Regional differences in myosin heavy chain isoform expression and maximal shortening velocity of the rat vaginal wall smooth muscle

Maureen Basha,1 Shaohua Chang,1 Elaine M. Smolock,2 Robert S. Moreland,2 Alan J. Wein,1 and Samuel Chacko1,3

1Division of Urology, University of Pennsylvania, 2Department of Pharmacology and Physiology, Drexel University College of Medicine, and 3Department of Pathobiology, University of Pennsylvania, Philadelphia, Pennsylvania

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Address for reprint requests and other correspondence: S. Chacko, 3005 Ravdin-Courtyard, HUP, Univ. of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104 (e-mail: chackosk@mail.med.upenn.edu). Regional differences in myosin heavy chain isoform expression and maximal shortening velocity of the rat vaginal wall smooth muscle. Am J Physiol Regul Integr Comp Physiol 291: R1076–R1084, 2006. First published May 11, 2006; doi:10.1152/ajpregu.00118.2006.—Contractility of the rat vagina; smooth muscle; female sexual response


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Interpretation of the pattern of isometric force tracings from the proximal and distal vagina have lead to the suggestion that the proximal vagina may be phasic in nature compared with the tonic distal vagina (37). Smooth muscle with a relatively high velocity of shortening, poor maintenance of force, and a tendency to display spontaneous activity are considered phasic, and muscle with a slow velocity of shortening and a superior ability to maintain force are considered as tonic. One goal of this study was to determine the MHC isoform expression in the proximal and distal vagina. Given the compelling evidence for a functional significance of different MHC isoforms, a second goal of this study was to determine the MHC isoform expression in the proximal and distal vagina.

MATERIALS AND METHODS

Animals. Animal use and the experimental protocol were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Female Sprague-Dawley rats (4–5 mo old, 250–300 g) were obtained from a commercial supplier and housed in a temperature (25°C) and light-controlled (12:12-h light-dark) room with free access to food and water. Histological examination of daily vaginal smears using the Papinocolo method was performed to monitor the estrus cycle of rats through visual assessment of sloughed epithelial cell morphology. To control for possible effects of estrus cycle stage and to ensure animals were cycling normally, all animals were killed the day of estrus following two consecutive cycles by an intraperitoneal injection of an overdose of ketamine (>75 mg/kg) and xylazine (>10 mg/kg).

Tissue preparation. The vaginal tube was isolated and dissected from the urogenital tract, cleaned of connective tissue, and cut open longitudinally. For histological and immunohistochemical procedures, sections from the intact vagina (n = 3) were placed in Histochoice fixative (Amresco, Solon, OH). For functional and molecular studies, the vagina was divided into a proximal (approximately upper two-thirds) and distal segment (approximately lower one-third) under a dissecting microscope (Fig. 1). The vaginal segments were either snap frozen in liquid nitrogen and stored at −80°C or placed in ice-cold MOPS-buffered physiological salt solution (PSS) for same-day physiological studies. The PSS solution contained (in mM) 140 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.6 CaCl₂, 1.2 Na₂HPO₄, 2.0 MOPS, 5.0 d-glucose, and 0.02 Na₂-EDTA.

RNA extraction/quantification. Frozen tissue (50–100 mg) was ground into a fine powder with a liquid nitrogen prechilled mortar and pestle (n = 6). RNA was extracted from tissue powder using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Briefly, 1 ml of TRIzol reagent was added to vaginal tissue powder, and the mixture was homogenized with a minielectric homogenizer. Following centrifugation, the resultant supernatant was mixed with 0.2 ml of chloroform and centrifuged for phase separation. The aqueous phase was then isolated, and RNA was precipitated by the addition of isopropl alcohol. The RNA pellet formed following centrifugation was washed with 75% alcohol and subsequently dissolved in 0.1% diethyl pyrocarbonate-treated water. RNA concentration was quantified at 260/280 with a UV-visible spectrophotometer (BioSpec-mini, Shindzu).

