Vascular Smooth Muscle Contraction Evoked by Cell Volume Modulation: Role of the Cytoskeleton Network

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Key Words

Microfilaments • Microtubules • Cell volume • Smooth muscle • Contraction

Abstract

Previously, we reported that hyposmotic swelling evoked transient vascular smooth muscle cell (SMC) contraction that was completely abolished by L-type Ca^{2+} channel blockers. In contrast, sustained contraction revealed in hyper- and isoosmotically-shrunk SMCs was insensitive to L-type channel blockers and was diminished in Ca^{2+} -free medium by only 30-50%. Several research groups reported cell volume-dependent cytoskeleton network rearrangements. This study examines the role of cytoskeleton proteins in cell volume-dependent contraction of endothelium-denuded vascular smooth muscle rings (VSMR) from the rat thoracic aorta. Hyperosmotic shrinkage and hyposmotic swelling were triggered by modulation of medium osmolality; isosmotic shrinkage was induced by VSMR transfer from hypo- to isosmotic medium. The relative content of globular (G) and fibrillar (F) actin was estimated by fluorescence microscopy. Hyperosmotic shrinkage and hyposmotic

swelling led to elevation of the F-actin/G-actin ratio by 2.5- and 1.8-fold respectively. Contraction of shrunk and swollen VSMR was insensitive to modulators of microtubules such as vinblastine, colchicine and docetaxel. Microfilament disassembly by cytochalasin B resulted in dramatic attenuation of the maximal amplitude of contraction of hyperosmotically-shrunk and hyposmotically-swollen VSMR, and almost completely abolished the contraction triggered by isosmotic shrinkage. These data suggest that both L-type Ca^{2+} channel-mediated contraction of swollen vascular SMC and Ca^{2+} -insensitive contractions of shrunk cells are triggered by reorganization of the microfilament network caused by elevation of the F-actin/G-actin ratio.

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Introduction

It has been well-documented that skeletal and cardiac muscle contractions are triggered by elevation of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) due to the activation

of several subtypes of voltage-gated channels, interaction of Ca^{2+}_i with troponin C and activation of actin-dependent myosin ATPase. Unlike striated muscle, smooth muscle cell (SMC) contraction is caused by Ca^{2+} /calmodulin-dependent phosphorylation of myosin light chain (MLC). Alternatively, SMC contractions may be evoked by protein kinase C and Rho-kinase mediated phosphorylation of MLC kinase that, in turn, leads to elevation of enzyme affinity for Ca^{2+} /calmodulin complex and contractions under baseline $[\text{Ca}^{2+}]_i$ values [1, 2].

Like other nucleated eukaryotic cells, myocytes contain a 3-dimensional cytoskeleton network formed by microfilaments, microtubules and intermediate filaments. Microfilaments are made up of actin polymers (F-actin) which are in dynamic equilibrium with globular actin monomers (G-actin). Importantly, in striated muscle, monomers contribute to only ~10% of total actin content whereas in SMC, the F-:G-actin ratio varies from 1:1 to 2:1 [3], suggesting a special role of the cytoskeleton in the regulation of SMC contractions. Several pharmacological tools have been developed to examine the involvement of cytoskeleton proteins in the regulation of cellular function. Thus, it has been shown that sustained treatment with colchicine, vinblastine or nocodazole results in microtubule disassembly via highly-selective binding to α - and β -tubulin that inhibits their polymerization. Docetaxel stabilizes microtubules and prevents their depolymerisation preferentially at the plus end of the microtubule. Latrunculin-A and cytochalasin-B prevent actin polymerization by capping of G- and F-actin, respectively, culminating in microfilament disruption.

Data on cytoskeleton-active compounds strongly indicate that side-by-side with unique mechanisms of intracellular signaling, excitation-contraction coupling in SMC is affected by stimuli leading to cytoskeleton rearrangements. Thus, in several types of SMC it has been shown that microfilament disassembly evoked by cytochalasins and latrunculin sharply reduces myogenic tone [4] and contractions triggered by K^+_o -induced depolarization [5, 6], α -adrenergic [6, 7] and m-cholinergic [8-10] agonists, angiotensin II [11], protein kinase C activators [12] and mechanical stretch [13]. In contrast, inhibitors of microtubule polymerization, such as colchicines, vinblastine and nocodazole, did not affect [8, 12], and even slightly potentiated, the actions of vasoconstrictors [14-16].

