Contraction of Stress Fibers Extracted from Smooth Muscle Cells: Effects of Varying Ionic Strength*

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Abstract

Actin stress fibers (SFs) play a key role in regulation of cell adhesion, but the biochemical and biophysical properties intrinsic to SFs remain unclear. Here we extracted SFs from rat embryonic smooth muscle cells by deroofing, and evaluated the effects of varying ionic strength and temperature on their intactness. Wash buffers with ionic strength ranging from 90 to 490 mM were prepared, and the extracted SFs were incubated in a buffer with a particular ionic strength for 10 min or 24 h. Light and electron microscopy revealed that the extracted SFs comprised tightly packed straight bundles at low ionic strengths that became looser and exhibited a ragged pattern at high ionic strengths. The expression of α -actinin associated with the extracted SFs decreased with the increase in ionic strength. Unexpectedly, non-muscle myosin II and smooth muscle myosin in the extracted SFs displayed comparable expression levels over the different ionic strengths. ATP-induced contractility was better preserved at low ionic strengths, including the physiological ionic strength of 170 mM. The rate of ATP-induced enzymatic activity increased with increase in temperature, but the difference was not statistically significant. These results demonstrate that low ionic strength produces extracted SFs that are more intact with regard to structure and function.

Key words: Stress Fibers, Smooth Muscle Cells, Ionic Strength, Contractility

1. Introduction

Stress fibers (SFs) play a key role in regulation of cell adhesion^(1,2) and tensional homeostasis^(2,3). Myosin II (MII), the main constituent of SFs, is considered to be essential to their function. Characterization of the biochemical and biophysical properties of SFs is important with a focus on the involvement of MII activity.

Samples obtained by homogenization of cells into fragments may obscure properties specific to SFs. To overcome this problem, Katoh et al.⁽⁵⁾ developed a method for extracting SFs from cultured cells. In this study, ATP-induced contraction of extracted SFs, driven by the actin–MII interaction, was demonstrated. Extracted SFs have been used by our group to characterize their tensile properties^(6–8). Phosphate buffer solutions are generally used in the extraction procedure to wash the samples. The ionic strength of the buffers used is typically within the physiological range (i.e., ~150 mM). Ionic strength, which is a function of the concentration of all ions present, generally affects protein

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function and stability; however, its effect on extracted SFs has not been investigated in previous studies.

In our earlier studies, we modified Katoh's method and prepared alternative wash buffers using imidazole^(9–11). Imidazole is a cationic buffer used within the physiological intracellular pH range. The aim of the present study was to investigate the effect of an imidazole-based buffer when the ionic strength was changed from 90 to 490 mM. Since enzymatic activities such as the ATP-consuming contractile process are generally dependent on temperature, the rate of ATP-induced contraction was measured at different temperatures. Our results demonstrated that the use of buffers with low ionic strengths produces SFs that are more intact with regard to structure and function.

2. Materials and Methods

2.1 Cells and solutions

A7r5 rat embryonic aortic smooth muscle cell lines (ATCC, CRL-1444) were cultured in DMEM (Invitrogen) supplemented with 10% FBS (JRH Biosciences) and 1% penicillin–streptomycin (Invitrogen) in a 5% CO₂ incubator at 37°C. TEA solution used for extraction of SFs contained 2.5 mM triethanolamine (Wako), 1 mM DTT, 1 µg/ml leupeptin, and 1 µg/ml pepstatin (Peptide Institute). Wash buffers containing 20 mM imidazole (MP Biomedicals), 2 mM EGTA (Dojindo), and 2.2 mM MgCl₂ (pMg 2.7) were adjusted to an ionic strength of 90, 100, 123, 170, 250, 330, 410, or 490 mM at pH 7.2 by the addition of KCl and HCl. The activation buffer containing 20 mM imidazole, 2 mM EGTA, 2.0 mM MgCl₂ (pMg 2.7), 1 mM MgATP (Wako), and 2.0 mM CaCl₂ (pCa 4.5) was adjusted to an ionic strength of 94 mM at pH 7.1 by the addition of KCl and HCl. These different ionic strengths cannot be accurately achieved by simply adding water to the solutions. The buffers were prepared considering all the metal ions and ligands present and their equilibrium constants described in Fabiato and Fabiato⁽¹²⁾.

