

Differences in Cellular Contractile Protein Contents among Porcine Smooth Muscles

Evidence for Variation in the Contractile System

D. M. COHEN and R. A. MURPHY

From the Department of Physiology, University of Virginia School of Medicine, Charlottesville, Virginia 22901

ABSTRACT Cellular myosin, actin, and tropomyosin contents and ratios were determined for arterial (carotid, aorta, and coronary), intestinal (circular and longitudinal), esophageal, uterine, and tracheal smooth muscles in the pig. Tissue protein contents were estimated by densitometry of polyacrylamide gels after electrophoresis of sodium dodecyl sulfate-treated tissue homogenates. Cellular contractile protein contents were estimated by correction for extracellular spaces. Cellular myosin contents were similar in each tissue (average \pm 1 SEM = 19.6 ± 0.8 mg/g cell wet wt). However, the cellular contents of the thin filament proteins, actin and tropomyosin, were significantly higher in the arteries than in the nonarterial tissues. The calculated weight ratios of actin:myosin averaged 2.6 ± 0.2 in the three arterial tissues and 1.5 ± 0.1 in the nonarterial tissues, which may be compared with 0.36 in vertebrate striated muscles. The actin:tropomyosin weight ratios for all tissues were 3.7 ± 0.1 , a value comparable to the skeletal muscle ratio. The physiological implications of variations in the cellular thin filament protein contents are unknown, but these variations probably contribute to the observed differences in contractile function among various smooth muscles.

INTRODUCTION

Vertebrate smooth muscles exhibit a remarkable diversity in physiological function. Functional variation is especially prominent in the maximum force generating capacity (F_0) of different tissues with axial fibers at their optimum tissue length. After corrections for extracellular spaces, reported values of F_0 range from 0.3 to 4.0×10^5 N/m² (Murphy, 1976). This may be compared with more uniform values of 1.5 – 3.0×10^5 N/m² reported for mammalian skeletal muscles (Close, 1972).

Differences in tissue F_0 may reflect variations in tissue geometry, in metabolic capacities, in membrane processes regulating intracellular Ca^{++} concentration and excitation-contraction coupling mechanisms, or in the contractile apparatus, per se. Although contractile protein contents are quite constant in vertebrate striated muscle cells having the same general filament geometry, considerable differences in actin:myosin ratios or contents have been reported among different types of smooth muscles from different species (Tregear and Squire,

1973; Murphy et al., 1974; Small and Sobieszek, 1977). Such differences would be presumptive evidence that the contractile apparatus must be included among the factors contributing to physiological diversity among smooth muscles. Inasmuch as differences in methodology and species complicate comparisons, this study of the myosin, actin, and tropomyosin contents in a variety of porcine muscles was made. A brief preliminary report summarizing some of these data has been presented (Murphy et al., 1977).

METHODS

Smooth Muscle Preparations

All tissues were obtained from sexually mature pigs (80–90 kg) at slaughter and stored in a physiological salt solution (4°C) containing (in millimolars): NaCl, 117.8; KCl, 6.0; NaHCO₃, 24.3; NaH₂PO₄, 1.2; Na₂-ethylenediamine tetraacetic acid (EDTA), 0.027; CaCl₂, 1.6; MgSO₄, 1.2; and D-glucose, 5.6; and bubbled with 95% O₂-5% CO₂ (pH 7.4). Smooth muscle layers were isolated from several different tissues. After removing thin intimal layers, the media were peeled from the opened aorta, carotid, and left anterior descending coronary arteries. The trachealis muscle was isolated from the cervical end of the trachea using methods described by Stephens et al., 1969. A portion of the small intestine (jejunum) was slit longitudinally, the muscularis mucosa separated, and segments of the circular or longitudinal muscle layers were carefully dissected. Esophageal smooth muscle layers of the lower fourth of the muscularis externa were isolated from the outer layer of connective tissue. Myometrial tissues were collected from a uterine horn, 5–10 cm from the uterine body.

