Differences in contractile protein content and isoforms in phasic and tonic smooth muscles

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[1]*Center for Swallowing and Motility Disorders, Harvard Medical School, West Roxbury Veterans Affairs Medical Center, West Roxbury, Massachusetts 02132; and* [2]*Department of Molecular Physiology and Biophysics, College of Medicine, University of Vermont, Burlington, Vermont 05405-0068*

Szymanski, Pawel T., Thomas K. Chacko, Arthur S. Rovner, and Raj K. Goyal. Differences in contractile protein content and isoforms in phasic and tonic smooth muscles. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C684–C692, 1998.—The basis of tonic vs. phasic contractile phenotypes of visceral smooth muscles is poorly understood. We used gel electrophoresis and quantitative scanning densitometry to measure the content and isoform composition of contractile proteins in opossum lower esophageal sphincter (LES), to represent tonic muscle, and circular muscle of the esophageal body (EB), to represent phasic smooth muscle. The amount of protein in these two types of muscles is similar: ~27 mg/g of frozen tissue. There is no difference in the relative proportion of myosin, actin, calponin, and tropomyosin in the two muscle types. However, the EB contains ~2.4-times more caldesmon than the LES. The relative ratios of α- to γ-contractile isoforms of actin are 0.9 in the LES and 0.3 in EB. The ratio between acidic (LC17a) and basic (LC17b) isoforms of the 17-kDa essential light chain of myosin is 0.7:1 in the LES, compared with 2.7:1 in the EB. There is no significant difference in the ratios of smooth muscle myosin SM1 and SM2 isoforms in the two muscle types. The level of the myosin heavy chain isoform, which contains the seven-amino acid insert in the myosin head, is about threefold higher in the EB compared with LES. In conclusion, the esophageal phasic muscle in contrast to the tonic LES contains proportionally more caldesmon, LC17a, and seven-amino acid-inserted myosin and proportionally less α-actin. These differences may provide a basis for functional differences between tonic and phasic smooth muscles.

lower esophageal sphincter; esophageal smooth muscle; visceral smooth muscle; actin; myosin; calponin; caldesmon; tropomyosin; opossum

THE LOWER ESOPHAGEAL sphincter (LES) smooth muscle is characterized by its ability to sustain contraction as opposed to the esophageal body (EB) circular muscle, which contracts only transiently when stimulated. The LES and EB represent tonic and phasic visceral smooth muscles, respectively (9, 12). Because they are directly adjacent, and yet have such unique physiological properties, these tissues offer a unique opportunity to examine the differences between tonic and phasic smooth muscles.

The force maintenance in sphincteric and other tonic muscles has been ascribed to several factors, including maintained stimulation by excitatory neurohormonal agents and myogenic factors involving the depolarized state of the smooth muscle membrane, enhanced basal Ca^{2+} entry and higher intracellular Ca^{2+} levels, and increased sensitivity of the contractile proteins to Ca^{2+} (12, 14, 19). Tonic muscles develop sustained tone when K^+ depolarized in vitro, whereas phasic muscles do not (37). Tonic muscles may have lower levels of myosin light chain kinase and myosin light chain phosphatase than phasic muscles (19). It has been also suggested that the content and isoform composition of the contractile proteins differ in tonic and phasic muscles (7, 9, 13, 14, 19).

The main contractile proteins are myosin and actin. The level of actin and the expression of its isoform have been reported to vary in different smooth muscle types (9, 14). Differences in isoform ratios of light and heavy chains of myosin as well as of actin have been found in muscles (1, 3, 14, 19). Moreover, tonic and phasic phenotypes have been attributed to proteins associated with thin filaments such as caldesmon (5, 26), calponin (10, 48), and tropomyosin (36).

A myosin molecule consists of two heavy chains, two 17-kDa essential light chains, and two 20-kDa regulatory light chains. The essential light chain exists in two isoforms, referred to as "acidic" (LC17a) and "basic" (LC17b) (3, 16). The LC17a-to-LC17b ratio is thought to influence shortening velocity (3, 15, 17, 25); however, heavy chain isoforms may be more important in determining actomyosin ATPase (41).

The gene for the single smooth muscle myosin heavy chain has two alternative splicing sites that give rise to different molecules. The first occurs at the extreme COOH terminus of the protein coding region and gives rise to isoforms referred to as SM1 and SM2, which differ slightly in molecular weight and thus can be separated on low-cross-linking polyacrylamide gels (8, 22, 27, 33). The alternative splicing causes SM1 to end in a unique, 43-amino acid nonhelical tailpiece, whereas the SM2 isoform contains 9 unique amino acids at its COOH terminus. It has been suggested that an increased proportion of SM1 over SM2 may correlate with phasic contractions (18, 38). More recently, a second site of alternative splicing has been described, which leads to the insertion or omission of seven amino acids in the NH_2-terminal region, near the ATP binding pocket (2, 23, 46). Presence of the seven-amino acid insert has been associated with the phasic phenotype of the smooth muscles (7) increased actomyosin ATPase and actin filament movement in an in vitro motility assay (23, 41).

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Smooth muscle actin also exists in several isoforms, namely, α, β, and γ (9, 14, 42). The β-isoform is a cytoskeletal actin, whereas α and γ colocalize within the contractile apparatus. Vascular smooth muscles predominantly contain the α-isoform, whereas visceral or phasic muscles contain a predominance of γ-actin (9, 14), suggesting that α- and γ-actins may also influence tonic vs. phasic phenotype of smooth muscles. It has been also shown that the actin-binding proteins such as tropomyosin (36), caldesmon (5, 26) and calponin (10, 47) play a role in the thin filament-based regulation of smooth muscle contractility.

