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Differences in calmodulin and calmodulin-binding proteins in phasic and tonic smooth muscles

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Szymanski, Pawel T., Grazyna Szymanska, and Raj K. Goyal. Differences in calmodulin and calmodulin-binding proteins in phasic and tonic smooth muscles. *Am J Physiol Cell Physiol* 282: C94–C104, 2002. First published September 5, 2001; 10.1152/ajpcell.00257.2001.—To determine whether densities of calmodulin (CaM) and CaM-binding proteins are related to phasic and tonic behavior of smooth muscles, we quantified these proteins in the opossum esophageal body (EB) and lower esophageal sphincter (LES), which represent phasic and tonic smooth muscles, respectively. Gel electrophoresis, immunoprecipitation, Western blot, and hemagglutinin epitope-tagged CaM (HA-CaM) overlay assay with quantitative scanning densitometry and phosphorylation measurements were used. Total protein content in the two smooth muscles was similar (~30 mg protein/g frozen tissue). Total tissue concentration of CaM was significantly (25%) higher in EB than in LES ($P < 0.05$). HA-CaM-binding proteins were qualitatively similar in LES and EB extracts. Myosin, myristoylated alanine-rich C kinase substrate protein, Ca^{2+}/CaM kinase II, and calponin contents were also similar in the two muscles. However, content and total activity of myosin light chain kinase (MLCK) and content of caldesmon (CaD) were three- to fourfold higher in EB than in LES. Increased CaM and MLCK content may allow for a wide range of contractile force varying from complete relaxation in the basal state to a large-amplitude, high-velocity contraction in EB phasic muscle. Increased content of CaD, which provides a braking mechanism on contraction, may further contribute to the phasic contractile behavior. In contrast, low CaM, MLCK, and CaD content may be responsible for a small range of contractile force seen in tonic muscle of LES.

caldesmon; calcium/calmodulin kinase II; hemagglutinin epitope-tagged calmodulin; lower esophageal sphincter; myristoylated alanine-rich C kinase substrate protein; myosin light chain kinase; visceral smooth muscles

ON THE BASIS of their functional behavior, visceral smooth muscles have been classified into sphincteric and nonsphincteric smooth muscles that represent tonic and phasic muscles (11, 19, 33). Phasic muscle shows a very wide range of contractile activity that varies from a fully relaxed basal state to a large-amplitude rapid contraction and rapid relaxation response. In contrast, tonic muscles remain contracted at rest and have small-amplitude slow contraction and slow relaxation responses (11). Tonic smooth muscles also differ from phasic muscles in maintaining tonic contraction that is thought to be due to the "latch" cross bridges that produce economical force at a low actomyosin ATPase activity (35, 46). The phasic and tonic behavior of smooth muscles may be related to differences in content and isoform composition of contractile proteins and intracellular signaling pathways including calcium, calmodulin (CaM), and CaM-binding proteins that regulate activities of contractile proteins (4, 6, 19, 31, 33, 38). Intracellular Ca^{2+} is a primary regulator of contraction and relaxation in smooth muscles (19), although Ca^{2+}-independent contractions have been described in certain smooth muscles including the esophageal smooth muscles (19, 32, 33, 44, 45). Phasic smooth muscles are electrically quiescent in the resting state and show electrical spikes, which cause enhanced Ca^{2+} influx and contraction, when stimulated. On the other hand, tonic smooth muscles have depolarized cells with or without low-level continuous spike activities that result in a small but continuous inflow of Ca^{2+} in the basal state (3, 11, 33). Ca^{2+} signals in smooth muscles are transduced into contractile activity by CaM. CaM, on binding to Ca^{2+}, modulates activity of many enzymatic and nonenzymatic proteins that regulate smooth muscle contractility (23, 33). Tansey and colleagues (40) suggested that, contrary to common belief, intracellular levels of free CaM may also be limiting factor in Ca^{2+}-mediated responses. Moreover, cytosolic CaM has been shown to be modulated by a CaM-binding protein, myristoylated alanine-rich C kinase substrate (MARCKS) protein (30). It is not known whether CaM and MARCKS protein are quantitatively different in phasic and tonic muscles.

CaM-binding proteins affect both thick and thin filament-based contractile mechanisms. The Ca^{2+}/CaM-binding enzyme myosin light chain kinase (MLCK) regulates phosphorylation level of the 20-kDa regulatory light chain (RLC) of myosin and hence the activity

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of actomyosin ATPase, myosin cross-bridge cycling, and force of muscle contraction. Although MLCK content and activity have not been compared in sphincteric and nonsphincteric visceral smooth muscles, studies have shown that MLCK activity in some phasic smooth muscles is significantly higher than in tonic muscles (10). It has been suggested that higher activity of MLCK may be responsible for accelerated phosphorylation of RLC, leading to a more rapid cross-bridge cycling rate and a faster contractile velocity in phasic muscles (10, 33). Ca^{2+}/CaM kinase II phosphorylates MLCK in the CaM binding region and reduces the affinity of MLCK for interaction with Ca^{2+}/CaM. Ca^{2+}/CaM kinase II may provide a mechanism for Ca^{2+} desensitization that contributes to relaxation that follows a contraction (33). It is not known whether there are any differences in content of CaM kinase II in phasic and tonic muscles.

Recent studies suggested that CaM-binding thin filament-associated proteins such as caldesmon (CaD) and calponin (CaP) also play an important role in smooth muscle contractility (5). CaD prevents contraction by inhibiting the activity of actomyosin ATPase and disengaging myosin heads from actin (28). On the other hand, CaP inhibits actomyosin ATPase and slows detachment rate of myosin from actin (14). Relative contents of CaD and CaP may be important in phasic and tonic behavior of smooth muscles (38). Interaction of CaD and CaP with CaM may lead to their disinhibition and muscle contraction. The physiological importance of these interactions, however, is not clear because of the low affinity of these proteins for CaM (25). Protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) also disable CaD and CaP from interfering with the actin-myosin interaction leading to the muscle contraction (25). PKC and MAPK regulation of CaD and CaP by phosphorylation is thought to be physiologically relevant (25, 28).

The purpose of the present study was to test the hypothesis that mechanical behavior of phasic and tonic smooth muscles is related to differences in the content of CaM and the CaM-binding proteins. Smooth muscles from the opossum esophageal body (EB) and lower esophageal sphincter (LES) were used to represent phasic and tonic smooth muscles, respectively (37). CaM-binding proteins were recognized using recombinant hemagglutinin epitope-tagged CaM (HA-CaM) (36). Contents of CaM and CaM-binding proteins MLCK and CaD were higher in EB than LES. Higher contents of MLCK and CaD may provide a dual (thick and thin filament based) mechanism for phasic behavior of EB circular muscle compared with the tonic contraction of LES muscle.

MATERIALS AND METHODS

Materials. The gel apparatus and material for electrophoresis were from Hoefer (San Francisco, CA) and Bio-Rad (Richmond, CA). All commonly used reagents and most of the protein antibodies were from Sigma (St. Louis, MO). Monoclonal antibody against MARCKS protein was from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody against the HA epitope YPYDVPDYA was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Horseradish-conjugated anti-mouse secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Actin (34), CaP (39), and myosin RLC (43) were isolated from chicken gizzard. Chicken gizzard CaD was a generous gift from Dr. Albert L.-O. Wang (Boston Biomedical Research Institute, Watertown, MA), and recombinant skeletal muscle MLCK (rMLCK) was from Dr. Zenon Grabarek (Boston Biomedical Research Institute).