We noted that vascular SMC relaxation evoked by β -adrenoceptor agonists and other activators of cAMP signaling is at least partially mediated by disassembly of microfilaments that, in turn, elicits the inhibition of L-type

Ca^{2+} channels [17] and $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter [18, 19], i.e. an electroneutral ion carrier involved in regulation of SMC contraction via modulation of $[\text{Cl}^-]_i$ and the Cl^- -dependent component of resting membrane potential (for recent review, see [20]). This hypothesis is consistent with data demonstrating that cytochalasin and activators of c-AMP signalling caused similar reorganization of the cytoskeleton network with inhibition of ^{86}Rb and ^{45}Ca fluxes mediated by $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter and L-type Ca^{2+} channels, respectively [17-19]. Later on, inhibition of L-type Ca^{2+} channels in cytochalasin-treated vascular SMC was also detected with the patch-clamp technique [21]. Importantly, both isoproterenol-induced cytoskeleton reorganization and inhibition of the above-mentioned ion transporters were abolished in the presence of vinblastine and colchicine [17-19].

Salt intake and salt reabsorption by ion transporters in proximal tubules sharply affect the osmolality of interstitial fluid in the juxtaglomerular apparatus that may be involved in blood pressure regulation via cell volume-dependent modulation of mesangial cell and vascular SMC functions [22]. The role of cell volume modulation in the regulation of myocyte contraction is widely disputed [23, 24] and supported by several studies performed in vitro with SMC. (i) Takeda and co-workers were the first to report that hyperosmotic stress induced MLC phosphorylation comparable to that in SMC treated with potent vasoconstrictors, such as arginine vasopressin and endothelin-1 [25]. (ii) Wagner and co-workers observed that elevation of medium osmolality blunted the vasoconstrictory responses of rabbit arterial rings triggered by K^+_o -induced depolarization and activation of α -adrenoceptors with phenylephrine (PE) [26]. (iii) Shrinkage in hyper- and isosmotic conditions was sufficient to evoke sustained contraction of endothelium-denuded vascular smooth muscle rings (VSMR) from the rat aorta. These phenomena persisted in the presence of L-type Ca^{2+} -channel blockers and were only partially attenuated in Ca^{2+} -free medium [27]. In contrast to cell shrinkage stimuli, swelling in hyposmotic medium resulted in transient VSMR contraction that was completely abolished by Ca^{2+} -channel blockers and by omission of extracellular Ca^{2+} [27].

Numerous researchers have reported that cell volume perturbation leads to tissue-specific reorganization of the cytoskeleton network. Thus, in lymphocytes, HL-60, renal epithelial and Ehrlich ascites tumor cells, cell swelling and shrinkage are associated with decreases and increases of F-actin content, respectively, whereas in hepatocytes, both swelling and shrinkage result in

Fig. 1. Effect of cytochalasin B (10 μ M) on isometric contractile force recording from VSMR subjected to K^+ -induced depolarization, hyperosmotic shrinkage caused by the addition of 150 mM sucrose, hypotonic swelling triggered by the transfer of VSMR from isotonic to hypotonic medium containing 120 and 40 mM NaCl, respectively, and to isosmotic shrinkage evoked by VSMR transfer from hypo- to isosmotic medium