Reverse transcription. Three micrograms of RNA were reverse transcribed with oligo(dT) primer (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcription (RT) (Invitrogen, Carlsbad, CA) at 37°C for 60 min. The reaction was stopped by heating to 90°C, and the cDNA was stored at −20°C.

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed with 1 μl of cDNA in 49-μl reaction mixture containing the following: upstream and downstream primer, 10× PCR buffer (Applied Biosystems), dNTP (Roche Applied Science, Germany), and Taq polymerase (Perkin-Elmer, Foster City, CA). The 5’ to 3’ sequences of the upstream and downstream primers were as follows. SMA/SMB: CCA CCA GGG CAA GAA GAC AGC (upstream); TCC GGC GAG CAG GTA GAA GA (downstream). SMI/SM2: AGC AGG CAG AGA AAG GAA ACA CCA (upstream); GAA GTC TGA GTG CCG AGC GTC CAT (downstream).

Western blot analysis. Protein was extracted from 50–100 mg of pulverized tissue from frozen vaginal segments (n = 4) in a buffer containing 0.05 M Tris, 20% glycerol, 1% SDS, 25 mM dithiothreitol, 2% bromophenol blue, and protease inhibitors (0.8 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin, 1 μM antipain, and 0.1 mg/ml soybean trypsin inhibitor). Before the addition of dithiothreitol and bromophenol blue, which interfere with the Bio-Rad DC assay, 25 μl of the supernatant were set aside for protein quantification. To verify accurate protein concentration measurements and confirm that no proteolytic breakdown of protein occurred, 40 μg of protein were separated by 10% SDS-PAGE and stained with Coomassie blue for scanning with a GS800 Bio-Rad calibrated densitometer (Hercules, CA). For total myosin, SM-B, and β-actin analysis, 5 μg of protein were separated by SDS-PAGE with a highly porous gel (7.5%), and the protein was transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was then blocked with 5% nonfat milk for 2 h in PBS containing 0.1% Tween 20 (PBST). As an internal control, the membrane was incubated in a 1:5,000 dilution of mouse anti-β-actin monoclonal antibody (A5316, Sigma, St. Louis, MO) for heating to 90°C, and the cDNA was stored at −20°C.
1 h followed by incubation in a 1:3,000 dilution of peroxidase-linked sheep anti-mouse antibody (NA931 V, Amersham Biosciences, Buckinghamshire, UK) at room temperature for 1 h. For analysis of MHC, the membrane was incubated for 1 h in a 1:50,000 dilution of a polyclonal antibody generated in rabbits against bovine aorta (a generous gift of Dr. R. Adelstein) followed by incubation in a 1:3,000 dilution of peroxidase-linked donkey anti-rabbit antibody (NA934 V, Amersham Biosciences) at room temperature for 1 h. For SM-B analysis, the membrane was incubated in a 7-amino acid-specific SM-B antibody generated in our laboratory (10) for 2 h followed by incubation in a 1:3,000 dilution of sheep anti-mouse antibody at room temperature for 1 h. Membranes were washed five times for 3–5 min in PBST between incubations. MHC, SM-B, and β-actin protein were detected using an enhanced chemiluminescence kit from Amersham Biosciences. Resultant autoradiograms were analyzed by scanning densitometry with quantitation software (Quantity One, Biorad, Hercules, CA).

Measurement of $V_{\text{max}}$. Maximal shortening velocity was measured as follows. Longitudinal strips measuring 1.5 mm × 6 mm were cut from the proximal and distal vagina (n = 5). Each strip was mounted on one end to a micrometer via a plastic clip, for control of muscle length. The other end was attached to an aluminum foil tube connected to a servo-lever (model 300H, Cambridge Technology) interfaced to a Linux operating system-based microcomputer. The preparation was immersed in a large water-jacketed muscle chamber containing PSS at 37°C and aerated with 100% O2. Strips were equilibrated for 90 min, during which time they were repeatedly contracted by the addition of 110 mM KCl (equimolar substitution for NaCl) to the water bath. Between contractions, the strips were stretched to a length that approximates the optimal length for maximal active contraction. For estimation of maximal velocity of shortening, each strip was stimulated with 110 mM KCl (equimolar substitution for NaCl) and then subjected to a series of isotonic quick releases to afterloads equaling 5, 10, 15, and 20% of the force at the time of release. The change in length at each afterload was fit by a double exponential equation, and a tangent to the fit at 100 ms after release was taken as the isotonic shortening velocity at that afterload. Isotonic shortening velocities at the various afterloads listed above were used to extrapolate velocity at zero load for calculation of $V_{\text{max}}$. $V_{\text{max}}$ was estimated at both the point of peak force of contraction and during steady-state conditions (~6 min following KCl stimulation).