Tissue harvesting and preparation. Experiments were performed in opossum (*Didelphis virginiana*) because its esophagus is composed of smooth muscles (11). Adult animals were anesthetized by intra-abdominal injection of pentobarbital sodium (40 mg/kg). The abdomen was opened by a midline incision. The lower esophagus including the gastroesophageal junction area was removed, and the animal was euthanized with additional doses of pentobarbital sodium. The experimental protocol was approved by the Animal Care Committee of the Department of Veterans Affairs Boston Healthcare System. 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). Mucosa, muscularis mucosa, and connective tissue were dissected away from the underlying muscle layers. LES was identified as a circumferential ridge at the gastroesophageal junction. A prominent part of the muscle ridge (~3 mm) was isolated and dissected away from the adjacent muscle layer with scissors. EB circular muscle segments, ~5 mm × 10 mm at ~1 cm above the LES, were obtained after carefully separating them from the longitudinal smooth muscle layer as described previously (37).

Total muscle protein extracts. Pooled tissue samples (0.3–0.6 g) were ground in a liquid nitrogen-cooled mortar and pestle and homogenized with 3 vols of extraction buffer/g frozen tissue. Extraction buffer consisted of 0.25 M NaCl, 40 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, pH 7.4, 2% sodium dodecyl sulfate (SDS), 5 mM ethylenediaminetetraacetic acid (EDTA), 4 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 5 mM NaN_3. Homogenization was performed, first by hand in a Pyrex 7727 glass homogenizer and then three times for 1 min each using a Brinkmann Polytron (PT 3000, Kinematica, Bern, Switzerland). Homogenization buffer was supplemented with a cocktail of protease inhibitors (Boehringer, Mannheim, Germany). After homogenization, samples were immediately heated at 100°C for 5 min and then cooled down to room temperature. To enhance the yield of extraction, samples were kept in extraction buffer for another 90 min and then centrifuged at 10,000 *g* for 10 min. Supernatants were collected, and remaining pellets were extracted two more times. All supernatants were pooled, frozen in liquid nitrogen, and stored at −80°C. To determine residual proteins in the pellets, the final pellets were subjected to SDS-PAGE. Proteins were examined by Coomassie blue staining and Western blots using specific antibodies and horseradish-conjugated secondary antibody. The substrate was visualized using enhanced chemiluminescence (ECL) reagent (Pierce Laboratories, Rockford, Il). No proteins were found in the residual pellets, indicating high-yield extraction.

Crude MLCK extracts. Crude MLCK extracts were prepared by a slight modification of the procedure originally used for chicken gizzard (1). Briefly, pooled tissue samples (2–3 g) were ground in a liquid nitrogen-cooled mortar and pestle and weighed. Homogenization of grounded material was performed in 3 vols of an ice-cold 20 mM Tris·HCl pH 6.8

buffer containing (in mM) 40 NaCl, 1 $MgCl_2$, 5 EGTA, and 1 dithiothreitol (DTT), plus a cocktail of protease inhibitors, with a PT 3000 Brinkman Polytron. Tissue homogenates were supplemented with 0.05% *t*-octylphenoxypolyethoxyethanol (Triton X-100) and centrifuged at 15,000 *g* for 15 min. Pellets were resuspended in homogenization buffer and centrifuged again. This procedure was repeated three times. Final pellets of myofibrils were resuspended in 3–4 vols of the ice-cold 40 mM Tris·HCl (pH 7.5) buffer containing 60 mM NaCl and 25 mM $MgCl_2$ and centrifuged for 30 min at 15,000 *g*. Supernatants were collected, whereas pellets were resuspended in the same buffer and centrifuged again. This extraction procedure was repeated two more times. Pooled supernatants were supplemented with glycerol (50% final concentration) and stored at −80°C.

Identification and quantification of CaM. Tissue extracts were separated on a 6–20% polyacrylamide continuous SDS-PAGE in the presence of 2 mM $CaCl_2$ or 5 mM EGTA. The mobility of CaM in Ca^{2+}-free conditions shifted slightly from that in the presence of Ca^{2+}. This procedure helped minimize potential errors in identifying and quantifying CaM caused by *1*) changes in CaM migration in the electric field associated with its folding/unfolding due to CaM binding to Ca^{2+} and *2*) overlapping of CaM with other muscle proteins, such as MLC 17 and a recently described 17-kDa PKC-potentiated inhibitory protein (CPI) (22). Once separated, tissue extracts were electrophoretically transferred onto Immobilon P membranes (Millipore, Bedford, MA). CaM was localized in muscle extracts by Western blot using monoclonal anti-CaM antibody (1:1,500) and peroxidase-conjugated secondary antibody (1:3,000). To quantify CaM contents in tissue extracts, a serial dilution of CaM standard and increasing amounts of tissue samples were separated on the 6–20% SDS-PAGE. Gels were stained with Coomassie blue and scanned densitometrically.

Preparation of recombinant HA-CaM. Epitope-tagged CaM containing the HA epitope YPYDVPDYA at its NH_2 terminus was prepared from N-4830 strain of *Escherichia coli* containing a temperature-sensitive cI repressor protein (36). To induce recombinant HA-CaM synthesis, bacterial cultures were grown overnight at a permissive temperature of 30°C. The bacterial cultures were diluted to an optical density of 0.4–0.5 at 600 nm with the medium prewarmed to a nonpermissive temperature of 41°C to induce HA-CaM synthesis. After growth at 41°C for 2 h, bacteria were pelleted by centrifugation for 5 min at 8,000 *g*. The bacterial pellet was resuspended in 0.02 vol of the ice-cold lysis buffer consisting of (in mM) 5 NaCl, 10 Tris·HCl pH 7.4, 1 EDTA, 0.5 DTT, and 0.1 phenylmethylsulfonyl fluoride with 0.25 mg lysozyme/ml solution. HA-CaM was purified with the use of a phenyl-Sepharose column (29). Fractions eluted from the column with EDTA were applied to the additional phenyl-Sepharose fractionation (1 ml of packed resin/ml of pooled fractions) equilibrated in 0.5 M NaCl, 50 mM Tris·HCl pH 7.4, and 5 mM $CaCl_2$. The column was washed with 5 vols of starting buffer, and HA-CaM was eluted with the buffer containing 1 mM EDTA.

HA-CaM overlay assay. Whole tissue protein extracts (5–25 μg/lane) were separated on the 6–20% SDS-PAGE and electrophoretically transferred onto Immobilon P membranes. Membranes were blocked with Tris-buffered solution (TBS) containing 5% bovine serum albumin for 1 h at room temperature. TBS contained 110 mM NaCl, 25 mM Tris-glycine pH 7.4, and 0.1% polyoxyethylene sorbitan monolaurate (Tween-20). The membranes were then extensively washed in TBS (3 times for 15 min each) and incubated in TBS containing HA-CaM (0.05–0.30 μM) and 2 mM $CaCl_2$ or 5 mM EDTA for 1 h at room temperature. They were again washed extensively in appropriate TBS (3 times, 15 min each) and exposed to monoclonal anti-HA antibody in appropriate TBS, with a dilution factor of 1:1,000, for 1 h at room temperature. After extensive washes, membranes were incubated with peroxidase-conjugated secondary anti-HA-CaM antibody in TBS, with a dilution factor of 1:2,000, for 1 h at room temperature. The membranes were washed one more time in TBS and exposed to the ECL. They were wrapped in a polyvinyl chloride foil and covered with autoradiographic film (Kodak X-OMAT, XAR-5). Films were developed using a Konica Medical Film Processor (model QX-70).