Additions, μ M	Maximal VSMR contraction, %				PE
	Hyperosmotic shrinkage	Isosmotic shrinkage	Hypotonic swelling	K^+ -induced depolarization	
1. None (control)	51.8 $\pm$ 9.0 (n=81)	21.6 $\pm$ 8.7 (n=10)	75.7 $\pm$ 8.9 (n=18)	100	103.1 $\pm$ 4.9 (n=12)
2. Vinblastine, 10	33.7 $\pm$ 3.4 (n=6)	22.3 $\pm$ 6.4 (n=4)	72.3 $\pm$ 7.7 (n=4)	104.0 $\pm$ 6.5 (n=6)	97.7 $\pm$ 10.3 (n=6)
3. Cytochalasin B, 10	17.2 $\pm$ 3.8 (n=6)	6.5 $\pm$ 1.5 (n=6)	14.3 $\pm$ 5.0 (n=6)	40.0 $\pm$ 4.9 (n=6)	20.6 $\pm$ 3.7 (n=6)
$p_{1,2}$	<0.05	NS	NS	NS	NS
$p_{1,3}$	<0.01	<0.01	<0.01	<0.001	<0.001

Table 1. Effect of vinblastine and cytochalasin B on VSMR contraction evoked by distinct stimuli: VSMR were subjected to K^+ -induced depolarization (addition of 30 mM KCl), activation of α -adrenoceptors with PE (1 μ M), hyperosmotic shrinkage caused by the addition of 150 mM sucrose, hypotonic swelling triggered by VSMR transfer from isotonic to hypotonic medium containing 120 and 40 mM NaCl, respectively, and to isosmotic shrinkage evoked by VSMR transfer from hypo- to isosmotic medium. K^+ -induced contractile responses were taken as 100%. Means $\pm$ S.E. values obtained in n experiments are shown.

attenuation of G-actin (for review, see [28-32]). We designed this study to examine the role of the cytoskeleton in VSMR contractions evoked by cell volume modulation.

Materials and Methods

Endothelium-denuded VSMR were obtained from the thoracic aortae of 11- to 13-week-old Wistar rats euthanized

under deep intraperitoneal anesthesia with sodium pentobarbital (Nembutal, 70 mg/kg) in accordance with institutional animal care guidelines. The isolated aorta was placed in physiologically-balanced salt solution (PSS) containing 120.4 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.5 mM glucose, and 15 mM Tris/HCl (pH 7.4). Connective tissue and fat were excised with scissors, whereas the endothelium was removed by careful rotation of a wooden manipulator inside the VSMR lumen just before the experiments. VSMR of 2 to 3 mm were either used immediately or stored at 4°C for up to 24 hr. In preliminary experiments, we documented

Fig. 2. Effect of vinblastine (10 μ M) on isometric contractile force recording from VSMR subjected to K^+ -induced depolarization, hyperosmotic shrinkage caused by the addition of 150 mM sucrose, hypotonic swelling triggered by the transfer of VSMR from isotonic to hypotonic medium containing 120 and 40 mM NaCl, respectively, and to isosmotic shrinkage evoked by VSMR transfer from hypo- to isosmotic medium.

that 24 hr storage did not affect VSMR contractile responses evoked by K^+ -induced depolarization and activation of α -adrenoceptors with PE. The absence of functionally-active endothelium was confirmed by the absence of a relaxing action of acetylcholine [27].

VSMR were mounted in 1-ml baths with stainless steel hooks inserted into the vascular ring orifice. One hook was fastened to a MX2B mechanical force transducer (Tomsk, Russia) with silk thread and recorded with an XY recorder (Carl Zeiss, Jena, Germany). The tissues were bathed in PSS, buffered with TRIS at pH 7.4 (37°C) and bubbled with 95% O_2 /5% CO_2 at a volumetric speed of ~1 ml/min. To control the contractile response, the VSMR were equilibrated for 1 hr at a tension of 0.5-1 g and exposed to K^+ -induced depolarization caused by isosmotic substitution of 30 mM NaCl with KCl. Isosmotic and hyperosmotic shrinkage was triggered by VSMR transfer from hypo- to isotonic medium and by elevation of medium osmolality with 150 mM sucrose, respectively. Cell swelling was evoked by VSMR transfer from iso- to hypotonic solution containing 40 mM NaCl. With ^{14}C -labeled urea, we observed that these procedures led to the modulation of intracellular water content within $\pm 20\%$ of its control values. For more details, see [27].

