Regulation of Contraction and Thick Filament Assembly-Disassembly in Glycerinated Vertebrate Smooth Muscle Cells

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ABSTRACT   Isolated smooth muscle cells and cell fragments prepared by glycerination and subsequent homogenization will contract to one-third their normal length, provided Ca^{++} and ATP are present. Ca^{++}-independent contraction was obtained by preincubation in Ca^{++} and ATPγS, or by addition of trypsin-treated myosin light chain kinase (MLCK) that no longer requires Ca^{++} for activation. In the absence of Ca^{++}, myosin was rapidly lost from the cells upon addition of ATP. Glycerol-urea-PAGE gels showed that none of this myosin is phosphorylated. The extent of myosin loss was ATP- and pH-dependent and occurred under conditions similar to those previously reported for the in vitro disassembly of gizzard myosin filaments. Ca^{++}-dependent contraction was restored to extracted cells by addition of gizzard myosin under rigor conditions (i.e., no ATP), followed by addition of MLCK, calmodulin, Ca^{++}, and ATP. Function could also be restored by adding all these proteins in relaxing conditions (i.e., in EGTA and ATP) and then initiating contraction by Ca^{++} addition. Incubation with skeletal myosin will restore contraction, but this was not Ca^{++}-dependent unless the cells were first incubated in troponin and tropomyosin. These results strengthen the idea that contraction in glycerinated cells and presumably also in intact cells is primarily thick filament regulated via MLCK, that the myosin filaments are unstable in relaxing conditions, and that the spatial information required for cell length change is present in the thin filament-intermediate filament organization.

In the best understood motility systems (i.e., striated muscle contraction and flagellar beat) the development of a demembranated model system was an essential step in relating the biochemistry of isolated contractile proteins to their role in vivo (reviewed in references 7, 18). It is possible that a similar strategy could lead to an increased understanding of smooth muscle structure and function. Although glycerinated smooth muscle tissues and detergent-treated single cell preparations have been used for ultrastructural studies, they have not been used for biochemical investigations (15, 20, 21, 35, 36, 37). In this report we describe a simple method for preparing a glycerinated cell model from chicken gizzards, a smooth muscle that has been extensively used in this and other laboratories for biochemical studies. This cell model has the advantage that response to various physiological manipulations can be monitored by both biochemical methods and by light or electron microscopy, on the same preparation.

In vertebrate smooth muscle, there is good evidence that the regulation of actomyosin interactions involves a Ca^{++}-dependent phosphorylation of the 20,000 M_r light chains of myosin (1, 21, 37). Ca^{++} initiates actin-myosin interactions by activating a specific calmodulin-dependent kinase (2, 12) that phosphorylates the light chains. This scheme is supported by a variety of studies on vertebrate smooth muscle strips and skinned muscle fibers (5, 6, 9, 13, 17, 23). This phosphorylation scheme is not, however, universally accepted. Ebashi and co-workers (25, 26) consider that actomyosin regulation is thin filament based and that Ca^{++}-sensitive regulation is mediated by a factor called leiotonin interacting with smooth muscle tropomyosin. Several workers have suggested that both thick and thin filament regulation may be involved in smooth muscle contraction (10, 24).

Another controversy concerns the organization and stability of thick filaments in smooth muscle in different physiological states (reviewed in references 34, 37, 38). Somlyo (38), and Small and Sobieszek (37), in their detailed ultrastructural

studies on vertebrate smooth muscle, suggest that the thick and thin filaments are organised into stable "mini-sarcomeres" or "contractile units." Other ultrastructural studies, however, have implied that the myosin component of vertebrate smooth muscle exists in a labile state of organization (11, 14, 21, 27, 33). In vitro studies on vertebrate smooth muscle and nonmuscle myosin filament assembly by Watanabe and co-workers (40) and subsequently by Kendrick-Jones and co-workers (21, 22, 29, 30) have demonstrated that light chain phosphorylation may play a role in regulating thick filament assembly. These biochemical studies suggest that in relaxed smooth muscle cells, i.e., in low Ca^{++} ($<10^{-7}$ M), high ATP, and with nonphosphorylated light chains, the myosin would be present in a disassembled form. However, electron microscopy of rapidly frozen vascular smooth muscle in a relaxed state demonstrates that in this tissue thick filaments are present even though the myosin is nonphosphorylated, i.e., the light chains are completely nonphosphorylated (39). The organization and stability of myosin filaments in vertebrate smooth muscle in different physiological conditions therefore still remains to be clearly established and may vary from one type of smooth muscle to another, depending on the functional requirements of that particular muscle.

In this report we describe experiments using glycerinated chicken gizzard smooth muscle fragments. We are able to use this system (a) to confirm the role of thick filament regulation during contraction and (b) to study whether assembly-disassembly of myosin thick filaments occurs during the contraction cycle. In these experiments, we are able to monitor the following: (a) the ability of the cell model to contract (by light microscopy), and (b) the presence of myosin in the tissue (by SDS gel electrophoresis and electron microscopy), and (c) the state of phosphorylation of the myosin 20,000 M_r light chain (by glycerol gel electrophoresis). We are also able to reconstitute a functional contractile smooth muscle cell model by the addition of myosin and appropriate regulatory proteins to glycerinated cells whose myosin had been previously extracted.

MATERIALS AND METHODS

Materials and Protein Preparation: ATP and ATPγS were obtained from the Boehringer Corporation (London) Ltd., Lewes, Sussex. Trypsin inhibitor (Soyabean), trypsin (from bovine pancreas), and Nonidet P-40 (NP40) were obtained from the Sigma Chemical Co. Ltd, Poole, Dorset.

Gizzard myosin and myosin light chain kinase (MLCK)[1] were prepared as previously described (2, 22). Unregulated kinase was prepared by tryptic digestion of MLCK in the presence of bound calmodulin (3). Kinase at 0.65 mg/ml in 100 mM NaCl, 25 mM Tris HCl pH 7.5, 0.2 mM CaCl₂, 2 mM dithiothreitol with excess calmodulin was digested with trypsin at 250:1 for 1 h on ice before the digestion was terminated with a 10-fold excess of trypsin inhibitor. Calmodulin was prepared from bovine brain as described previously (30), whilst troponin and tropomyosin were prepared using the methods of Greaser et al. (16) and Bailey (4), respectively. Skeletal myosin was kindly donated by Brian Pope (MRC Laboratory of Molecular Biology).

Protein concentrations were estimated spectrophotometrically using $E^{0.1\%}_{280 nm}$ (E, extinction coefficient) for myosin of 0.56 (2) and MLCK of 1.08 (2), troponin of 0.48 and tropomyosin of 0.38 (16).

