms in rabbits (8, 11, 27). Figure 3A is a histogram of percent SM2 mRNA for 46 carotid SMCs isolated from the adluminal and luminal regions of the media. Ratios were less variable in the luminal (0–40% SM2 mRNA) compared with the adluminal carotid medial SMCs (0–70% SM2 mRNA); however, average percent SM2 mRNA was not statistically different in SMCs isolated from the adluminal (24.6 \pm 3.4 % SM2) and luminal (17.02 \pm 2.3% SM2) regions of the carotid media. In contrast, MLC₁₇ ratios were more variable in luminal carotid medial SMCs (0–50% MLC_{17b} mRNA)

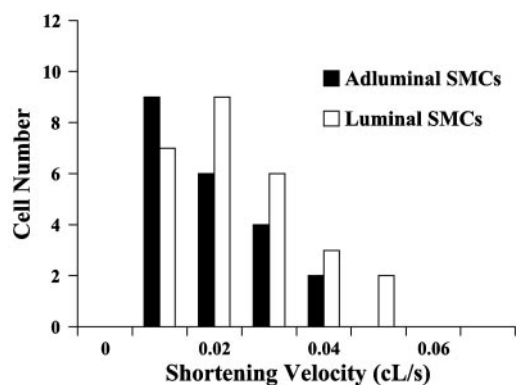


Fig. 1. Unloaded shortening velocities of smooth muscle cells (SMCs) isolated from different regions of the carotid media. Unloaded shortening velocity was measured as described in METHODS to examine differences in unloaded shortening velocities of SMCs isolated from the adluminal and luminal regions of the carotid media. Histogram of 21 adluminal (0.014 \pm 0.002) and 27 luminal (0.018 \pm .002) carotid SMCs shows the distribution of unloaded shortening velocities.

Table 2. Characteristics of smooth muscle cells isolated from arteries along the length of the arterial tree

SMC Origin	Initial Length, μm	Final Length, μm	% Cell Shortening	Shortening Velocity, $L/s \times 1,000$	% SM2 mRNA	% MLC _{17b} mRNA	Artery % SMB mRNA
Carotid	195.9 \pm 4.6*	65.8 \pm 1.8†	65.8 \pm 1.0	16 \pm 2	21.2 \pm 2.2‡	29.4 \pm 1.7§	11 \pm 8‡
Femoral	223.3 \pm 7.1	85.35 \pm 3.4	61.0 \pm 1.8‡	14 \pm 2‡	27.8 \pm 5.6	33.9 \pm 2.1§	13 \pm 8‡
Saphenous	202.9 \pm 8.4	68.1 \pm 4.4†	66.1 \pm 2.3	24 \pm 3	43.2 \pm 7.5	12.8 \pm 2.4	54 \pm 10

Values are means \pm SE; $n = 45\text{--}54$ (carotid), $24\text{--}33$ (femoral), $17\text{--}37$ (saphenous), 3 (artery). *Difference from femoral is significant ($P < 0.05$). †Difference from femoral is significant ($P < 0.001$). ‡Difference from saphenous is significant ($P < 0.05$). §Difference from saphenous is significant ($P < 0.001$).

compared with the adluminal SMCs (20–50% MLC_{17b} mRNA) (Fig. 3B). Average percent MLC_{17b} mRNA was not statistically different in SMCs isolated from the adluminal (31.9 \pm 1.7% MLC_{17b}) and luminal (28.1 \pm 2.6% MLC_{17b}) regions of the carotid media.