Most studies have described one or two components of contractile proteins that may be related to tonic or phasic behavior of vascular and visceral smooth muscles in different animal species (7, 9, 13, 17, 26, 36). There are no systematic studies of contractile proteins in sphincteric and nonsphincteric regions of the gut. The purpose of the present study was to comprehensively compare the content and isoform-subunit composition of all five major contractile proteins in the opossum tonic LES and the adjacent, phasic, nonsphincteric EB circular muscle (9, 12, 44). Our studies show four major differences in the contractile proteins in these two smooth muscle phenotypes. The EB circular muscle has *1*) higher proportions of LC17a relative to LC17b, *2*) higher proportions of myosin heavy chain isoform with seven-amino acid insert relative to the noninserted form, *3*) higher proportions of γ-actin relative to α-actin, and *4*) higher caldesmon content compared with LES. These differences may provide a basis for the functional differences in sphincteric and nonsphincteric muscles in the gut and tonic and phasic contractility in general.

MATERIALS AND METHODS

Materials. Gel apparatus and materials for electrophoresis were from Hoefer (San Francisco, CA) and Bio-Rad (Richmond, CA), respectively. All commonly used reagents were from Sigma. All antibodies were from Sigma except the antibody that recognizes the seven-amino acid insert in smooth muscle heavy chain of myosin. The latter antibody was produced as follows. A 12-residue peptide was synthesized with the sequence Q-G-P-S-F-A-Y-G-E-L-E-C, with the former 7 residues representing the sequence of the insert in the 25/50-kDa junctional loop in rat smooth muscle heavy chain (46). The purified peptide was linked to keyhole limpet hemocyanin via the cysteine residue and used to immunize rabbits for antibody production (HTI Bioservices). The specificity of the resultant antiserum has been confirmed by Western blotting vs. baculovirus-expressed smooth muscle myosin molecules containing or lacking the insert. It has also been extensively characterized in immunocytochemical analysis of various smooth muscle tissues of the lung (R. B. Low, J. Mitchell, J. Woodcock-Mitchell, A. S. Rovner, and S. L. White, unpublished observations). In addition, Western blots of myosin isoforms either lacking or containing the seven-amino acid insert expressed in baculoviral system (32) have confirmed the high degree of specificity of this antibody preparation.

Standard proteins. Myosin (20), the 17-kDa essential light chain (43), and calponin (40), used as standards, were isolated from chicken gizzard according to established procedures. Chicken gizzard caldesmon was a generous gift from Dr. Samuel Chacko (Division of Urology, University of Pennsylvania). Chicken gizzard actin and tropomyosin were from Sigma.

Tissue harvesting preparation. Experiments were performed in opossums (*Didelphis virginiana*). The adult animals were anesthetized by intra-abdominal injection of pentobarbital sodium (40 mg/kg). The abdomen was opened by midline incision. The esophagus was removed, and the animal was euthanized with additional doses of pentobarbital sodium. The esophagus was placed in a modified Krebs solution (4°C) containing (in mM) 118 NaCl, 4.69 KCl, 25 $NaHCO_3$, 1.01 NaH_2PO_4, 2.52 $CaCl_2$, 0.58 $MgSO_4$, and 11.1 D-glucose and bubbled with 95% O_2-5% CO_2 (pH 7.35). The mucosa, muscularis mucosa, and connective tissues were dissected away from the underlying muscles. The LES was identified as a circumferential ridge at the gastroesophageal junction. The prominent part of the muscle ridge (~3 mm) was isolated and dissected away from the adjacent muscle layer using sharp scissors. We have previously shown that this muscle ridge develops spontaneous tone and relaxes in response to the intramuscular inhibitory nerve stimulation that constitutes typical in vitro behavior of LES (34, 35). The EB circular segments, ~5 mm × 10 mm at ~1 cm above the LES, were obtained after carefully separating them from the longitudinal smooth muscle layer.

Total cell protein extract. Pooled tissue samples (0.3–0.6 g) were ground in a liquid nitrogen-cooled mortar and pestle, weighed again, and homogenized with 1.5 ml of buffer/0.5 g frozen tissue in extraction buffer. The buffer consisted of 0.5 M NaCl, 40 mM Tris·HCl (pH 7.2), 2% SDS, 5 mM EDTA, 4 mM EGTA, and 5 mM NaN_3. Homogenization was performed by hand in a Pyrex 7727 glass homogenizer in a thorough but very gentle manner to minimize loss of protein(s) through foaming (6). To minimize proteolytic degradation, the homogenization buffer was enriched in a cocktail of protease inhibitors composed of (in mM) 0.5 benzamidine, 1 phenylmethylsulfonyl fluoride (PMSF), 1 *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 1 *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.3 leupeptin, and 0.3 chymostatin. After homogenization, samples were immediately heated at 100°C for 5 min and cooled to room temperature. To enhance the yield of extraction, samples were kept in extraction buffer for another 90 min (13) and then centrifuged at 10,000 *g* for 10 min. Supernatants were collected, and pellets were extracted twice as described above. Collected supernatants were pooled, frozen in liquid nitrogen, and stored at −80°C. To determine any residual contractile proteins in the pellets, the pellets were suspended in the Laemmli buffer (24) and subjected to gel electrophoresis and Western blot analysis. Contractile proteins were looked for with Coomassie blue staining of the polyacrylamide gels and by antibodies directed against all five contractile proteins together with visualization of substrate by enhanced chemiluminescence (ECL). No contractile proteins were looked for in the residual pellets, showing that the extraction protocol effectively extracted all of the five contractile proteins.