Muscle Glycerination and Cell Models: Chicken gizzards were cleaned of fat and connective tissue and sliced with a razor-blade "comb" to produce slivers of muscle 0.5 mm thick, before being immersed in ice-cold 50% glycerol, 50 mM KCl, 10 mM Na glycerophosphate, 5 mM EGTA, 10 mM 2-(N-Morpholino) ethanesulphonic acid (MES) pH 6.5, 2 mM dithiothreitol, 75 mg/liter phenylmethyl sulfonyl fluoride, 10 mg/liter soyabean trypsin

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[1] *Abbreviations used in this paper:* MLCK, myosin light chain kinase; NPM, 100 mM NaCl, 10 mM PIPES pH 7.0, 2 mM MgCl, 2 mM dithiothreitol.

inhibitor, 10 mg/liter L-1-tosylamide-2-phenyl-ethylchloromethylketone, 10 mg/liter benzyl arginylmethyl ester, 1 mg/liter leupeptin, 1 mg/liter pepstatin, 0.1% NP40, 0.1 mM ATP. Time elapsed from sacrificing the chicken to immersion in glycerination medium was <15 min. It was later found that the presence or absence of ATP and the detergent NP40 made no difference in the performance of the cell model, although the freshness of the muscle and the enzyme inhibitors did. After 24 h on ice the glycerinated muscle was stored at −20°C for 7 d before use. These preparations were stable for several months. Myosin lability was noticeably affected by prolonged storage.

We have successfully prepared functional contractile models from chicken gizzard muscles that were glycerinated in a variety of salt solutions. Provided the tissue is fresh (<15 min after removal from the chicken), proteolytic inhibitors are added to the medium, and the tissue is chopped into small pieces to facilitate rapid infusion of the glycerol medium; the composition and pH of the medium are not critical for preparation of cell models.

Cell and tissue fragment preparations for the cell model were prepared by homogenizing in glycerination medium with a Polytron (Kinematica, Luzern, Switzerland) for 4 1-s bursts and centrifuging in a bench-top centrifuge (IEC Centra 3) for 2–5 s at 2,000 rpm, and discarding the pellet of large tissue fragments. The supernatant was centrifuged for 2–5 s at 4,000 rpm and this pellet of small tissue fragments and single cells was then resuspended in 100 mM NaCl, 10 mM PIPES pH 7.0, 2 mM MgCl, 2 mM dithiothreitol (NPM medium) containing 40% glycerol. The supernatant, containing cell debris and soluble proteins, was discarded.

Myosin solubility assays used 500-μl aliquots of the cell and tissue fragment preparations. These were centrifuged at maximum rpm for 3 s in a microcentrifuge (Quickfit Instrumentation, Stone, Staffs, England), the supernatant discarded, and the pellet washed with NPM plus 20% glycerol and finally NPM alone. The pellet was solubilized in 200 μl assay medium by gentle vortexing, and drawing through an Eppendorf tip. 10% of supernatants and 5% of pellets were loaded onto the SDS PAGE gels. "Extracted" cell preparations were produced by incubating the samples in NPM plus 2 mM EGTA, 0.1 mM ATP for 5 min before extensive washing and adding back proteins in NPM plus 0.2 M NaCl (final concentration 0.3 M NaCl) and 2 mM EGTA. Samples were incubated on ice for half an hour with myosin at 0.7–1.0 mg/ml, and tropomyosin and troponin at ~0.2 mg/ml.

Gel Electrophoresis: Gel electrophoresis was carried out on SDS-10% polyacrylamide slab gels using a discontinuous 0.1 M Tris-bicine, pH 8.1 buffer system, and staining with Coomasie Brilliant Blue R250. Glycerol-urea polyacrylamide gel electrophoresis was performed using the system described by Perrie and Perry (28). For densitometry, a Joyce-Lobel Microdensitometer 3CS was used in conjunction with a tablet on a PDP 11/45 computer using a program by Terry Horsnell (MRC Laboratory of Molecular Biology) to estimate the area of peaks where necessary.

Microscopy: Microscope slides were precoated with 5 μl of 0.1 mg/ml polylysine and immediately rinsed in running deionized water. Flow-through chambers were made by placing "Parafilm" (American Can Co., Greenwich, CT) spacers between the coverslip and slide to hold about 50 μl medium and the coverslips were held in place by valap (a 1:1:1 mixture of vasoline, lanolin, and paraffin). After the cells and fragments were stuck down, they were washed through with two volumes of NPM plus 20% glycerol, followed by NPM alone. Solutions were flushed under the coverslip by using filter paper as a wick as described previously (8). Preparations were examined by phase and Nomarski optics using a Zeiss standard microscope. Tri-X film was developed with Aculux (Paterson Products Ltd., London).

Fresh and glycerinated muscle was fixed with 2.5% glutaraldehyde, 0.1 M Na cacodylate pH 7.0, 0.1 M KCl, 1 mM EGTA, 1 mM MgCl₂ overnight before being washed in buffer for 30 min, and then postfixed in 0.8% osmium tetroxide for 1 h and washed in buffer and distilled H₂O. The specimen was then block-stained in uranyl acetate for 1–½ h, washed with distilled H₂O, and dehydrated through alcohol before embedding in araldite. Sections were examined in a Phillips EM 300 electron microscope.

RESULTS

Cell Model Contraction

After glycerination and homogenization, chick gizzard cell and tissue fragments adhering to glass slides coated with polylysine contracted to one-third of their original length within ~1 min, provided ATP and Ca^{++} are present in the NPM medium (Figs. 1 and 2, *d–f*). Although the population of cell models consisted of single cells, fragments of cells, and small pieces of tissue containing ~50–100 cells, all cells and fragments in the field contracted to a similar extent (Fig. 2 *f*), provided that the concentration of polylysine used to stick

the cells to the glass slide was not above ~0.15 mg/ml.

The largest individual cells observed before contraction were several hundred microns long. After contraction, individual cells assumed a cuboid shape and the cell surfaces were
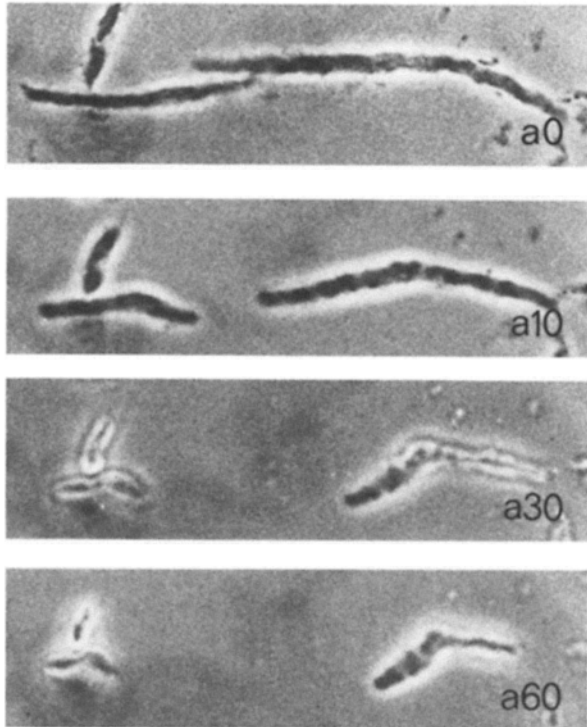

FIGURE 1 Photomicrographs showing contraction of the glycerinated cells. Sequential phase-contrast photomicrographs of the contractile response of individual smooth muscle cell fragments (a0) before and (a10) after 10 s, (a30) after 30 s and (a60) after 60 s in 0.1 mM Ca^{++}, 0.5 mM ATP. There is a threefold reduction in length. × 570.

thrown into many folds (Fig. 1). In some cells, cytoplasm appeared to be extruded into the medium during contraction and the supernatant contained aggregates of protein. Cells and cell fragments in tissue fragments underwent similar shape changes after contraction and individual contracted cells embedded in the collagen matrix gave the tissue fragment a "cobblestone" appearance (Fig. 2 f).