Tissue samples (0.2–0.3 gm) were homogenized by hand at 0°C in Ten Broeck tissue grinders with 1.5 ml/0.1 gm tissue wet wt of a solution containing 25 g/liter sodium dodecyl sulfate (SDS), 25 ml/liter β-mercaptoethanol, and 10 mM sodium phosphate buffer, pH 7.0. The samples were heated (100°C) for 30 min to denature the proteins into their constituent polypeptides. More rapid grinding with a motorized apparatus caused foaming and possible loss of protein. However, proteolysis is unlikely as we have quantitatively recovered pure skeletal muscle myosin and actin after adding these proteins to smooth muscle tissues, homogenizing, and incubation in SDS (Seidel and Murphy, 1978). In some cases the tissues were homogenized in 8.0 M urea and incubated for 2 hr at 20°C to obtain the polypeptides (see Results). The subsequent treatment of urea or SDS tissue homogenates was identical (below). Total protein concentrations in the SDS tissue homogenates were determined by micro-Kjeldahl methods, assuming a 16% protein nitrogen content.

Contractile Protein Preparations

Myosin was prepared from carotid arteries or intestinal segments, as described by Megerman and Murphy (1975). Arterial or intestinal tropomyosins were prepared by methods used for rabbit skeletal muscle by Hartshorne and Mueller (1969), as modified by Driska and Hartshorne (1975) for smooth muscle. Actin (modified from Bárány and Bárány, 1959) was obtained from arterial or intestinal tissues by mincing, homogenizing with 6 vol of acetone (–20°C) and centrifuging at 2,000 g for 5 min. The resulting residue was washed four times with acetone to obtain a dry powder. Actin was extracted at pH 7.0 with 0.3 M KI and 1 mM ATP (10 ml/g powder). The extract was diluted with water to give 0.1 M KI, filtered through paper pulp, and dialyzed overnight against 0.1 M KCl, 1 mM MgCl₂, and 10 mM morpholinopropane sulfonic acid (MOPS) buffer (pH = 7.0).

Solid KCl was then added to achieve a final concentration of 0.6 M, and the actin was sedimented by centrifugation for 1 h at 103,500 g. The isolated proteins showed < 10% contamination on SDS-polyacrylamide gels.

In some cases the isolated proteins were incubated in 8.0 M urea at 20°C for 2 h, before incubation in an SDS-buffer solution and electrophoresis, as described in the following section.

Disk Electrophoresis

The purity of the myosin, actin, and tropomyosin preparations and the contractile protein contents of the different tissues were determined by electrophoresis of SDS-treated proteins on SDS-polyacrylamide gels. The gels were prepared with 0.15% ammonium persulfate, 0.025% N,N,N',N'-tetramethylethylenediamine (TEMED), 5.6% acrylamide, and 0.2% N,N'-methylene-bis-acrylamide, 1% SDS, 40 mM tris (hydroxymethyl) aminomethane (Tris), 20 mM sodium acetate, and 2 mM EDTA. The degassed gel solution was cast in 5-mm × 10-cm tubes and overlaid with a degassed solution consisting of 1% SDS, 0.15% ammonium persulfate, and 0.05% TEMED. Aliquots of the SDS-treated tissue samples or purified proteins were prepared for electrophoresis by adjusting the total protein concentration to 1 mg/ml in a solution containing 1 g/100 ml SDS, 5 g/100 ml D-glucose, 10 mM Tris, 1 mM EDTA, 40 mM dithiothreitol (DTT), and 0.001% pyronin Y tracking dye. The samples were then incubated at 45°C for 15 min. From 2 to 20 μg of the SDS-treated pure proteins and 20–30 μg of tissue homogenates were applied to the gels and subjected to an electrophoresis current of 5 mA/tube. Gels were stained overnight in 0.04% (vol/vol) Coomassie Blue, 25% isopropyl alcohol, and 10% acetic acid. Destaining by diffusion occurred in two steps: (a) 6 h in 0.002% Coomassie Blue, 10% isopropyl alcohol, and 10% acetic acid, and (b) overnight, or until background staining was minimal, in 10% acetic acid (Fairbanks et al., 1971). Densitometry of the gels was performed on an ISCO (Instrumentation Specialities Co., Lincoln, Nebr.) gel scanner (λ = 580), and peak areas were determined with a planimeter.