Preparation of myofibrils. The myofibrils from the LES and EB were prepared according to an established procedure (28). Specifically, freshly removed smooth muscles were thoroughly fragmented by mincing twice with a Brinkmann Polytron, model PT-MR 3000 (Kinematica, Litteu, Switzerland), at low speed (~3,500 rpm) in four volumes (vol/wt) of 10 mM imidazole-HCl (pH 7.0) buffer containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM NaN_3, 10 μg/ml of leupeptin, 1 mM trypsin inhibitor, and 1 mM PMSF. The muscle residues were collected by centrifuga-

tion at 15,000 *g* for 5 min. This washing step was repeated five times. Triton X-100 was added in the washing solution during the first three steps at decreasing concentrations of 1% to 0.5% (1, 1, 0.5%). After the last washing, pellets were solubilized in 0.5 M NaCl, 40 mM Tris·HCl, pH 7.2 buffer, 2% SDS, 5 mM EDTA, 4 mM EGTA, and 5 mM NaN_3 supplemented with protease inhibitors. Then, samples were immediately heated at 100°C for 5 min and clarified by centrifugation at 10,000 *g* for 10 min. Supernatants were collected and stored at −80°C.

Isolation of myosin. To quantitate myosin isoforms, myosin was prepared according to a detailed procedure (16). Minced LES and EB tissues were homogenized in a solution containing 20 mM $MgCl_2$, 25 mM HEPES (pH 7.0), 2 mM EGTA, 0.1 mM PMSF, and 0.1 mM DTT and were washed with three volumes of the same solution. The washed muscles were suspended in three volumes of an extraction solution containing (in mM) 40 NaCl, 25 HEPES (pH 7.0), 10 EDTA, 2 EGTA, and 5 ATP. After the solution was stirred for 1 h, the supernatant was collected by centrifugation at 10,000 *g* for 20 min. $CaCl_2$ was added to the supernatant to reach a final concentration of 30 mM, while pH was adjusted to 7.2. The actomyosin precipitate was collected and dissolved in 0.2 M $MgCl_2$, 20 mM sodium phosphate buffer (pH 7.0), and 1 mM EGTA. After removal of actin by addition of ATP to 5 mM and ultracentrifugation at 40,000 rpm for 1 h, the supernatant was diluted with nine volumes of 1 mM $NaHCO_3$. The myosin precipitate was collected and dissolved in 0.5 M KCl. The solutions were again diluted with four volumes of 1 mM $NaHCO_3$, and the remaining actomyosin preparations were precipitated by centrifugation at 12,000 *g* for 20 min. The supernatants were diluted fourfold with 1 mM $NaHCO_3$, and $MgCl_2$ was added to 10 mM. After the solution was allowed to stand for 2 h, the myosin precipitate was collected again and dissolved in 0.5 M NaCl and stored at −80°C after mixing with an equal volume of glycerol.

Electrophoresis. SDS-PAGE was carried out under varying conditions to allow the best separation and quantification of specific proteins. For most proteins 10%, 12%, or a continuous gradient of 6–20% polyacrylamide was used. In all cases, the procedure of Laemmli (24) was followed.

To monitor the myosin doublet heavy chains pattern (SM1/SM2), isolated myosin was electrophoresed on a gel of 5% polyacrylamide and 1% *N*,*N*′-methylene-bis-acrylamide gels.

PAGE in the presence of 8 M urea (urea-PAGE) was carried out on isolated myosin to separate the isoforms of the 17-kDa essential light chains according to an established procedure (31).

Two-dimensional gel electrophoresis was carried out to separate isoforms of actin and calponin. This was performed as follows: tissue samples in homogenization buffer were diluted (1:3, vol/vol) in a solution containing 8 M urea, 2% Nonidet P-40, 20 mM DTT, 2% ampholytes of various pH ranges: pH 4–6 supplemented with 20% of the pH 3–10 for actin and pH 9–11 plus 40% of pH 3–10 for calponin. Aliquots of 70–140 μg of protein extracts were isoelectrically focused on 5% polyacrylamide in cylindrical gels (2 × 100 mm) in the first dimension (29) and then electrophoresed on 10% polyacrylamide SDS-PAGE in the second dimension (24). Proteins were visualized on gels using the Coomassie blue staining protocol or rapid silver staining kit according to the protocol recommended by the manufacturer (ICN Biomedicals, Cleveland, OH).

Western blotting and autoradiography. The tissue samples separated by SDS-PAGE were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA). Proteins were identified as follows. Briefly, nonspecific binding sites were blocked by incubating membranes in Tris-buffered saline plus 0.5% Tween 20 containing 2% BSA. The mouse primary antibodies directed against a particular protein or its isoforms were used in the following dilutions: 1:10,000 for actin; 1:5,000 for calponin, 1:10,000 for caldesmon, 1:400 for tropomyosin, 1:1,000 for 20-kDa myosin light chain, and 1:1,000 for the myosin heavy chain isoform that contains the seven-amino acid insert. The secondary antibody, a peroxidase-conjugated goat affinity pure anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA), was applied using a dilution of 1:75,000. Substrates were visualized by using ECL according to the manufacturer's instructions (Amersham, Arlington Heights, IL) and by exposing the membranes to autoradiographic film (Kodak X-OMAT, XAR-5).

Identification of protein(s) on gel electrophoresis. Actin, tropomyosin, myosin heavy chain, the 17-kDa essential light chain, caldesmon, and calponin on polyacrylamide gels were identified in two ways. First, in pilot experiments, tissue extracts in different concentrations and with or without added standard proteins were separated electrophoretically using different experimental conditions. Simultaneously, the standard proteins were separated under identical conditions in adjacent wells. This approach allowed localization of proteins and their isoforms in tissue samples and also provided information on optimal experimental conditions for making determinations. Second, tissue samples run in the presence and absence of standard proteins on gel electrophoresis were electrophoretically transferred into Immobilon-P membranes, and the location of the proteins was made using antibodies and ECL.

