Myosin Essential Light Chain Isoforms Modulate the Velocity of Shortening Propelled by Nonphosphorylated Cross-bridges*

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The differential effects of essential light chain isoforms (LC17a and LC17b) on the mechanical properties of smooth muscle were determined by exchanging recombinant for endogenous LC17 in permeabilized smooth muscle treated with trifluoperazine (TFP). Co-precipitation with endogenous myosin heavy chain verified that 40–60% of endogenous LC17a could be exchanged for recombinant LC17, or LC17b. Upon addition of MgATP in Ca2+-free solution, recombinant LC17 exchange induced slow contractions unaccompanied by regulatory light chain (RLC) phosphorylation only in TFP-treated, but not in untreated, permeabilized smooth muscle; the shortening velocity and rate of force development were approximately 1.5 and 2 times faster, respectively, in response to LC17a than LC17b. Additional incubation with recombinant, thiophosphorylated RLC increased the shortening velocity, independent of the LC17 isoform exchanged. The LC17-induced contractions of TFP-treated muscles were abolished by prior addition of nonphosphorylated cross-bridges. We suggest that LC17 stiffens the actomyosin motor. The muscle-specific differences in actomyosin kinetics have been ascribed to the expression of the two myosin heavy chain isoforms, SM-1 and SM-2 (2) that consequently, rate of cooperative cycling of nonphosphorylated cross-bridges.

The markedly different rates of contraction and relaxation in fast, phasic and slow, tonic smooth muscles reflect differences not only at the level of the membrane potential, signal transduction, rates of myosin light chain phosphorylation and dephosphorylation, but also in the kinetic properties of the actomyosin motor. The muscle-specific differences in actomyosin kinetics have been ascribed to the existence of specific myosin isoforms, but their relative contributions to the mechanical properties of smooth muscles have not been unequivocally demonstrated (reviewed in Ref. 1). The expression of the two myosin heavy chain (MHC) isoforms, SM-1 and SM-2 (2) that differ by, respectively, the presence or absence of a 34-amino acid extension of the carboxyl terminus (3), does not correlate with phasic or tonic kinetics of contraction. On the other hand, motility assays show different rates of movement propelled by MHC isoforms that are the products of an alternative splicing mechanism resulting in the presence or absence of a 7-amino acid insert near the nucleotide-binding region of the myosin head (4, 5). Disparate results have been obtained concerning the effects on contractile kinetics of the two LC17 isoforms, acidic (LC17a) and basic (LC17b), that differ in 5 of the 9 COOH-terminal amino acid residues and are products of a single gene generated by an alternative splicing mechanism (6). The faster kinetics of phasic, smooth muscle in situ (7–9) correlates with the expression of the LC17a isoform (10, 11), albeit the proportion of myosin heavy chain containing the insert was also variant in these muscles, whereas in vitro studies have produced conflicting results. Increasing the LC17a content of isolated aortic myosin has been reported to increase ATPase activity (12), but motility assays failed to detect a difference in the velocity of actin movement as a function of LC17 isoform, although they showed faster movement propelled by myosin containing the 7-amino acid insert in the motor domain (4, 13). Thus, it is possible that the LC17a isoform, the motor domain insert, or a combination of both (1) are responsible for the different kinetic properties of phasic and tonic smooth muscle actomyosin in situ. The aim of the present study was to determine whether a change in the ratio of LC17 isoforms alone can alter the contractile kinetics of smooth muscle. To that end, we exchanged the basic isoform, LC17b, into bladder and amnion smooth muscles, both of which have fast, phasic properties and express only the LC17a isoform (Ref. 10 and the present study).

We used TFP, a hydrophobic cation that facilitates extraction and/or exchange of light chains from isolated myosin (12, 14, 15) and single cells (16), to promote light chain exchange in permeabilized smooth muscle. Our results show a significant LC17 isoform-specific effect on the unloaded shortening velocity of nonphosphorylated, slowly cycling cross-bridges, but no detectable effect on muscles in which the RLCs are thiophosphorylated.