2.2 Extraction of SFs and light microscopy

Ventral SFs were extracted from A7r5 cells based on a previously described method⁽⁹⁾. In brief, cells were washed twice with cold PBS and deroofed by repeated exposure to ice-cold TEA solution. The extracted SFs were incubated in wash buffer with an ionic strength of 90, 100, 123, 170, 250, 330, 410, or 490 mM at 4°C for 24 h. F-actin in the extracted SFs was stained with 3 nM Alexa 488–phalloidin for 1 min and observed under a microscope (IX-71; Olympus) equipped with a camera (ORCA-R2; Hamamatsu Photonics).

2.3 Electron microscopy

For negative staining electron microscopy (EM), the extracted SFs were incubated in wash buffer with an ionic strength of 90, 170, or 490 mM at 4°C for 24 h. SFs were then scraped off the dish using a rubber scraper, put on an EM grid with a Formvar support film, stained with 4% uranyl acetate solution, quickly dried, and observed under an electron microscope (H-7600; Hitachi) at an accelerating voltage of 100 kV.

2.4 SDS-PAGE and immunoblotting

The extracted SFs were incubated in wash buffer with an ionic strength of 90, 123, 170, 250, 330, 410, or 490 mM at 4°C for 24 h. For SDS-PAGE, the extracted SFs and intact cells were separately solubilized in SDS sample buffer, boiled for 5 min, and fractionated on 10% polyacrylamide gels. Equal amounts of total protein (5 µg) were loaded in each lane. Gels were stained with Coomassie Brilliant Blue G-250 (CBB; Bio-Rad Laboratories). Samples were transferred onto polyvinylidene difluoride membranes (Millipore) for immunoblotting. Membranes were incubated with an antibody against non-muscle MIIA

(NMIIA; 1:1000, Covance), non-muscle MIIB (NMIIB; 1:1000, Abcam), smooth muscle myosin (SMM; 1:1000, Abcam), α -actinin (monoclonal, 1:1000, Abcam and polyclonal, 1:200, Santa Cruz Biotechnology), fascin (1:200, Santa Cruz Biotechnology), α -smooth muscle actin (α -SMA; 1:1000, Abcam), β -actin (1:5000, Applied Biological Materials), and myosin light chain (MLC; 1:200, Santa Cruz Biotechnology). The primary antibodies were detected using HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (1:10000, Bio-Rad Laboratories) and enhanced chemiluminescence (Immobilon Western, Millipore).

2.5 Contractility assay

Cells were cultured on 18-mm square cover glasses placed in a plastic dish. The SFs were extracted essentially according to the abovementioned method but under 3 different sets of conditions shown in Table 1. Experiments A and B were designed to investigate how buffer treatment time may affect contractility. Experiment C was designed to investigate the effect of varying temperature. In Experiment A, the extracted SFs were treated with wash buffer having an ionic strength of 90, 123, 170, 250, 330, 410, or 490 mM for 24 h; in Experiment B, SFs were treated with buffer having an ionic strength of 90, 123, 170, 250, 330, 410, or 490 mM for 10 min; and in Experiment C, SFs were briefly washed twice with buffer having an ionic strength of 100 mM. A perfusion chamber with a channel was made from the cover glass supporting the extracted SFs ⁽⁹⁾. The chamber was mounted on the microscope stage to capture time-lapse images and was perfused with activation solution from one side of the chamber via capillary action. SFs were reactivated with activation solutions containing MgATP at the following concentrations and temperatures: 2 mM at room temperature (25°C) for Experiment A, 1 mM at 25°C for Experiment B, and 1 mM at either 4, 25, or 37°C for Experiment C. After reactivation with MgATP for either 3 min (Experiment A) or 5 min (Experiment B), F-actin was stained with 3 nM Alexa 488 conjugated-phalloidin in PBS for 1 min. Data were quantified using ImageJ (NIH).

Table 1 Experimental conditions of contractility assay.