Calculation of protein content. The amount of proteins in samples was quantified by scanning densitometry of the Coomassie blue- or silver-stained gels and autoradiographic films of Western blots (Kodak X-OMAT, XAR-5) using a Sony XC-77 charge-coupled device video camera interfaced with a Macintosh computer (39). Gel images were analyzed with the program Image from the National Institutes of Health, Research Series Branch. Standard curves for all proteins analyzed were constructed to establish the linear concentration ranges. For myosin, actin, tropomyosin, caldesmon, and calponin, the following amounts of proteins were found to be within the linear range when loaded on polyacrylamide gels (μg/lane): 0–7.5, 0–6.25, 0–4, 0–2.5, and 0–0.75, respectively. The heavy chain of myosin was used for the calculation of total myosin concentration.

One of the potential problems in accurately estimating the contents of the contractile proteins is the presence of other protein(s) in the tissues with similar mobility on the polyacrylamide gel. We used several approaches to minimize such errors. We enhanced the probability of separation of proteins that migrate similarly by performing long runs using separation gels of 11 cm in length. Moreover, we determined the content of only one protein at a time. This allowed us to optimize gel loading and thereby sharpen protein bands of interest. We also modified conditions for separation, using gel densities varying from 10% to 12% in addition to the 6–20% continuous polyacrylamide gradient as well as running gels in the presence and absence of 2 mM $CaCl_2$ (21). Proteins such as calponin and tropomyosin, when used in small amounts, were fully transferred onto membranes during Western blotting because of their small masses. The contents of these proteins were determined in membranes using Western blotting analysis combined with densitometry of autoradiographic films. These quantitative data compared well with that obtained from densitometry of Coomassie blue-stained gels.

Protein determination. Protein contents in tissue samples were determined colorimetrically by bicinchoninic acid protein assay according to the protocol of the manufacturer

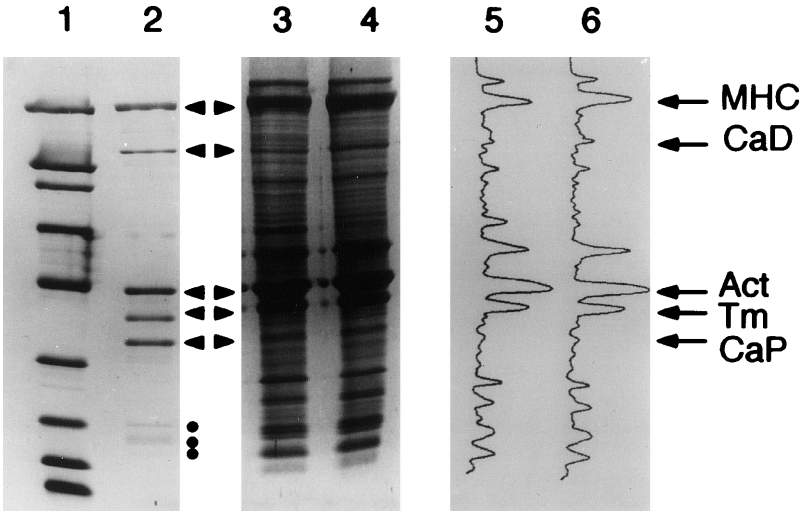

Fig. 1. Coomassie blue-stained 6–20% polyacrylamide SDS-PAGE patterns of total protein extracts (*lanes 3* and *4*) derived from the lower esophageal sphincter (LES) and esophageal body (EB) of opossum, respectively. *Lane 1*, molecular mass standards, from *top* to *bottom*, of 200, 116, 97, 66, 45, 31, 21, 14, and 6 kDa, corresponding to myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin, respectively. *Lane 2* (from *top* to *bottom*), positions of standards of myosin heavy chain (MHC), caldesmon (CaD), actin (Act), tropomyosin (Tm), and calponin (CaP). *Lanes 5* and *6*, tracings of densitometer scans of 6–20% polyacrylamide SDS-PAGE from LES and EB, respectively. ● (from *top* to *bottom*), 20-kDa regulatory light chain of myosin, calmodulin, and 17-kDa essential light chain of myosin, respectively. Note that the band labeled as tropomyosin in standards lane is the β-subunit; α-subunit comigrates with actin.

(Pierce, Rockford, IL) using BSA as a standard. Protein concentrations were also determined spectrophotometrically, using absorption coefficient (1%, 1 cm) values of 4.5 for myosin (30), 10.9 for actin (44), and 3.3 for caldesmon (4) at 280 nm and 2.4 for tropomyosin and 11.3 for calponin (47) at 277 nm.

Statistical analysis. All values are expressed as means ± SE. Student's *t*-test was used for statistical analysis, and a confidence level of $P < 0.05$ was chosen as indication of statistical significance.

RESULTS

Content of contractile proteins. Our initial efforts sought to determine whether differences in the tissue contents of the five major contractile proteins could be related to the functional differences between the LES and EB. To do this, we developed techniques to thoroughly extract and electrophoretically measure the relative amounts of these proteins. We extracted nearly the same amounts of protein from the LES and EB per frozen weight of tissue, and the respective values (in mg/g of frozen tissue) were 26.8 ± 0.8 ($n = 3$) and 27.4 ± 1.3 ($n = 3$) (n refers to no. of determinations). Figure 1 shows the positions of the five major contractile proteins, including actin, myosin, tropomyosin, caldesmon, and calponin on the 6–20% continuous gradient of polyacrylamide gel in the tissue extracts from the LES and EB (*lanes 3* and *4*, respectively), compared with individual standard proteins mixed together before separation on the gel (*lane 2*). As shown, three of five proteins, myosin heavy chains, caldesmon, and actin, migrate with identical mobility in extracts of the LES (*lane 3*) and EB (*lane 4*) compared with standard proteins (*lane 2*). These three proteins appeared on the gel as single, sharp bands that allowed their proper quantification.