**EXPERIMENTAL PROCEDURES**

**Tissue Preparation and Light Chain Exchange**

Bundles of bladder detrusor muscle (3 mm × 200–400 mm) from male, New Zealand rabbits weighing 2–3 kg or chicken amnion sheets (3 × 3 mm) from 10–13 day eggs were dissected and the ends tied with monofilament silk, and attached to a fixed hook and a force transducer (AE 801; AME, Horten, Norway) for recording isometric tension at room temperature. The chicken amnion also expresses 100% LC17a and was used in preliminary studies to optimize conditions for LC17 exchange in smooth muscle, because of its thinness and lack of connective tissue and gel electrophoresis; CaM, calmodulin; LC17a, and LC17b, light chain acidic and basic, respectively.
basement membrane on the surface smooth muscle cells (17). However, the smooth muscle fibers in bladder bundles were better (more parallel) oriented and, unless otherwise stated, all mechanical experiments reported were performed using rabbit bladder detrusor muscle strips. Strips were stretched by 10% of their resting length and, after determining a high K+, permeabilized in a 0.5% 1 Triton X-100 (0.05%, v/v) and A23187 (10 µm), to deplete internal Ca2+ stores, for 15–20 min before washing in relaxing solution and incubation in a rigor solution designed to facilitate light chain exchange and based on that used previously (16), it contained (mM): 1 TFP, 10 CDTA, 150 potassium methanesulfonate, and 20 PIPES (pH 6.5) for 15 min at 30 °C (see Fig. 7A for illustration of this protocol). The remainder of the experiment was conducted at room temperature (22–23 °C). After washing, strips were incubated in solutions containing recombinant LC17 (0.75 mg/ml) for up to 60 min. The compositions of the solutions were (mM): relaxing solution (47 potassium methanesulfonate, 4.72 magnesium methanesulfonate, 5 KEGTA, 2.23 MgATP, 20 imidazole (pH 7.1)). The ionic strength of these solutions was 0.12. In addition, all incubation solutions contained 10 mM dithiothreitol (DTT), 1 mM AEBSF (Calbiochem, La Jolla, CA), and 0.1 mM leupeptin. All chemicals were obtained from Sigma unless otherwise stated.

Expression, Purification, and Thio phosphorylation of Myosin Light Chains

Recombinant light chains, from a gizzard library, were expressed in Escherichia coli as described previously (18). In some experiments, thio phosphorylated recombinant RLC was added to muscle strips. These were prepared by incubating recombinant RLC (1.0 mg/ml) with 20 µg/ml myosin light chain kinase (MLCK, generous gift of Dr. D. J. Hartshorne), 5 µM calmodulin (Calbiochem), 2 mM ATP-S (Boehringer Mannheim, Indianapolis, IN), 2.4 mM MgCl2, and 0.6 mM CaCl2 overnight at 4 °C, followed by extensive dialysis against zero Ca2+–rigor solution. Greater than 90% thio phosphorylation of recombinant RLC was confirmed by SDS-PAGE using urea/glycerol gels (19).

Photolysis of Caged ATP

Muscle strips were treated according to the TFP protocol and incubated in zero Ca2+–rigor solution containing either LC17a or LC17b, for 60 min. This was replaced by photolysis solution containing 5 mM caged ATP (1-(2-nitrophenyl)-ethyl-ATP; Molecular Probes, Eugene, OR), and 5 mM MgCl2, in zero Ca2+–rigor solution (73.2 potassium methanesulfonate, 23.5 MgCl2, 5 KEGTA, and 20 imidazole (pH 7.1)). The ionic strength of these solutions was 0.12. In addition, all incubation solutions contained 10 mM dithiothreitol (DTT), 1 mM AEBSF (Calbiochem, La Jolla, CA), and 0.1 mM leupeptin. All chemicals were obtained from Sigma unless otherwise stated.

Analysis of Light Chain Exchange

Muscle strips were rapidly frozen and stored in liquid nitrogen for subsequent analysis of protein composition. To discriminate between true exchange and nonspecific binding to the tissue, the extent of myosin light chain isom form exchange was determined by either immunoprecipitation of myosin using anti-myosin heavy chain antibody (generous gift from Drs. C. Kelley and R. Adelstein) or by sedimentation of myosin in low salt buffer and centrifugation, followed by SDS-PAGE and transfer to polyvinylidene difluoride membranes for subsequent Western blotting using anti-LC17a antibody (clone 7B5.1) and anti-RLC antibody (generous gift from Dr. K. Kann). The immunoprecipitated pellets were washed from three to six times and the supernatant of the final wash treated with trichloroacetic acid to precipitate remaining protein. The entire precipitate was loaded on the gel. No LCs were found in the final wash supernatant. Contaminants, if present, were detected using an anti-calmodulin antibody generously provided by Dr. M. Walsh. Using one-dimensional gel electrophoresis (15% polyacrylamide; 16 × 14 cm), the LC17a and LC17b isoforms could be readily distinguished according to their mobility. In experiments involving incubations with recombinant, thio phosphorylated RLC, its level of exchange with endogenous, nonphosphorylated RLC could not be accurately determined, since both species migrate to the same position on an isoelectric focusing gel, as a consequence of an NH2-terminal 4-amino acid tag on the recombinant RLC (18).

