Single rabbit stomach smooth muscle cell myosin heavy chain SMB expression and shortening velocity

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Eddinger, Thomas J., and Daniel P. Meer. Single rabbit stomach smooth muscle cell myosin heavy chain SMB expression and shortening velocity. Am J Physiol Cell Physiol 280: C309–C316, 2001.—Isolated single smooth muscle cells (SMCs) from different regions of the rabbit stomach were used to determine a possible correlation between unloaded shortening velocity and smooth muscle (SM) myosin heavy chain (MHC) S1 head isoform composition (SMA, no head insert; SMB, with head insert). α-Toxin-permeabilized isolated single cells were maximally activated to measure unloaded shortening velocity and subsequently used in an RT-PCR reaction to determine the SMA/SMB content of the same cell. SM MHC SMA and SMB isoforms are uniquely distributed in the stomach with cells from the fundic region expressing little SMB (38.1 ± 7.3% SMB; n = 16); cells from the antrum express primarily SMB (94.9 ± 1.0% SMB; n = 16). Mean fundic cell unloaded shortening velocity was 0.014 ± 0.002 cell lengths/s compared with 0.036 ± 0.002 for the antrum cells. Unloaded shortening velocity in these cells was significantly correlated with their percent SMB expression (r² = 0.58). Resting cell length does not correlate with the percent SMB expression (n = 32 cells). Previously published assays of purified or expressed SMA and SMB heavy meromyosin show a twofold difference in actin filament sliding speed in in vitro motility assays. Extrapolation of our data to 0–100% SMB would give a 10-fold range of shortening velocity, which is closer to the ~20-fold range reported from various SM tissues. This suggests that mechanisms in addition to the MHC S1 head isoforms regulate shortening velocity.

Functional relevance for these isoforms continues to be controversial, with the possible exception of the SMA and SMB MHC isoforms. Using biochemical and molecular approaches, a number of researchers have shown that the myosin ATPase activity and in vitro actin filament sliding speed for SMB myosin or heavy meromyosin (HMM) is approximately twofold higher than for SMA (7, 15, 26). These differences were independent of the MLC₁₇ isoform present. This difference is only approximately one-tenth the difference reported for unloaded shortening velocity of various intact SM tissue preparations (22). In vitro ATPase and motility studies are valuable because they are “biologically simple” and there is little room for misinterpretation of results. This does, however, leave room for doubts about the physiological relevance to the intact system. In this study, we have used the “intact” SM cell as our model. The permeabilized SM single cell maintains the three-dimensional organization and composition of the in vivo cell that is lost in molecular studies while eliminating the intercellular variability of tissue studies. The results indicate that in the rabbit stomach, the SMA and SMB expression varies from 12–100% in cells from the fundus to the antrum and shows a significant correlation with unloaded cell shortening velocity. Extrapolation to 0 and 100% SMB gives a 10-fold difference in unloaded shortening velocity. Which is closer to the data to 0–100% SMB would give a 10-fold range of shortening speed in in vitro motility assays. Extrapolation of our meromyosin show a twofold difference in actin filament sliding speed for SMB myosin or heavy meromyosin (HMM) is approximately twofold higher than for SMA (7, 15, 26). These differences were independent of the MLC₁₇ isoform present. This difference is only approximately one-tenth the difference reported for unloaded shortening velocity of various intact SM tissue preparations (22). In vitro ATPase and motility studies are valuable because they are “biologically simple” and there is little room for misinterpretation of results. This does, however, leave room for doubts about the physiological relevance to the intact system. In this study, we have used the “intact” SM cell as our model. The permeabilized SM single cell maintains the three-dimensional organization and composition of the in vivo cell that is lost in molecular studies while eliminating the intercellular variability of tissue studies. The results indicate that in the rabbit stomach, the SMA and SMB expression varies from 12–100% in cells from the fundus to the antrum and shows a significant correlation with unloaded cell shortening velocity. Extrapolation to 0 and 100% SMB gives a 10-fold difference in unloaded shortening velocity.
loaded shortening velocity. Thus it is possible that expression of the SMB MHC isoform can explain approximately half of the in vivo unloaded shortening velocities reported for SM tissues.