Experiment	Ionic strength	Treatment time	Temperature	[MgATP]
Experiment A	90, 123, 170, 250, 330, 410, or 490 mM	24 h	25°C	2 mM
Experiment B	90, 123, 170, 250, 330, 410, or 490 mM	10 min	25°C	1 mM
Experiment C	100 mM	2 washes	4, 25, or 37°C	1 mM

3. Results

3.1 Effect of varying ionic strength on SF morphology

The extracted SFs were observed using phase-contrast microscopy (PM), fluorescence microscopy (FM), and EM (Fig. 1). PM revealed extracted SFs with a sharp and straight morphology at an ionic strength of 90 mM. Furthermore, SFs with this morphology were found only at ionic strengths below 250 mM, and this morphology was absent at high ionic strengths. The SF outlines became less defined as the ionic strength was increased, and at more than 410 mM they were barely observed. Particularly straight actin filaments labeled with fluorescent phalloidin were observed by FM at an ionic strength of 90 mM. As the ionic strength increased, the fluorescent actin bundles appeared thicker in width. The loosely packed structure of the actin bundle was particularly prominent at an ionic strength of 490 mM. EM showed that at an ionic strength of 90 mM, isolated SFs displayed a tightly packed structure with several filamentous constituents bundled together. As the ionic strength increased, the bundles became looser with larger distances between the

components. At the highest ionic strength examined (490 mM), the continuous arrangement of straight filamentous constituents was no longer found, but rather, loosely packed, punctuate patterns were observed along the length of the isolated SFs. We quantified the bundle thickness of the isolated SFs from two representative EM images and found that the average thickness was 428 nm (for 90 mM), 1029 nm (170 mM), and 2003 nm (490mM) (Table 2).

Fig. 1 The effects of varying the ionic strength of buffers on extracted SFs. PM (Phase), multiple cells. FM (F-actin), single cells. EM, single SFs. Scale bars: Phase, 50 μ m; F-actin, 20 μ m; EM, 200 nm.

Table 2 Bundle thickness of individual SFs.

Ionic strength (mM)	Thickness (nm)
90	428 $\pm$ 48
170	1029 $\pm$ 111
490	2003 $\pm$ 272

Means $\pm$ s.d. ($n = 8$).

3.2 Effect of varying ionic strength on SF composition

SDS-PAGE was performed to examine the effects of varying ionic strength on the composition of extracted SFs (Fig. 2). In comparison to whole cell lysates, the extracted SFs contained a darker band around 42 kDa, which corresponds to actin. Because samples containing equal amounts of total protein were loaded in each lane, the relative amounts of actin were higher in SFs than in whole cells. A distinct decrease in band intensity was observed at approximately 75 kDa in CBB-stained gels as the ionic strength increased from 90 to 490 mM. A total of 8 proteins (NMIIA, NMIIB, SMM, α -actinin, fascin, α -SMA,

Fig. 2 Comparison of protein concentrations. (A) CBB-stained SDS-PAGE and (B) western blot analyses of whole cell lysates and extracted SFs at ionic strengths ranging from 90 to 490 mM. The gel and blots are representative of 2 experiments except for α -actinin which was obtained from a single experiment for each antibody. Note that the different blots do not indicate the absolute amount of proteins present in SFs.

β -actin, and MLC) were identified by immunoblotting. Fascin, a globular F-actin cross-linking protein that is essential for filopodia formation, was only present in whole cell lysates, suggesting that the material we extracted from cells was indeed SFs and not actin bundle filopodia. The expression levels of all proteins, except α -actinin, were comparable regardless of the ionic strength. Two different antibodies (monoclonal and polyclonal) were used to detect α -actinin, and similar decreases with an increase in ionic strength were detected.