Figure 4 is a histogram of percent SM2 mRNA levels in 46 carotid, 24 femoral, and 17 saphenous SMCs. Carotid SMC mRNA ratios ranged from 0 to 70% SM2 and were less variable than both femoral and saphenous SMC ratios (0–100% SM2, Fig. 4A). Saphenous SMCs had higher levels of the SM2 mRNA (43.2 \pm 7.5% SM2) than carotid (21.1 \pm 2.2% SM2; $P < 0.05$) but not femoral (27.8 \pm 5.6% SM2) SMCs. Figure 4B is a histogram of percent MLC_{17b} levels in 46 carotid, 24 femoral, and 30 saphenous SMCs. All arteries examined displayed similar variability in MLC_{17b} mRNA ratios, with ranges from 0 to 50% MLC_{17b} in carotid, from 20 to 60% MLC_{17b} in femoral, and from 0 to 50% MLC_{17b} in saphenous SMCs. However, saphenous SMCs had significantly less MLC_{17b} (12.8 \pm 2.4% MLC_{17b}; $P < 0.001$) than carotid (29.4 \pm 1.7% MLC_{17b}) and femoral (33.9 \pm 2.1% MLC_{17b}) SMCs. Intercellular percent SM2 and MLC_{17b} mRNA levels were not correlated in carotid ($r^2 = 0.01$, $n = 45$), femoral ($r^2 = 0.05$, $n = 24$), and saphenous ($r^2 = 0.12$, $n = 18$) SMCs (Fig. 5). RT-PCR analysis was performed with SMA/SMB specific primers on whole tissue samples from the carotid, femoral, and saphenous arteries. The saphenous artery had significantly more SMB mRNA (54 \pm 10%

SMB, $n = 3$, $P < 0.05$) than both the carotid (11 \pm 8% SMB) and femoral (13 \pm 8% SMB) arteries (Table 2).

Linear regressions fit to percent SM2 mRNA and unloaded shortening velocity of single SMCs from the carotid ($r^2 = 0.05$, $n = 25$), femoral ($r^2 = 0.12$, $n = 12$), and saphenous arteries ($r^2 = 0.21$, $n = 14$) reveal that there is no correlation between the percent SM2 mRNA and unloaded shortening velocity in single arterial SMCs (Figure 6A). Linear regressions fit to percent MLC_{17b} mRNA vs. unloaded shortening velocity of single SMCs from the carotid ($r^2 = 0.01$, $n = 25$), femoral ($r^2 = 0.01$, $n = 15$), and saphenous arteries ($r^2 = 0.1$,

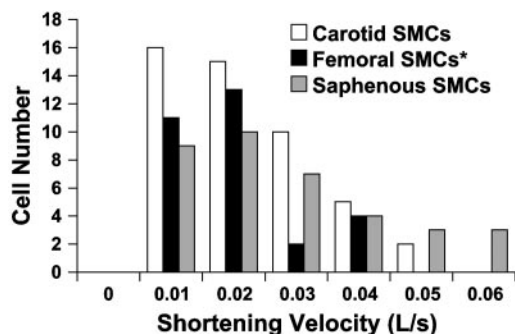


Fig. 2. Unloaded shortening velocities of SMCs isolated from arteries along the arterial tree. Unloaded shortening velocity was measured as described in METHODS to examine differences in unloaded shortening velocities of SMCs isolated from arteries located longitudinally within the arterial tree. Histogram of 48 carotid (0.016 ± 0.002 L/s), 33 femoral (0.014 ± 0.002 L/s), and 35 saphenous (0.024 ± 0.003 L/s) SMCs showing the distribution of unloaded shortening velocities. *Femoral SMCs shortened significantly slower ($P < 0.05$) than saphenous SMCs. L/s, cell length/s.