METHODS

General. New Zealand White rabbits were killed by CO$_2$ asphyxiation according to an approved protocol. The stomach was removed, opened, cleaned of blood and adipose, loose connective tissue and nonmuscle tissue mucosa, and stored in physiological salt solution (PSS; in mM): 140 NaCl, 4.7 KCl, 1.2 Na$_2$HPO$_4$, 1.2 MgSO$_4$, 1.6 CaCl$_2$, 5.6 glucose, 0.02 EDTA, and 2.0 3-(N-morpholino)propanesulfonic acid, pH 7.2 at 4°C until further processed.

The stomach was laid out flat with the esophageal sphincter to the right and the pyloris to the left (Fig. 1). Starting at the fundus adjacent to the esophageal sphincter and progressing along the greater curvature of the stomach to the pyloris, the stomach was divided into nine regions. In addition, region 10 was taken from the lesser curvature of the stomach near the esophagus (see Fig. 1). Samples were taken from each of these regions for myosin isoform composition comparisons.

Protein gel electrophoresis. Tissues for protein analysis were homogenized in sample buffer [0.125 M Tris, 2% SDS (wt/vol), 30% glycerol (vol/vol), 0.01% bromphenol blue (wt/vol), and 30 mM dithiothreitol (DTT)]. MHC were resolved on low cross-linking SDS gels using the methods of Giulian et al. (13). For some experiments, the protein on the gels was transferred to nitrocellulose (4), and SMB-specific antibodies (gift from A. S. Rovner, Univ. of Vermont; Ref. 29) were used to identify the presence of this isoform.

Tissue RNA extraction. Total RNA was extracted from tissues using RNA$_{acol}$ B (Tel-Test B, Friendswood, TX). Samples for protein and RNA comparisons were collected from the same tissue samples by first extracting the RNA and subsequently extracting the protein. Extracted RNA (1.0–1.5 μg) was added to the RT reaction mix [2 μl of 5× RT buffer (U.S. Biochemical, Cleveland, OH), 20 μg/ml random hexamers (Promega, Madison, WI; for the MHC tail isoforms, SM1 and SM2, and the MLC$_{17\alpha/b}$ isoforms, oligo(dT) is used in place of random hexamers], 2 U/μl RNasin (Promega), 0.1 μg/μl acetylated bovine serum albumin, 500 μM dNTP, 1.25 mM DTT, and 10 U/μl Moloney murine leukemia virus-RT (U.S. Biochemical)] to a final volume of 10 μl and incubated at 37°C for 2 h.

Cell isolation. Individual cells were isolated from the fundus or the antrum using the method of Driska and Porter (8). Briefly, before digestion, a region of fundus or antrum (including part of the esophagus or small intestine, respectively) was incubated in no-Ca$^{2+}$ PSS (in mM: 140 NaCl, 4.7 KCl, 1.2 Na$_2$HPO$_4$, 2.4 MgSO$_4$, 0.0 CaCl$_2$, 5.6 glucose, 0.02 EDTA, and 2.0 MOPS, pH 7.4) for 15 min before digestion to reduce activation due to mechanical perturbation during isolation. A 5-ml plastic syringe was cut down to include the Luer end and ~1 cm of the barrel. The barrel end was heated to form a flange. The stomach region was pulled over the barrel flange and tied securely so it would not slip off. The esophagus or small intestine, depending on the stomach region, was also tied off. A plastic Luer fitting (female Luer X 3/32 hose barb, Cole-Parmer) was attached to one end of ~30 cm of the primed-inch-C-flex tubing (Cole-Parmer); and a 0.5-ml pipette tip was attached to the other. The stomach region and the tubing were both filled with the digesting solution to avoid air pockets and then attached via the Luer lock. The stomach region was ballooned and bathed in a no-Ca$^{2+}$ PSS solution that contained papain (20 U/ml) and DTT (2 mM) at 37°C with 40 cm H$_2$O head pressure applied to balloon the vessel. After ~10–20 min of digestion, the tissue was removed from the enzyme solution and placed in fresh no-Ca$^{2+}$ PSS. The tissue was removed from the syringe barrel and thin strips of the muscle were teased away, liberating single smooth muscle cells (SMCs). To produce cDNA from a single SMC, a single living cell was attached to a glass micropipette (2-μm tip) under a microscope using a micromanipulator. The pipette was transferred to a clean microcentrifuge tube. The tip of the micropipette was broken into the microcentrifuge tube, and the cell was lysed by a freeze-thaw cycle. Ten microliters of RT mix were added to each microcentrifuge tube, vortexed briefly, and incubated at 37°C for 2 h.