Identification of CaM-binding proteins. Two experimental protocols were used to identify the nature of CaM-binding proteins. The first protocol involved comparing relative mobility of the proteins in the extract with authentic protein standards. The tissue extracts and protein standards were electrophoretically separated on the 6–20% continuous SDS-PAGE and stained with Coomassie blue. The protein standards were included in the same well as the tissue extract and in the well adjacent to the muscle extract. This method was suitable for identification of proteins that are expressed in relatively large quantities. Myosin, CaD, and CaP were identified using this protocol. The second protocol involved Western blot analysis with primary antibodies, horseradish-conjugated secondary antibody, and ECL. This method was suitable for identification of proteins that are expressed in relatively low quantities and are not visible on Coomassie blue-stained polyacrylamide gels. MLCK, Ca^{2+}/CaM kinase II, and MARCKS protein were identified using this protocol. This protocol was also useful in detecting degradation products of proteins.

Quantification of CaM-binding proteins. To calibrate scan density as a function of protein content, density-content relationships of authentic proteins in the linear range were established. Content ranges of proteins in the extracts that yielded a linear content-density relationship were established, and content of proteins in the extracts was determined. The data points were also used to perform linear regression analysis, assuming density to be proportional to content, with Prism GraphPad version 2.0 software, (GraphPad Software, San Diego, CA). Myosin, CaD, and CaP were quantified by densitometry of the Coomassie blue-stained gels. MLCK, MARCKS protein, and Ca^{2+}/CaM kinase II were quantified by densitometry of the Western blots. For MLCK, monoclonal anti-MLCK antibody (1:10,000) and the secondary antibody (1:20,000) were used. The calibration line was constructed from rMLCK. For Ca^{2+}/CaM kinase II, anti-Ca^{2+}/CaM kinase II antibody (1:1,500) and the secondary antibody (1:3,000) were used. For MARCKS protein, MARCKS protein immunoprecipitates of the tissue extracts were separated on the 6–20% SDS-PAGE. They were stained with anti-MARCKS protein antibody (1:500) and the secondary antibody (1:1,500). The MARCKS protein immunoprecipitate, instead of total muscle protein extracts, was used because *1*) total content of MARCKS protein is very small, representing ~0.4% of the total protein content (9), and *2*) anti-MARCKS protein antibody interacted with many protein bands in total muscle protein extract.

Immunoprecipitation of MARCKS protein. MARCKS protein immunoprecipitate was prepared as described by Thelen and associates (41). Briefly, proteins in the muscle extract were precipitated with 20% trichloroacetic acid and centrifuged. Sedimented proteins were rinsed with cold water and acetone and suspended by gentle sonication in buffer solution consisting of 20 mM Tris·HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% sodium deoxycholate. Mono-

clonal anti-MARCKS-protein antibody was added (20 μl) to the sample (50 μg protein) and incubated for 3 h with constant shaking in a cold room. Immunocomplexes were pelleted by low-speed centrifugation after 50 μl of protein A-agarose (50% slurry) was added. Pellets were extensively washed in reaction buffer and applied for Western blot analysis.

MLCK activity. MLCK activity in the crude MLCK extracts of the muscles was determined by measuring rates of Ca^{2+}/CaM-dependent phosphorylation of isolated chicken gizzard RLC in the presence of excess CaM as a function of both time and protein concentration. Reaction mixtures of a final volume of 100 μl contained MLCK extract (0.01–0.2 mg muscle protein/ml), 10 mM $MgCl_2$, 110 mM NaCl, 30 mM Tris·HCl pH 7.5, 10 μM microcystin-LR, 1 mg/ml CaM, 20 μM isolated myosin RLC, and either 1 mM $CaCl_2$ or 5 mM EGTA. One millimolar [γ-^{32}P]ATP was added to start the reaction and to yield a specific activity of 800–1,200 cpm/pmol ATP. The extent of incorporation of $^{32}P_i$ into myosin RLC was measured by the filter paper method (42). Kinetic data were fitted with the single-phase exponential association model with Prism GraphPad version 2.0 software for Macintosh.

Other assays. Scanning densitometry of the Coomassie blue-stained gels and autoradiographic films exposed over Western blots were taken using a Sony XC-77 charge-coupled device video camera interfaced with a Macintosh computer. They were analyzed using the program Image from the National Institutes of Health Research Series Branch. Protein contents in tissue extracts and MLCK preparations were determined by bicinchoninic acid protein assay according to the protocol of the manufacturer (Pierce Laboratories). Protein concentrations were also determined spectrophotometrically using absorption coefficient (1%, 1 cm) values of 4.5 for myosin, 3.3 for CaD, and 1.5 for myosin RLC, all at 280 nm; 11.3 for CaP at 277 nm; and 1.3 for CaM at 276 nm.

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

RESULTS

Total protein content. The total protein contents in milligrams of protein per gram of frozen tissue were 30.12 ± 0.81 ($n = 3$) in EB and 29.45 ± 0.92 ($n = 3$) in LES. This difference was not significant ($P > 0.05$).

Identification and quantification of CaM in muscle extracts. SDS-PAGE (6–20%) resolved proteins into a large number of bands. These bands were similar in EB and LES (Fig. 1*A*). CaM was positively identified by Western blot analysis using anti-CaM antibody. CaM migrated faster in the presence than in the absence of Ca^{2+}. Whereas in the absence of Ca^{2+} (5 mM EGTA) CaM migrated with 17-kDa proteins, in the presence of Ca^{2+} it migrated at ~14 kDa. Therefore, to distinguish CaM from other Ca^{2+}-independent 17-kDa proteins such as MLC-17 and CPI-17 (22), CaM quantification was performed in the presence of 2 mM $CaCl_2$. Figure 1*B* shows scan densities of the increasing amounts of CaM standard. In Fig. 1*C*, the inset shows the calibration line for CaM standard obtained from data shown in Fig. 1*B*. Figure 1*C* shows regression lines of density vs. content of CaM in the smooth muscle extracts. The slope of the regression line was 2.89 ± 0.080 ($n = 3$) for EB and 2.04 ± 0.43 ($n = 3$) for LES, indicating that the CaM content of EB was higher than that of LES. The total content of CaM (in μg/mg frozen tissue) was also 25% higher in EB than LES [0.98 ± 0.03 ($n = 9$) vs. 0.79 ± 0.04 ($n = 10$); $P < 0.05$; Table 1]. With a molecular mass of 16.7 kDa for CaM, total molar concentration was determined to be 58.40 ± 1.80 μM in EB vs. 47.18 ± 2.25 μM in LES ($P < 0.05$; Table 1).