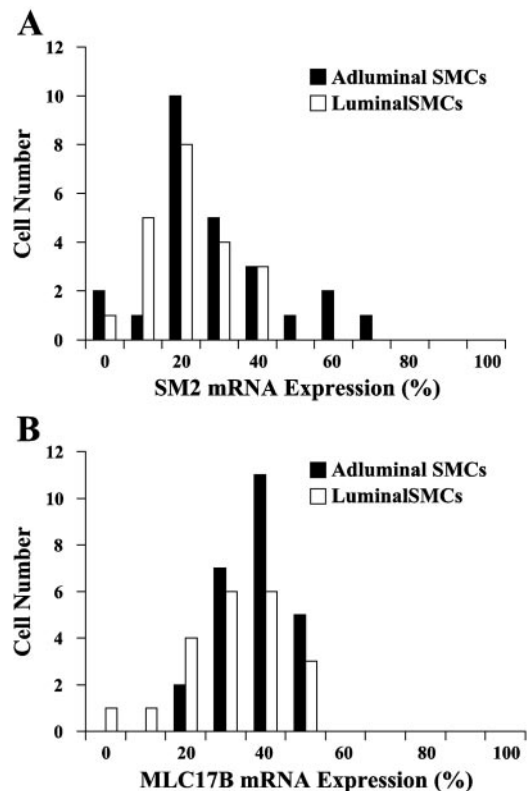


Fig. 3. Myosin isoform PCR products of SMCs isolated from the adluminal and luminal regions of the carotid media. Isolated SMCs were utilized for RT-PCR as described in METHODS to examine the heterogeneity of smooth muscle myosin heavy chain (MHC) and myosin light chain (MLC) expression in carotid luminal and adluminal SMCs. A: histogram of 25 adluminal ($24.6 \pm 3.4\%$ SM2) and 21 luminal ($17.0 \pm 2.3\%$ SM2) carotid SMCs showing the distribution of myosin isoform PCR products. B: histogram of 25 adluminal ($31.9 \pm 1.7\%$ MLC_{17b}) and 21 luminal ($28.1 \pm 2.6\%$ MLC_{17b}) carotid SMCs showing the distribution of MLC₁₇ PCR products.

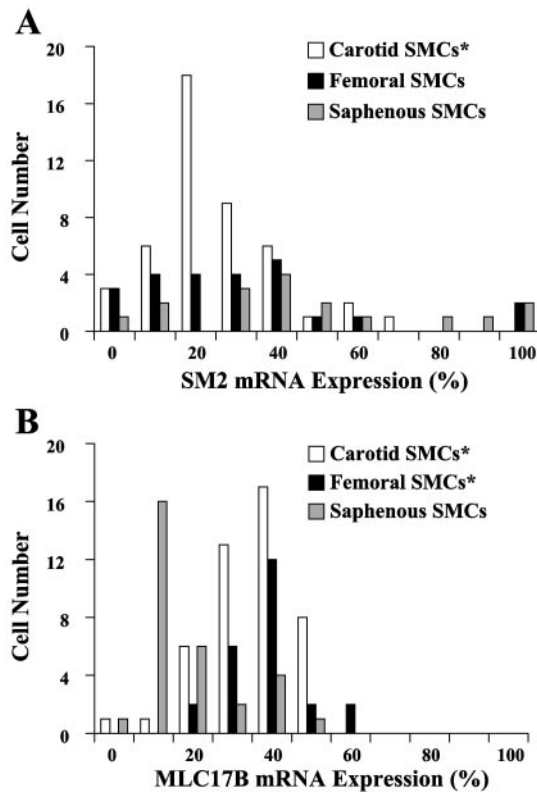


Fig. 4. Myosin isoform PCR products of SMCs isolated from arteries located longitudinally within the arterial tree. Isolated SMCs were utilized for RT-PCR as described in METHODS to examine the heterogeneity of smooth muscle MHC and MLC expression longitudinally within the arterial tree. **A:** histogram of 46 carotid ($21.3 \pm 2.2\%$ SM2), 24 femoral ($27.8 \pm 5.6\%$ SM2), and 17 saphenous ($43.2 \pm 7.5\%$ SM2) SMCs showing the distribution of SM1/SM2 PCR products. *Carotid SMCs express significantly less ($P < 0.05$) SM2 than saphenous SMCs. **B:** histogram of 46 carotid ($29.4 \pm 1.7\%$ MLC_{17b}), 24 femoral ($33.9 \pm 2.0\%$ MLC_{17b}), and 30 saphenous ($12.8 \pm 2.4\%$ MLC_{17b}) SMCs showing the distribution of MLC₁₇ PCR products. *Carotid and femoral SMCs express significantly more MLC_{17b} ($P < 0.001$) than saphenous SMCs.

$n = 21$) shows that there is no correlation between percent MLC_{17b} and unloaded shortening velocity in single arterial SMCs (Fig. 6B).

To ensure that the mechanical measurements did not alter the cellular expression of the MHC SM1/SM2 and MLC_{17a}/MLC_{17b}, RT-PCR of the mRNA for these isoforms was analyzed in SMCs without contracting the cells. Thirty-one carotid SMCs analyzed in RT-PCRs without prior mechanical measurements had mRNA expression profiles for the SM1/SM2 ($26.6 \pm 1.8\%$ SM2) and MLC_{17a}/MLC_{17b} isoforms (33.7 ± 1.2) similar to the 46 carotid SMCs with prior mechanical measurements ($21.1 \pm 2.2\%$ SM2 and $29.4 \pm 1.7\%$ MLC_{17b}). These results suggest that the contractile measurements performed in this study did not alter mRNA expression of the MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms.