PCR. Two oligonucleotide primers (corresponding to rabbit uterus myosin; nucleotide positions 610–629 and 806–787; Operon, Alameda, CA; GenBank M77812; Ref. 25) were used in the 50-μl PCR reaction [5 μl of DNA polymerase 10× reaction buffer (Promega); 1.5 mM MgCl$_2$, 200 μM dNTP, 2 μM 5’ primer, 2 μM 3’ primer, and 1 unit of Taq polymerase (Promega)]. Two microliters of the RT reaction were used for cDNA amplification of tissue RNA samples. After a 2-min, 94°C hot start, the PCR temperature protocol was 94°C denaturation (1 min 30 s), 55°C (2 min), and 72°C (3 min) primer extension for 35 cycles. An Ericomp thermoelectric thermocycler was used for these studies (USA/Scientific Plastics, Ocala, FL). These primers flank the 21-nucleotide exon that encodes the difference region that defines the SMA and SMB isoforms. The PCR products generated with the use of these primers are 197 and 218 base pairs (bp), corresponding to SMA and SMB, respectively. Quantitation problems due to differential primer annealing are obviated by using a single pair of primers to generate both SMA- and SMB-specific PCR products. The SMA/SMB PCR primers flank intervening sequence in the genomic DNA; therefore, only cDNA products generate appropriately sized PCR products.

For single cell analysis, the entire RT reaction mixture (10 μl) was routinely used for PCR amplification (60 cycles). The PCR fragment corresponding to the SMB isoform derived from rabbit stomach was purified from gel bands and sequenced with the use of the dideoxy chain termination method (27). For the SM1/SM2 and LC$_{17\alpha/b}$ isoforms, the

Fig. 1. Schematic of rabbit stomach showing regions 1–10, small intestine, and esophagus, for tissue sampling. Isolated cells for mechanical measurements and single cell RT-PCR were obtained from either the region of the fundus (regions 1 and 2) or the antrum (regions 7–9).
primers used were the same as previously reported (9, 21, respectively).

Single cell contractions. Mechanical measurements were performed in a flow-through chamber consisting of a depression slide with three inputs and one output. The concave depression on the slide was surrounded by a wax ring to prevent fluid dispersal during the experiment. The gravity-feed flow through the input tubes was monitored by inclusion of fast green dye (0.002% wt/vol) in the perfusion solutions. Outflow from the perfusion chamber was controlled by a peristaltic pump (Rabbit Miniplus 2; Rainin Instrument, Emeryville, CA). Output was matched to input by adjusting the speed of the peristaltic pump so that the chamber fluid meniscus remained at a constant level.

Single SMCs were isolated from the stomach by the methods detailed in Cell isolation. The SMCs were transferred from coated glass isolation dishes (Sigma-cote; Sigma, St. Louis, MO and Slickcoat; Intermount Scientific, Kaysville, UT) via wide-bore pipettes to the depression slides. Cells were allowed to settle to the bottom of the slide (5 min) before solution exchange from a no-Ca2+-PSS to relaxing buffer (pCa 9.0) [relaxing buffer contained (in mM): 14.5 creatine phosphate, 7 EGTA, 20imidazole, 1 free Mg2+, 4 free MgATP, 5.42 MgCl2, 79.16 KCl, 4.74 ATP, and 16.33 μM CaCl2, pH 7.0]. Once cells were in the relaxing buffer, permeabilization of the SMC membrane was accomplished by a 7-min incubation in relaxing buffer plus 250 hemolytic U/ml Staphylococcus aureus α-toxin (20) to form small pores (2–3 nm) in the plasma membrane, which allow passage of small substances (<4 kDa) (16). After permeabilization, all SMCs were washed with relaxing buffer (2 min).