Anatomical Measurements

Extracellular spaces were estimated by weighing cutout cell profiles from × 1,800 electron micrographs of 10–20 random fields in each of the tissues. The tissues were fixed in 2% glutaraldehyde (made isosmotic with CaCl₂ and cacodylate buffer), washed in cacodylate buffer, postfixed with osmium tetroxide, and imbedded in Epon (Epoxy Resins, Shell Chemical Co., Houston, Tex.). Sections were stained with uranyl acetate and lead citrate. Values for extracellular spaces obtained by this method tend to be smaller than those obtained with marker solutes (Paton, 1975), probably because micrographs used for study are often selected to include muscle bundles (Jones et al., 1973). With this in mind, we selected fields which were representative of the tissue as a whole. Although technique artifacts such as tissue shrinkage might alter absolute extracellular space values, identical handling of the different tissues should yield valid comparisons.

Statistical Analysis

The procedure for quantitative estimation of proteins on polyacrylamide gels is fairly sensitive to a number of parameters, including gel characteristics, staining, and destaining. The contribution of such errors to the results was minimized by collecting samples of each tissue type, simultaneous processing, and simultaneous electrophoresis in the same gel batch. In each experiment, duplicate gels were run to ensure reproducible results. The average protein content estimates from six experiments were tested for

significant differences, using the Student's *t* test. With this approach, differences between the means due to experimental error were minimized, although experimental variations were reflected in larger values for the SEM.

RESULTS

To determine if a direct proportionality exists between applied protein and peak areas on stained gels, arterial and intestinal actin, myosin, and tropomyosin were isolated, and peak areas were determined after electrophoresis of known amounts of protein (Fig. 1). These tissues were chosen as typical of the two distinct types of smooth muscle seen in our results. Linear regression analysis showed that staining was directly proportional to the amount of the three applied proteins for both tissues. Furthermore, the slopes of least square lines

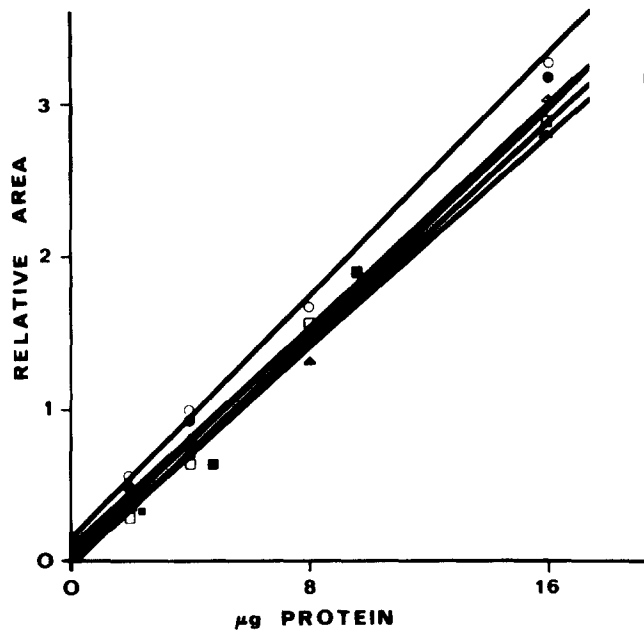


FIGURE 1. Relationship between areas under the peaks of densitometer tracings of gels as a function of amounts of contractile proteins applied to the gels. (●), Arterial myosin, y (area) = $0.189x$ (μg) + 0.121; (■), arterial actin, $y = 0.171x - 0.026$; (▲) arterial tropomyosin, $y = 0.178x - 0.018$; (○) intestinal myosin, $y = 0.194x + 0.167$; (□) intestinal actin, $y = 0.186x - 0.043$; (△) intestinal tropomyosin, $y = 0.185x + 0.059$. Each point represents the mean value for three gel scans on each of three preparations.

generated from the analysis were not significantly different ($P > 0.05$) for any of the contractile proteins, indicating that differential staining did not occur. This uniformity was a sensitive function of the procedures and was not obtained with modifications in staining and destaining.

Stained gels exhibited three major peaks occurring at apparent mol wt of 200,000 for myosin heavy chains, 45,000 for actin, and about 37,000 for tropomyosin after electrophoresis of each tissue SDS-homogenate (Fig. 2).