Immunoprecipitation of Myosin—Treated strips were homogenized in ice-cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM AEBSF, 0.1 mM leupeptin, and 10 mM Tris-HCl (pH 7.1)). Cellular debris was pelleted by centrifugation at 800 × g (4 °C) and 10 mM MgATP was added to collected supernatant for 15 min with bovine polyvalent anti-myosin heavy chain antibody, which recognizes both SM-1 and SM-2 heavy chain isoforms, for 60 min at room temperature. The homogenate was incubated for a further 1 h at room temperature with protein A-10% agarose (v/v) (Santa Cruz, Santa Cruz, CA) and immune complexes were collected by centrifugation at 300 × g for 5 min and washed 4 times with ice cold lysis buffer. The final supernatant was run as a control after precipitation with 20% trichloroacetic acid in acetone and several washes with acetone. Both immune complex and final supernatant pellets were resuspended in 2 × Laemmli sample buffer.

Sedimentation of Myosin—Treated strips were homogenized in ice-cold lysis buffer (1% Nonidet P-40, 500 mM NaCl, 1 mM AEBSF, 0.1 mM leupeptin, and 10 mM Tris-HCl (pH 7.1)). Cellular debris was pelleted by centrifugation at 300 × g (4 °C) and 10 mM MgATP added to the supernatant for 15 min before centrifugation at 80,000 × g (4 °C) for 20 min. The collected supernatant was diluted with 9 volumes of ice-cold buffer containing 1 mM MgCl2 and 10 mM MOPS and allowed to stand for 30 min to precipitate myosin before centrifugation at 105,000 × g (4 °C) for 30 min. After removal of the supernatant, the pellet was resuspended in low salt buffer and the last centrifugation step repeated before resuspension of the pellet in 2 × Laemmli sample buffer.

RLC Phosphorylation in Situ

Phosphorylation levels of endogenous RLC in Table I were determined by two-dimensional gel electrophoresis, transfer to nitrocellulose membranes, and staining with colloidal gold as described previously (23).

Effect of TFP on Light Chain Content, RLC Phosphorylation, and Circular Dichroism (CD) of LC17 in Vivo

Turkey gizzard myosin was prepared as described previously (24). To determine the effects of TFP on isolated myosin using the same (1 µM) concentration of TFP as used in experiments on skinned fibers, myosin (12 µg) was incubated in 500 mM KCl, 10 mM EDTA, 1 mM ATP, 1 mM DTT, and 20 mM PIPES (pH 6.5) for 15 min at 30 °C. In the presence or absence of 1 mM TFP. The solution was diluted with 9 volumes of ice-cold buffer containing 1 mM MgCl2 and 10 mM MOPS, and allowed to stand for 30 min to precipitate myosin before centrifugation at 105,000 × g (4 °C) for 30 min and the pellet resuspended in 2 × Laemmli buffer. For comparison, myosin precipitates were also prepared following treatment with 5 mM TFP, a concentration known to inhibit the high-performance liquid chromatography of the trough solution at the completion of force measurements. Contaminant ATP and ADP were removed from the caged ATP stocks using 100 µg/ml 1-4 appyrase (grade V; Ref. 10). The set-up used for laser flash photolysis has been previously described (20, 21).

Slack-test Protocol

Unloaded shortening velocity (V0) was determined using the slacktest method (22). Briefly, strips were attached to hooks via aluminum T-clips or, in some earlier experiments, with small loops. After reaching steady state isometric tension, length changes of varying amplitude, sufficient to just reduce tension to zero, were applied to one end of the muscle strip using an isotonic lever (Model 308, Cambridge Technology Inc., Watertown, MA, maximum step time 300 µs). The time taken for the strip to shorten against zero load and take up the slack was recorded and plotted against the length change. The slope of this relationship was taken as unloaded shortening velocity. Linear regression analysis was used to obtain the best fit straight line. In order to compare the effects of LC17–isoforms on V0, strips were treated according to the TFP protocol and incubated with the appropriate LC17 isoform in zero Ca2+–rigor for 60 min before force activation in relaxing solution and determination of V0 as described above. In some experiments, V0 was also measured in LC17– exchanged muscles after an additional 45-min incubation in zero Ca2+–rigor containing recombinant thio phosphorylated RLC (0.7 mg/ml).
LC17 Isoforms Modulate Cycling of Nonphosphorylated Cross-bridges