Visualization of CaM-binding proteins using HA-CaM overlay assay. Appearance of the CaM-binding proteins in HA-CaM overlay assay depended on the concentration of HA-CaM, the presence of Ca^{2+} in the incubation medium, and the duration of exposure of the autoradiographic films. In Fig. 2, *lane 3* shows only one high-affinity protein band of molecular mass of ~130 kDa. This band was seen when HA-CaM concentration was low (0.05 μM), the exposure time was short (5–20 s), and the medium contained Ca^{2+}. When the concentration of HA-CaM increased to 0.3 μM and the time of exposure was extended to 1–5 min, a large number of protein bands were seen (Fig. 2, *lane 4*). These bands had molecular masses of 280, 200, 150, 130, 120, 105, 87, 71–61, 58–54, 42, 38–35, 32, 19–16, and 14–12 kDa. The bands were qualitatively similar in EB and LES (data not shown). When Ca^{2+} was chelated with EGTA (5 mM) all except one ~42-kDa band disappeared (Fig. 2, *lane 5*). This band, showing Ca^{2+}-independent association with CaM, represents denatured actin. This conclusion is based on several observations. First, this protein band was recognized by anti-actin antibody. Second, the protein standard of actin, when resolved on identical SDS-PAGE, also interacts with CaM in the HA-CaM overlay assay (data not shown). Third, when cosedimented with F-actin, HA-CaM stayed in supernatant regardless of the presence of Ca^{2+}, showing that native actin does not interact with CaM (data not shown). Fourth, the cDNA sequence of actin lacks a CaM-binding sequence. Fifth, although actin has been found to stick to the CaM-Sepharose 4B column, this association has been shown to represent a nonspecific interaction of actin with Sepharose matrix (17).

Identification and quantification of MLCK. Western blot analysis revealed that ~130-kDa MLCK is the highest-affinity CaM-binding protein in LES or EB. CaM binding to MLCK disappeared in the absence of Ca^{2+} (Fig. 3*B*). Anti-MLCK antibody also recognized two other protein bands of molecular masses of ~87 and ~61 kDa that are thought to represent degradation products of MLCK (data not shown). MLCK is known to be very susceptible to proteolytic cleavage (13). Figure 3*B* shows an autoradiograph of the 130-kDa MLCK bands visualized by anti-MLCK antibody and their density scans in the extracts from EB and LES. The inset in Fig. 3*C* shows a linear calibration curve for increasing amounts of skeletal muscle rMLCK. Figure 3*C* shows fitted regression lines of data on optical density of MLCK with different amounts of the muscle extracts. The value of the slope for EB is 8.77 ± 0.16 ($n = 3$), which is approximately threefold larger than the value (2.91 ± 0.5; $n = 3$) for LES. MLCK contents in EB and LES are summarized in

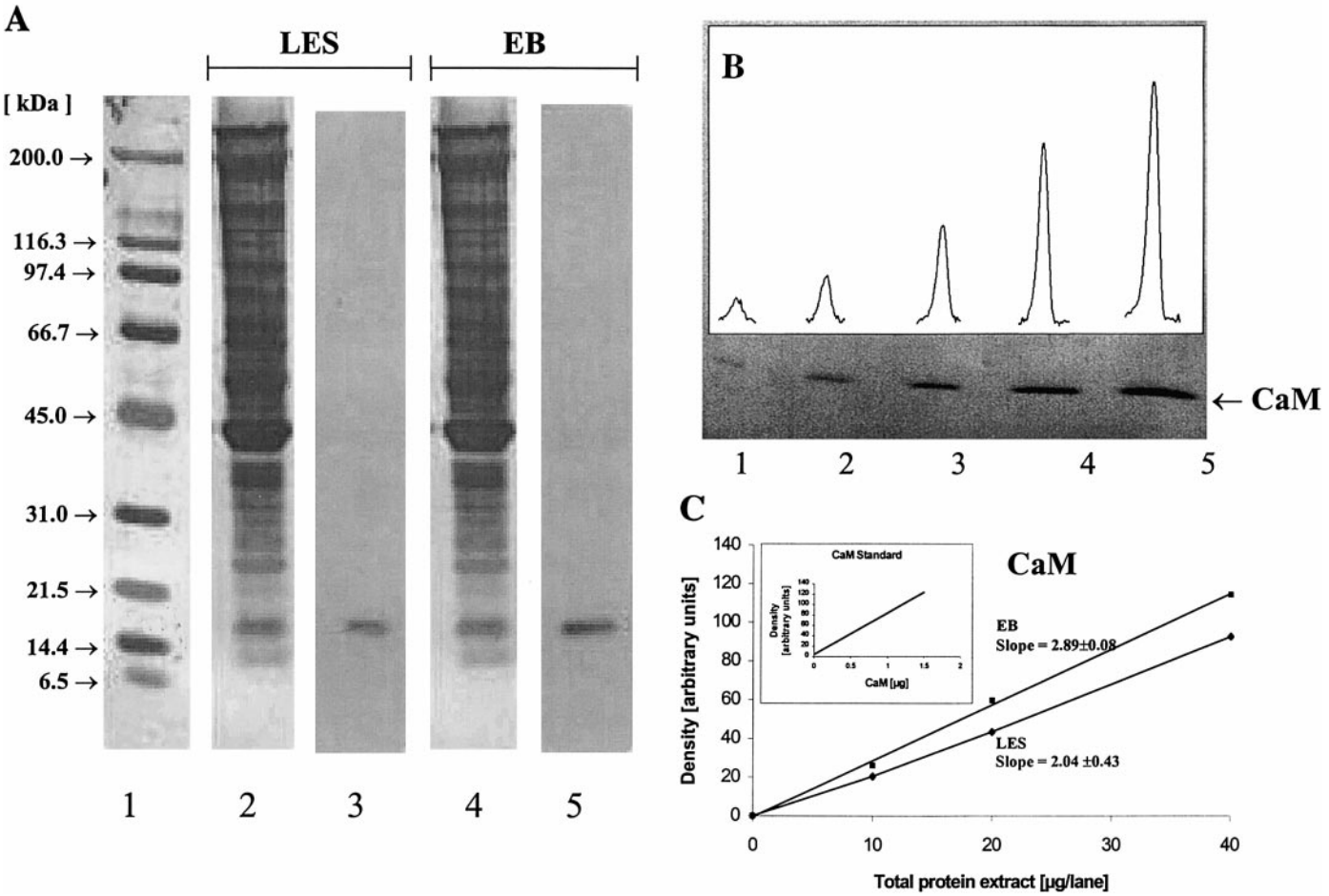

Fig. 1. *A*: protein bands, including calmodulin (CaM), in esophageal body (EB) and lower esophageal sphincter (LES) smooth muscles. *Lane 1* represents Coomassie blue-stained 6–20% SDS-PAGE of molecular mass standards. *Lanes 2* and *4* represent total protein extracts from LES and EB, respectively. *Lanes 3* and *5* are Western blots of electrophoretically resolved total protein extracts of LES and EB. Note that the 17-kDa protein band reacts with CaM antibody. Molecular mass standards (in kDa), from *top* to *bottom*, are myosin heavy chain (MHC; 200.0), β-galactosidase (116.3), phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31,0), soybean trypsin inhibitor (21.5), lysozyme (14.4), and aprotinin (6.5). *B*: Coomassie blue-stained 6–20% SDS-PAGE of increasing amounts of CaM standard (*lanes 1–5*). *Inset*, densitometric scans of the bands of CaM standards. *C*: calculation of CaM content. *Inset*, linear fitted line, from densitometric values of the increasing amounts of CaM standard, that was used for calibration. Graph shows computer-fitted lines of densitometric values of CaM bands obtained with increasing amounts of protein extracts from EB and LES. Note that the value of the slope for CaM is 25% higher for EB than for LES, indicating higher levels of CaM in EB than LES.