Force/cross-sectional area. At the end of each experiment, strips were placed in Histochoice fixative (Amresco, Solon, OH) and embedded in paraffin. For each muscle strip, 10 cross sections, 5 μm thick, were taken at intervals of 50 μm and stained with hematoxylin and eosin. Images of cross sections were captured with a RT slider SPOT camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed for cross-sectional area of muscle and total cross-sectional area of strip using Image Pro Plus software, version 4.5 (Media Cybernetics, Silver Spring, MD). The amplitude of each contraction at peak force and during steady state was then normalized to force/cross-sectional area of tissue strip (tissue stress) and force/cross-sectional area of smooth muscle (muscle stress) using the values obtained for percent muscle mass.

Histology/immunohistochemistry. Cross sections of 5-μm thickness were taken from the proximal and distal end of the paraffin-embedded vaginal tube. Morphometric analysis of Masson’s Trichrome-stained cross sections of the vaginal tube was carried out as described for morphometric analysis of tissue strips. For immunohistochemistry, sections were deparaffinized in a xylene substitute (Shandon, Pittsburgh, PA), followed by descending grades of alcohol, and washed three times in PBS. Tissue sections were first incubated for 1 h in 1% BSA at room temperature to block nonspecific binding. Subsequently, sections were incubated in a 1:300 dilution of anti-α-smooth muscle actin antibody generated in mouse (M-7786, Sigma) for 1 h, washed 3× in PBST, followed by a 1-h incubation in a 1:400 dilution of anti-mouse IgG-Cys3 (C2181, Sigma). Following a final washing (3× PBST), a drop of mounting medium (Aqua-Mount, Lerner Laboratories, Pittsburgh, PA) and a cover slip were placed on the section, and the edges sealed. Images viewed under a Nikon (Melville, NY) Eclipse E800 fluorescence microscope were captured using a RT Slider SPOT camera, described earlier.

Statistical analysis. All data are expressed as means ± SD. Paired t-tests were used to compare differences between proximal and distal vaginal measurements except for $V_{\text{max}}$ and amplitude of force which were analyzed using a mixed effects model to consider repeated measures within each rat (SAS, version 9.1). Differences were considered significant at $P < 0.05$.

RESULTS

Masson’s trichrome staining and α-actin immunostaining demonstrated that the vaginal muscularis was not uniformly distributed and did not form a continuous layer in the vaginal wall (Fig. 2). Where two layers of muscularis were present, it appeared that the inner layer was oriented in a longitudinal arrangement, and the outer layer of the muscularis was arranged circularly (Fig. 2, B and C). In the distal vagina, the thin muscularis layer projected into the mucosal folds (Fig. 2D). Despite these irregularities, there were differences observed between the proximal and distal vagina, most notably that the muscularis was more abundant in the proximal vagina compared with the distal vagina. Morphometric analysis of cross sections indicated that the muscularis comprised 8.80 ± 2.71% of the proximal vaginal wall compared with 3.13 ± 0.21% of the distal vagina.