The Effect of TFP on the Light Chain Content and Properties of Isolated Myosin and Myosin in Smooth Muscle—The effects of TFP treatment were explored on isolated myosin for comparison with the in situ effects. Unlike experiments on isolated myosin (14) or single cells (16), treatment of Triton-permeabilized rabbit bladder strips with TFP caused no detectable loss of the RLC, LC17, or calponin, as determined in whole homogenates (Fig. 1A) or MHC immunoprecipitates (Fig. 1B). To verify whether this apparent discrepancy was the result of method-based differences, we examined the effects of two different treatment protocols on LC removal from isolated gizzard myofibrillar protein. A concentration of TFP (1 mM TFP, 15 min at 30 °C) which did not extract light chains from the muscle strips also did not extract light chains from isolated myosin in solution (Fig. 2). Loss of both RLC and LC17, however, was observed with the method (5 mM TFP, 1 h at 4 °C) used by Trybus et al. (14) to extract the RLC (Fig. 2).

To assess the plausibility of TFP having a direct effect on the extent of RLC thiophosphorylation was determined using one-dimensional isoelectric focusing mini-gels (7.5% polyacrylamide, 2% pH ampholytes 4.0–5.4 (Pharmalyte, Pharmacia, Piscataway, NJ)).

CD spectra of LC17 were obtained to determine whether TFP had a direct effect on LC17 structure. LC17a (17 μM), in the presence of absorption spectrum of TFP (100 μM), was incubated in 50 mM Tris buffer (pH 7.0) at 30 °C for 15 min and kept at 20 °C for approximately 30 min. CD spectra were then recorded in a 0.5-mm fused silica cuvette at 20 °C using a J-600 spectropolarimeter (JASCO, Tokyo, Japan). Five scans were accumulated per sample and spectra are presented as difference spectrum, i.e. spectrum recorded in the presence or absence of TFP minus spectrum of TFP or Tris buffer alone. Measurements were repeated three times with different solutions and the results were reproducible. The α-helical content was estimated from the mean residue molar ellipticity at 220 nm using the DICHROPROT v. 2.4 analysis program (available at http://www.ibcp.fr/DICHROPROT.html).

Fluorescent Labeling

In order to determine the intracellular distribution of LC17 added to skinned muscle fibers, LC17a, was fluorescently labeled at an approximate molar stoichiometry with tetramethylrhodamine 5-iodoacetamide (Molecular Probes). Labeling was performed for 2 h at room temperature followed by extensive dialysis to remove free tetramethylrhodamine 5-iodoacetamide. Labeling of LC17a was confirmed by SDS-PAGE.

Electron Microscopy

Strips of bladder muscle were carried through the standard permeabilization protocol, followed by treatment with TFP. Untreated permeabilized control strips were carried through the same solutions, minus TFP. Following TFP treatment the muscles were incubated in relaxing solution or zero calcium rigor solution for 15 min and subsequently fixed in 2% glutaraldehyde in 0.075 M sodium cacodylate buffers with 4% sucrose plus 0.2% tannic acid for 2 h, followed by fixation in 2% osmium tetroxide and en bloc staining with saturated uranyl acetate, dehydration in alcohol, and embedment in Spurr’s resin. Thin longitudinal or transverse sections were sampled throughout the thickness of the muscle strips.

Statistics and Data Analysis

Data are presented as the mean ± S.E., and n refers to the number of muscle strips. Statistical significance was determined (p < 0.05) with unpaired Student’s t test. Unless otherwise stated, all gels presented are representative of at least three similar experiments. The data from the flash photolysis and slack test experiments were collected using LabView 3.1.1 data acquisition software (National Instruments, Austin, TX) and curve fitting of force transients was performed using Sigma Plot 2.0 software (Jandel Scientific, San Rafael, CA).

RESULTS

Fig. 1. Protein content of Triton-permeabilized rabbit bladder strips after TFP treatment, showing no detectable loss of myosin light chains or calponin compared with intact strips using SDS-15% polyacrylamide gels. Panel A, Western blots of calponin, RLC and LC17 content of whole homogenate. Note that all lanes are from the same blot and that RLC runs as a doublet. Numbers refer to the total protein load in each lane, determined by the Bradford assay. Panel B, Coomassie Blue stain of MHC and corresponding Western blots of RLC and LC17, transferred from the same gel as shown for MHC, after immunoprecipititation of myosin using anti-MHC antibody. TFP-treated samples are shown in duplicate.