Table 2. With Western blot analysis, protein content of MLCK was found to be 1.47 ± 0.15 μg/mg frozen tissue (n = 5) in EB and 0.47 ± 0.06 μg/mg (n = 5) in LES ($P > 0.05$). A similar approximately threefold higher content of MLCK was found in EB over LES with the HA-CaM overlay assay (data not shown). Calculation of protein content of MLCK in tissue samples is based on the assumption that native smooth muscle MLCK and rMLCK are identically recognizable by smooth muscle anti-MLCK antibody (21). With a molecular mass of 130 kDa for smooth muscle MLCK (8), molar concentrations of MLCK in EB and LES were determined to be 11.35 ± 1.16 (n = 5) and 3.62 ± 0.45 (n = 5) μM, respectively ($P < 0.05$; Table 2; molecular mass of rMLCK is 125 kDa based on its cDNA sequence).

Table 1. *Total content and concentration of CaM in EB and LES muscles*

	EB	LES	*P*-Value
Content, μg/mg frozen tissue	0.98 ± 0.03	0.79 ± 0.04	<0.05
Concentration, μM	58.4 ± 1.8	47.18 ± 2.25	<0.05
No. of observations	9	10	

Values are means ± SE. CaM, calmodulin; EB, esophageal body; LES, lower esophageal sphincter.

MLCK activity. These studies were performed with a large excess of CaM (CaM in >800-fold excess over MLCK) to negate the influence of differences in CaM content in different tissues. The myosin RLC phosphorylation rate in the absence of Ca^{2+} (in the presence of 5 mM EGTA) was 5–9% of that in the presence of 2 mM $CaCl_2$. Phosphorylation rates of RLC with crude MLCK extracts from LES (0–1 mg/ml) and EB (0–0.3 mg/ml) were linear and similar. The maximal RLC

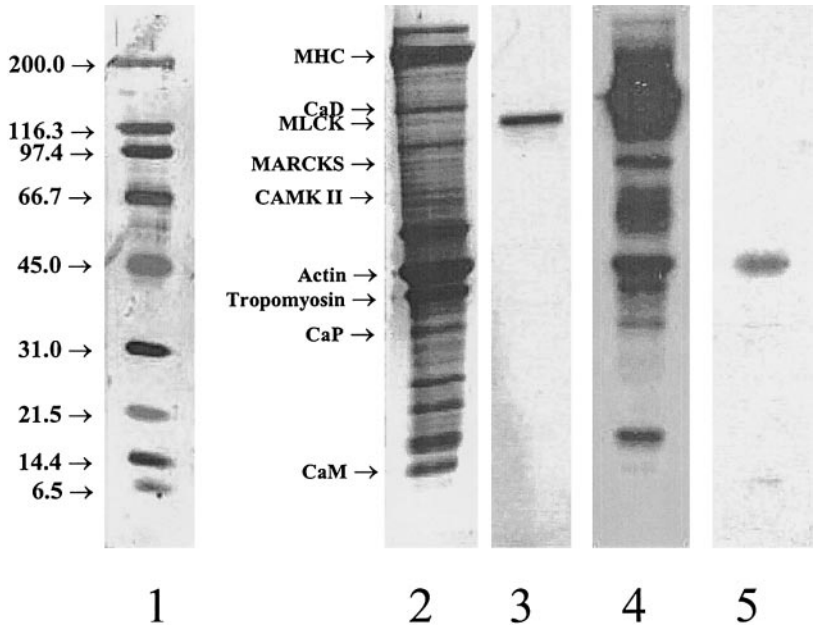

Fig. 2. Visualization of the CaM-binding proteins in EB smooth muscle by hemagglutinin epitope-tagged CaM (HA-CaM) overlay assay, showing effects of concentration of HA-CaM, exposure time, and Ca^{2+} on HA-CaM binding to muscle proteins. Total muscle protein extract (30 μg protein/lane) was resolved in electric field on 6–20% SDS-PAGE and then electrophoretically transferred onto Immobilon P membranes. *Lanes 1* and *2* show Coomassie blue-stained electrophoretically resolved molecular mass standards and the EB protein extract, respectively. *Lane 3* shows autoradiographs of HA-CaM overlay assay with short exposure (5–20 s) in the presence of a low concentration of HA-CaM (0.05 μM) and 2 mM $CaCl_2$. Note that only a 130-kDa protein band, representing high-affinity CaM-binding myosin light chain kinase (MLCK), is seen. *Lane 4* shows that with long exposure (1–5 min) in the presence of a high concentration of HA-CaM (0.3 μM) and 2 mM $CaCl_2$, a large number of the HA-CaM-binding proteins appear. *Lane 5* shows the result in the presence of 0.3 μM HA-CaM and 5 mM EGTA to chelate Ca^{2+}. Note that in the absence of Ca^{2+} all CaM-binding protein bands disappear, except for 1 having a molecular mass of ~42 kDa, which was recognized to be a nonspecific CaM binding to actin. Arrows, from *top* to *bottom*, indicate positions of MHC, caldesmon (CaD), MLCK, myristoylated alanine-rich protein kinase C substrate (MARCKS), Ca^{2+}/calmodulin kinase II (CaMKII), calponin (CaP), and CaM.

phosphorylation rates were 2.7-fold higher in EB than LES, being (in μmol P_i·mg protein extract^{-1}·min^{-1}) 0.084 ± 0.001 (n = 3) for EB and 0.031 ± 0.005 (n = 4) for LES ($P < 0.05$; Table 2). Figure 4 shows fitted curves of phosphorylation of isolated myosin RLC as a function of time. As shown, addition of MLCK extract from EB produced rapid phosphorylation of RLC that reached a plateau within ~2.5 min. In contrast, MLCK extract from LES produced slow phosphorylation of RLC that achieved a plateau in ~9 min. The amounts of protein in crude MLCK extracts from the two muscle types were similar, being 8.71 ± 0.91 μg/mg frozen tissue (n = 3) in LES and 9.58 ± 0.92 μg/mg frozen tissue (n = 3) in EB ($P > 0.05$). The values of the rate constant k obtained from the fitted data were 0.88 for EB and 0.18 for LES, indicating that RLC phosphorylation by the MLCK extract from EB was faster than that from LES (Fig. 4). These data indicate that EB has three to fivefold higher activity of MLCK than LES.

Nature of low-affinity CaM-binding proteins. Using SDS-PAGE and/or Western blot analysis of the CaM-binding proteins recognized by HA-CaM overlay assay, we identified a protein band of ~200-kDa molecular mass as myosin heavy chains, an ~150-kDa band as CaD, an ~71-kDa band as MARCKS protein, an ~58-kDa band as Ca^{2+}/CaM kinase II, and an ~32-kDa band as CaP. The anti-CaD antibody also recognized a faint protein band of ~120-kDa molecular mass (data not shown). This was thought to represent a degradation product of CaD. CaD is known to be highly susceptible to proteolysis.

Contents of lower-affinity CaM-binding proteins. Anti-Ca^{2+}/CaM kinase II antibody recognized only one ~58-kDa protein band whose density was similar in EB and LES. Anti-MARCKS protein antibody recognized two (~71 and ~63 kDa) protein bands. This doublet is similar to that found in bovine brain MARCKS protein immunoprecipitate (2). EB and LES have similar relative contents of ~71-kDa and ~63-kDa proteins.