Positive identification of these peaks was obtained by the addition of purified proteins to the tissue homogenates (Murphy et al., 1974).

Incubation of tissue homogenates in 8 M urea followed by 1% SDS resulted in a translocation of a substantial fraction of the protein in the tropomyosin-containing peak to a region of the gel corresponding to an apparent mol wt of 55,000 (Fig. 2). This translocated protein is tropomyosin, because pure smooth muscle tropomyosin subunits, preincubated with 8 M urea, migrate with a

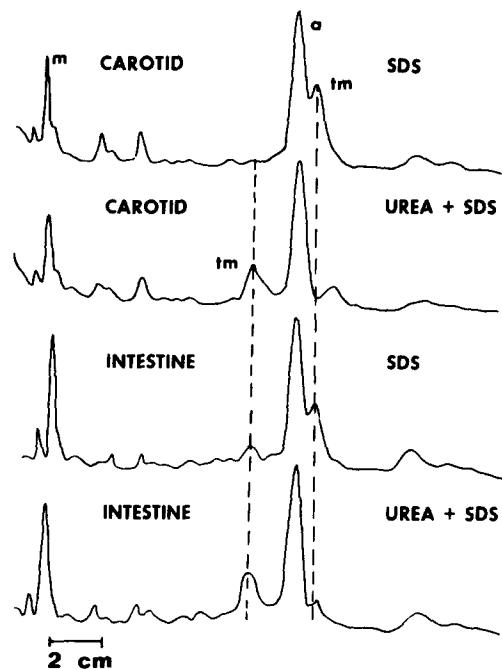


FIGURE 2. Tracings of densitometer scans of SDS-treated tissue homogenates from carotid media and intestinal tissue in the presence and absence of 8 M urea on 1% SDS gels. Origin of each gel is at the left. Vertical dashed lines correspond to positions of peptides nominally migrating with a mobility of 55,000 and 37,000 daltons.

mobility corresponding to a 55,000 dalton peptide in the SDS gel system (Fig. 3). The same phenomenon has been described by Sender (1971) for tropomyosin isolated from skeletal muscle. Thus, tropomyosin content estimates were based on the area of the material shifted to a region on the gels corresponding to an apparent mol wt of 55,000. It was critical to use both urea-treated and untreated samples to determine tropomyosin contents, as staining in the 37,000 dalton area included substantial amounts of an unknown protein (particularly in arteries), whereas staining in the 55,000 dalton region included the intermediate filament subunit (prominent in visceral tissues) (Cooke, 1976; Small and Sobieszek, 1977).

Maximal estimates of actin, myosin heavy chain, and tropomyosin contents of the various pig tissues are given in Table I. Tissue protein contents varied and

the only trend appeared to be somewhat lower myosin contents in the arterial tissues. These values, however, do not take into account substantial differences in extracellular spaces among the tissues. The fractions of the tissue cross sections composed of smooth muscle cells are given in Table II. Dividing the contractile protein tissue contents (Table I) by the fractional area of smooth muscle cells of the tissues (Table II) yields estimates of cellular protein contents (Table III). The cellular myosin contents were fairly uniform in all of the smooth muscles. It was difficult to accurately quantify light chains using our procedures. Thus, the values for myosin content were multiplied by 1.19 to correct for light chain weights in Table III. Arterial actin and tropomyosin contents were significantly higher ($P < 0.05$) than their nonarterial counterparts. This difference was significant when any arterial tissue was compared