After the 2-min wash, a permeabilized SMC was selected on the basis of morphology (11) and birefringence of the cell membrane using phase-contrast microscopy. The cell was attached to a small-bore (1- to 3-μm tip) glass micropipette by close apposition of the pipette tip and gentle suction. Once attached, the SMC was lifted off the chamber bottom to ensure absence of adhesion. The cell was stimulated to contract with an activation cocktail that consisted of 1 μM phenylephrine and 1 μM histamine in activating buffer (pCa 6.0) [activating buffer contained (in mM): 14.5 creatine phosphate, 7 EGTA, 20imidazole, 1 free Mg2+, 4 free MgATP, 5.31 MgCl2, 68.55 KCl, 4.79 ATP, and 4.91 CaCl2, pH 7.0]. Previous control experiments in arterial cells included 1 μM okadaic acid (an MLC phosphatase inhibitor) in the activating solution and gave similar results when it was absent (9, 20).

For these studies, we also performed control experiments in stomach cells with okadaic acid to test for possible variable phosphorylation. The isolated cells were incubated in 1 μM okadaic acid for 7 min during the α-toxin permeabilization, and the okadaic acid was also included in the activating solution. One micromolar okadaic acid has been reported to give complete inhibition of type I phosphatase (MLC-1) (6).

Cell length data acquisition. Before and throughout the single SMC contraction, cell length was continuously monitored by a charge-coupled device (CCD) camera (XC-75; Sony, Park Ridge, NJ) attached to an inverted microscope (Fluovert Leitz, Overland Park, KS). The output from the CCD camera was recorded by a video recorder (EV-S3000; Sony, Park Ridge, NJ) on 8-mm videotape. SMC images from the 8-mm videotape were captured by a frame grabber (Data Translation, Marlborough, MA) in conjunction with NIH Image software (National Institutes of Health, Bethesda, MD) at regular intervals encompassing the SMC contraction. Image analyses were performed using NIH Image software (version 1.59). Each individual frame of the cell from successive time points during the contraction was projected on the monitor. The ends of the cell were identified on the frame. With the use of the mouse and starting at one end of the cell, the midpoint of the cell’s width was marked along its entire length at 5- to 10-μm intervals. The length of the line connecting these points gave total cell length for each frame. This method gives an accurate measurement of cell length independent of its linearity as long as the cell remains in the plane of focus (20).

Untethered velocity measurements from single SMCs. Video images from the CCD camera were recorded on a videocassette recorder, digitized, imported into a personal computer, and analyzed with respect to length over a discrete time frame using NIH Image software (version 1.59). Thirty-two cells exhibited the following criteria: 1) characteristic morphology of contracted SMCs (i.e., evaginations in cell membrane postcontraction) (11), and 2) a smooth sigmoidal decrease in length over time, indicating unconstrained contraction within the plane of focus of the microscope. Unloaded shortening velocity was taken from the steepest slope of a plot of length vs. time for these cells. After its contraction, each cell from which mechanical measurements were made was transferred to a microcentrifuge tube so that the SMA/SMB mRNA ratio could be determined. Both the mechanical measurements and the RT-PCR were performed on the same single cell, thus allowing possible correlations between them to be determined.

Electrophoresis and densitometry. PCR products were separated by electrophoresis on 8% polyacrylamide gels (13) and visualized under ultraviolet light after staining with ethidium bromide (1 μg/ml). Band intensity was quantified by image analysis of gel photographs using Ambis 2000 software (Ambis, San Diego, CA). Extensive controls for the validity of these methods for relative quantitation of the RT-PCR products have been reported previously (21).