Similarly, morphometric analysis of tissue strips used for physiological studies indicated a significantly greater percentage ($P < 0.001$) of smooth muscle in the proximal vagina wall compared with the distal vagina (Table 1). The proximal vagina’s phasic-like response to KCl stimulation compared with a toniclike response of the distal vagina was strikingly consistent (Fig. 3). During the period of peak force following KCl stimulation, force/cross-sectional area of tissue strip was almost three times greater ($P < 0.001$) in strips from the proximal vagina compared with the distal vagina. However, normalization of force to cross-sectional area of the smooth muscle in the strip indicated no regional difference in contractile strength at peak force generation. Force generation remained virtually unchanged (~107 mN/mm² muscle) for distal strips from peak force to steady state. On the other hand, force generation of proximal strips decreased from 95.6 ± 6.6 mN/mm² muscle at peak force to 51.9 ± 17.9 mN/mm² muscle during steady-state conditions. Accordingly, although force was not different between regions at peak force, the distal vagina demonstrated greater force/mm² muscle ($P < 0.05$) at steady state compared with the proximal vagina (Fig. 4).

$V_{\text{max}}$ was almost threefold higher in the proximal vagina (0.11 ± 0.04 muscle lengths/s) compared with the distal vagina (0.04 ± 0.01 muscle lengths/s) at peak force ($P < 0.01$). The distal vagina $V_{\text{max}}$ decreased by ~10%, whereas the $V_{\text{max}}$ of the proximal vagina decreased 37% from peak force to steady-state conditions, and no significant regional difference in $V_{\text{max}}$ was detected during steady-state conditions (Fig. 5).

MHC protein detected by Western blotting was ~1.6× higher in the proximal vagina compared with the distal vagina ($P < 0.01$). The protein expression of the SM-B isoform of MHC was also shown to be regionally different in the vagina (Fig. 6). There was a threefold higher ($P < 0.001$) expression
RT-PCR analysis of mRNA transcripts for SM-A and SM-B isoforms of MHC indicated a predominance of the relative expression of SM-B isoform in the proximal vagina compared with an approximately equal expression level of SM-A and SM-B in the distal vagina (Fig. 7A). Semiquantitative analysis of the mRNA transcripts for MHC isoforms indicated that the expression of SM-B protein in the proximal vagina compared with the distal vagina.

Table 1. Isometric force of contraction of proximal and distal rat vaginal strips detected using a dual-mode servo-lever (model 300H, Cambridge Technology)

<table>
<thead>
<tr>
<th></th>
<th>Cross-Sectional Area of Tissue Strip, mm²</th>
<th>Peak Force/Total Cross-Sectional Area of Tissue Strip, mN/mm²</th>
<th>Cross-Sectional Area Muscle/Total Cross-Sectional Area of Strip, %</th>
<th>Peak Force/Cross-Sectional Area Muscle, mN/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal vagina</td>
<td>1.14±0.219</td>
<td>11.13±1.57</td>
<td>11.69±0.65</td>
<td>95.62±6.64</td>
</tr>
<tr>
<td>Distal vagina</td>
<td>1.00±0.17</td>
<td>4.47±0.92*</td>
<td>4.16±0.23*</td>
<td>107.32±19.67</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5. Strips were formalin fixed and paraffin embedded following physiological studies for subsequent morphometric analysis (Image ProPlus, version 5.1) of hematoxylin- and eosin-stained cross sections. *The cross-sectional area of muscle was greater (P < 0.001) in the proximal vagina as well as the force/cross-sectional area of strip (P < 0.001). However, normalization of force to cross-sectional area of muscle in the strip indicated no difference in force generation between the proximal and distal vagina. NS, not significant.
percent SM-B \([\text{SM-B}/(\text{SM-B} + \text{SM-A})]\) in the proximal vagina \((73.81 \pm 4.44)\) was significantly greater \((P < 0.001)\) than that of the distal vagina \((50.59 \pm 4.45)\) (Fig. 7B).

RT-PCR analysis of the mRNA transcripts for the SM-1 and SM-2 isoforms of MHC indicated a predominance of the SM-1 isoform in both the proximal \((69.41 \pm 1.57\%)\) and distal vagina \((71.82 \pm 1.24\%)\). The percent SM-1 \([\text{SM-1}/(\text{SM-1} + \text{SM-2})]\) in the proximal vagina was not statistically different than that in the distal vagina.