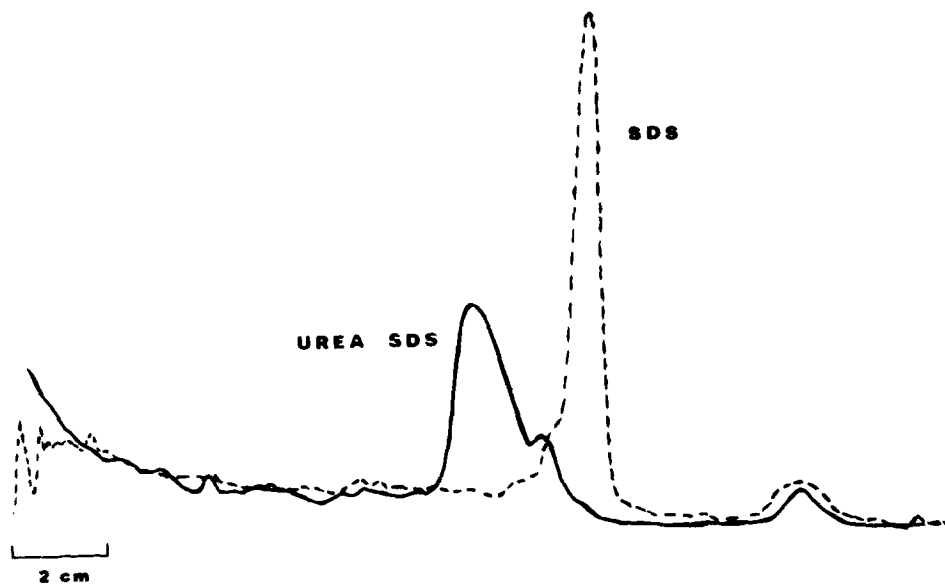


FIGURE 3. Superimposed densitometry tracings obtained using an intestinal tropomyosin preparation after electrophoresis of samples that had been preincubated with (solid tracing) and without (broken tracing) 8 M urea (see Methods). Urea-treated tropomyosin polypeptides exhibit a lowered mobility. Two minor peaks representing contaminant peptides were relatively unaffected. The areas of the tropomyosin peaks with and without urea pretreatment were 83 and 87% of the total area under the curve, respectively.

with any of the remaining tissues. Each smooth muscle had a very high actin:myosin ratio compared with skeletal muscle (0.36; Murphy et al., 1974). Furthermore, each arterial actin:myosin ratio was significantly greater ($P < 0.05$) than each of the nonarterial ratios. Actin:tropomyosin weight ratios, on the other hand, were not significantly different among any of the muscle types (Table III) and were similar to striated muscle (Murphy et al., 1974).

DISCUSSION

Validity of the Methods

Quantitative densitometry of stained polyacrylamide gels is a widely used technique for estimating subunit composition of pure proteins. In special cases, this technique can also be used to estimate the contents of individual proteins in complex mixtures such as tissue homogenates. In practice, this requires that the proteins of interest constitute a high fraction of the total protein in the homogenate and have mobilities differing from other significant constituents. We have shown that the smooth muscle of the pig carotid artery is suitable for this procedure (Murphy et al., 1974). The structural proteins, myosin, actin, tropomyosin, elastin, and collagen, constitute the great majority of the total protein in the tissue homogenate, and elastin and collagen do not enter the gels.

The practical homogeneity of the peak containing myosin was demonstrated by two independent estimates of myosin content in the carotid artery (Murphy et al., 1974). However, the peak containing tropomyosin has an additional component(s). Tropomyosin was independently assessed using its altered mobil-

TABLE I
ESTIMATES OF PROTEIN CONTENT OF PIG TISSUE (mg/g TISSUE WET WEIGHT) BY DENSITOMETRY OF SDS-POLYACRYLAMIDE GELS*

	Myosin (heavy chains)	Actin	Tropomyosin
Arterial			
Carotid	9.3±1.5	26.7±4.4	7.4±1.0
Aorta	6.8±0.4	18.0±1.6	5.5±0.8
Coronary	7.8±1.3	27.4±4.6	7.2±1.1
Nonarterial smooth muscle			
Esophagus	16.2±3.6	27.9±5.4	6.7±1.2
Uterus	11.3±1.4	14.4±1.7	4.9±1.2
Trachea	10.0±1.5	17.8±3.1	5.1±0.8
Circular intestine	13.7±1.0	25.3±3.8	6.8±1.0
Longitudinal intestine	12.2±1.6	24.4±2.4	6.4±0.8

* Values = mean ± SEM for six preparations.