Fig. 2. Effect of TFP treatment on light chains of gizzard smooth muscle myosin in vitro. Coomassie Blue stain showing light chain content after TFP treatment and precipitation of myosin in low salt followed by high-speed centrifugation using 4–20% gradient SDS-PAGE. Note that the 70-kDa band appearing in the 5 mM TFP-treated lanes is bovine serum albumin, included in the incubation solution. Std, 12 μg of whole myosin. Note that at 1 mM TFP for 15 min at 30 °C, the same conditions used for the permeabilized muscle strips, light chains are not removed, similar to the findings in situ. However, treatment with 5 mM TFP did result in light chain extraction. All samples are shown in duplicate.

Fig. 3. Concentration (1 mM) that did not extract RLC or LC17 from myosin in situ (see below) or in vitro, as described above, inhibited thiophosphorylation of RLC of isolated myosin by a Ca2+/CaM-independent MLCK in vitro. The TFP concentration (1 mM) that did not extract RLC or LC17 from myosin in situ (see below) or in vitro, as described above, inhibited thiophosphorylation of RLC of isolated myosin by a Ca2+/CaM-independent MLCK in vitro, suggesting that TFP made threonine and serine unavailable for thiophosphorylation
rather than through inhibition of CaM-MLCK. A similar result was obtained using full-length MLCK in the presence of Ca\(^2^+\)/CaM (data not shown). Paired controls of non-TFP-treated myosin showed the expected phosphorylation of RLC by MLCK and Ca\(^2^+\)/CaM-independent MLCK (Fig. 4B).

Efficiency of Exchange of Recombinant Light Chains—Experiments were carried out to exchange the endogenous LC\(_{17a}\) isoforms in bladder and chicken amnion cells with the recombinant LC\(_{17b}\) and to characterize the efficiency and extent of exchange. Although no selective extraction of either LC\(_{17}\) or RLC was observed after 1 mM TFP treatment, subsequent incubation with recombinant LC\(_{17}\) led to significant exchange of recombinant for endogenous LC\(_{17}\) as determined by immunoprecipitation (Fig. 5A) or sedimentation of myosin (Fig. 5B).

The amount of recombinant LC\(_{17b}\) associated with myosin heavy chain, expressed as percent of total LC\(_{17}\), was 50 ± 1% (\(n = 6\)) in the rabbit bladder and 44 ± 5% (\(n = 6\)) in the chicken amnion. No LC\(_{17b}\) was detectable in myosin precipitates (Fig. 5B) or in the final wash of immunoprecipitates if the TFP solution was omitted from the exchange protocol. Further evidence of TFP facilitating LC\(_{17}\) exchange was obtained using confocal microscopy. Incubation with fluorescently labeled LC\(_{17b}\) according to the exchange protocol followed by extensive washing revealed a diffuse cytoplasmic signal, excluding nuclei and the extracellular matrix (Fig. 6A), whereas in paired experiments without TFP pretreatment the fluorescent signal obtained with identical detection parameters was markedly less intense (Fig. 6B). These findings indicate that added LC\(_{17}\) can freely diffuse into and out of the filament lattice, but binds to the myosin heavy chain only after TFP treatment. Similar results were found for the RLC, where ~50% of total RLC was exchanged by recombinant RLC (data not shown). This was easily discernible, as the recombinant and endogenous non-phosphorylated RLCs ran separately on one-dimensional isoelectric focusing gels.

The Effects of Light Chain Exchange on Contractile Properties—Surprisingly, in all smooth muscles treated with TFP, incubation with either recombinant LC\(_{17a}\) or LC\(_{17b}\), in relaxing solution (5 mM EGTA, 2 mM MgATP) produced large (~30% of initial high K\(^+\)-induced force), but slow, Ca\(^2^+\)-independent force generation (Fig. 7A). In approximately 50% of strips examined, transfer to relaxing solution without added LC\(_{17}\) also induced a small contraction (9 ± 1% of initial high K\(^+\); \(n = 9/19\) fibers), but subsequent addition of LC\(_{17a}\) or LC\(_{17b}\) caused force development irrespective of whether this smaller, initial con-
LC17 Isoforms Modulate Cycling of Nonphosphorylated Cross-bridges