In contrast, identification of the tropomyosin bands in tissue extracts was more difficult. Initially, when we coelectrophoresed tropomyosin standards on the same gel with actin standards, we found that the α-subunit of tropomyosin comigrated with actin, whereas β-tropomyosin was significantly lower on the gel. However, antibody staining of Western blots indicated that, in tissue extracts, both subunits of tropomyosin migrate significantly below the actin band. This discrepancy was resolved by rerunning the purified standards in the presence of 2 mM $CaCl_2$ on our 6–20% polyacrylamide gels. Under these conditions, both subunits of tropomyosin had greater mobility than actin, and a better separation of the α- and β-subunits was achieved (data not shown).

The purified calponin standard migrated slightly faster than calponin in whole tissue extracts on the 6–20% continuous polyacrylamide gradient. However, the mobility of the calponin standard became identical to that in the tissues when electrophoresis was performed in the presence of 2 mM $CaCl_2$ (21) (data not shown). These procedures reassured us that no other protein contributed to calponin quantification.

Using these different experimental protocols, we were able to identify and accurately quantify single protein peaks in tissue samples. Table 1 lists the contents of all contractile proteins examined. These data indicate that the amounts of actin, myosin, tropomyosin, and calponin are similar in these two types of muscles, whereas the amount of caldesmon is 2.4-fold

Table 1. *Content of contractile proteins in total tissue protein extract of the lower esophageal sphincter and the esophageal body of opposum*

	Lower Esophageal Sphincter	*n*	Esophageal Body Circular Muscle	*n*	Significance
Myosin	3.9 ± 0.1 (8.1 ± 0.1)	15	4.0 ± 0.1 (8.4 ± 0.2)	15	NS
Actin	10.1 ± 1.0 (241.2 ± 24.5)	8	10.9 ± 1.3 (260.5 ± 30.7)	8	NS
Tropomyosin	1.5 ± 0.2 (23.7 ± 3.7)	7	1.6 ± 0.3 (24.3 ± 3.8)	7	NS
Caldesmon	0.3 ± 0.0 (3.9 ± 9.3)	8	0.8 ± 0.1 (9.4 ± 0.7)	8	$P < 0.05$
Calponin	0.4 ± 0.1 (10.6 ± 1.8)	4	0.3 ± 0.1 (10.0 ± 1.8)	4	NS

Values are means ± SE in μg/mg of frozen tissue; n = no. of determinations. Values in parentheses show values in μM concentration. $P < 0.05$, significantly different. NS, not significantly different from values in lower esophageal sphincter.

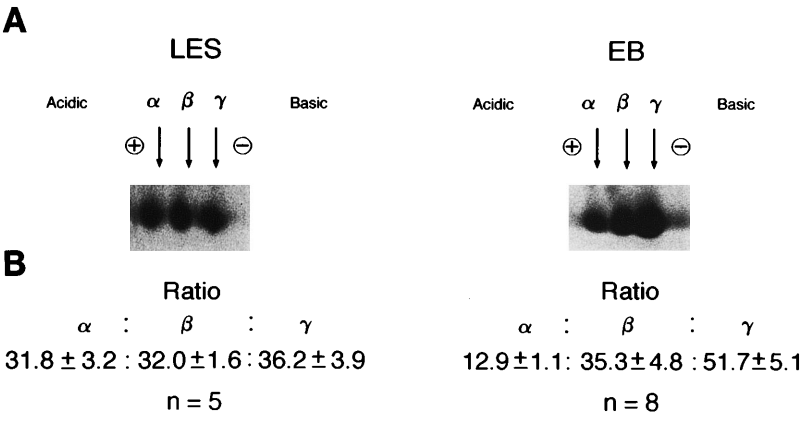

Fig. 2. *A*: analyses of the actin isoforms in the myofibril fractions (for details, see MATERIALS AND METHODS) from LES and EB, using Coomassie blue-stained 2-dimensional gels. *B*: summaries of relative ratios of α- to β- to γ-isoforms of actin as percentage of the total, obtained by densitometry of the 10% polyacrylamide SDS-PAGE.

higher in the EB compared with LES. With the use of molecular masses of 42,300, 480,000, 65,000, 87,000, and 34,000 Da for actin, myosin, tropomyosin, caldesmon, and calponin, respectively, the molar concentrations of these proteins were calculated. The values for these two muscle types are shown in Table 1. The relative molar proportions of myosin, actin, tropomyosin, and calponin were similar in the two types of muscles, that is, 1:30:3:1. However, there was twice as much caldesmon in the EB compared with LES. Thus the ratio of caldesmon to calponin was one in the EB and only one-half in the LES.

Actin isoforms. The molecular mass of three isoforms of actin (α, β, and γ) is the same, but they can be differentiated based on their mobilities in an electric field (42). We applied two-dimensional gel electrophoresis to determine the relative ratios of these three isoforms of actin in the myofibril preparations from the LES vs. EB. Actin isoform composition was determined in the myofibrils instead of the total protein extracts because myofibril preparations did not have contaminants that spread across all of three actin isoforms in second dimension. These data are presented in Fig. 2 and are also summarized in Fig. 2*B*. Thus expression of β, the so-called cytoskeletal actin, is similar in both smooth muscle types. These two muscles differ, however, in the relative content of the "contractile" actin isoforms α and γ. The LES contains ~2.5-fold more α-actin compared with EB, and the EB contains 43% more γ-actin than the LES. The calculated ratios of α-actins to γ-actins indicate that in the LES this proportion falls into the range of 0.9, as opposed to EB, where it reaches a value of ~0.3.

Tropomyosin subunits. Using the 6–20% polyacrylamide gradient SDS-PAGE, we separated two subunits of tropomyosin in the LES and EB (Fig. 3). The subunit that migrates more slowly has been termed α-tropomyosin, and the one that migrates faster is called β-tropomyosin (36). Applying quantitative scanning densitometry of Coomassie blue-stained gels, we found that the relative ratios of α- to β-subunits of tropomyosin in both muscle types examined are not different, and the respective values for the LES and EB are summarized in Fig. 3*B*.