Addition of NPM medium and ATP in the absence of Ca^{++} did not induce relaxation (or increase in length) of partially contracted or fully contracted cells. Cell models exposed to relaxing conditions at neutral pH (NPM medium + ATP + EGTA) rapidly lost their ability to contract if subsequently challenged with Ca^{++} (Fig. 2, a–c). Addition of calmodulin and MLCK did not restore Ca^{++}-dependent contraction to these cells. However, at pH 6.5, the contractile machinery was more stable and cells contracted upon subsequent Ca^{++} addition even though they were exposed to EGTA and ATP for several minutes (Fig. 2, d–f). Exposure of cells to NPM medium plus EGTA or Ca^{++} in the absence of ATP did not lead to loss of ability to contract when ATP was subsequently added to the coverslip preparation. As we will demonstrate later, the labile component of the contractile machinery appeared to be myosin and its regulatory proteins (see Fig. 4).

The ultrastructure of fresh and glycerinated gizzard smooth muscle cells was very similar (see Fig. 7 a; unpublished data). Although membranes and membrane-bound organelles were distorted by storage in 50% glycerol for several weeks, thin, thick, and intermediate filaments and dense bodies were present in both preparations.

We noticed that the cell and tissue fragments after glycerination contained less tropomyosin than that assumed to be present in vivo, i.e., ~3:1 actin/tropomyosin molar ratio (see Fig. 4, lane Ap, vs. reference 37). It has been suggested that high levels of Mg^{++} favors tropomyosin binding to actin. Glycerination in a medium with varying Mg^{++} levels up to 10 mM, however, did not alter the level of tropomyosin found
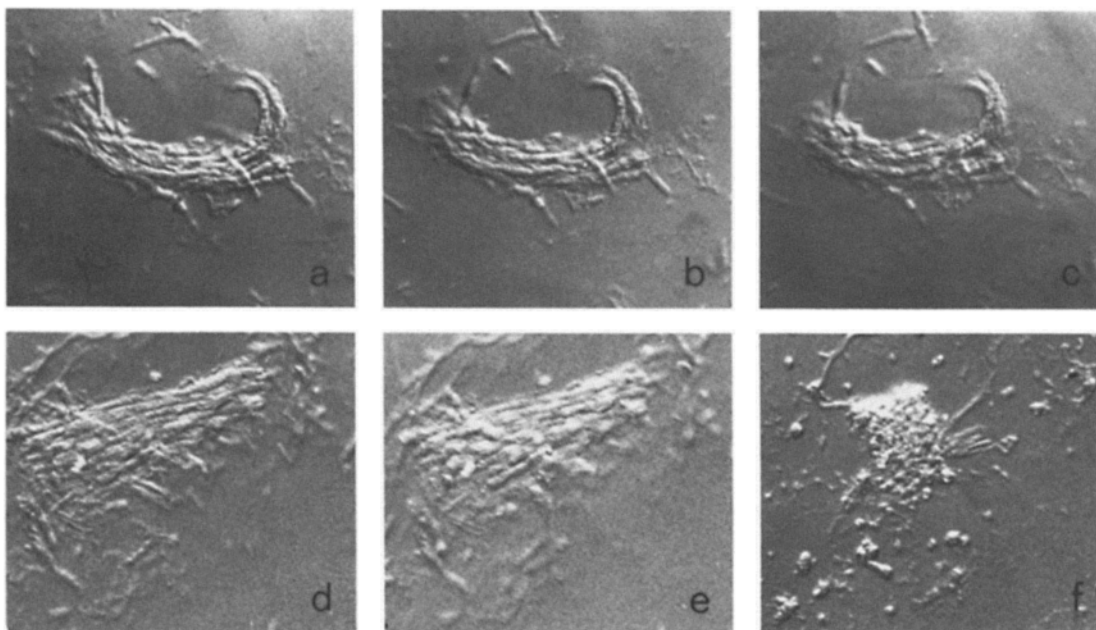

FIGURE 2 Contractile activity is lost after incubation in relaxing conditions. Cell preparations after mounting in flow-through chambers (a) and washing with 2 mM EGTA and 0.5 mM ATP at pH 7 (b) do not contract upon addition of 0.1 mM Ca^{++}, 0.5 mM ATP (c). If the preparation (d) is instead washed with 2 mM EGTA and 0.5 mM ATP at pH 6.5 (e) then contraction does occur on addition of 0.1 mM Ca^{++}, 0.5 mM ATP (f). × 220.

in this cell model. Cell preparations glycerinated in the presence of 2 mM Mg^{++} behaved the same as cells glycerinated in the absence of Mg^{++}.

Regulation of Contraction

To determine whether contraction in the cell preparations was regulated by myosin light chain kinase or by other regulatory systems such as troponin-tropomyosin, we studied the effects of ATPγS incubation on Ca^{++}-dependent contraction. As previously demonstrated on chemically skinned smooth muscle (9, 17), incubation of the cell models in ATPγS and Ca^{++} induces Ca^{++}-independent contractility (Fig. 3, a–b). An alternative method of obtaining Ca^{++}-independent contraction is to incubate cell models in unregulated MLCK (Fig. 3, c–d). After trypsin digestion (as described in Materials and

Methods) MLCK activity in the absence of calcium was elevated to the level observed in the presence of calcium before digestion, presumably because the regulatory site has been removed from the enzyme by proteolysis (3, 41, 42). This unregulated kinase triggers contraction in the cell model in the presence of EGTA overriding the endogenous regulatory system. However, it was necessary to run this experiment at pH 6.5, presumably to keep the contractile machinery stabilized during the incubation period.