Fig. 6. Confocal micrograph showing longitudinally oriented muscle cells in rabbit bladder strips after Triton permeabilization and incubation with tetramethylrhodamine B isothiocyanate (TRITC)-labeled LC17a, with (Panel A) or without (Panel B) TFP pretreatment. Imaging at different planes in the muscle strips showed fluorescence throughout the cytoplasm in all cells, excluding nuclei (N) only with TFP pretreatment. TRITC-labeled LC17a did not bind nonspecifically, as shown in Panel B. Scale bar, 10 μm.

traction was present (compare Fig. 7, A and B). Both contractions could be promptly relaxed by 3 mM vanadate (H2VO4; Fig. 7B), indicating that force was generated by cross-bridges. No significant increase in phosphorylation of endogenous RLC above basal levels was detectable at the peak of LC17-induced contraction (Table I), whereas in paired, untreated strips, Ca2+-induced contractions of a similar magnitude induced the expected increase in RLC phosphorylation. An inhibitor of myosin light chain kinase, ML-9 (300 μM), had no effect on the contraction induced by exogenous LC17 in TFP-treated muscles (n = 2; data not shown), although it relaxed control fibers contracted with pCa 5.0. These findings imply that the function of the RLC has been altered, leading to a loss of its repressor role allowing the unregulated head with the functional, exchanged LC17 to generate force upon addition of MgATP. Addition of nonphosphorylated RLC relaxed both the LC17-induced force (data not shown) and the initial, variant small contraction sometimes observed upon transfer to relaxing solution (Fig. 7C).

Muscles preincubated with nonphosphorylated RLC failed to develop force in response to LC17 (data not shown), suggesting that the ability of nonphosphorylated RLC to switch off actomyosin ATPase activity had been regained. Recombinant thiophosphorylated RLC produced a qualitatively similar contraction (Fig. 7D) to that observed in response to LC17. None of the recombinant light chain species had any effect on force if TFP was omitted from the protocol detailed under “Experimental Procedures,” consistent with the premise that TFP treatment enables subsequent exchange of recombinant light chains.

The Differential Effects of Exchanged Light Chain Isoforms on Unloaded Shortening Velocity (Vus)—We next wished to determine whether the Vus propelled by nonphosphorylated, cycling cross-bridges during force generation induced by recombinant LC17 (see above) were affected by the type of LC17 (a or b) isoform. To avoid time-dependent changes in Vus (25, 26), we determined it from the linear component during the initial 150 ms, and found it to be significantly (p < 0.05) faster after treatment with LC17a (0.27 ± 0.03 lengths s−1) than with LC17b (0.17 ± 0.02 lengths s−1; Fig. 8 and Table II, rows a and c). In LC17-exchanged fibers, additional incubation with recombinant, thiophosphorylated RLC significantly increased Vus, indicating that the relatively slow Vus following LC17 exchange was not due to rundown of the preparation. However, Vus under these conditions was independent of the LC17 isoform exchanged (Table II, rows b and d).

Effects of Light Chain Exchange on the Rate of Force Development—Photolysis of caged ATP was utilized to compare the relative rates of force development and circumvent diffusional delays. Following incubation of TFP-treated strips with either LC17a or LC17b in the absence of ATP, the initial rate of force development following photolytic release of ATP, determined by the time taken for 20% of the maximal force to develop (t20%), was significantly (p < 0.01) faster in LC17a-treated muscles than in the LC17b group (Table III). The maximal force generated after flash photolysis was not significantly different between the two groups.

Morphology—Electron microscopy of TFP-treated muscles viewed in transverse section showed a normal distribution of myosin filament arrays across the muscle bundle, consistent with the gels of intact and permeabilized tissues showing retention of contractile proteins with the permeabilization protocol utilized. Cell packing and outlines were normal.

DISCUSSION

The major findings of our study are that: 1) essential light chain exchange with the acidic isoform, LC17a, results in a significantly faster Vus and rate of force development by nonphosphorylated cross-bridges compared with exchange with the basic isoform, LC17b; this is the first demonstration of an effect solely attributable to LC17 isoforms in situ; 2) in the presence of thiophosphorylated RLC, shortening velocities of TFP-treated fibers are restored and this effect is independent of the type of LC17 isoform exchanged; and 3) TFP can directly affect the properties of LC17 and RLCs in vitro. The major advantage of the current approach is that it allows a direct assessment of the mechanical effects of isoform exchange within the physiological context of organized, oriented, and strained cross-bridges capable of developing force and may be a useful model system for investigating cycling of nonphosphorylated cross-bridges in situ.
Following exposure to 1 mM TFP, endogenous RLC and LC17 remained stoichiometrically associated with myosin heavy chain both in situ (Fig. 1B) and in vitro (Fig. 2). However, both light chains appeared to be modified: RLC could no longer be thiophosphorylated in situ (Fig. 4A) and the endogenous LC17 could now exchange with recombinant LC17 (Fig. 5). The refactoriness of RLC to thiophosphorylation was not due to inhibition of calmodulin by TFP (remaining in skinned fibers), because TFP also inhibited phosphorylation of isolated myosin (Fig. 4B) by a Ca<sup>2+</sup>/calmodulin-independent myosin light chain kinase (42). Similarly, direct modification of LC17 by TFP was demonstrated by its effect on the CD spectrum (Fig. 3).