DISCUSSION

The major hypothesis guiding this study is that myosin isoform expression patterns influence muscle con-

tractile properties. Muscle contractile properties are characteristically studied in whole tissue or purified proteins. However, the mechanical effects of myosin isoform expression patterns may be difficult to resolve with these techniques. In whole tissue studies, each individual tissue strip may contain several thousand SMCs, each expressing unique ratios of the individual myosin isoforms, thus confounding the ability to distinguish the unique mechanical properties of the individual myosin isoforms from the tissue strip mechanical properties. In purified protein assays, the contractile proteins may not have the same three-dimensional organization as in vivo. Isolated single SMCs were used in this study to obtain SM contractile information without the complexities of whole muscle or isolated protein studies. In vivo, medial SMCs are believed to have intercellular connections with other medial SMCs, and the removal of these connections during isolation may influence the contractile properties of single SMCs. However, studies of contractile properties in isolated SMCs suggest that enzymatic isolation does not compromise contractile function (12). In addition, in Table 2, average percent shortening is 66% (carotid), 61% (femoral), and 68% (saphenous) of initial cell length, and shortening velocities reported here are similar to unloaded shortening velocities reported previously in SM tissues (6) and single SMCs (36). In this study, myosin isoform mRNA levels and contractile properties were measured in the same single SMC. This laboratory has previously shown that mRNA levels correlate with the protein levels for the MHC SM1/SM2 (27), SMA/SMB (11), and MLC_{17a}/MLC_{17b} isoforms (8). Thus this study is designed to examine myosin isoform expression (by measuring mRNA levels) and cell mechanical properties in the same single SMC, thus allowing us to make correlations between myosin isoform expression and SMC contractile properties.

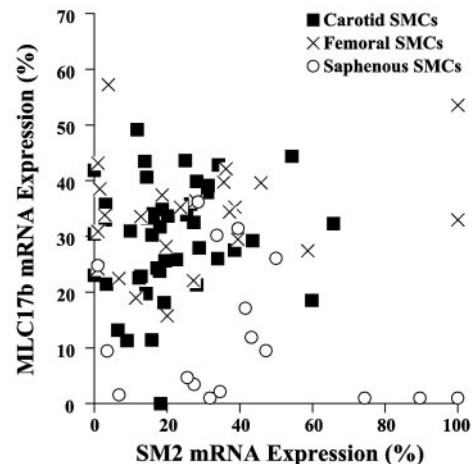


Fig. 5. Products of RT-PCR with two sets of primers as described in METHODS were performed on single SMCs. Linear regressions were fit to %SM2 and %MLC_{17b} RT-PCR products in single carotid, femoral, and saphenous SMCs. R values of least-squares regression: carotid ($r^2 = 0.01$), femoral ($r^2 = 0.05$), and saphenous ($r^2 = 0.12$) SMCs.