We found that smooth muscle cell models will not contract at low ATP concentrations (~25 μM ATP), even though Ca^{++} was present (Fig. 3, e–f). This is in marked contrast to the Ca^{++}/ATP-dependent contraction observed in glycerinated skeletal myofibrils, which will occur in ATP concentrations as low as 10 μM. It is possible to overcome the high ATP requirement for contraction by preincubation in ATPγS
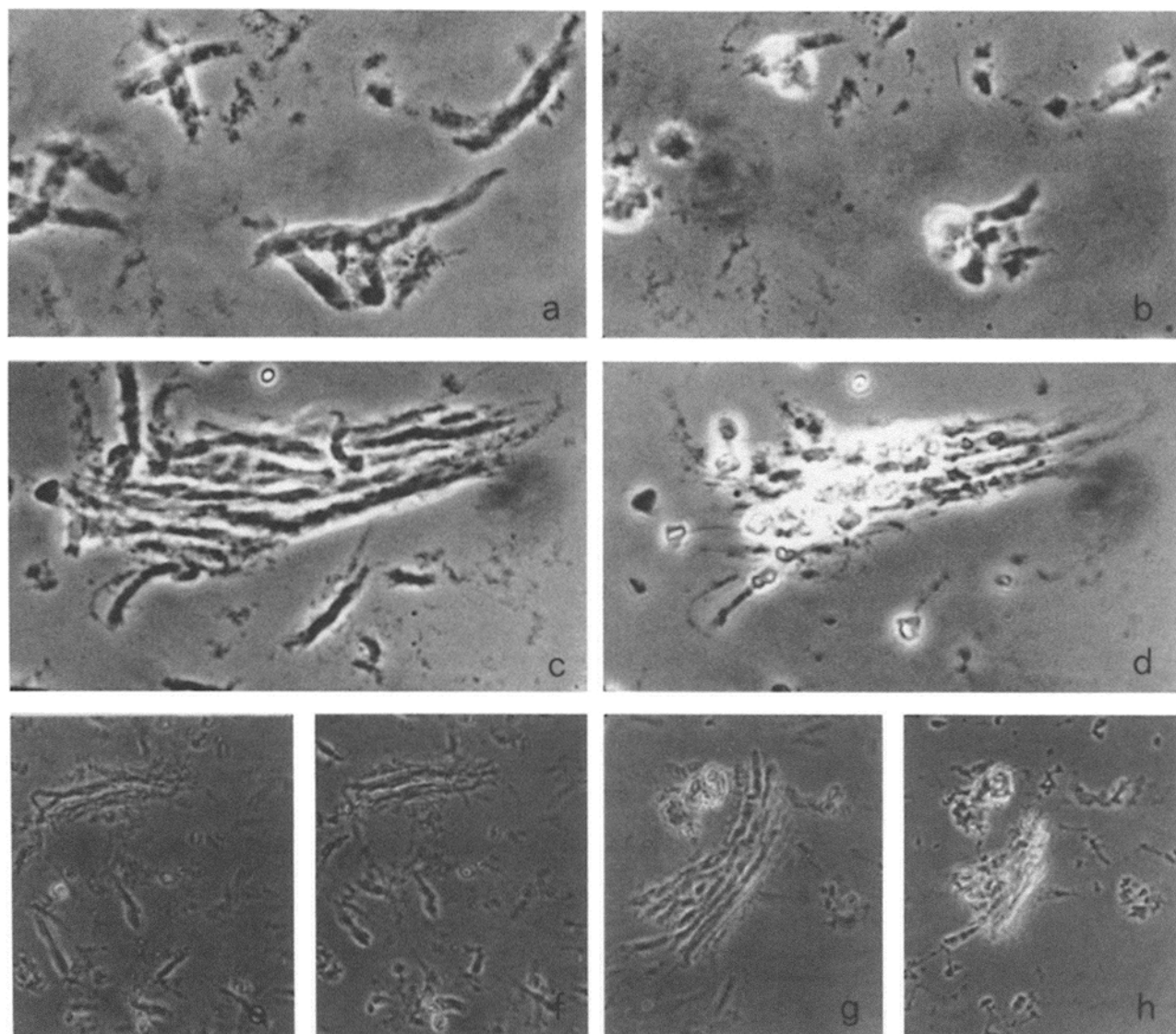

FIGURE 3   Phase-contrast photomicrographs demonstrating the role of myosin light chain phosphorylation during contraction. Cell preparations preincubated in NPM pH 7.0, 0.1 mM Ca^{++}, 0.5 mM ATPγS (a) do not contract but the addition of 2 mM EGTA, 0.5 mM ATP (b) will result in contraction. If unregulated MLCK is preincubated with the cell preparation (c), then Ca^{++} is not a requirement for contraction. Such cells contract upon addition of 2 mM EGTA, 0.5 mM ATP in NPM medium, pH 6.5 (d). Photomicrographs of cell fragments before (e) and after (f) addition of 0.1 mM Ca^{++}, 10 μM ATP, NPM pH 7.0 show no contraction. If the cell preparation is first preincubated with 0.1 mM Ca^{++}, 0.5 mM ATPγS (g), subsequent addition of 10 μM ATP (h) will trigger contraction. All preincubations were carried out in NPM in the flow-through chamber on the microscope slide. a–d, × 570. e–h, × 220.

and Ca^{++} (Fig. 3, g–h). This result demonstrated that the step that requires elevated ATP levels is the phosphorylation of myosin light chains.

Regulation of Myosin Disassembly

SDS PAGE gels of glycerinated chick gizzard cells demonstrated that the most abundant proteins present in these preparations are myosin, the 65-kdalton protein desmin, which is the major subunit of the intermediate filaments of this type of smooth muscle (37), actin, and tropomyosin (Fig. 4, lane Ap; Table I). Extensive washing of cell and tissue suspensions with NPM medium ± Ca^{++} or with Ca^{++} and ATPγS (not hydrolyzed by myosin) (Fig. 4, lanes A–B; Table I, and unpublished data) did not lead to extraction of these components and their appearance in the supernatant fraction, with the exception of actin.

As described in Fig. 2, addition of ATP in the absence of Ca^{++} leads to a loss of contractile activity when the cell models were subsequently challenged with Ca^{++}. This loss of contractility is pH dependent. After addition of ATP and EGTA to cell suspensions, myosin and actin were extracted but the intermediate filament protein remained with the cell pellet fraction (Fig. 4, lanes C–H). The levels of solubilized actin are independent of pH, but the level of extracted myosin increases with the increase in pH over the range from pH 6.2–7.0 (Table I). At pH 7, 86% of the myosin was extracted into the supernatant, but at pH 6.2 only 38% was extracted. We have previously examined the effects of pH on in vitro myosin thick filament disassembly, as monitored by turbidity and confirmed by electron microscopy (22, 29). We repeated these experiments using chick gizzard myosin in NPM medium. At pH 7.0, thick filament disassembly occurred rapidly and was essentially complete within minutes, but at pH 6.5 and pH 6.2 little or no disassembly occurred (unpublished data).

The extent of myosin solubilization that occurs in the cell

suspensions is also dependent on the ATP concentration in the suspension medium, and the greatest concentration of myosin extracted (82%) occurs in the highest ATP levels used (Fig. 4, lanes F–H; Table I). Approximately one-half of the myosin was extracted even at ATP levels that will not support contraction (10 μM ATP, Fig. 4, lane F, Table I) and this

TABLE I

Densitometry of Major Peaks in Fig. 4

		Myosin	Percent myosin in supernatant	Intermediate filaments	Actin	Percent actin in supernatant
A	p	3.3*		1.0	4.3	
	s	—		—	0.1	
B	p	5.6		2.4	6.1	
	s	0.6	10	0.1	2.3	38
C	p	2.1		0.8	2.0	
	s	1.3	38	—	1.8	47
D	p	1.3		0.7	1.9	
	s	2.7	68	—	2.5	57
E	p	0.8		0.8	2.1	
	s	4.8	86	—	2.2	51
F	p	3.4		2.7	5.4	
	s	4.3	56	—	3.1	37
G	p	2.5		3.4	6.5	
	s	5.9	70	—	4.5	41
H	p	1.3		2.6	4.9	
	s	6.1	82	—	4.7	49
I	p	3.0		1.9	5.5	
	s	2.7	47	—	1.7	24
J	p	4.3		3.3	6.4	
	s	2.4	36	—	3.5	35
K	p	2.5		1.3	3.3	
	s	2.4	49	—	4.5	56

* Peak areas in square centimeters.