3.3 Effect of varying ionic strength on SF contractility

Two separate sets of experiments were performed to induce actin–MII cross-bridge cycling. In Experiment A, 2 mM MgATP was added to the extracted SFs initially treated with wash buffer of a particular ionic strength for 24 h. In Experiment B, 1 mM MgATP was added to SFs initially treated with wash buffer of a particular ionic strength for 10 min. Three minutes after the addition of 2 mM MgATP in Experiment A or 5 min after the addition of 1 mM MgATP in Experiment B, PBS containing fluorescent phalloidin was perfused. Thus, ATP-induced shortening was subdued since ATP was removed by PBS perfusions. In Experiment A, majority of the extracted SFs shortened at ionic strengths less than 170 mM. It was difficult to observe SFs at high ionic strengths, since there were only a few distinct filamentous structures visible in PM (Fig. 3). At high ionic strengths, only peripheral regions of the cells, which were deroofed in order to expose the SFs to the solutions, shrank in response to ATP. FM confirmed that all SFs shortened and collapsed into a tight mass at ionic strengths below 123 mM. Meanwhile, SFs presented as rough, ragged actin structures with almost no shortening at ionic strengths above 410 mM. The responses of SFs obtained in Experiment A can be categorized into the following groups: one in which all SFs shortened in response to ATP at low ionic strengths and the other in which some ragged actin structures, which are insensitive to ATP, remained, but

Fig. 3 Active contraction of extracted SFs initially treated with buffers of different ionic strengths for 24 h. (A) Shortening of SFs initially treated with buffer at ionic strength of 90 mM on the addition of 2 mM MgATP (at $t = 0$ s). The lower panels show magnified sequential images of a boxed area in the upper panel. Arrowheads indicate the translocation of points on SFs over time to show shortening. Note that SFs shortened to finally collapse into an F-actin-associated mass as visualized by FM. Other filamentous structures (such as those shown by arrows) were left on the substrate after SF shortening. They were not associated with phalloidin-detecting F-actin, and thus were probably the extracellular matrix. (B) Shortening of SFs initially treated with buffers of different ionic strengths. Note that the SFs almost completely shortened at an ionic strength of 123 mM or less, while many non-contractile F-actin structures remained even after 180 s at high ionic strengths. Scale bars: (A) 50 μ m; magnified images in (A) 20 μ m; (B) 50 μ m.

unidentified peripheral regions of the cell shrank at high ionic strengths. In Experiment B, we quantified the proportion of cells that shortened to completely collapse into a mass within the 5-min interaction with ATP. At an ionic strength of 170, 250, or 330 mM, all SFs shrank in response to ATP. However, at an ionic strength of 90, 123, 410, or 490 mM only some cells (on average 60%–80% of the total) exhibited a contractile response (Fig. 4). SFs in the non-shortened cells initially treated at an ionic strength of 90 or 123 mM displayed a straight morphology. Meanwhile, SFs in the non-shortened cells initially treated at an ionic strength of 410 or 490 mM displayed ragged actin structures with few fibers showing a straight morphology. The responses of SFs obtained in Experiment B, can be categorized into the following 3 groups (Fig. 4B): (I) group in which some straight SFs remain without shortening even in the presence of ATP; (II) group in which all SFs shorten in the presence of ATP; and (III) group in which some ragged actin structures, which are insensitive to ATP, remain.

3.4 Effect of varying temperature on the shortening rate of SFs

In Experiment C, the effects of varying temperature on cell contractility was investigated using SFs extracted at an ionic strength of 100 mM which lies within region I of Fig. 4B. Many SFs began to shorten on the addition of 1 mM MgATP at $t = 0$ (Fig. 5). The shortening rate normalized to the initial length of SFs gradually decreased over time

Fig. 4 Active contraction of extracted SFs initially treated with buffers of different ionic strengths for 10 min. (A) Shortening of SFs (initially treated with buffer at ionic strength of 90, 170, or 490 mM) after the addition of 1 mM MgATP at $t = 0$ s. The panels on the right represent a magnified view of the boxed areas. Note that SFs completely shortened at an ionic strength of 170 mM, while some SFs (such as those shown by arrow heads) did not shorten and remained unchanged at other ionic strengths. (B) The histogram shows the proportion of contractile and non-contractile cells. The responses of SFs can thus be categorized into 3 groups, denoted as regions I, II, and III.

Fig. 5 The effects of temperature on SF contractility. (A) Time-course change in the length of SFs normalized to their initial length. Data are means $\pm$ s.d. ($n = 6-7$ experiments). (B) Time-course change in the shortening rate. (C) Shortening rates measured at $t = 0-5$ s (V_1) and 5-10 s (V_2). Data are means $\pm$ s.d.