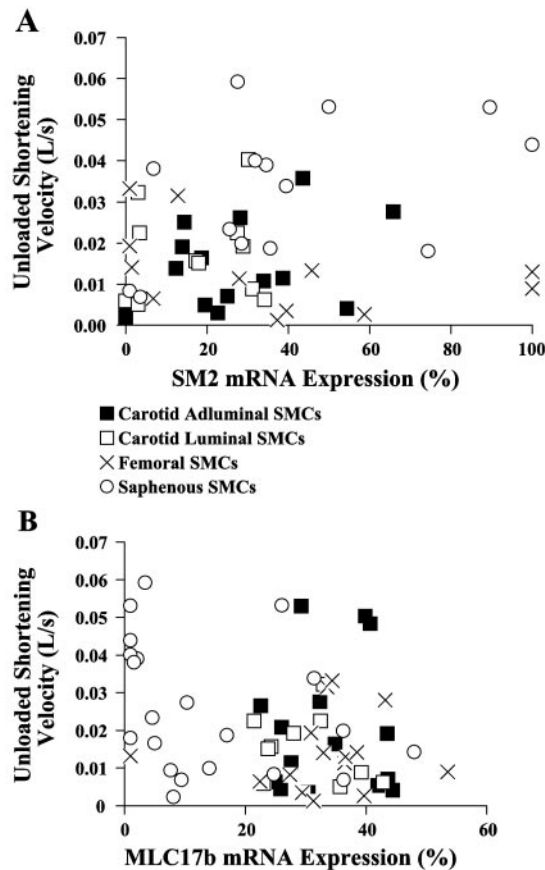


Fig. 6. Linear regression correlations between %SM2 and %MLC_{17b} and unloaded shortening velocity in carotid, femoral, and saphenous SMCs. Unloaded shortening velocity and RT-PCRs with two sets of primers were performed as described in METHODS to examine the correlation between MHC and MLC isoform expression and unloaded shortening velocity. A: linear regressions fit to %SM2 RT-PCR products and unloaded shortening velocity in SMCs in single SMCs. *R* value of least-squares fit regression: carotid ($r^2 = 0.05$), femoral ($r^2 = 0.12$), and saphenous ($r^2 = 0.21$) SMCs. B: linear regressions fit to %MLC_{17b} RT-PCR products and unloaded shortening velocity measured in single SMCs. *R* value of least-squares fit regression: carotid ($r^2 = 0.01$), femoral ($r^2 = 0.01$), and saphenous SMCs ($r^2 = 0.1$).

SMCs isolated from the luminal and adluminal regions of the carotid artery had similar initial cell lengths ranging from 90.5 to 274.2 μm (Table 1). In response to histamine/phenylephrine/calcium stimulation, adluminal and luminal SMCs shortened to a similar percent of initial length. However, after shortening, carotid luminal SMCs were significantly shorter than the carotid adluminal SMCs. This result may be explained by the observations of Van Citters et al. (34). Using immunohistochemistry to visualize SMCs within the canine mesenteric arterial media, they suggested that, with maximal vasoconstriction, the SMCs adjacent to the internal elastic lamina (luminal) were more deformed than SMCs in the outer (adluminal) region of the artery. Thus luminal SMCs may be required to attain shorter final lengths than SMCs in the adluminal region of the arterial media. This may be important to accommodate tissue volume as the artery diameter decreases and the arterial wall thickness increases.

In this study, SMCs isolated from the adluminal and luminal regions of the carotid media were shown to have similar normalized unloaded shortening velocities. Our work is consistent with the work of Garland and Keatinge (14). In bovine carotid arterial strips, they suggested that the inner and outer muscle layers of arterial strips were equally sensitive to high concentrations of histamine but that there was a difference in the constrictor response of outer and inner muscle layers to acetylcholine. In contrast, Villaschi et al. (35) suggested that cultured SMCs from the intimal side of the media, but not from the remainder of the media, rapidly contracted collagen gels in response to stimulation with endothelin-1. However, our work suggests that there is no difference in the mechanical properties of luminal and adluminal carotid medial SMCs. Differences in these results may be explained by differences in cell sources, freshly isolated vs. cultured, and the agonist stimulation, acetylcholine or endothelin-1 vs. histamine/phenylephrine/calcium. Further work needs to be done to characterize differences in the pharmacological responses of SMCs from different regions of the arterial media.

Isolated, unstimulated femoral SMCs were significantly longer than carotid SMCs (Table 2). In response to agonist stimulation, femoral SMCs shortened less than saphenous SMCs, resulting in femoral SMCs being significantly longer than both carotid and saphenous SMCs after contraction. Both femoral and saphenous SMCs shortened over a wider range [ranging from 40 to 90% (femoral) and 30–90% (saphenous); percent final length/initial length] than carotid SMCs (ranging from 40 to 80%). The physiological demands of tissues supplied by the femoral and saphenous arteries may require that SMCs from these arteries have the capacity to shorten over a wider range than the carotid SMCs. These results suggest that some of the unique functional characteristics of arteries may be due to the inherent characteristics of the SMCs within the media.