Our results suggest that lower (0.1–1 mM) concentrations of TFP directly interact with LC17 and RLC, also increasing their exchangeability in situ. More prolonged treatment and/or high concentrations (e.g., 5 mM) of TFP remove light chains from the heavy chain (14, 16, 27).

We suggest that the exchange of recombinant with endogenous, TFP-modified LC17 (present study) enabled the lever arm to convert unregulated actomyosin ATPase activity into active tension, possibly by stiffening the regulatory domain of S1. Transient electrical birefringence studies of skeletal S1 suggest that essential light chain and RLC stiffen S1 (28), and removal of light chains reduces the force developed by isolated myosin molecules (29). Thiophosphorylation of RLC increases rigor stiffness of permeabilized smooth muscle, consistent with a regulatory effect of light chains on the mechanical properties of the myosin heavy chain (43).

The effects of both thiophosphorylated and nonphosphorylated LC17 isoforms on tension in Triton-permeabilized rabbit bladder strips are shown in Fig. 7. Following permeabilization and TFP treatment (Panel A), incubation with LC17 in relaxing solution (RS) induces a contraction (Panels A and B). Note that in B an additional, small contraction develops in RS before addition of LC17, and both contractions are promptly relaxed by addition of 3 mM vanadate. Incubation with nonphosphorylated RLC can inhibit the small initial force developed in RS; 50% of preparations when transferred to RS after TFP treatment (Panel C). Incubation of TFP-treated muscles with thiophosphorylated RLC induced a contraction (Panel D).

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Table II

Unloaded shortening velocity in Triton-permeabilized, TFP-treated rabbit bladder strips following exchange of light chains

After TFP treatment, muscles were treated with recombinant light chains followed by activation of force by transfer to zero Ca2+ containing solution and determination of Vus, as described under "Experimental Procedures." Rows 1 and 2, incubation in rigor solution containing LC17a or LC17b (0.75 mg/ml) for 60 min; rows 3 and 4, incubation in LC17a or LC17b, respectively, followed by incubation in rigor solution containing recombinant thiosphorylated RLC (0.7 mg/ml) for 45 min. Numbers in parentheses refer to number of data points. Rows 1 versus 2, 3 versus 4, and c versus d are significantly different, p < 0.05. Note the ability of the thiosphorylated RLC to restore Vus.

| Exchanged light chains | n | Vus (lengths · s⁻¹) |
|------------------------|---|---------------------|
| a LC17a                | 8 | 0.27 ± 0.03 (51)    |
| b LC17a-Thio-RLC       | 6 | 0.43 ± 0.03 (39)    |
| c LC17b                | 8 | 0.17 ± 0.02 (43)    |
| d LC17b-Thio-RLC       | 6 | 0.40 ± 0.04 (44)    |

Table III

Effect of LC17 isoform incubation on the kinetics of force development by nonphosphorylated cross-bridges

Rabbit bladder strips, Triton-permeabilized and TFP-treated, were incubated with LC17a or LC17b (0.75 mg/ml) for 60 min in zero Ca2⁺-rigor solution. Force development was initiated by laser flash photolysis of caged ATP as described under "Experimental Procedures."

| Exchanged light chain | n | Delay (ms) | Maximum force (mN) |
|-----------------------|---|------------|--------------------|
| LC17a                 | 5 | 32 ± 5     | 2.9 ± 0.4b         |
| LC17a                 | 7 | 39 ± 7     | 6.3 ± 5.0a         |

* t20%, time to reach 20% of maximal force.

b, c, p < 0.01.

lated recombinant RLC on force can also be explained by a process of exchange of recombinant with endogenous, TFP-modified RLC. Exchange with the thiosphorylated species would result in constitutively active actomyosin ATPase activity which correlates with the observed force generation, whereas the inhibitory effect of nonphosphorylated RLC on force is the expected result of restoration of regulation of contraction, consistent with the view that nonphosphorylated RLC is a repressor (Ref. 14 and reviewed in Ref. 30). The formation of nonphosphorylated, slowly cycling cross-bridges has also been demonstrated in permeabilized, single smooth muscle cells (16), from which calponin had been extracted; however, we did not find detectable loss of calponin from bundles of smooth muscle (present study).