Statistics. Comparisons between sample means were tested for significance by a two-tailed t-test, and significance of the correlation coefficient from zero was tested for by a t-test with two degrees of freedom.

RESULTS

The rabbit stomach is a U-shaped chamber that is readily divided into distinct anatomic regions (Fig. 1). DNA and protein were isolated sequentially from samples taken from regions 1–10. The protein was run on polyacrylamide gels and stained for visualization (Fig. 2A) or Western blotted to nitrocellulose and reacted with an SMB-specific antibody (Fig. 2B). The first four lanes are alternating samples of homogenized fundus and antrum at two different loadings, followed by regions 1–9. The Coomassie brilliant blue-stained gel-stained gel (Fig. 2A) shows that there is approximately equal amounts of both the SM1 and SM2 MHC isoform in all these regions of the stomach. Figure 2B shows that the expression of the SMB MHC isoform is much less uniform. Regions 1–4 show very little reactivity with the SMB antibody, whereas regions 5–9 show a dramatic increase in reactivity across this area.

RT-PCR on the RNA from regions 1–10 (Fig. 1) was performed using primers for the SMA/SMB (Fig. 3A) MHC isoforms as well as the MLC-1α and -β isoforms (Fig. 3B). The SMA/SMB cDNA from the PCR products were sequenced to verify their identity. The nucleotide sequences for both the SMA and SMB MHC were iden-
tical with the exception of the 21-nucleotide insert in the SMB. Both of these sequences matched published sequence data for the rabbit visceral sequence (1). We have performed similar sequence analysis for the MLC17a/b isoforms (9). Similar to the shift in expression from low to high observed with the SMB antibody in regions 1–9 of the stomach, the percent SMB mRNA message shows distinct shift from <10% in regions 1–4, to 40–60% SMB in regions 5–7, and to ~100% in regions 8 and 9 (Fig. 3A). Region 10 on the lesser curvature of the stomach showed ~85% SMB mRNA.

When primers for the MLC17a/b isoforms were used on the mRNA from these same stomach regions, the percent MLC17b mRNA decreased from 20% in the fundic and body regions (Fig. 3B, regions 1–6) to 10% in the antral regions (Fig. 3B, regions 7–9). Region 10 also expressed a minimal amount of MLC17b (<5%). The expression of the SM1/SM2 MHC isoforms was determined from the protein gels (see Fig. 2A) and illustrated a very slight decrease in percent SM1 protein expression levels [from 46–51% in the fundus and body (regions 1–6, Fig. 3C) to 40–46% in the antrum (regions 7–9, Fig. 3C)]. Region 10 also showed ~42% SM1 expression. Thus the expression of the SMB MHC isoform shifts from 5–100% from regions 1–9 of the stomach, while both the SM1 MHC and MLC17b isoform vary <15% over these same regions.

Single SMCs were isolated from either the fundus or antrum by papain digestion, permeabilized with α-toxin, and stimulated with an activating cocktail (Fig. 4). The time series shows a single SMC at various time points following addition of the activating cocktail. Following the cell contraction, the cell is transferred to a microcentrifuge tube for RT-PCR. Figure 5 shows the results of the RT-PCR on seven individual SMCs using primers to distinguish between the SMB (top, 220 bp) and SMA (bottom, 207 bp) isoforms. Lane
1 is a 123-bp ladder. The first five cells are from the fundus and the last two cells are from the antrum. Similar to the tissue SMB mRNA results (Fig. 3A), the fundic cells shown here have low and variable SMB expression (10–50%), whereas the antral cells express almost exclusively the SMB mRNA.