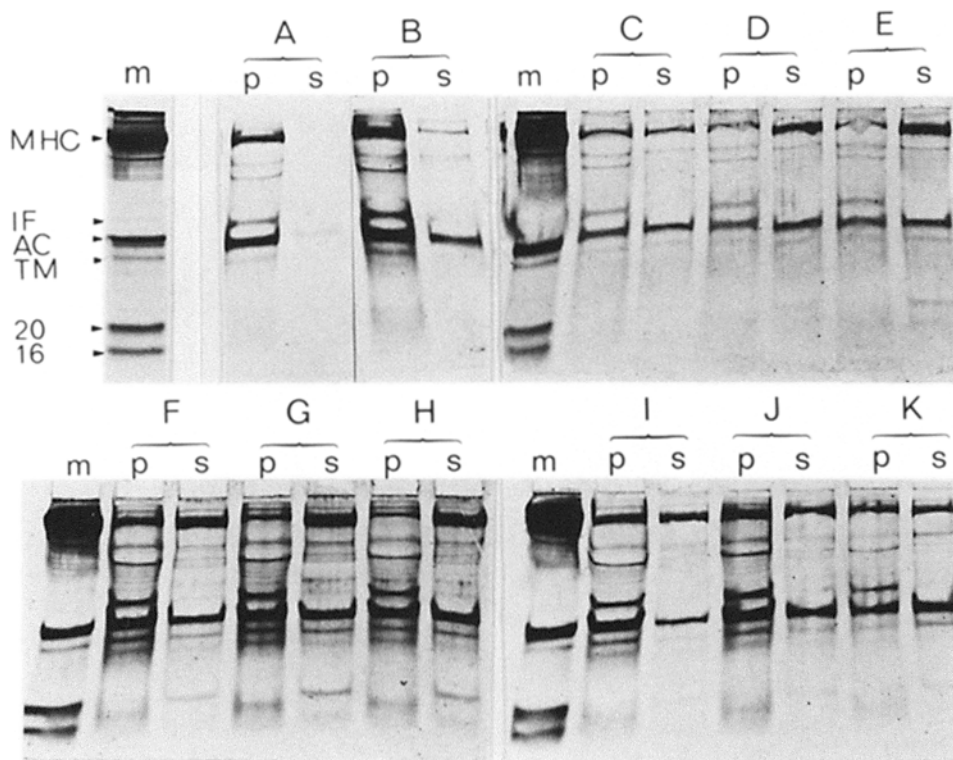FIGURE 4 Effect of pH and myosin light chain phosphorylation on the stability of the myosin in cell preparations. SDS PAGE of pellets (p) and supernatants (s) from the myosin solubility series after preincubation in NPM pH 7.0 and: (A) 2 mM EGTA; (B) 0.1 mM Ca^{++}, 0.5 mM ATPγS; (C) 2 mM EGTA, 0.5 mM ATP, 10 mM MES, final pH = 6.2; (D) 2 mM EGTA, 0.5 mM ATP, 10 mM MES, final pH = 6.5; (E) 2 mM EGTA, 0.5 mM ATP, pH 7.0; (F) 2 mM EGTA, 10 μM ATP; (G) 2 mM EGTA, 100 μM ATP; (H) 2 mM EGTA, 1 mM ATP; (I) 0.1 mM Ca^{++}, 10 μM ATP; (J) 0.1 mM Ca^{++}, 1 mM ATP; (K) 0.1 mM Ca^{++}, 0.5 mM ATPγS, followed by 0.1 mM Ca^{++}, 1 mM ATP. Actomyosin standards (m) included for reference; MHC, myosin heavy chain; IF, intermediate filament protein; AC, actin; TM, tropomyosin; 20-kdalton (20) and 16-kdalton (16) myosin light chains.

level of extraction occurred regardless of whether Ca^{++} was present in the suspension medium (Fig. 4, lane I, and Table I).

Under contraction conditions, as much actin as myosin was released into the supernatant (Fig. 4, lane J) and the level of myosin released (36%) was considerably less than at comparable nucleotide levels in the absence of Ca^{++} (82%, Fig. 4, lane H; Table I). The level of myosin released does not depend on the ATP concentration in the contraction medium (unpublished data) nor does it depend on whether the cell preparation has been incubated in ATPγS before ATP addition (Fig. 4, lane K; Table I). Very little myosin was released into the supernatant when only ATPγS and Ca^{++} were present (Fig. 4, lane B; Table I).

The extent of phosphorylation of myosin 20,000 M_r light chains was monitored by urea-glycerol gel electrophoresis (Fig. 5). During these experiments we discovered that there was residual phosphatase activity in the pellet fraction that was not destroyed by the trichloroacetic acid and 8 M urea treatment (unpublished data). This activity, in the absence of ATPγS (Fig. 5, lanes A–D), may have reduced the level of phosphorylated myosin in the pellet fraction in some of these experiments. <10% of the myosin was phosphorylated in the initial glycerinated cell preparations, and after incubation in EGTA, ATP ± ATPγS, this level of phosphorylation was unchanged (Fig. 5, lanes B and F). The myosin light chains were fully phosphorylated when incubated in the presence of Ca^{++}, ATPγS, and ± ATP (Fig. 5, lanes D and G). When these experiments were performed in the absence of ATPγS the levels of phosphorylation observed in the pellet fractions were significantly less (Fig. 5, lane C), due to the presence of the residual phosphatase activity. Phosphorylated myosin was lost to the supernatant after contraction (Fig. 5, lanes C and G), however these supernatants contained protein aggregates that were not pelleted by the low speed centrifugations used in these experiments. No phosphorylated myosin was lost to the supernatant in lane D with only Ca^{++} and ATPγS addition and in this condition no contraction took place. In these experiments, as in the experiments described in Fig. 4 and Table I, more myosin was released into the supernatant in the absence of Ca^{++} than in the presence of Ca^{++} (Fig. 5, lanes B and F vs. C, D, and G).

Supernatants from these experiments were examined by negative staining in the electron microscope for the presence of thick filaments and by dark field light microscopy. Although collagen fibers, thin filaments, and aggregates of protein were found in these preparations, no myosin thick filaments were found. The supernatants from suspensions washed with ATP and Ca^{++} were turbid and contained large clumps of aggregated protein. The supernatants of cells washed in ATP and EGTA were clear and upon Ca^{++} addition, these supernatants rapidly turned cloudy and large clumps of aggregated protein were visible to the naked eye and by dark field microscopy.