(Fig. 5A and 5B), and was therefore quantified over 2 different time intervals. V_1 and V_2 were calculated from the rate for $t = 0-5$ s and 5-10 s, respectively (Fig. 5C). The average value of the shortening rates tended to increase with an increase in temperature for both V_1 and V_2 . The differences were analyzed using the Student's t -test, but no significant differences were observed between the shortening rates at different temperatures for both V_1 and V_2 .

4. Discussion

Experiments for SFs in intact cells ^(2,4,13) are often only suggestive rather than demonstrative of the molecular mechanisms underlying their specific contributions. The inherent mechanisms at the molecular level may be studied with SFs extracted from live cells. A method for mass isolation of functional SFs from cells was introduced by Katoh et al. ⁽⁵⁾, in which the cell membranes and cytoplasmic constituents were removed by procedures similar to those used in the present study. The absence of the surrounding cytoplasmic structures permits controlled solutions direct access to the extracted SFs that remain on the substrate. However, these *in vitro* studies may overlook some of the regulatory mechanisms present in intact cells, which could be lost or altered in the extracted systems. Thus, we investigated how structure, molecular composition, and contractility of SFs could differ during the extraction procedure when the ionic strength of the wash buffer and temperature were altered. The imidazole-based buffer we used was a modification of that described by Fabiato and Fabiato ⁽¹²⁾, taking advantage of accumulated techniques developed for skinned muscle cells or fibers that were first introduced by Natori ⁽¹⁴⁾.

Our results suggest that the extracted SFs may be more intact with regard to structure (Fig. 1), composition (Fig. 2), and contractility (Fig. 3) at low ionic strengths. The three experiments (i.e., morphological observation, composition analysis, and contractility measurement) employed the SFs treated with buffer at a particular ionic strength for 24 h. A long exposure time was selected in order to achieve the desired effect. Meanwhile, we examined the contractility of SFs treated with buffer at a particular ionic strength for 10 min (Fig. 4). The selection of this short exposure time (in Experiment B) was to evaluate the effects of ionic strength within a practical treatment time, i.e., when extracted from cells, SFs are usually exposed to buffers within a few minutes ⁽⁹⁾. In Experiment B, the extracted SFs exhibited the most extensive shortening at an intermediate ionic strength corresponding to region II (Fig. 4). We speculate that the protein structure of the adhesion–matrix complex and contractility may remain intact in region I. This is consistent with the fact that MII is known to form an anti-parallel folded dimer, suitable for contractile activity, at low or physiological ionic strengths ^(15,16). It is possible that the SFs were not able to shorten in length because the intact adhesions continued to function as anchors even after extraction. Because the extracted SFs maintained a straight morphology in region I as seen in live cells, they may have been undergoing an isometric contraction (with an unchanged macroscopic length of SFs) in the presence of ATP. At intermediate ionic strengths, all cells shortened to collapse into a mass. This suggests that the adhesion–matrix complex and/or matrix itself may have denatured even during the short treatment period of 10 min, and it was subsequently unable to function as an anchor to prevent shortening.

In contrast, all SFs shortened even at low ionic strengths when treated for 24 h in Experiment A (Fig. 3). This result suggests that the adhesion–matrix complex and/or matrix itself may have denatured even at the low stabilizing ionic strength when treated for a long time. Fibronectin, a principal constituent of the matrix, has been reported to undergo an ionic strength-dependent change in conformation between a compact and an extended form ⁽¹⁷⁾. In addition, disulfide bonds in matrix proteins could be reduced in the extracted state due to the presence of oxygen-removal reagents in the solutions. Thus, further study is required for exclusion of the possible involvement of the matrix and cell adhesions. Development of a novel system for physically isolating functional SFs from the substrate is underway in our group to definitively characterize the contractile properties intrinsic to SFs.