The exchange of recombinant LC17b for endogenous LC17a was verified by co-immunoprecipitation and co-sedimentation with myosin heavy chains and ranged between 40 and 60%. In tonic smooth muscles, such as rabbit femoral artery and aorta and porcine aorta, expression of LC17b ranges between 43 and 58% of total LC17 content (10, 11, 31). Therefore, the level of exchange achieved in the present study was consistent with the LC17a/LC17b ratio that correlates with the lower ATPase activity and slower Vus and rates of force development by tonic smooth muscle (10, 11, 31).

The Vus is thought to be rate-limited by the dissociation of MgADP from cross-bridges (32). MgADP affinity for actomyosin cross-bridges is higher in tonic, than in phasic, smooth muscles (8, 10) and slower cycling is attributed to the slower off-rate of MgADP from cross-bridges. This greater sensitivity of tonic smooth muscle to MgADP correlates with a higher relative content of the LC17a isoform. Therefore, we suggest that the approximately 1.5-fold difference in Vus between LC17a- and LC17b-induced contractions is due to the relatively greater MgADP affinity conferred by the LC17b isoform upon these (nonphosphorylated) cross-bridges that also contain the heavy chain insert, but in which the LC17a isoform was the only variable modified in our study. Although the velocities obtained under conditions of LC17 exchange are relatively slow, this does not reflect a deterioration and rundown of the muscle fibers, since additional incubation with recombinant, thiosphorylated RLC significantly increased the Vus of LC17-exchanged fibers. Our results are in agreement with Lowey et al. (33), who showed that both essential light chain and RLC of skeletal myosin were required for propelling actin filaments at maximal sliding velocity. The absence of a difference in Vus between LC17a- and LC17b-treated fibers containing thiosphorylated recombinant RLC is consistent with the finding that complete replacement of LC17b with the LC17a isoform does not change the in vitro motility of thiosphorylated heavy meromyosin containing the 7-amino acid insert (13). However, unlike these thiosphorylated myosins, under physiological conditions, even at maximal steady-state force, RLC phosphorylation is in the range of only 20–30% or even lower in smooth muscles.

The initial rate of force development (time to reach 20% of maximal force), thought to be determined by the rate of P1, but not ADP, release (reviewed in Ref. 34), was approximately two times faster in LC17a- than LC17b-exchanged muscles. This may have been due to a difference in the rate of internal shortening affecting the rate of force development, or, because contractions were initiated from a rigor state, due to an effect of MgADP slowing the detachment of rigor bridges (20) prior to transition into force-generating states (20, 35). Essential light chain-deficient myosins have a reduced unloaded duty cycle, reflecting altered rates of myosin attachment and detachment from actin (29). It is worth noting that the rate of force development by the nonphosphorylated cross-bridges (present study) was considerably slower than the rate of force developed by phosphorylated or thiosphorylated cross-bridges in phasic smooth muscles (7). This finding is consistent with the view that: 1) nonphosphorylated cross-bridges can cycle, and 2) they do so at slower rates than phosphorylated cross-bridges.

Although results obtained from cycling cross-bridges which are either maximally thiosphorylated or nonphosphorylated could be considered "nonphysiological," a physiological latch or catch-like state of tonic smooth muscles is thought to reflect slow cycling nonphosphorylated cross-bridges (Refs. 36–38 and reviewed in Refs. 39 and 40). Since myosin in tonic smooth muscles contains a greater proportion of LC17b, has a lower Kᵣ for MgADP, and lacks the heavy chain insert, the present finding that the LC17 isoforms can modulate the Vus of nonphosphorylated, cycling cross-bridges may have implications concerning the molecular origin(s) of the latch or catch-like state. Furthermore, under most physiological conditions RLC are only partially phosphorylated, and cooperative cycling of nonphosphorylated cross-bridges under these conditions (20, 41) could also be modulated by the LC17 isoform. We propose that the coordinate expression of a particular LC17 isoform and the presence or absence of the 7-amino acid insert loop near the catalytic site of the motor domain, leads to the different affinities for MgADP and underlies the tonic (slow) or phasic (fast) contractile phenotypes of smooth muscle (1).

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