Reconstitution of Contractile Cell Models by Addition of Myosin

Using cells whose myosin was extracted by ATP in the absence of Ca^{++}, we have investigated the conditions required for restoration of contraction. Two very different strategies were used successfully in the myosin add-back experiments, i.e., addition under rigor conditions or addition under relaxing
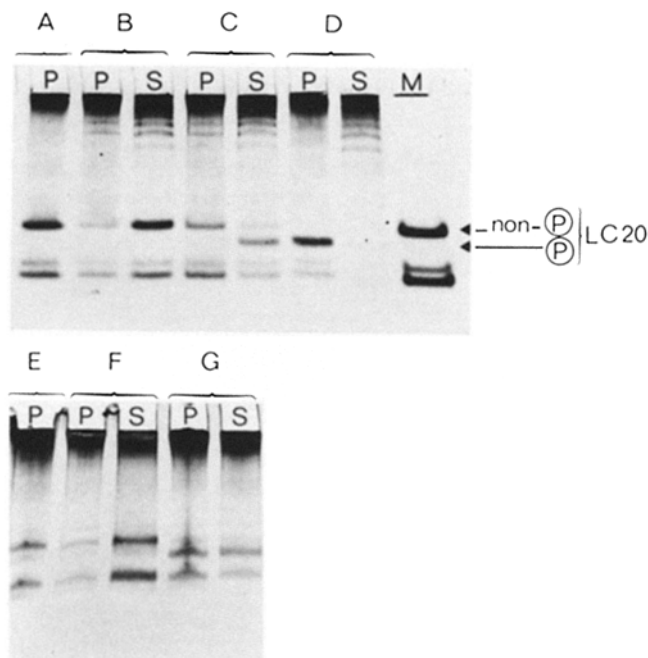

FIGURE 5 Urea/glycerol gel analysis of the level of phosphorylation of the 20,000 M_r light chain of the myosin in pellet (p) and supernatant (s) fractions of the glycerinated gizzard muscle cells. The samples were preincubated before centrifugation in: (A) NPM pH 7.0 and 2 mM EGTA; (B) NPM, 2 mM EGTA, and 1 mM ATP; (C) NPM, 0.5 mM Ca^{++} and 1 mM ATP; (D) NPM, 0.5 mM Ca^{++} and 1 mM ATPγS; (M) purified gizzard myosin; (E) NPM and 2 mM EGTA; (F) NPM, 2 mM EGTA, 0.5 mM ATP and 0.5 mM ATPγS; (G) NPM, 0.5 mM Ca^{++}, 0.5 mM ATP and 0.5 mM ATPγS. After centrifugation the supernatants were removed and the pellets resuspended in the initial solutions outlined above. The pellet and supernatant fractions were processed for electrophoresis by precipitation with a final concentration of 3% trichloroacetic acid (wt/vol) and the precipitated proteins centrifuged and washed with cold acetone. They were "dissolved" in 9 M urea, 20 mM Tris, 122 mM glycine buffer pH 8.6 solution and run on 40% glycerol, 10% polyacrylamide gels (28). Non-Ⓟ LC 20 indicates position of the nonphosphorylated M_r 20,000 light chain and Ⓟ LC 20 its position when phosphorylated.

conditions (Fig. 6; Table II). In neither case was it necessary to add back actin for restoration of contraction. First, cells were incubated in the absence of ATP with skeletal or gizzard myosin kept in a nonfilamentous state by elevated salt (0.3 M NaCl). Excess myosin was removed by extensive washing of the cell pellets with NPM medium without ATP. Myosin in vast excess was retained in these pellets (Fig. 6, gel), presumably by the formation of rigor bonds with the actin in the cell models. Cells incubated in gizzard myosin alone would not contract upon Ca^{++} and ATP addition unless MLCKinase and calmodulin were added at the same time (Fig. 6a and b; Table II). As described previously (Fig. 3), when the cells were first incubated in ATPγS and Ca^{++}, before ATP addition, contraction was independent of Ca^{++}.

Electron microscopy of cell pellets treated with EGTA and ATP show that the thick filaments that were visible in cells bathed solely in NPM medium (Fig. 7a) are no longer present after 5 min exposure to this relaxing medium (Fig. 7b). However, thin and intermediate filaments were still present in extended arrays. After addition of myosin in high salt and extensive washing, no obvious decoration of thin filaments
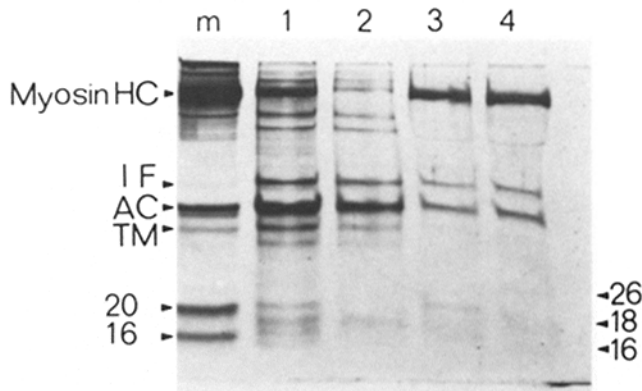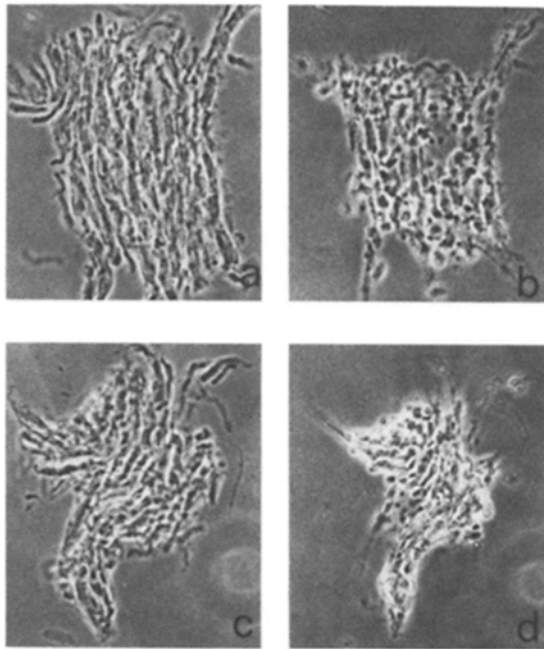
FIGURE 6 The restoration of the ability of "extracted" cell preparations to contract by addition of native and skeletal myosins and regulatory proteins. The cell preparations were "extracted" by 5-min exposure to 2 mM EGTA, 0.5 mM ATP, NPM pH 7.0. When 1 mg/ml gizzard myosin in NPM + 0.2 M NaCl is added back (a), followed by subsequent washings in NPM as described in the text, contraction occurs upon addition of 0.1 mM Ca^{++}, 0.5 mM ATP, 5 μg/ml MLCK, and 50 μg/ml calmodulin (b). If ~1 mg/ml rabbit skeletal myosin in NPM + 0.2 mM NaCl is added to "extracted cell preparations" (c) as described in the text, calcium-independent contraction will occur in 2 mM EGTA, 0.5 mM ATP, NPM pH 7.0 (d). SDS-10% polyacrylamide gels of (1) initial cell preparation in NPM pH 7.0; (2) the cell preparation after "extraction" with NPM pH 7.0, 2 mM EGTA, and 0.5 mM ATP and washing in the same medium; (3) the "extracted" preparations after incubation with gizzard myosin and subsequent washing with NPM pH 7.0; (4) the "extracted" cell preparation incubated with rabbit skeletal myosin and washed with NPM pH 7.0; m, "impure" gizzard myosin standard. Myosin HC, myosin heavy chain; IF, intermediate filament protein; AC, actin; TM, tropomyosin; 20, M_r 20,000 myosin light chain; and 16, M_r 16,000 myosin light chain. The faint bands at 26,000, 18,000, and 16,000 M_r are the skeletal myosin light chains. (a–d) × 220.

was observed, although the arrangement of thin filaments appeared to be more clumped and some short thick filament-like structures may be present (Fig. 7c). After addition of Ca^{++}, calmodulin, MLCK, and ATP, the thin and interme-

diate filaments were highly disordered and large swirls of filaments were present (Fig. 7d). Even at this stage, few thick filament-like structures could be observed.