TABLE II
SMOOTH MUSCLE FRACTION OF TISSUE CROSSSECTIONS DETERMINED FROM LOW POWER ELECTRON MICROGRAPHS*

Tissue	Cellular fraction
Carotid artery	0.56±0.02
Aorta	0.44±0.03
Coronary artery	0.45±0.01
Esophagus	0.87±0.02
Uterus	0.60±0.02
Trachea	0.71±0.04
Circular intestine	0.84±0.02
Longitudinal intestine	0.87±0.02

* Values = mean ± SEM from 10-20 fields for each tissue.

ity on SDS gels after urea pretreatment (Fig. 3). An additional control experiment suggested that the actin estimates did not contain a major error. Arterial and intestinal tissue homogenates were subjected to isoelectric focusing (IEF) in the presence of urea, followed by electrophoresis in the second dimension in an SDS-acrylamide slab gel (O'Farrell, 1975). Actin was identified by its position in the slab, which is a function of both its isoelectric points and polypeptide size (Izant and Lazarides, 1977; Rubenstein and Spudich, 1977). In both tissues, actin constituted about 90% of the total protein migrating with a molecular weight corresponding to 45,000 daltons. The remaining protein of this size, but differing in its apparent isoelectric point, might be "trapped" actin (Wilson et

TABLE III
CELLULAR MYOSIN CONTENT, ACTIN/MYOSIN WEIGHT RATIOS, AND
ACTIN/TROPOMYOSIN WEIGHT RATIOS OF VARIOUS PORCINE
SMOOTH MUSCLES

Tissue	Cellular protein contents			Weight ratios	
	Myosin*	Actin	Tropomyosin	Actin/myosin	Actin/tropomyosin
		<i>mg/g cell</i> <i>wet wt</i>			
Arterial					
Carotid	19.8±3.3	48.5±6.1	13.5±1.8	2.6±0.3	3.5±0.2
Aorta (thoracic)	18.3±1.2	40.9±3.6	12.5±1.9	2.3±0.3	3.7±0.6
Coronary (left anterior descending)	20.6±3.4	60.6±11.9	16.0±2.6	2.9±0.1	3.4±0.3
Nonarterial					
Esophagus	22.1±4.9	30.4±5.4	7.7±1.4	1.5±0.1	4.2±0.3
Trachea	16.7±2.5	25.1±4.4	7.2±1.2	1.5±0.1	3.5±0.2
Intestine					
Circular	19.4±1.5	29.0±4.4	7.8±1.2	1.6±0.2	3.8±0.3
Longitudinal	16.8±2.2	29.2±2.8	7.4±0.9	1.7±0.1	4.0±0.2
Uterus	22.5±2.7	23.9±2.9	8.1±2.0	1.1±0.1	3.5±0.5
Means					
Arterial	19.6±0.7	50.0±4.7	14.0±1.2	2.6±0.2	3.5±0.1
Nonarterial	19.5±1.3	27.5±1.8‡	7.7±0.6‡	1.5±0.1‡	3.8±0.1

* Calculated by multiplying the myosin heavy chain estimates by 1.19 to correct for light chain weights.

‡ Significantly different ($P < 0.05$) from the mean of arterial tissues.

al., 1977) rather than an unknown contaminant (as it was found at the top of the IEF gel after focusing). These results indicate a maximum overestimate of actin contents of about 10%. They also suggest that differences in actin:myosin ratios between carotid and intestinal tissues were not compromised by unknown proteins in the actin bands. The actin:tropomyosin weight ratios did not differ significantly among the tissues. Because the calculated molar ratios of about six actin monomers for each tropomyosin molecule were similar to that in skeletal muscle (Lehman et al., 1972), the actin values are reasonable, assuming that all vertebrate muscles have similar thin filament structures. This assumption is supported by X-ray diffraction evidence (Vibert et al., 1972).

Another potential source of error is the contractile protein contribution of nonmuscle cells present in the tissue samples. Although it appears that the only cell type present in the media of large mammalian arteries is smooth muscle (Pease and Paule, 1960; Wissler, 1968), it is possible that nonmuscle cells, e.g., fibroblasts, constitute a small portion of some other tissues. Such nonmuscle cells have very high actin:myosin ratios (Pollard and Wehling, 1974). The error introduced by such contamination is unknown. However, the effect of this type of error would be to increase the actin:myosin ratio in the nonarterial tissues. The difference observed between arterial actin:myosin ratios and those of the other tissues would only be magnified if this potential artifact were eliminated.