Calponin isoforms. Three major isoelectric variants of calponin (α, β, γ) with pI values ranging from 9.9 to 9.4 of a similar molecular mass around 34-kDa have been identified using two-dimensional gel electrophoresis (11). Using silver-stained two-dimensional gels, we determined the relative proportions of these three isoforms of calponin in myofibril preparations (Fig. 4). Myofibril preparations were used instead of total muscle protein extract to minimize contamination by other protein(s) that may overlap with calponin isoforms.

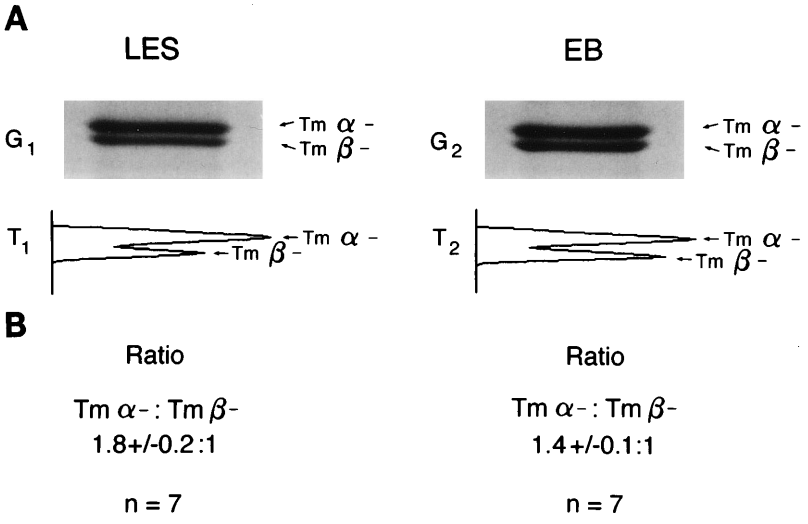

Fig. 3. *A*: Coomassie blue-stained 6–20% gradient polyacrylamide SDS-PAGE patterns of tropomyosin subunits in total cell protein extract (for details, see MATERIALS AND METHODS) from the LES (G_1) and EB (G_2). T_1 and T_2, original tracings of densitometer scans of respective gels. Tm α and Tm β, α- and β-subunits of tropomyosin, respectively. *B*: summaries of the relative ratios of α- to β-tropomyosin subunits.

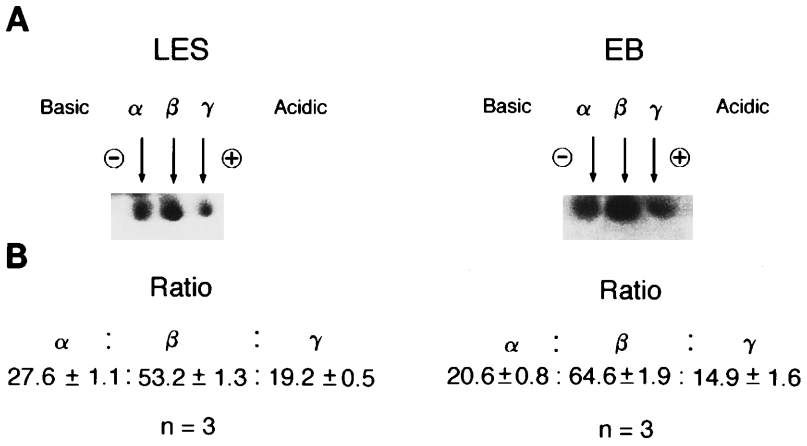

Fig. 4. *A*: analyses of calponin isoforms in the myofibril fractions (for details, see MATERIALS AND METHODS) from LES and EB, using silver-stained 2-dimensional gels. *B*: summaries of the relative ratios of α- to β- to γ-isoforms of calponin as a percentage of the total obtained by densitometry of 12% polyacrylamide SDS-PAGE.

These data show that LES and EB contain similar amounts of and similar isoform compositions of calponin, which are summarized in Fig. 4*B*.

Myosin heavy chain subunits. The relative content of the myosin heavy chain isoform that contains the seven-amino acid insert in the NH_2-terminal region was measured using an antibody raised against a peptide that mimics the sequence of the seven-amino acid insert (see MATERIALS AND METHODS). Because we found no significant difference in the amount of myosin in the LES and EB (Table 1), we determined whether these tissues express different amounts of the alternatively spliced myosin isoforms. First, we investigated the levels of expression of the isoform with the seven-amino acid insert in the head region of the heavy chain. We used the 6–20% polyacrylamide gradient SDS-PAGE to separate the myosin heavy chains as a single sharp band. Then, using the antibody that specifically recognizes the insert, we quantified the amount of the inserted isoform using quantitative ECL. Using these techniques, we found that the myosin heavy chain from the EB contains 2.8 ± 0.5 (n = 3) times more insert than that from LES (Fig. 5*A*, and Table 2).

To compare the amounts of the COOH-terminal SM1 and SM2 isoforms, we used the low-cross-linking 5% polyacrylamide SDS-PAGE system in an extra tall format with low loadings to achieve optimal separation of the two variants. Using scanning densitometry of the Coomassie blue-stained gels, we determined the relative amounts of these two isoforms in both smooth muscles. Our data show that, in myosin derived from LES and EB, the relative proportions of SM1 to SM2 are similar, and the respective values are 3.8 ± 0.6:1 (n = 4) and 3.7 ± 0.4:1 (n = 3) (Fig. 5*B*, and Table 2).

Finally, to examine the combined expression of the head and tail isoforms, we used the insert-specific antibody to quantitate immunoblotting on samples in which SM1 and SM2 had been separated on the 5% gels. The data presented in Fig. 5*B* and summarized in Table 2 show that SM1 of the EB contains 2.3 times more insert than the SM1 of LES and SM2 of EB contains ~1.5 times more insert than LES.