Cells incubated in skeletal myosin will contract without addition of calmodulin and MLCK, but this contraction was not Ca^{++} dependent (Fig. 6c and d; Table II). In a limited number of experiments, Ca^{++}-dependent contraction was obtained with skeletal myosin when cells were incubated with skeletal troponin and tropomyosin before addition of myosin (Table II). Unlike the original cell preparations, these cell models did not lose their contractile activity upon incubation in ATP in the absence of Ca^{++}.

An alternative method for restoring contractile function was to incubate cell models in excess gizzard myosin in the presence of ATP and EGTA (Table II). As described previously (22) when the myosin is nonphosphorylated, no myosin thick filament assembly occurs under these conditions even when MLCK and calmodulin are present in the incubation medium. Contraction is initiated in these preparations upon addition of excess Ca^{++} (Table II). Cells did not contract when either calmodulin or MLCK were omitted from the incubation medium.

DISCUSSION

The glycerinated cell model described here provides a useful alternative to the preparations of smooth muscle contractile proteins used in biochemical studies. Unlike studies performed on highly purified proteins, in the glycerinated cells we may have preserved regulatory systems of muscle structure or function that are lost or altered during normal biochemical preparative procedures. In addition, by using glycerinated cells we are able to introduce proteins into cells, an option not available in physiological studies using intact muscle strips. We have used a simple light microscopy assay of cell length change to monitor maintenance of muscle function and SDS-PAGE gel electrophoresis and electron microscopy to assay maintenance of muscle structure. Our studies using glycerinated cells confirm the importance of myosin light chain phosphorylation in the regulation of Ca^{++}-dependent contraction and demonstrate the lability of myosin thick filaments under defined but physiologically relevant conditions, i.e., under relaxing conditions. What is striking about our results is how well they conform to previous biochemical studies, from this and other laboratories, on regulation of thick filament assembly-disassembly and interaction with actin by myosin light chain phosphorylation (see reviews 1, 21, 37).

The studies using glycerinated cells demonstrate that myosin light chain phosphorylation is the primary and perhaps the only step required for Ca^{++}-dependent regulation of contraction. We can override the normal Ca^{++} requirement for initiation of contraction either by the irreversible thiophosphorylation of myosin done in the presence of ATPγS by endogenous MLCK (9, 17, 31) or by the addition of an unregulated MLCK that no longer requires Ca^{++} for activation (3, 41, 42). The central role of myosin light chain phosphorylation is also demonstrated by the relatively high ATP requirement for contraction, which mirrors the low affinity of MLCK for ATP (50 μM, reference 2). These results are inconsistent with the possibility that an inhibitory thin filament regulatory system such as the tropomyosin-troponin system found in skeletal muscle is involved in Ca^{++}-dependent contraction in smooth muscle. However, our assay of smooth
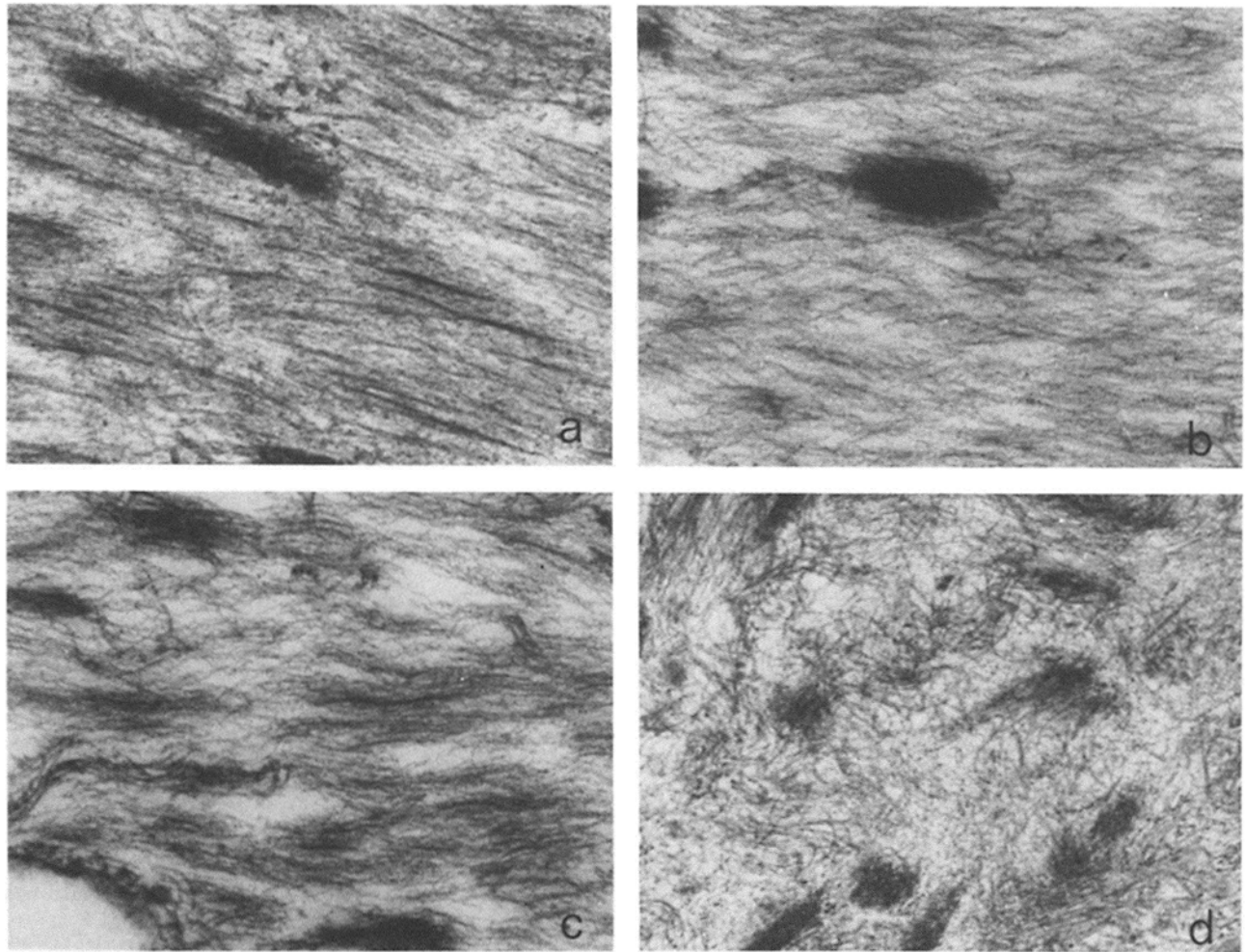
FIGURE 7   Electron micrographs of cell pellets from a myosin reconstitution experiment. (a) Cell fragments in NPM pH 7.0, 2 mM EGTA contain thick filaments. (b) Cell fragments after 5-min incubation in NPM, 2 mM EGTA, 0.5 mM ATP, followed by washes in NPM do not contain thick filaments although thin and intermediate filaments are present. (c) Cell fragments after incubation in ~1 mg/ml gizzard myosin in NPM, 0.2 M NaCl for 30 min. Several small thick filament-like structures are visible (lower right) but the thin filaments do not look decorated. (d) After addition of 5 μg/ml MLCK, calmodulin, 0.1 mM Ca^{++}, and 0.5 mM ATP, cell fragments contract as described in Fig. 6 a–b, and thin filaments and intermediate filaments are thrown into large swirls. No thick filaments are apparent in this section. × ~40,000.

muscle function, i.e., cell length change measures contractility rather than development of tension. This assay may not be sensitive enough to detect the presence of a positive regulatory mechanism such as the leiotonin system postulated by Ebashi and co-workers (25, 26), which would potentiate actomyosin interactions, or the presence of noncycling cross bridges (latch bridges) as postulated by Dillon et al. (13). Nevertheless, such systems, if present, would supplement but could not replace the MLCK regulation of contraction that we observe here.