Stimulated or unstimulated VSMR were submerged in ice tissue-embedding medium (Neg 52, Richard-Allen Scientific, Kalamazoo, MI) precooled on dry and stored at -20°C. Sections were cut with a cryostat, and 8 μ m-thick samples were collected directly onto slides. Usually, 2 sections per slide were placed well apart on each slide. The sections on the slides were thoroughly air-dried at room temperature and stained the same day. First, the sections were fixed in 4% formaldehyde with

TBS (0.05 M Tris, 0.9% NaCl, pH 7.6) for 30 min at room temperature. Then, they were rinsed 3 times with TBS for 5 min each time and treated with 0.2% Triton X100 for 5 min. After washout in TBS (3 x 5 min), all the sections were incubated with TBS/2% bovine serum albumin (BSA) for 30 min at 37°C in a sealed humidity chamber to reduce nonspecific signals. To stain F-actin, the sections were incubated with Rhodamine-Phalloidin (Molecular Probes, Eugene, OR) diluted 1:100 in TBS/2% BSA for 1 hr at 37°C in a sealed humidity chamber. Then, the slides were rinsed 3 times for 5 min in TBS. After that, the sections were incubated with G-actin-specific fluorescent dye DNase I-Alexa Fluor 488 conjugate (Molecular Probes) diluted 1:100 in TBS/2% BSA for 1 hr at 37°C in a sealed humidity chamber. The slides were rinsed 3 times for 5 min in TBS, air-dried at room temperature, and sealed with Fluoromount-G (SouthernBiotech, Birmingham, AL). Incubation, fixation, and staining were always performed in parallel for all sections on a slide. Actin pools were visualized under a fluorescence microscope (Leica DM/RB, Wetzlar, Germany). The images were captured and stored with a MagnaFire digital camera using MagnaFire software (Meyer Instruments, Houston, TX). The total and background intensity of Rhodamine-Phalloidin and DNase I-Alexa Fluor 488 conjugate was analyzed with the Multi Gauge V3.0 program. The background of fluorescence intensity from each image was subtracted and filamentous-to-globular (F/G) ratios were calculated. To control for day-to-day variations in staining intensity, control tissues were always compared with stimulated VSMR rings on the same microscope slide, as tissues on the same slide undergo identical fixation, staining, and microscopy conditions, allowing meaningful comparisons between samples.

Fig. 3. Representative photographs of VSMR stained with F- and G-actin-specific fluorescent dyes in control conditions and subjected to 10-min hyperosmotic shrinkage or hyposmotic swelling caused by the addition of 150 mM sucrose and by transfer of VSMR from isotonic to hypotonic medium containing 120 and 40 mM NaCl, respectively.

Table 2. Effect of hyperosmotic shrinkage and hyposmotic swelling of VSMR on the F-actin/G-actin ratio: Shrinkage and swelling were triggered by 10-min exposure of VSMR to hypertonic (PSS containing 150 mM sucrose) and hypotonic (PSS containing 40 mM NaCl) media, respectively. The F-actin/G-actin ratio under control conditions was taken as 100%. Means $\pm$ S.E. values obtained in 5 (shrinkage) and 3 (swelling) experiments are shown. NS – non-significant.

Conditions	F-actin/G-actin ratio (%)
1. Control	100
2. Hyperosmotic shrinkage	267 $\pm$ 54
3. Hyposmotic swelling	178 $\pm$ 50
$p_{1,2}$	<0.001
$p_{1,3}$	<0.05

Chemicals were obtained from Sigma (St. Louis, MO). The data, presented as means $\pm$ SE, were analyzed by Student's t-test or t-test for dependent samples as appropriate. Significance was defined as $p < 0.05$.

Results

Exposure of VSMR from the rat aorta to cytochalasin B resulted in the inhibition of $K^+_{\circ}$ - and PE-induced

contractions by ~ 60 and 80% , respectively (Table 1). This observation is consistent with the inhibitory actions of cytochalasins and other compounds that lead to microfilament disassembly on $K^+_{\circ}$ - and PE-induced contractions documented in guinea-pig taenia coli [5], chicken gizzard [6] and rat mesenteric arteries [7]. Cytochalasin B also sharply attenuated the contractions of VSMR subjected to hyper- and isosmotic shrinkage as well as transient contractions of VSMR evoked by hyposmotic swelling (Fig. 1, Table 1).