**DISCUSSION**

The results of this study are the first demonstration of a regional heterogeneity of \(V_{\text{max}}\) and MHC isoform expression of the vaginal wall smooth muscle. We report that the proximal vagina of the rat displays a significantly higher \(V_{\text{max}}\) and a greater abundance of the SM-B isoform of myosin, which has a higher ATPase activity and a 7-amino acid insert in the
NH₂-terminal. We have shown the higher content of the SM-B isoform in the proximal vagina compared with the distal vagina at both the message and protein levels.

Animal models of female sexual arousal have illustrated in vivo differences in the contractility of the proximal and distal vagina, demonstrating a functional significance of our findings (15, 31, 34, 38). Decreased intraluminal pressure of the distal vagina following pelvic nerve stimulation of rabbits was first reported by Park et al. (38). In addition to a decrease in distal luminal pressure, Min et al. (34) reported that pelvic nerve stimulation induced an increase in luminal pressure and repeated contractions of the proximal vagina. The data presented in our report indicate a myogenic contribution to the regional difference in the contractility of the vaginal wall smooth muscles. It is noteworthy that luminal pressure within the vagina may be influenced by changes in muscle contractility within the wall of the vagina and also by changes in activity of the surrounding striated muscle of the pelvic floor and abdominal and rectal pressure. However, it has been demonstrated that the measured increase in wall tension and luminal pressure of the proximal vagina of the rat in response to pelvic nerve stimulation was blocked by atropine, suggesting that striated muscles, at least, are not involved in the noted increase (15). Furthermore, in an earlier study of female sexual arousal, McKenna et al. (31) recorded bursts of electrical activity within the vaginal wall (location unspecified), corresponding to an increase in intravaginal pressure in response to urethral stimulation, providing more direct evidence of vaginal contraction.

Previous studies have demonstrated that phasic muscle such as bladder (48) and intestine (16, 24) contain predominantly the SM-B MHC isoform. In vitro motility assays (12, 24, 40) also have indicated that the presence or relative abundance of a 7-amino acid insert in the motor domain near the ATP binding site of the SM-B isoform (3, 16) is correlated with a high velocity of shortening of smooth muscle. Furthermore, regional differences in MHC isoform expression have also been detected in the vasculature, bladder, and stomach (8, 12, 18, 20, 24). Regional differences of the vaginal muscularis.
19, 45). Similarly, we have shown that the proximal vagina, with a greater maximal velocity of shortening, also displays a predominance of the SM-B isoform at the mRNA level, whereas the distal vagina was found to express approximately equal amounts of SM-B and the SM-A isoform (Fig. 7). Furthermore, semiquantitative analysis of densitometric readings of Western blots indicated significantly greater \((P < 0.001)\) amounts of SM-B protein in the proximal vagina compared with the distal vagina (Fig. 6). The greater expression of SM-B in the proximal vagina compared with the distal vagina provides a second line of evidence that the proximal vagina is a phasic tissue, distinct from the tonic qualities of the distal vagina.

SM-1/SM-2 expression in the proximal and distal vagina did not parallel changes in SM-A/SM-B expression. This is in agreement with other investigations, suggesting that the NH2-terminal- and COOH-terminal-based isoforms of myosin are independently regulated (8, 12, 32). We report that the vagina displays a predominance of the SM-1 isoform in both regions of the vagina. Additionally, the percent SM-1 was similar in the proximal \((69.41 \pm 1.57\%)\) and the distal \((71.82 \pm 1.24\%)\) vagina. This would suggest that SM-1/SM-2 expression does not contribute to differences in shortening velocity or contractile profile in the vagina. A similar lack of correlation between SM-1/SM-2 and contractile properties has been suggested in other smooth muscle tissues (23, 39, 45). In contrast, a positive relationship between percent SM-1 and \(V_{\text{max}}\) has been reported in the myometrium of estrogen-treated ovariecctomized rats (17). Morano et al. (35) demonstrated an increase in expression of myosin light chain 17\(_{\text{a}}\) and concomitant increase in \(V_{\text{max}}\) in the hypertrophied myometrium of pregnant women. Thus it is possible that the results of Hewett et al. (17) may reflect differences in myosin light chain expression rather than heavy chain differences. SM-1/SM-2 isoform expression is under ovarian hormone control in the myometrium (7, 17, 35, 42) and bladder (44). This information, coupled with the fact that mechanical properties of the vagina are altered by ovariecctomy (26), makes it interesting to speculate if SM-1/SM-2 expression is altered by ovarian hormomes.