Unloaded shortening velocity was significantly different in SMCs isolated from arteries along the length of the arterial tree (Fig. 2). There was no difference in the mean unloaded shortening velocities of SMCs isolated from the saphenous and carotid arteries; however, saphenous SMCs shortened significantly faster than femoral SMCs. DiSanto et al. (6) demonstrated a larger maximal velocity (V_{max} ; about 2-fold) in saphenous compared with aortic tissue strips. The difference in these results suggests there may be differences in the mechanical properties of the carotid artery and aorta. This is the first study to suggest differences in the mechanical properties of the femoral and saphenous arteries.

Analyzed in RT-PCR, SMCs isolated from the luminal and adluminal regions of the carotid media express similar amounts of MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms (Fig. 3). This laboratory has previously shown that mRNA levels correlate with the protein levels for the MHC SM1/SM2 (27) and MLC_{17a}/MLC_{17b} isoforms (8). Thus these results suggest that the cellular expression of the MHC SM1/SM2 and

MLC_{17a}/MLC_{17b} isoforms is similar in SMCs from the luminal and adluminal regions of the carotid artery. This is the first study to analyze both MHC SM1/SM2 and MLC_{17a}/MLC_{17b} expression in distinct regions of an artery. The results of this study contribute and extend previous observations by suggesting that MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoform expression does not vary as a gradient with distance from the lumen of the carotid artery as proposed by Eddinger and Meer (9). Further work needs to be performed to determine whether SMCs within the media heterogeneously express the myosin isoforms in a random intercellular pattern or in a random pattern of cell clusters. Thus our results demonstrate that SMCs from the adluminal and luminal sides of the carotid artery have similar unloaded shortening velocities and similar expression levels of the MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms.

In this study, SMCs were isolated from the adluminal and luminal sides of the arterial media by flushing solution through the center of the digested artery. Cryosections of digested and flushed arteries confirmed that flushing solution through the center of a digested artery released SMCs from only the inner (2–4) layers of the arterial media (data not shown).

SMCs isolated from arteries along the length of the arterial tree express different levels of the MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms (Fig. 4). The SMCs isolated from the saphenous artery were found to have more SM2 mRNA than SMCs isolated from the carotid artery. DiSanto et al. (6) demonstrated that the aorta and femoral/saphenous arteries express similar amounts of the SM1 and SM2 MHC isoforms at the protein level. They also measured the mRNA for the SM1 and SM2 MHC isoforms in the aorta, femoral, and saphenous arteries, suggesting that mRNA levels were similar to protein levels for these isoforms in rabbit arteries. In their study, percent SM2 mRNA for the saphenous artery (58%) was higher than percent SM2 protein for the femoral/saphenous arteries (41%). Differences in these results are not interpreted to be due to protein vs. mRNA levels for these isoforms, because previous work suggests that mRNA levels correlate with protein levels for the SM1/SM2 isoforms (27) in rabbit SM tissues. In this study percent SM2 expression in femoral SMCs was intermediate between carotid and saphenous SMCs. Thus these differences may be due to their methods of pooling tissues from the saphenous and femoral arteries, precluding them from detecting the unique MHC SM1/SM2 expression of the saphenous artery (Fig. 4A).

Saphenous SMCs were found to have lower levels of the MLC_{17b} mRNA than carotid and femoral artery SMCs. DiSanto et al. (6) reported that the femoral/saphenous arteries express less MLC_{17b} (18% MLC_{17b}) than the aorta (45% MLC_{17b}), concluding that the large elastic arteries express more MLC_{17b} than the smaller elastic arteries. However, because they pooled tissues from the femoral and saphenous arteries, they were unable to detect the differences in MLC₁₇ isoform ex-

pression between the femoral and saphenous arteries (Fig. 4B).

Results from these experiments suggest that there is no preferential association of either SM1 or SM2 and the MLC_{17a} or MLC_{17b} myosin isoforms, as determined from the levels of mRNA transcripts, in single SMCs. The MHC SM1/SM2 and the MLC_{17a}/MLC_{17b} isoforms result from the alternative splicing of the genes encoding these proteins (1, 30). Alternative splicing may be regulated by the presence or absence of splicing factors. These results suggest that the alternative splicing processes resulting in the MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms are not coordinated in single SMCs. Previous work has demonstrated that the mRNA expression of these isoforms correlates with their protein expression (11, 27); thus these results suggest that expression of these MHC and MLC isoforms are not coregulated. Additional work needs to be done at the single cell level to determine whether the alternative splicing processes resulting in the MHC SM1/SM2 and SMA/SMB isoforms are regulated independently.