The extracted SFs were packed more loosely at high ionic strengths (Fig. 1). We originally speculated that denaturation of MII may be responsible for this loose packing because of a partial loss of SF contractility (Figs. 3 and 4). However, there was no significant change in the expression of MII (NM2A, NM2B, and SMM) and MLC (Fig. 2). Instead we found that α -actinin associated with the extracted SFs decreased in expression

with an increase in ionic strength. Incubation with the anti- α -actinin polyclonal antibody revealed multiple bands including one at approximately 75 kDa in addition to the known band at 105 kDa (Fig. 2). Each band became less intense as the ionic strength increased. The affinity between non-muscle α -actinin and actin is known to be significantly reduced in the presence of Ca^{2+} ⁽¹⁸⁾. However, since we removed Ca^{2+} from wash buffers with EGTA, its involvement in α -actinin dissociation is unlikely. Regardless of the mechanism of α -actinin dissociation, sarcomere structures might disintegrate at high ionic strengths, resulting in the loss of contractility and the appearance of ragged rather than straight patterns.

One critical point of regulation for contraction and maintenance of SFs is MLC phosphorylation. MLC phosphorylation is required for MII bipolar filament assembly and ATP-induced actin–MII cross-bridge cycling ⁽¹⁾. Katoh et al. ⁽⁵⁾ reported that no MLC phosphorylation was initially detected in SFs isolated from human foreskin fibroblasts. They observed a gradual increase in MLC phosphorylation after the addition of exogenous ATP to the isolated SFs with recombinant Rho-kinase and/or MLCK that phosphorylate MLC. The use of PBS might alter protein activities such as MLC phosphorylation. In contrast, we previously detected mono- and diphosphorylated MLC in the SFs extracted from A7r5 cells using an imidazole-based buffer with an ionic strength of 170 mM ^(9,10). We did not detect significant monophosphorylation of MLC in the present study at the physiological ionic strength (figure not shown). In the present study, the extracted SFs were washed with buffer for 24 h, whereas in the previous studies they were washed for a short period of less than 1 min. The long wash duration employed in the present study thus somehow decreased phosphorylation.

MgATP (1 mM) was added to the SFs extracted at an ionic strength of 100 mM at different temperatures to evaluate their contractility (Fig. 5). The resulting shortening could be hampered by the adhesion–matrix anchor (Fig. 4). Such physical restrictions may prevent or at least slow the initial shortening. We then focused on cells that were isolated from their surrounding neighbors, and thus, were physically independent of the effect of cell–cell interaction. The shortening rate was quantified at the following time intervals: V_1 being the initial rate measured for $t = 0$ –5 s and V_2 for 5–10 s. Since V_1 could be more susceptible to retardation due to local physical restrictions, V_2 might be regarded as being more independent of such surrounding restrictions and could thus be a more accurate measure of contractility. V_2 increased on an average with an increase in temperature, but the difference was not statistically significant (Fig. 5). Although a temperature-dependent difference exists for the ATPase rate of the MII enzyme itself ⁽¹⁹⁾, we did not detect a significant difference at the level of SFs. Thus, while the increase in ionic strength contributed to a positive entropy-driven change, the change in temperature may not have provided a significant enthalpy change in the structure of the extracted SFs.

Peripheral cell regions shrank in response to ATP in both Experiments A and B at all ionic strengths, while SFs shortened only at low ionic strengths (Figs. 3 and 4). The peripheral regions with a relatively rough morphology are distinct from SFs having a clear filamentous appearance. Although we did not identify the peripheral regions, we speculate that because they were labeled with phalloidin, they were the actin network-lined cell membranes left after the extraction procedure. MII and its contractile activity were probably present in the membranes, causing shrinkage of the rough structures on ATP addition (Figs. 3 and 4). The shrinkage of the actin–MII network in the membranes may not require α -actinin. On the other hand, α -actinin decreased in expression in the extracted SFs at high ionic strengths (Fig. 2), and they exhibited no contractility even with MII activity. Thus, the presence of α -actinin or its involvement in maintaining sarcomere integrity is implicated in the shortening of SFs.

5. Conclusions

We investigated the effects of varying the ionic strength of buffers and temperature on SFs extracted from cells. Light microscopy and EM revealed that SFs possessed more straight, packed bundles at low ionic strengths that lost contractility completely when treated with high ionic strength buffers for a long period. We did not detect a significant difference in contractility with a change in temperature at low ionic strengths. These results suggest that the extracted SFs are more intact with regard to structure and function at low ionic strengths, but this conclusion may only apply at the macroscopic level. The present study thus provides practical methodological information that could be useful for investigators interested in performing studies on extracted systems.

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