The 17-kDa myosin light chain isoforms. To determine the relative content of myosin LC17a and LC17b isoforms, we applied quantitative scanning densitometry of the Coomassie blue-stained urea-PAGE electrophoresis of purified myosins from these two types of muscles. This enabled us to distinguish the LC17a as a faster-migrating band from the basic, slower-migrating isoform (LC17b) (3). Scanning densitometry of our gels shows that the relative ratios between these two isoforms in the LES vs. EB were 0.7:1 and 2.7:1, respectively (Fig. 6). These data indicate that there is about three- to fourfold excess of the LC17a in the EB compared with the LES.

DISCUSSION

This study was guided by the hypothesis that differences between the mechanical behavior of tonic and

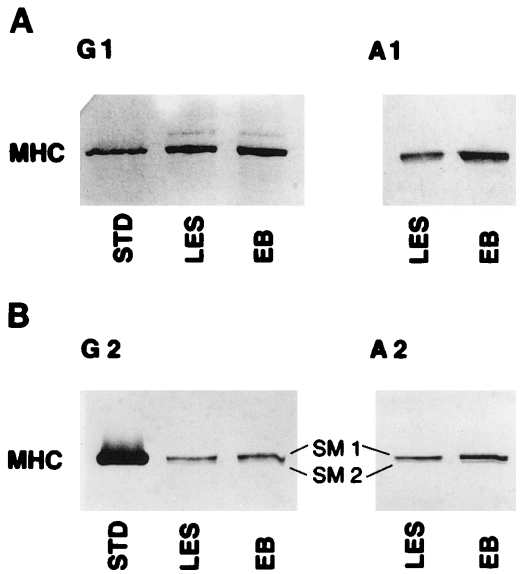

Fig. 5. *A*: G1, Coomassie blue-stained 6–20% polyacrylamide SDS-PAGE of the myosin heavy chain in isolated myosin preparations from the LES and EB (for details, see MATERIALS AND METHODS). A1, enhanced chemiluminescence (ECL) film image of immunoblots stained with antibody against 7-amino acid insert near the NH_2-terminal end of the heavy chain of myosin. *B*: G2, Coomassie blue-stained 5%, low-cross-linking polyacrylamide SDS-PAGE of myosin heavy chain isoforms, SM1 and SM2, of isolated myosin preparations from the LES and EB. A2, ECL film image of immunoblots of SM1 and SM2 isoforms stained with antibody against 7-amino acid insert of the NH_2-terminal end of myosin. STD and MHC, chicken gizzard myosin heavy chain run as a standard.

Table 2. *Relative content of myosin heavy chain isoforms in the lower esophageal sphincter and esophageal circular body*

	Lower Esophageal Sphincter	*n*	Esophageal Body Circular Muscle	*n*	Significance
7-AAI containing myosin, % of LES	100	3	284 ± 30	3	$P < 0.05$
SM1-to-SM2 ratio	3.8 ± 0.6:1	4	3.7 ± 0.4:1	3	NS
7-AAI, % of LES in					
SM1	100	3	231.7 ± 23.5	5	$P < 0.05$
SM2	100	3	144.8 ± 14.1	5	$P < 0.05$

Values are means ± SE; *n* = no. of determinations. 7-AAI, isoform containing the 7-amino acid insert in the NH_2-terminal end of heavy chain of myosin; LES, lower esophageal sphincter. $P < 0.05$, significantly different. NS, not significantly different from values in LES.

phasic visceral smooth muscles could be due to differences in the contractile proteins themselves. This investigation involved comprehensive studies of the content and isoform/subunit composition of five major contractile proteins, including myosin, actin, tropomyosin, caldesmon, and calponin, in smooth muscles of the two different contractile phenotypes from the opossum esophagus. Given that these tissues are from the same organ and animal species, this approach minimizes some of the variations that might occur when tissues from different organs and species are investigated (1, 3, 14, 19, 26, 36).

Our study shows that the contractile proteins of the LES differ from that of the EB in four major categories: *1*) isoform distribution of the 17-kDa essential light chain of myosin, *2*) relative amounts of the contractile actin isoforms, *3*) relative content of myosin isoform that contains the seven-amino acid insert in the NH_2-terminal region of heavy chain, and *4*) the content of caldesmon.

It has been reported that LC17a is the sole isoform in phasic muscles such as porcine gastric corpus and jejunum (17) and chicken gizzard (23) and the predominant isoform in guinea pig taenia coli (25). LC17b dominates in tonic smooth muscle of the aorta (15, 17, 25). DiSanto et al. (7) have shown that, whereas rabbit aorta contains equal quantities of the acidic and basic isoforms, small arteries contain a preponderance of the LC17a isoform, similar to visceral smooth muscles. The contribution of the 17-kDa light chain isoforms to the contractility of smooth muscle is not yet clear. Some studies, however, have documented an inverse correlation between the content of LC17b and the maximal shortening velocity (25). It has also been suggested that LC17b increases the affinity of the myosin head for actin while suppressing actomyosin ATPase (15, 17). Moreover, exchange of the LC17a into the aorta myosin is reported to be associated with an increased actomyosin ATPase activity (15). Our finding of a relative preponderance of LC17b in the LES is consistent with the role of LC17b in tonic smooth muscles. However, in vitro motility assay with myosin reconstituted to increase LC17a content does not show alterations in the velocity of actin filament movement over myosin heads (23, 41). Further studies are needed to establish the contribution of LC17a and LC17b isoforms to contractility in phasic and tonic muscle phenotypes.