Figure 6 is a histogram of percent SMB mRNA expression for 32 SMCs from the fundus and antrum. The percent SMB mRNA expression for the fundic cells is highly variable (12–100%) with an average of <40%. In contrast, the antral cells show uniformly high (80–100%) SMB mRNA expression. The unloaded shortening velocities for these cells is shown in Fig. 7, with the average velocity of the fundic cells being approximately one-third of the antral cells but having a similar variability. There is a significant correlation between the shortening velocity of these cells and their percent SMB mRNA expression (Fig. 8; \( r^2 = 0.58; P = 0.01 \)). All antral cells are included in the cluster of data points between 80–100% SMB, whereas the fundic cells are included over the whole range of percent SMB expression observed.

To rule out incomplete and/or variable phosphorylation of MLC20, which could also account for differences in unloaded shortening, two additional sets of cells were tested. Cells are shown (see Table 2) from the rabbit fundus that were preincubated with 1 μM okadaic acid for 7 min before being exposed to the activating solution with 1 μM okadaic acid vs. no okadaic acid. There are no significant differences between these two groups for the five variables measured.

Table 1 (top) gives a summary of the single cell data from the fundus vs. the antrum. Isolated cells from both these regions were the same length, but the antral cells shortened significantly more during unloaded shortening, giving them a shorter final length following

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**Fig. 4.** Contraction of a single smooth muscle cell (SMC). A temporal series of digitized images of a permeabilized SMC contracting in response to exposure to an activating solution containing Ca\(^{2+}\) (pCa 6.0), phenylephrine (1 μM), and histamine (1 μM) is shown. A segmented line drawn through the middle of the cell from end to end shows the measured length for each time point. The time and length for each frame are given below the frame.

**Fig. 5.** Polyacrylamide gel showing single SMC SMA/SMB PCR products. Individual cells from either the fundus or antrum of the stomach were utilized for RT-PCR as described in METHODS. RT-PCR product from 7 single cells showing the variable range of expression from ~10 to 100% SMB. The first lane is a 123-bp ladder showing bands from 123–369. The SMA RT-PCR product is 197 bp, whereas the SMB RT-PCR product is 218 bp. The first 5 cells (10.1–47.3% SMB) were isolated from the fundus, while the last 2 cells (100% SMB) were isolated from the antrum.
shortening and a greater percent length change. The antral cells expressed significantly more SMB MHC mRNA than the fundic cells and had unloaded shortening velocities significantly faster than the fundic cells. The table (bottom) describes similar data, but for cells with <30% SMB vs. >80% SMB as the separation criteria rather than anatomic region of origin. This allows the fundic cells (n = 3) expressing percent SMB mRNA levels similar to the antral cells (>80%) to be grouped with the antral cells as well as eliminating the two fundic cells with percent SMB mRNA expression at 33 and 52% from the analysis. The unloaded shortening velocity for the <30% SMB cell population is significantly slower than the fundic cell population, as would be predicted by the significantly lower percent SMB.

**DISCUSSION**

Defining SM myosin isoform function continues to be an active area of research. It remains unclear, however, how expression of these isoforms is regulated and what, if any, unique functional role they have in SM tissue. This study was performed to examine the unique distribution of SMB MHC expression in the stomach using new methods of single cell analysis (20, 21) to further our understanding of the possible unique functional roles for the SMA/SMB MHC isoforms. The rabbit stomach has both anatomically and physiologically distinct regions in spite of being a single organ. The fundus is a region of thin-walled SM that has “tonic” characteristics, whereas the antrum has a very thick SM wall with “phasic” mechanical characteristics. SMCs from these anatomically distinct regions of the stomach express significantly different amounts of SMB MHC mRNA and protein (Fig. 3, A–C). This heterogeneous expression is significantly greater at the cellular level than at the tissue level. Fundic cells vary in SMB MHC expression from ~10–100% (whereas tissue samples only vary between 2–10%). As one follows the greater curvature of the stomach from the fundus to the antrum, the amount of expressed SMB MHC mRNA goes from very low (<10% in regions 1–4), to a mixture (40–60% in regions 4–7), to nearly pure SMB isoform expression (95–100% in regions 8 and 9; Fig. 3A). The shift at the protein level parallels the mRNA shift (Fig. 3, A–C). We have previously reported that both the SM1/SM2 MHC isoform and the MLC17α/β isoform mRNA levels exhibit a very good correlation with protein expression (7, 17). Thus it is possible to predict protein expression based on mRNA expression. The results from this study suggest that the SMB mRNA expression can also be used to predict SMB protein expression (compare Fig. 2B and Fig. 3A).