Contents and concentrations of myosin, CaD, and CaP were determined by comparing the linear parts of density-content relationships of tissue proteins with those of authentic protein standards. Amounts of standard proteins that were in linear relationship with the densities were (in μg/lane) myosin 0–7.5, CaD 0–2.5, and CaP 0–0.75. Relationships between 5 μg and 18 μg of tissue proteins and optical densities were also linear. As shown in Table 3, myosin and CaP contents were not significantly different in EB and LES. However, CaD content in EB was 3.7-fold greater than in LES. CaD contents in EB and LES were 0.85 ± 0.09 (n = 3)

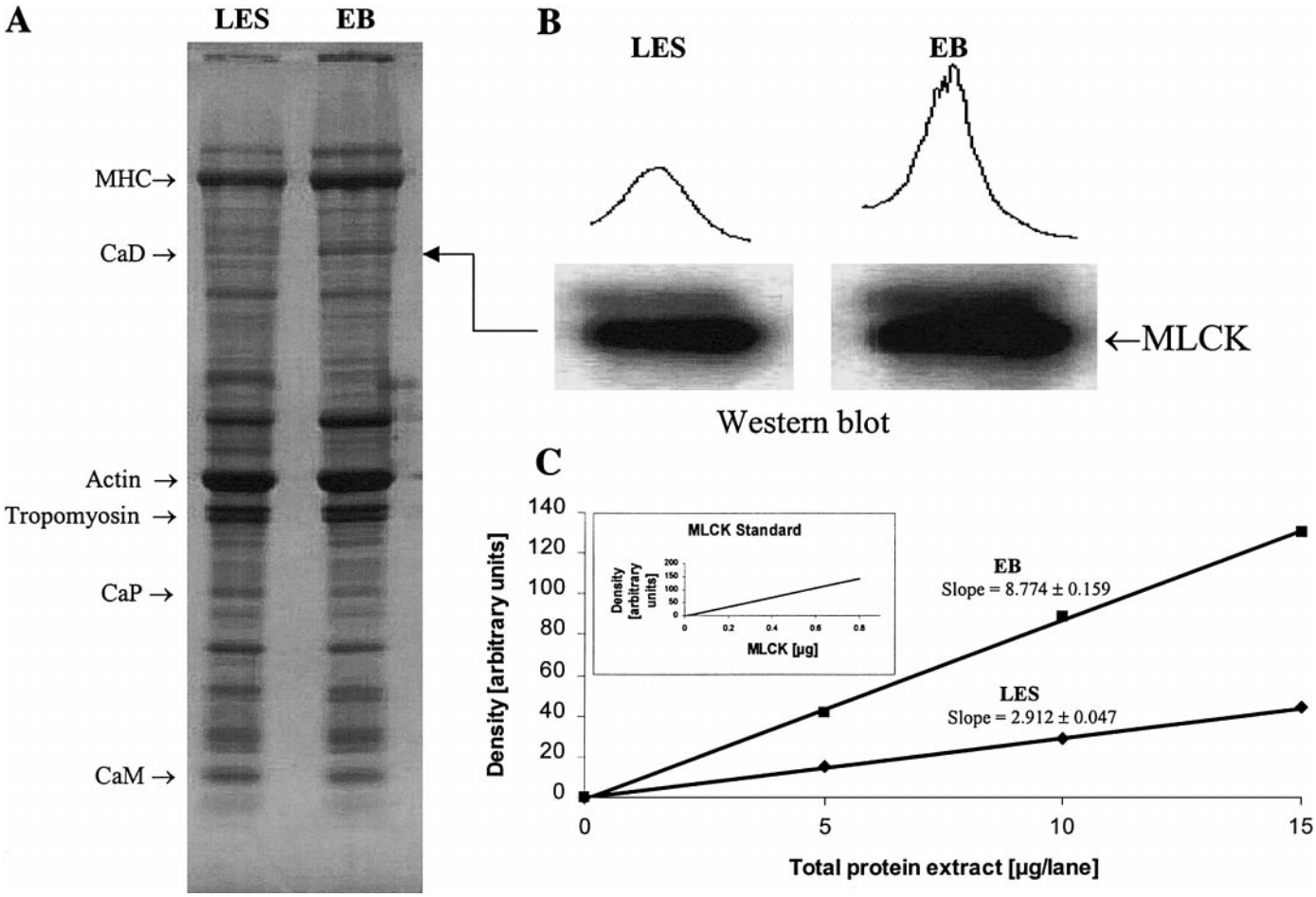

Fig. 3. MLCK content in EB and LES smooth muscles. *A*: Coomassie blue-stained 6–20% SDS-PAGE of protein extracts of EB and LES (18 μg protein/lane each). Note that because of the low abundance of MLCK, MLCK was not visible on Coomassie blue-stained SDS-PAGE. *B*: Western blots of 130-kDa MLCK stained with anti-MLCK antibody. Note that the relative protein content of MLCK appears to be higher in EB than in LES. *C*: computer-fitted lines of densitometric values of MLCK in increasing amounts of muscle protein extracts from EB and LES. Note that the value of the slope representing MLCK content is 3.5-fold higher in EB than LES. *Inset*, a linear fitted line from densitometric values of the increasing amounts of recombinant skeletal muscle MLCK (rMLCK) used as a standard that served for quantitative calibration.

and 0.23 ± 0.03 (n = 3) μg/mg frozen tissue, respectively ($P < 0.05$). With molecular masses of 480 kDa for myosin and 34 kDa for CaP, their calculated molar concentrations in EB and LES were 9.78 ± 1.04 (n = 3) and 2.64 ± 0.34 (n = 3) μM, respectively ($P < 0.05$; Table 3). The molar concentration of CaD was calculated based on its cDNA molecular mass of 87 kDa. Scanning densitometry images of Western blots of CaD

Table 2. *Content and concentration of MLCK, MARCKS protein, and Ca^{2+}/CaM kinase II in EB and LES muscles*

	EB	LES	*P*-Value
MLCK			
Content, μg/mg frozen tissue	1.47 ± 0.15	0.47 ± 0.06	<0.05
Concentration, μM	11.35 ± 1.16	3.26 ± 0.45	<0.05
No. of observations	5	5	
Activity, nmol P_i·mg protein^{-1}·min^{-1}	0.084 ± 0.001	0.031 ± 0.005	<0.05
No. of observations	3	4	
MARCKS protein			
Content, relative units	100	92 ± 8.1	>0.05
No. of observations	3	3	
Ca^{2+}/CaM kinase II			
Content, relative units	100	105 ± 9.2	>0.05
No. of observations	3	3	

Values are means ± SE. MLCK, myosin light chain kinase; MARCKS, myristoylated alanine-rich C kinase substrate.