In contrast to cytochalasin, inhibition of microtubule assembly with vinblastine reduced by ~40% contractions in hyperosmotically-shrunk cells and did not affect those triggered by K^+ -depolarization, PE as well as by isosmotic shrinkage and hyposmotic swelling (Fig. 2, Table 1). Similar results were obtained with VSMR subjected to inhibition of microtubule assembly with 100- μ M colchicine and to microtubule stabilization with 10- μ M docetaxel (data not shown).

Previously, it was demonstrated that contractions of rat cerebral and mesenteric arteries, evoked by acetylcholine- and intravascular pressure-induced myogenic responses, respectively, were accompanied by decreased G-actin without any significant modulation of total actin content [4, 10]. Despite numerous reports on cell volume-dependent microfilament reorganization (for review, see [28-31]), data on the modulation of F- and G-actin content in shrunken and swollen cells are limited to a few discrepant publications. Thus, in rat hepatocytes, the ratio of G-/total actin was attenuated by cell swelling rather than shrinkage [33]. In contrast, confocal microscopy experiments with human B-lymphocytes revealed that submembrane F-actin was disrupted in response to the hyposmotic gradient [34]. Negative correlation between F-actin and cell volume was noted in HL-60 cells [35], Ehrlich ascites tumor cells [36] and cells of the inner medullary collecting ducts [37]. We found that hyperosmotic shrinkage and hyposmotic swelling of VSMR resulted in 2.7- ($P<0.001$) and 1.8-fold ($P<0.05$) elevation of the F-actin/G-actin ratio, respectively (Fig. 3, Table 2).

Discussion

Previously, we reported that L-type Ca^{2+} -channel blockers completely abolished transient contractions in swollen VSMR from the rat aortae [27]. The inhibitory actions of these compounds and Ca^{2+} -free medium were also noted in studies of contractions in swollen rat cerebral and tail arteries [38, 39]. Recent investigations have demonstrated that contractions in swollen SMC are mediated by activation of Src nonreceptor tyrosine kinase [40] that might be involved in the augmented permeability of unidentified cation channels and sarcolemmal depolarization [38]. In pulmonary artery SMC, hypotonic swelling evoked PI3K-SGK cascade that, in turn, affected

downstream signaling molecules linked to activation of volume-sensitive anion channels [41]. In canine basilar arteries, hyposmotic challenge activates cation channels, which presumably cause Ca^{2+} influx, activation of Ca^{2+} -sensitive Cl^- channels, membrane depolarization and Ca^{2+} influx through voltage-dependent L-type channels and elicit contraction [42]. In epithelial cells, swelling-induced Ca^{2+} influx triggers massive release of UTP and ATP that can affect cellular functions via activation of P2Y receptors [43]. The role of this autocrine mechanism in contractions of swollen SMC as well as upstream intermediates of cell volume sensing remain poorly understood.

Unlike swollen VSMR, sustained contractions in shrunken VSMR were insensitive to the presence of L-type Ca^{2+} -channel blockers and only partially diminished in Ca^{2+} -free medium [27]. Moreover, Lee and co-workers reported that hyperosmotic medium led to contractions of VSMR after their fixation with glutaraldehyde [44]. It should be underlined, however, that despite the different mechanisms of force generation in shrunken and swollen SMC, their contractions were equally sensitive to actin cytoskeleton disruption. Importantly, even complete disruption of microfilaments with cytochalasin B did not affect $[Ca^{2+}]_i$ as well as MLC phosphorylation [5, 6, 10], which is consistent with the Ca^{2+} -independent mechanism of cytochalasin-sensitive contraction of shrunken SMC.

Conclusion

Our data strongly suggest that reorganization of the actin microfilament network resulting in elevation of F-actin/G-actin ratio plays a key role in VSMR contractions induced by cell volume modulations independently on signaling cascades triggering force generation. The precise mechanism of SMC contractions mediated by microfilament rearrangements requires further investigations.

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