Actin:Myosin Weight Ratio Differences

The results of this study show that differences sufficiently large to be demonstrated by electrophoresis of tissue homogenates exist at the contractile system level for different vertebrate smooth muscles. Two distinct actin:myosin distribution patterns are suggested by the data: arterial smooth muscles having actin:myosin weight ratios of 2.6, and nonarterial smooth muscles having ratios of 1.5. The lower ratio in uterine smooth muscle may reflect a combined error of slightly higher myosin and slightly lower actin estimates. Although the tissues surveyed suggest that there may be two distinct groupings of smooth muscle contractile systems, the number of tissues sampled is too few to exclude other ratios or small differences within these groupings.

Our actin:myosin ratios are higher and follow a more consistent pattern than those reported by Small and Sobieszek (1977). Inasmuch as no tissue or cellular content estimates were reported by those authors, it is difficult to determine where the discrepancy occurs. The pig carotid artery was included in both studies; thus, the discrepancy is not simply due to the different types of tissues sampled. The ratio differences may reflect the inclusion of a peak in Small and Sobieszek's (1977) myosin estimates, with a mobility somewhat less than that which we attributed to myosin heavy chains on the basis of our coelectrophoresis studies of pure myosin with tissue homogenates. However, in spite of quantitative differences, Small and Sobieszek (1977) do show a higher actin:myosin ratio in the pig carotid than in the four visceral tissues examined from guinea pig, pig, and chicken.

It is possible to estimate the expected ratio of thin:thick filaments from our contractile protein content data (Murphy et al., 1974). The resulting calculation of from 15–17 thin:thick filaments for the vascular tissue compares quite favorably with electron micrograph observations by Ashton et al. (1975). With the exception of the uterus, nonarterial tissue values ranged from 10–12, which also correspond well with filament counts made by Bois (1973) and Heumann (1969) from intestinal smooth muscle. Although these calculations assume comparable filament structures and the same relative lengths for thin:thick filaments, the close agreement between filament ratios calculated from our protein data and those of direct observation lends credibility to the results. An alternative explanation for the higher arterial actin:myosin ratios is that the thin filaments in these tissues are relatively long compared to the thick filaments (Mrwa et al., 1976).

Significance of the Actin:Myosin Ratio Differences

Our results show that various smooth muscles within one mammalian species have similar myosin contents, but exhibit substantial differences in the ratios of thin:thick filament proteins. All types of smooth muscle have low myosin and high thin filament protein contents compared with vertebrate striated muscles. This raises questions about the physiological significance of high actin:myosin ratios in smooth muscle in general, and of variations in this ratio among different types of smooth muscle. It is interesting to note that large arteries exhibit high force-generating capacities (reviewed in Murphy, 1976), and consistently had the highest actin:myosin ratios of the tissues studied. Maximum force generating capacities of cat intestinal, dog tracheal, and rabbit uterine smooth muscles are lower than those of arterial smooth muscle (Murphy, 1976). Moreover, in the pig tissues studied, intestinal, tracheal, and uterine smooth muscles had lower actin:myosin ratios than did those of arterial smooth muscles. Although the correlation between high F_0 and high actin:myosin ratios is interesting, it must be tested by performing biochemical and mechanical studies on the same tissues.

Any physiological basis for a relationship between thin:thick filament protein ratios and force generation is unknown. However, two suggestions have been made: (a) As the ratio of the numbers of thin:thick filaments is increased from 2.0 in vertebrate skeletal muscles, the probability that any given cross-bridge is in a position to interact with an actin site would increase (Auber, 1967), which would favor higher forces. (b) Alternatively, the axial force that filaments can bear may limit the total tension that can be developed. Huxley (1972, p. 382) suggested that the strength of thin filaments or their attachments may be limiting, and high thin:thick filament ratios could reduce the axial force in any individual thin filament in contractile systems that generate high axial forces in the thick filaments.

Our data suggest that there are consistent differences in the thin filament protein contents and actin:myosin ratios among smooth muscles. It seems likely that the functional diversity of vertebrate smooth muscles may depend to some degree on such differences, which have no counterpart in vertebrate striated muscles.

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