The significant increase in SMB MHC mRNA from the fundus to the antrum (Fig. 3A) is not paralleled by similar changes in the MLC17α/β or SM1/SM2 isoforms (Fig. 3, B and C). These data suggest that expression of
these isoforms is not coordinated in the rabbit stomach. However, these measurements were made from tissue pieces at each of nine locations along the greater curvature of the stomach and not from single cells. Because of the significant intercellular variability in SMB expression in the fundus, this comparison will need to be performed at the cellular level to confirm these findings.

Single SMC unloaded shortening velocity is significantly faster in the antral cells relative to the fundic cells. The initial length of cells from these two regions is not significantly different, but the antral cells have a significantly shorter final cell length following unloaded shortening and a significantly greater percent length change during shortening (Table 1). It is unclear what, if any, affect the higher percent SMB MHC in the antral cells play in their ability to shorten to a greater extent during activation. We have previously reported an inverse correlation between the SM2/SM1 ratio and final cell length following unloaded shortening in single SMCs (20). The very small decrease in percent SM2 expression in the antral relative to the fundus is in the opposite direction to what would be expected for an increased percent shortening based on our previous work. The small (~8%) decrease in percent SM2 from fundus to antrum may not be significant and would therefore not be relevant. Direct manipulation of expression of the SM1/SM2 isoform may be required to elucidate any causal relationship.

A possible alternative explanation for the differences in unloaded shortening velocity would be variable phosphorylation. Phosphorylation levels are critically correlated with shortening velocity in smooth muscle (22). These studies used an α-toxin-permeabilized cell that allows exchange of small (<4 kDa) molecular weight solutes without interfering with receptor-coupled activation (16). The activation cocktail (1 μM histamine, phenylephrine, and Ca²⁺) was designed to maximally activate the cells. In this study, and all single cell studies that we are aware of, there is no quantitation of MLC₂₀ phosphorylation because of technical limitations.

To test for incomplete or variable activation that could result in nonmaximal phosphorylation, 1 μM okadaic acid was used to inactivate MLCP (5, 6). Table 2 shows no difference in the results when okadaic acid is used. Most important, mean maximal unloaded shortening velocity with and without the okadaic acid is not significantly different, nor is the SE for these groups. These results are strong evidence that variable phosphorylation is not a factor in these studies.

A twofold difference in vitro motility and ATPase activity for purified myosin (7, 15) and expressed HMM (26) with and without the head insert (SMB/SMA) have been reported. This twofold difference is at least an order of magnitude less than the difference reported for intact SM strip/ring preparations from different SM tissues (22). In contrast, in the permeabilized intact SM cells used in this study, there is an ~10-fold difference in unloaded shortening velocity when one extrapolates from 0 to 100% SMB expression. This is fivefold greater than with the in vitro motility results (15, 26), but still less than half of the tissue studies. Thus in vitro motility studies on purified/expressed myosin or myosin subunits do not equate with unloaded shortening velocity measurements in intact cells because the in vitro motility values are lower by a factor of five. This could be because myosin needs to be in its filamentous form in a three-dimensional organization of the cell to be fully effective. It may also mean that other proteins can affect its function. The very small changes in the SM1/SM2 MHC and MLC₁₇α/β isoforms across the stomach regions and previous work from this lab (9, 20) suggest that these isoforms are not potential modulatory protein candidates for this explanation.