Stimulation of the distal vagina produced a monotonic increase in force to a stable value \((107.32 \pm 19.66 \text{ mN/mm}^2\text{ muscle})\). Steady-state force of the distal vagina was significantly higher than that produced by the proximal vagina, which contracted with a high transient peak before falling to a lower stable level (Fig. 4). \(V_{\text{max}}\) of the proximal vagina also transiently increased, followed by a decline of 37\% to achieve steady-state levels that were similar to those estimated during contraction of the distal vagina (Fig. 5). Peak force levels were similar during membrane depolarization-induced contractions of the proximal and distal vaginal smooth muscle when normalized to cross-sectional area of muscle. Therefore, if total myosin content were similar, one would not expect that the levels of SM-B or SM-A expression would influence peak force. This is in agreement with studies on purified myosin (29) and Triton X-100 permeabilized strips of bladder smooth muscle from SM-B knockout mice (22), all of which produced similar levels of force, regardless of the myosin isoform. However, in other studies using the same transgenic animals, it has been reported that loss of SM-B reduced maximal force generation of bladder (5), while increasing force generation of mesenteric artery and aorta (6). Differences in tissue preparation and modes of activation may be responsible for these conflicting results. There are a variety of normalizing parameters used to express isometric force. We determined the percent muscle mass of the vaginal tissues and therefore used muscle stress \((\text{force/cross-sectional area muscle})\) rather than the more typical tissue stress \((\text{force/cross-sectional area of total tissue})\). Normalization of force to total cross-sectional area of tissue strip indicated a significantly greater peak force of the proximal vagina, a reflection of the greater abundance of smooth muscle in the proximal vagina (Table 1). Using the normalizing parameter of muscle stress, we found no difference in peak force generation between the proximal and distal vagina, indicating that isoform composition did not influence peak force, as mentioned earlier. This clearly supports the importance of determining percent muscle mass when comparing the contractile properties of two smooth muscles or two areas within a single smooth muscle.

Distinct functional and molecular characteristics of the proximal and distal vaginal wall smooth muscle lend support to the theory of a dual origin of the vagina. It is widely accepted that the upper two-thirds of the vagina arises from the Mullerian ducts, whereas the distal vagina arises from the sinovaginal bulbs of the urogenital sinus (28). However, our findings of the predominance of the SM-B isoform within the proximal vagina compared with the distal does not correlate with the predominance of SM-A isoform expression of the uterus (30, 48), which also arises from the Mullerian ducts. Furthermore, our report of an approximately equal expression of SM-A/SM-B isoform expression in the distal vagina does not correlate with the predominant SM-B isoform expression of the urethra (19). Given that SM-A/SM-B isoform expression has been shown to be developmentally regulated in a tissue-specific manner (49), studies of myosin isoform expression of the vagina at different stages of development are required to more precisely relate vaginal SM-A/SM-B expression to its embryological source. Furthermore, recent research has suggested that the sinovaginal bulbs are actually the caudal tips of the Wolffian ducts and raises the possibility that the embryological pathway of vaginal development may need to be reconsidered (1, 11, 43).

In summary, our data provide a molecular explanation for some of the differences in contractile behavior of the proximal and distal vagina and strongly indicate that the proximal vagina is phasic compared with a tonic distal vagina. Furthermore, the results of this study highlight the inappropriateness of extrapolating data from one region of the vagina to the organ as a whole. Although many researchers emphasize the importance of vaginal relaxation during female sexual arousal, much of the evidence for relaxation stems from results of the distal vagina. The phasic nature of the proximal vagina raises the possibility that there may be a contractile component to the female sexual response, which warrants further investigation.

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