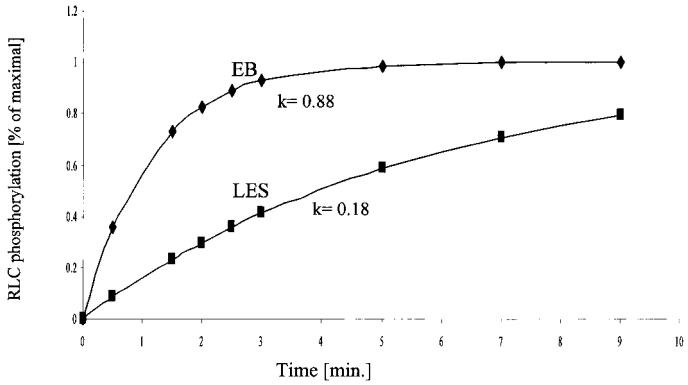

Fig. 4. MLCK activity in EB and LES smooth muscles. Phosphorylation of isolated chicken gizzard myosin regulatory light chain (RLC) by crude MLCK extracts of EB and LES muscles was determined in the presence of 2 mM $CaCl_2$ as a function of time. The amounts of protein in the crude MLCK extracts from the 2 tissues were similar ($P > 0.05$). The lines show fitted curves from the values of maximal phosphorylation rates of myosin RLC in the 2 muscles. The rate constant k was 0.88 for EB and 0.18 for LES, suggesting a 5-fold higher MLCK activity in EB than in LES smooth muscle.

show that relative content of CaD is higher in EB than LES (Fig. 5*B*). The inset in Fig. 5*C* shows a linear calibration curve of the increasing amounts of CaD standard. Figure 5*C* shows fitted regression lines of data on optical density of CaD with different amounts of muscle extracts. The CaD slope value for EB (1.83 ± 0.07; $n = 3$) is ~3.7-fold larger than for LES (0.49 ± 0.04; $n = 3$).

DISCUSSION

CaM content in opossum esophageal smooth muscles is in the range of 40–60 μM, which is similar to the reported values for cow aorta and rabbit uterus smooth muscles (16). Although differences in CaM contents in phasic and tonic muscles have not been reported before, total content and concentration of CaM are significantly higher in EB than LES. These results are consistent with the idea that Ca^{2+}/CaM signaling cascades may be more active in phasic than tonic muscles.

We used HA-CaM to identify the CaM-binding proteins in the esophageal smooth muscles. HA-CaM provides enhanced sensitivity in detecting CaM-binding proteins, minimizes steric problems, and introduces low background in the overlay assay. The HA epitope tag does not affect the ability of CaM to bind to Ca^{2+} or binding and enzymatic activities of the Ca^{2+}/CaM-regulated proteins (36). This study shows that, in overlay assays, HA-CaM appropriately reacts with different CaM-binding proteins. CaM-binding proteins were qualitatively similar in EB and LES. The goal of this study was to quantify those CaM-binding proteins that are known to play a role in smooth muscle contractility. These included MLCK, MARCKS protein, Ca^{2+}/CaM kinase II, myosin, CaD, and CaP. Of these, MLCK and CaD were found to occur in greater abundance in EB than in LES.

It is generally accepted that phosphorylation of myosin RLC by MLCK is the major mechanism responsible for contraction in smooth muscles. Phosphorylation of RLC removes its inhibitory effect on actomyosin ATPase and increases cross-bridge cycling that leads to contraction (38). It is also well known that MLCK interacts with Ca^{2+}/CaM with very high affinity, and the Ca^{2+}-CaM-MLCK complex phosphorylates RLC. MLCK content in EB was approximately threefold higher and phosphorylation of RLC approximately fivefold faster than in LES. These results are not consistent with the view that Ca^{2+}-CaM-MLCK is not a major kinase responsible for RLC phosphorylation in EB (32). Our observations are similar to the report that guinea pig ileum and portal vein smooth muscles have approximately threefold higher activity of MLCK than rabbit femoral artery (10). Guinea pig ileum and portal vein smooth muscles represent phasic muscles, whereas rabbit femoral artery represents tonic muscles. Higher concentrations of CaM and MLCK may be associated with faster phosphorylation of myosin RLC and an accelerated contraction in EB compared with LES. This observation may be applicable to other phasic and tonic smooth muscles (18, 33).

Phosphorylation of RLC by MLCK is counterbalanced by its dephosphorylation by myosin light chain phosphatase (MLCP) (10). Some excitatory agonists may act to inhibit MLCP via a G protein-coupled pathway that may involve certain PKCs and MAPK (44). These kinases act by phosphorylating a newly recognized 17-kDa PKC-potentiated inhibitor of myosin phosphatase, CPI-17 (22). Inhibition of MLCP may produce smooth muscle contraction in virtual absence of intracellular Ca^{2+} (21) because of unopposed activity of Ca^{2+}-independent activation of MLC resulting from autophosphorylation or involvement of an unknown Ca^{2+}-independent MLC kinase (44). These signaling pathways may explain PKC mediated Ca^{2+}/CaM-independent contractions reported in esophageal smooth muscle, particularly in the nonsphincteric smooth muscle of EB (32). Although MLCP activity in sphincteric and nonsphincteric smooth muscles have not been compared, MLCP activity has been reported to be higher in phasic muscles of ileum, portal vein, and gizzard than in tonic muscles of femoral artery and aorta (10, 13). Relatively higher activity of MLCP in

Table 3. *Content and concentration of myosin, CaD, and CaP in EB and LES muscles*

	EB	LES	*P*-Value
Myosin			
Content, μg/mg frozen tissue	4.23 ± 0.66	3.97 ± 0.41	>0.05
Concentration, μM	8.82 ± 1.38	8.27 ± 0.85	>0.05
No. of observations	3	3	
CaD			
Content, μg/mg frozen tissue	0.85 ± 0.0	0.23 ± 0.03	<0.05
Concentration, μM	9.78 ± 1.04	2.64 ± 0.34	<0.05
No. of observations	3	3	
CaP			
Content, μ/mg frozen tissue	0.30 ± 0.03	0.37 ± 0.04	>0.05
Concentration, μM	8.85 ± 0.89	10.94 ± 1.18	>0.05
No. of observations	4	4	

Values are means ± SE. CaD, caldesmon; CaP, calponin.