It has also been suggested that differential expression of myosin heavy chain isoforms may influence smooth muscle contractility (18, 38). It has been found for example that, during muscle hypertrophy due to urinary bladder obstruction, a reversible downregulation of SM2 is associated with a decrease in force maintenance and force generation (7). In our study, we

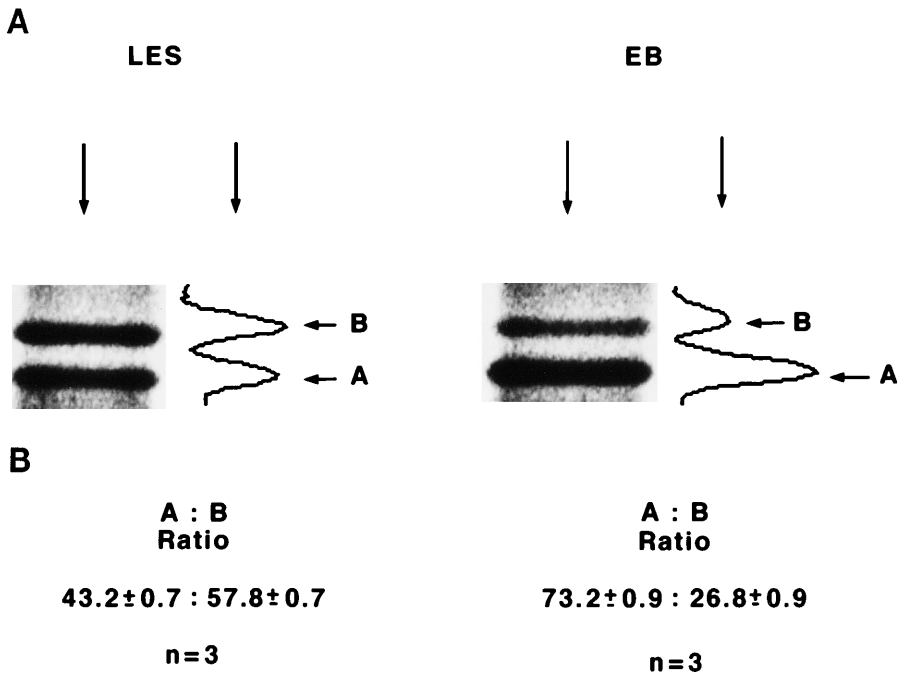

Fig. 6. *A*: urea-PAGE patterns of 17-kDa myosin light chain isoforms of isolated myosin preparations from the LES and EB (for details, see MATERIALS AND METHODS). Coomassie blue-stained urea-PAGE (*left*) and original tracings of densitometer scans (*right*) of respective gels are shown. A and B, acidic and basic isoforms of the myosin essential light chain isoforms, respectively. *B*: summaries of the relative ratios of acidic to basic isoforms of the 17-kDa myosin light chains as a percentage of the total.

found that both types of smooth muscle contained three- to fourfold more SM1 subunit than SM2.

The heavy chain isoforms based on the seven-amino acid insert in myosin subfragment-1 have been implicated to be important in determining mechanical properties of smooth muscles (23, 41). For example, Kelley et al. (23) who discovered that chicken gizzard, but not chicken aortic smooth muscle, contains myosin with the seven-amino acid insert showed that the presence of myosin with the inserted peptide correlated with a higher velocity of movement of actin filaments over myosin heads in the in vitro motility assay and higher actin-activated myosin ATPase. Our data showed that myosin isolated from the EB contains about three times more inserted form than that from LES. Recently, DiSanto et al. (7) investigated the levels of myosin heavy chain mRNA with a 21-nucleotide insert in the portion of the cDNA encoding the seven-amino acid insert in the myosin head. They found the inserted mRNA as a percentage of total myosin mRNA was 0% in rabbit aorta, >50% in femoral and iliac arteries, and >80% in more distal saphenous arteries (7). The comparison of various tissues indicates that the inserted myosin mRNA level increases in more distal muscular arteries that show phasic contractions compared with aorta with primarily tonic activity (7). This is consistent with the observations that the actomyosin ATPase and maximum shortening velocity are greater in the distal muscular arteries than aorta (7).

The total actin content in the LES vs. EB was similar. The relative content of β-actin, so-called "cytoskeletal" actin, is also similar in the two muscles examined. However, the expression level of individual contractile actin isoforms is different in these two muscle types. We found that there is about two to three times more α-actin in the LES than in EB, and the EB contains ~43% more γ-actin than LES. Our results agree with the early data of Fatigati and Murphy (9) showing a similar trend in expression of the α- and γ-actin isoforms in identical gastrointestinal muscles of opossum. The α-actin is predominantly found in the arterial smooth muscle, whereas γ-actin usually predominates in visceral smooth muscle (9). Further studies are needed to establish how contractile actin isoforms influence the mechanical behavior of smooth muscles.

There is now growing evidence that the thin filament-associated proteins such as calponin (10, 48), caldesmon (5, 26), and tropomyosin (36) play roles in smooth muscle contractility. The present study shows that, whereas the contents and patterns of calponin and tropomyosin subunits were not different in the EB and LES muscles, there is two- to threefold greater abundance of caldesmon in the EB compared with LES. Haeberle et al. (13) have shown that small muscles from guinea pig taenia coli, rat uterus, and rabbit ileum have more caldesmon than muscles isolated from bovine aorta or porcine carotid arteries. The caldesmon level in chicken gizzard smooth muscle is reported to be 23–33 μM, which is about twofold higher than in vascular smooth muscle with levels of 10–16 μM (13, 26). All these observations suggest that higher caldesmon covalent may play a role in phasic phenotypes of smooth muscles.

In summary, these studies show that phasic EB circular muscle differs from the tonic LES in its relative abundance of the *1*) ratio of acidic to basic isoform of the essential light chain, *2*) ratio of the seven-amino acid inserted to noninserted myosin isoform, *3*) γ- to α-actin ratio, and *4*) caldesmon-to-calponin content. However, these studies do not define how these differences in the contractile proteins result in the phasic vs. the tonic phenotype. Further studies are needed to define the roles of these proteins in determining the functional phenotype of smooth muscles and specifically to determine how these proteins function: whether they work independently or depend on each other to produce the tonic vs. phasic behavior.

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