We cannot eliminate the possibility that some thin filament regulatory proteins are lost during the glycerination process, especially since SDS gel electrophoresis demonstrates that there is less tropomyosin present in glycerinated cells than in whole muscle extracts (37). However, we think this possibility is unlikely for the following reasons: (a) the ultrastructure of glycerinated smooth muscle is similar to fresh tissue; (b) glycerination under conditions that should preserve tropo-myosin binding to actin filaments (i.e., in 2 mM Mg^{++}) does not alter the contractile properties of the glycerinated cells; (c) our results using ATPγS and unregulated MLCK are identical to those of Cassidy et al. (9) and Hoar et al. (17),

who used chemically skinned smooth muscle to investigate the role of myosin light chain phosphorylation in regulation of contraction, and those of Walsh et al. (41, 42) who used unregulated MLCK to trigger Ca^{++}-independent contraction in chemically skinned gizzard strips.

Although many studies of smooth muscle ultrastructure demonstrated that the state of myosin filament assembly changes with the contractile activity of the smooth muscle (11, 14, 19, 20, 27, 32, 33), several recent studies suggest that myosin may be organized into stable filaments arranged in "mini-sarcomeres" with actin (37, 38). However, biochemical studies on myosin assembly-disassembly from Watanabe's and our laboratories have shown that ATP binding to non-phosphorylated gizzard myosin promotes rapid filament disassembly and that phosphorylation of the myosin light chains promotes thick filament assembly (21, 22, 29, 30, 40). Our observations on myosin lability in glycerinated cells upon ATP addition in the absence of Ca^{++} support the claim that the in vitro studies of myosin stability may be of physiological significance. In cells glycerinated in the absence of divalent cations and at pH 6.5 thick filaments are present. Since these

Treatment	Percent cell or tissue length decrease*	
	2 mM EGTA/0.5 mM ATP	0.1 mM Ca^{++}/0.5 mM ATP
Extracted cells	0	0
Calmodulin, MLCK	0	0
Gizzard myosin	0	0
Calmodulin, MLCK, gizzard myosin	0	59
Rabbit skeletal myosin	46	40
Rabbit skeletal myosin, troponin, tropomyosin	0	43
‡ Calmodulin, MLCK, gizzard myosin, in relaxing medium	0	contracted

Cells were extracted by 5-min exposure to 2 mM EGTA, 0.5 mM ATP, NPM pH 7.0. In b-f myosin (~1 mg/ml) and regulatory proteins (see text and Fig. 6) were added back in NPM pH 7.0, + 0.2 M NaCl.

* Average of at least six cells taken from at least two separate experiments.

‡ 1 mg/ml gizzard myosin was added back in 2 mM EGTA, 0.5 mM ATP, NPM pH 7.0. After centrifugation, the pellet fraction was resuspended in 5 µg/ml MLCK, 50 µg/ml calmodulin, 0.5 mM ATP, NPM pH 7.0, and contraction was triggered by addition of 0.1 mM Ca^{++}.

are unphosphorylated, they are presumably held together and in place in these nonphysiological conditions by rigor bonds. After introduction of MgATP in the absence of Ca^{++} (i.e., relaxing conditions), the thick filaments fall apart and are readily extracted. The pH and ATP dependence of myosin stability under these conditions exactly parallels that observed in vitro when ATP is added to nonphosphorylated thick filaments (22). The loss of myosin observed after contraction (Ca^{++} and ATP) does not show the same ATP dependence and does not depend on the phosphorylation state of the myosin. Circumstantial evidence suggests that this myosin is "squeezed" out of the contracting cells since large protein aggregates are observed in the cloudy supernatants after contraction. Since the myosin present in thick filaments in glycerinated cells has not been exposed to cycles of polymerization-depolymerization by exposure to high/low salt, we have thus eliminated the possibility that the in vitro lability of myosin thick filaments is due to the loss of a special stabilizing or "capping" protein that is not retained during purification of smooth muscle myosin by cycles of filament assembly-disassembly.

How then can one explain the recent demonstration that myosin with nonphosphorylated light chains exists in filaments in relaxed vertebrate smooth muscle (39)? One possibility is that actomyosin interactions even in the absence of light chain phosphorylation stabilize the thick filament to some extent. A second possibility is that dephosphorylated thick filaments may be in equilibrium with a monomer pool of myosin. Unlike the intact cell, in the cell model monomers would diffuse out of the cell in relaxing conditions driving disassembly of thick filaments. A third possibility is that the conditions that we have defined in vitro and with our glycerinated cells as relaxed (high ATP in the absence of calcium) do not correspond exactly to any physiological state normally

observed in smooth muscle, even in the absence of any apparent tension generation. That is, in some vertebrate smooth muscles some tension may always be maintained and hence some thick filaments are always present. Finally it is possible that myosins from different vertebrate smooth muscles have different stability properties. However, preliminary observations on glycerinated rabbit uterus and uterine myosin, and glycerinated guinea pig taena coli and taena coli myosin suggest that the contractile and myosin thick filament lability properties of these other smooth muscles are similar to those described here for gizzard muscle (W. Z. Cande, P. J. Tooth, and J. Kendrick-Jones, unpublished data).

One possible objection to the physiological relevance of the thick filament lability observed in vitro is that stable thick filaments may be required for repeated cycles of contraction and relaxation of smooth muscle cells. However, the myosin add-back experiments demonstrate that all of the spatial information required for cell length change during contraction is present in the thin filament-intermediate filament organization. After extraction of the myosin thick filaments, the other filament arrays, at least at an ultrastructural level, do not appear to be greatly disturbed. It is unlikely that the thick filaments formed during the reconstitution experiments are similar in structure to those observed in normal or glycerinated cells, especially since they are not very apparent in electron micrographs. By definition this must be true in those experiments using skeletal myosin. It may even be that thick filaments as small as myosin dimers may be responsible for some of the cell length change. However, this appears to make little difference since contraction similar in extent occurs after reconstitution, regardless of whether myosin is added back under either rigor or relaxation conditions. Therefore the actin thin filaments in glycerinated cells (and also maybe in vivo) must be arranged such that contraction can occur as soon as thick filaments are formed and activated by light chain phosphorylation.

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