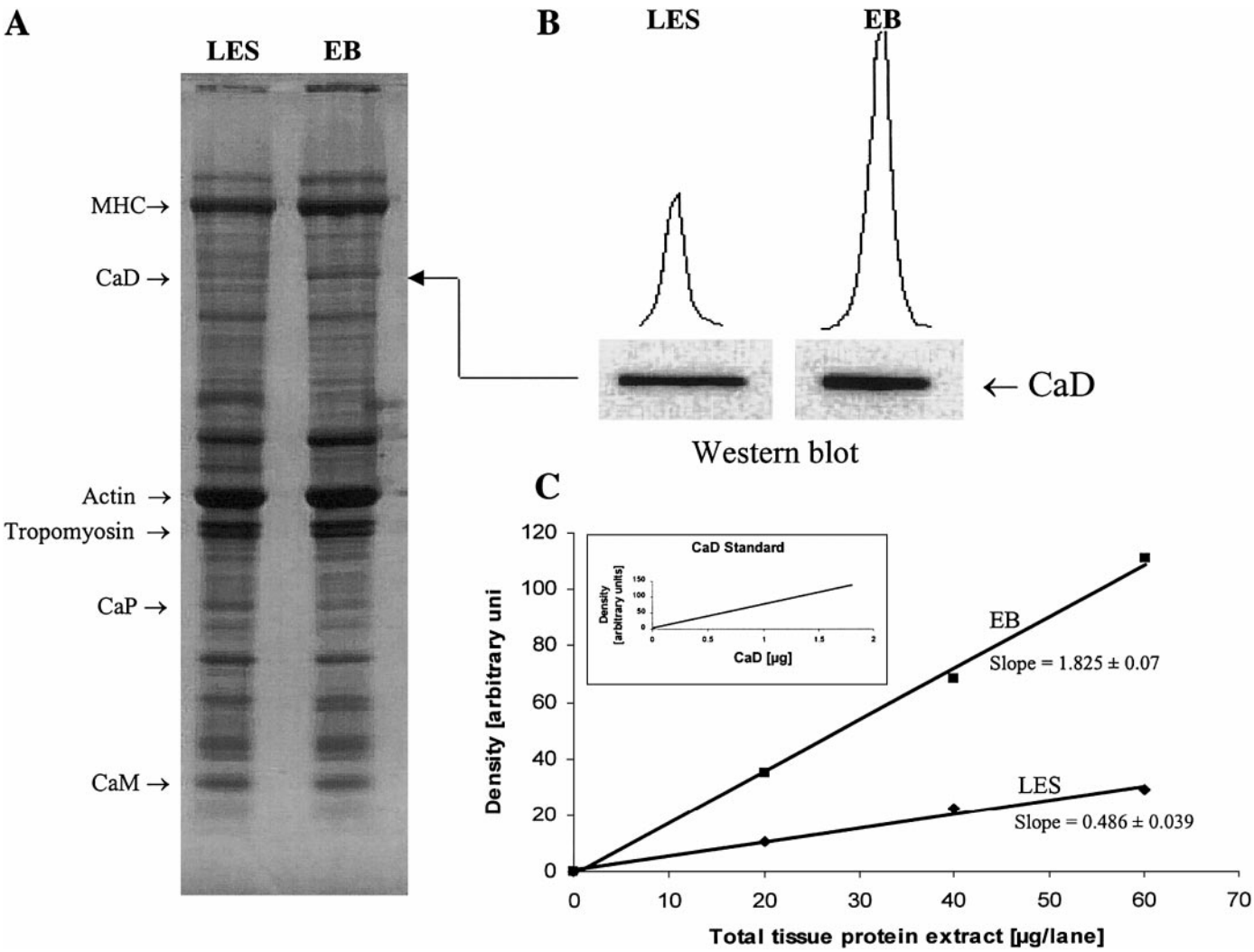

Fig. 5. CaD content in EB and LES smooth muscles. *A*: Coomassie blue-stained 6–20% SDS-PAGE of total protein extracts from EB and LES (18 μg protein/lane). Note the presence of a sharp protein band of relative mobility corresponding to ~150 kDa, representing CaD. *B*: Western blot analyses of CaD bands with anti-CaD antibody in protein extracts from LES and EB. *C*: computer-fitted lines of density values of CaD content in tissue extracts from EB and LES loaded on the gel in increasing amounts. Note that the value of the slope of the line that represents CaD is >3-fold greater for EB than LES, indicating that EB has greater CaD content than LES. *Inset*, a calibration curve represented by a linear fitted line from the density values of the increasing amounts of CaD standard.

phasic smooth muscles may contribute to the lack of steady-state RLC phosphorylation and lack of basal tone in these muscles. Moreover, higher MLCP activity may also contribute to an accelerated rate of dephosphorylation of RLC and accelerated relaxation in phasic smooth muscles (10, 18, 33). These observations suggest that, along with the Ca^{2+}/CaM-dependent signaling pathway, Ca^{2+}/CaM-independent PKC/CPI-17/MLCP-mediated signaling of smooth muscle contraction may also be more prominent in phasic than in tonic smooth muscles. Further studies are needed to test this possibility.

Differences in rates of phosphorylation/dephosphorylation of myosin RLC cannot fully explain the divergent mechanical behavior of phasic and tonic smooth muscles. For example, phasic and tonic muscles, in which RLC is fully phosphorylated by irreversible thiophosphorylation, show different rates of force development and maximal shortening velocity (33). These observations show that RLC phosphorylation and actomyosin ATPase activity can be dissociated. It has been shown that the thin filament-based regulatory proteins CaD and CaP exert an inhibitory effect on actomyosin ATPase and suppress contraction. CaD also disengages myosin heads from actin and causes muscle relaxation. CaD content is higher in EB than LES. These results are similar to those reported earlier (15, 37). Higher CaD contents in phasic smooth muscles may facilitate accelerated relaxation and lack of basal tone. On the other hand, dissociation of CaD from F-actin allows myosin heads to interact with actin filaments and muscle to contract (5, 25). Dissociation of CaD from actin is thought to occur because of its phosphorylation by PKC and MAPK (25). Although

interaction of CaD with Ca^{2+}/CaM also removes the inhibitory effect of CaD on actomyosin ATPase, the role of Ca^{2+}/CaM in regulating CaD binding to F-actin under physiological conditions is unclear. This is because of the low affinity of Ca^{2+}/CaM for interaction with CaD, which is 2–3 orders of magnitude lower than for MLCK (25). Therefore, a 10- to 20-fold molar excess of CaM over CaD is necessary to inhibit CaD binding to actin filaments. However, the cytosolic levels of CaM during the contractile cycle of smooth muscle are not known.

Recently, it was shown that PKC may act to increase cytosolic CaM via the phosphorylation of MARCKS protein (30). It is suggested that MARCKS protein forms a complex with CaM and localizes to cell membrane (12). On activation of smooth muscle by certain contractile agonists, cytosolic PKC translocates to plasma membrane, where it phosphorylates MARCKS protein. Phosphorylation of MARCKS protein causes its dissociation from the complex with CaM, and CaM is released into the cytoplasm (30). Therefore, it is suggested that phosphorylation of MARCKS protein by PKC provides an important mechanism for increases in cytosolic CaM level (30). An important finding of this study is the demonstration of presence of MARCKS protein in the esophageal smooth muscles. These observations suggest that PKC may regulate the levels of cytosolic CaM via MARCKS protein in esophageal smooth muscle.

The relationship between actomyosin ATPase activity and force has been shown to be different in phasic and tonic muscles. Tonic muscles are thought to develop latch cross bridges that maintain force with very low actomyosin ATPase activity. The latch cross bridges are associated with slower detachment rate than normal cross bridges (33). The CaM-binding protein CaP has been reported to slow down the detachment rate (14) and inhibit actomyosin ATPase without disengaging myosin heads from actin (27). Moreover, the recent report of faster unloaded shortening velocity in smooth muscle of CaP knock-out mice is also consistent with the regulation of the cross bridge cycle by CaP (26). Therefore, CaP has been suggested to play a role in force maintenance in tonic smooth muscles(14). However, total contents of CaP are similar in EB and LES. It is possible that the action of CaP is evident in tonic muscles but not in phasic muscles because of the overriding inhibitory influence of high levels of CaD and MLCP in these muscles. Further studies are necessary to define the role of CaP in smooth muscle contractility. The slower detachment rate of cross bridges in tonic muscles may also be related to greater abundance of α-actin, basic myosin essential light chain, and the myosin heavy chain isoform lacking the 7-amino acid insert (7, 19, 20, 24, 37).

In summary, these studies show that EB smooth muscle with phasic contractile behavior is characterized by a greater abundance of CaM, CaD, and MLCK than LES smooth muscle with tonic behavior. The greater abundance of CaM and MLCK may explain the faster rate of force development in phasic than in tonic muscles. Moreover, the greater abundance of CaD may be responsible for accelerated and full relaxation after a contraction in the phasic muscle. In contrast, the lower abundance of CaM, CaD, and MLCK in LES is consistent with low amplitude and slow contractile activity in tonic smooth muscles. These results suggest that both the thick and thin filament-based regulatory CaM-binding proteins may influence phasic vs. tonic behavior of smooth muscles.

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