The ~10-fold difference reported in this study is still less than half of the difference reported for intact SM tissues (22). This suggests that the remaining variability must result from other mechanisms. The list of possibilities is seemingly endless and includes every-

### Table 1. Single SMCs grouped by region of origin or %SMB expression

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Length, μm</th>
<th>Final Length, μm</th>
<th>%Length Change</th>
<th>%SMB</th>
<th>Velocity, cell lengths/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fundus cells (n = 16)</td>
<td>330.0 ± 26.8</td>
<td>120.2 ± 13.1</td>
<td>61.5 ± 3.9</td>
<td>38.1 ± 7.3</td>
<td>0.0144 ± 0.0025</td>
</tr>
<tr>
<td>Antrum cells (n = 16)</td>
<td>318.3 ± 11.4</td>
<td>72.3 ± 3.3</td>
<td>77.2 ± 0.7</td>
<td>94.1 ± 1.4</td>
<td>0.0386 ± 0.0024</td>
</tr>
<tr>
<td>P value (&lt;)</td>
<td>0.687</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>&gt;30% SMB (n = 11)</td>
<td>297.6 ± 28.0</td>
<td>134.9 ± 16.5</td>
<td>53.9 ± 3.7</td>
<td>21.9 ± 1.7</td>
<td>0.0094 ± 0.0021</td>
</tr>
<tr>
<td>&gt;80% SMB (n = 18)</td>
<td>338.4 ± 18.6</td>
<td>75.6 ± 4.8</td>
<td>77.6 ± 0.7</td>
<td>94.9 ± 1.0</td>
<td>0.0357 ± 0.0026</td>
</tr>
<tr>
<td>P value (&gt;)=</td>
<td>0.217</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Population means for single smooth muscle cells (SMCs) grouped by the region of stomach from which they were isolated (fundus vs. antrum) or by percent expression of the SMB myosin heavy chain (<30% vs. >80% SMB). With the exception of the initial length measurements for both groupings, all comparisons are significantly different (P ≤ 0.01).

### Table 2. Stimulated single SMCs

<table>
<thead>
<tr>
<th>Group</th>
<th>Activation cocktail</th>
<th>Cocktail + okadaic acid</th>
<th>P Value, ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fundus Cells (n = 10)</td>
<td>334.8 ± 28.7</td>
<td>326.9 ± 18.3</td>
<td>0.82</td>
</tr>
<tr>
<td>Fundus Cells (n = 11)</td>
<td>112.8 ± 10.3</td>
<td>106.9 ± 10.3</td>
<td>0.68</td>
</tr>
<tr>
<td>Final length, μm</td>
<td>%Length change</td>
<td>%SMB</td>
<td>Velocity, cell lengths/s</td>
</tr>
<tr>
<td></td>
<td>64.1 ± 2.7</td>
<td>61.9 ± 3.8</td>
<td>0.012 ± 0.002</td>
</tr>
<tr>
<td>%SMB</td>
<td>26.6 ± 5.0</td>
<td>19.3 ± 3.7</td>
<td>0.011 ± 0.003</td>
</tr>
<tr>
<td>P Value, ≤</td>
<td>0.82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Population means for single SMCs stimulated with the activation cocktail with or without okadaic acid (see METHODS).
thing from different receptors or receptor subtypes to all the second messenger pathway components, including G proteins, phospholipase C, d-myo-inositol trisphosphate, kinases, phosphatases, etc., and other contractile proteins (i.e., calponin, caldesmin) (for reviews, see Refs. 3, 22, and 28). Further work is needed to examine each of these before a complete understanding of mechanical variability will be understood.

In conclusion, this paper shows that there are distinct expression patterns of the SM MHC SMA/SMB isoforms in different regions of the stomach and at the cell level. The SMB MHC expression is significantly correlated with unloaded shortening in single SMCs and percent length change during shortening. Expression of the SMA/SMB MHC isoforms is not coupled to expression of the SM1/SM2 MHC of MLC17s, isoforms in the stomach at the tissue level. The 10-fold difference in unloaded shortening velocity reported here, which may be attributable to variable SMB expression, is fivefold greater than that reported by in vitro motility assays and less than half that reported for SM isomyosin light chain mRNAs are encoded by a single-copy gene in the chicken. Biochim Biophys Res Commun 170: 53–58, 1990.