No correlations were found between the mRNA levels of the SM1/SM2 isoforms and unloaded shortening velocity in arterial SMCs, thus suggesting that expression of the MHC SM1/SM2 isoforms does not correlate with maximal unloaded shortening velocity in single SMCs. In this study, the saphenous artery had significantly more SM2 mRNA than the carotid artery; however, unloaded shortening was similar for these two arteries. These results are in agreement with Kelley et al. (23) and Meer and Eddinger (28), suggesting that expression of the SM1/SM2 isoforms does not correlate with velocity of shortening. In single SMCs isolated from the rabbit carotid and aorta, they reported no correlation between SM1/SM2 isoform expression and unloaded shortening velocity.

In this study, no correlations were found between MLC₁₇ mRNA levels and maximal unloaded shortening velocity in SMCs from the carotid, femoral, and saphenous arteries. These data are in agreement with results reported previously in rabbit carotid and aortic SMCs (8). Although we demonstrate that saphenous SMCs have significantly less MLC_{17b} mRNA than both the carotid and femoral SMCs, differences in unloaded shortening velocities were found only between the femoral and saphenous cells. The mechanisms behind these contractile differences remain undefined. There may be another factor masking the correlation between myosin isoform expression and unloaded shortening velocity, but it is beyond the scope of this study to examine myosin isoform expression and unloaded shortening velocity in single SMCs.

Several studies using purified proteins (23a, 33) and single SMCs (11) suggest that differences in SMA/SMB expression correlate with unloaded shortening velocity. Thus to test whether the differences in unloaded shortening velocity may be due to differences in SMA/SMB isoform expression, RT-PCR was used to analyze MHC SMA/SMB mRNA levels in carotid, iliac/femoral, and saphenous tissue samples. The saphenous artery was found to have higher SMB mRNA levels than the

iliac/femoral and carotid arteries. Because expression of MHC SMA/SMB was analyzed in tissue samples and myosin isoform expression is heterogeneous within the SMCs of the arterial media, it was not possible to test for a correlation between unloaded shortening velocity and SMA/SMB isoform expression within single SMCs. The shift in SMB mRNA levels is consistent with the faster shortening velocities measured in the saphenous SMCs being due to a greater expression of the SMB MHC isoform expression. However, there was no difference in unloaded shortening velocities of carotid and saphenous SMCs, but saphenous SMCs had significantly more SMB mRNA than carotid SMCs. These results suggest that SMB expression may not be the only factor regulating unloaded shortening velocity in arterial SMCs. The most appropriate experiments to resolve this issue may be the single SMC studies where the contractile structures are intact and the results are not befuddled by the heterogeneous protein expression of hundreds of other SMCs. Work is underway to examine this correlation in arterial SMCs.

In this study, no correlation was found between MHC SM1/SM2 and/or MLC_{17a}/MLC_{17b} isoform expression and initial cell length, final cell length, or percent of cell shortening. This is in contrast to the results of Meer and Eddinger (28). They reported that increased SM2 expression enabled the SMCs to attain a shorter final length following agonist contraction. The difference in these results is not clear but may be due to the larger number of cells examined in this study.

In conclusion, our results reveal that 1) myosin isoform expression and unloaded shortening velocity does not vary as a gradient across the carotid arterial media, 2) the saphenous artery expresses more SMB and MLC_{17a} than the carotid and femoral arteries and more SM2 than the carotid artery, 3) intracellular expression of the MHC SM1/SM2 and MLC_{17a}/MLC_{17b} isoforms is not coordinately regulated, and 4) no correlation exists between the MHC SM1/SM2 or MLC_{17a}/MLC_{17b} isoform cellular expression ratios and unloaded shortening velocity.

We thank Leah Carboneau for extracting RNA and performing RT-PCRs to resolve expression of the MHC SMA/SMB isoforms in rabbit arterial tissues samples.

This work was supported by National Heart Lung and Blood Institute Grant HL-62237-02, National Arthritis and Musculoskeletal and Skin Diseases Institute Grant R15-AR-45294-03 (to T. J. Eddinger), and American Heart Association Predoctoral Fellowship 9910142Z (to J. J. Sherwood).

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